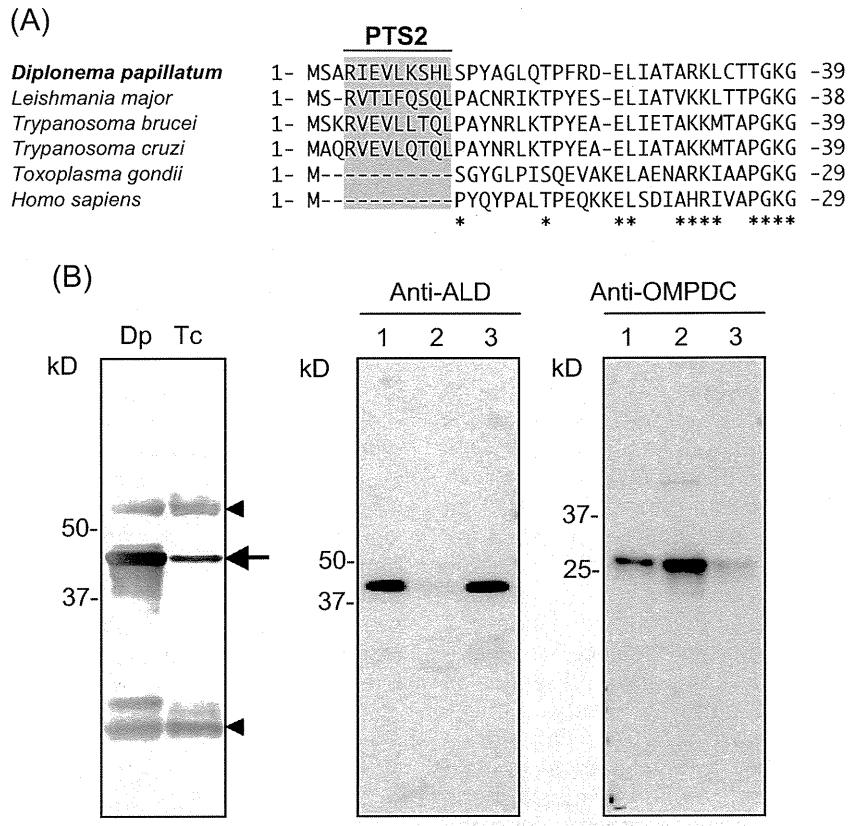

Figure 1. Characterization of aldolase (ALD) of *Diplonema papillatum*. (**A**) Amino acid sequence alignment of Class I fructose-1,6-bisphosphate aldolases. The asterisks indicate the same or equivalent amino acids. The N-terminal peroxisomal targeting signals (PTS2), consisting of a consensus nonapeptide (R/K)-(L/V/I)-X_5-(Q/H)-(L/A), are shaded. (**B**) Western blot analysis of ALD (41 kD) (left and middle panels) and orotidine-5'-monophosphate decarboxylase (OMPDC, 25 kD) (right panel) of *D. papillatum*. The anti-ALD antibody specifically recognizes ALD of *Trypanosoma cruzi* (Tc) and *D. papillatum* (Dp) (left panel). The crude cell lysate (lane 1), supernatant (lane 2) and precipitate (lane 3) of a 105,000 × *g* preparation were reacted with the specific antibodies. The arrow and arrowheads indicate the bands for ALD (41 kD) and the precipitated antibodies, respectively.

the formation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate from the substrate, fructose-1,6-bisphospate (data not shown). The most striking feature of *D. papillatum* ALD is the presence of a consensus N-terminal peroxisomal targeting signal (PTS2) present in trypanosomatid ALDs (Gabaldón 2010; Michels et al. 2005), suggesting that *D. papillatum* ALD is likely to localize in peroxisome-related compartments (Fig. 1A).

D. papillatum ALD Has a Common Evolutionary Origin as Glycosomal ALDs of Kinetoplastids

We examined whether the targeting of ALD to peroxisome-related organelles occurred in the common ancestor of diplonemids and kinetoplastids or evolved independently in each lineage. Phylogenetic analysis of Class I ALDs clearly showed monophyly of diplonemid and trypanosomatid ALDs within the eukaryotic clade with strong bootstrap support (Fig. 2). The evolutionary origin of euglenoid Class I ALD is unclear, due mainly to the lack of resolution. It is worth noting that euglenoids possess both Class I (plastid) and Class II (cytosolic) ALDs (Plaumann et al. 1997). The finding, that euglenoid Class I ALD did not fall into the diplonemid/kinetoplastid clade, suggests their different origin, the former originating probably from a secondary endosymbiont (an algal lineage) that gave rise to the plasmid. We conclude that the addition of PTS2 to ALD may have occurred in the

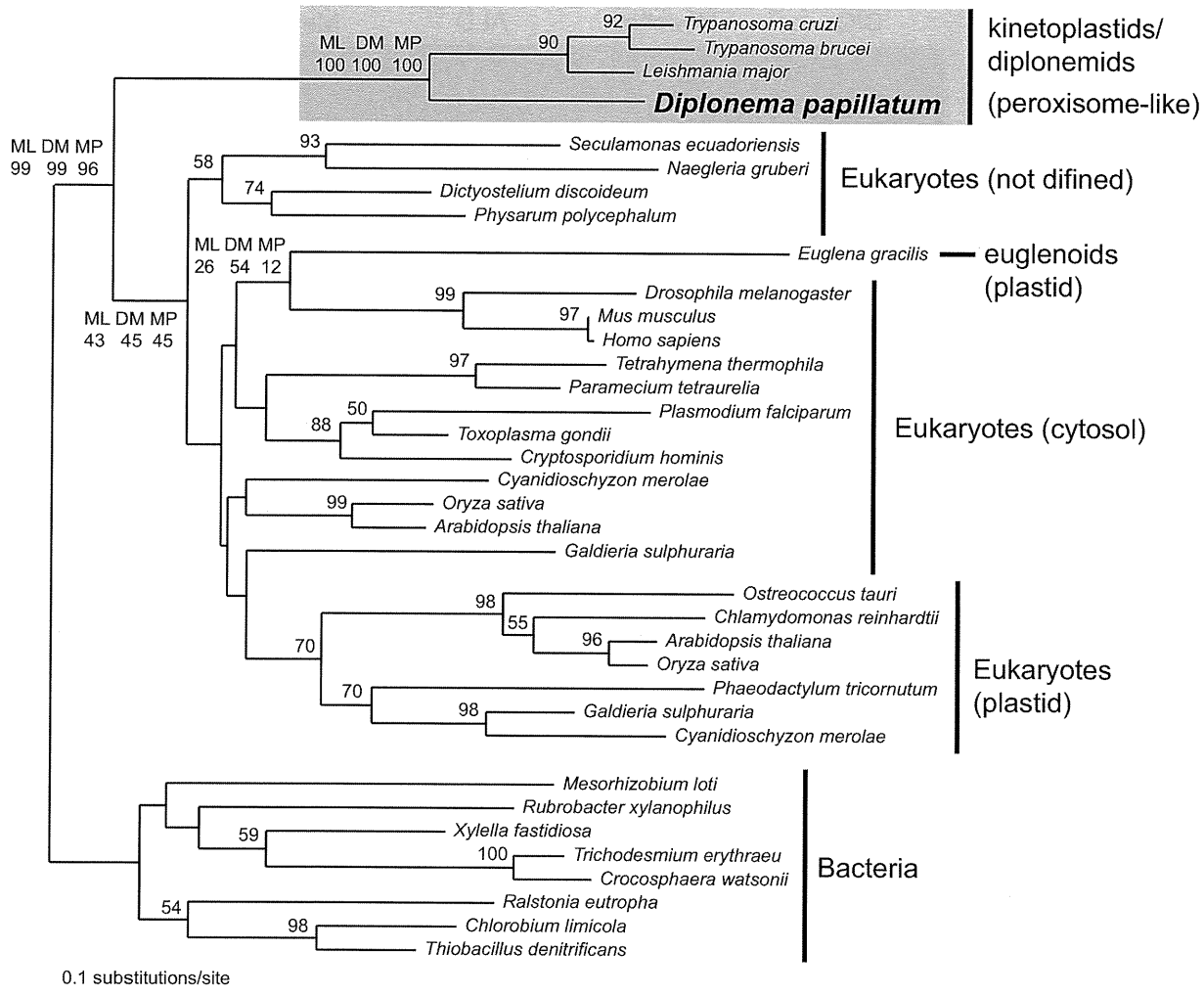

Figure 2. Phylogenetic reconstruction of Class I fructose-1,6-bisphosphatae aldolase (ALD). The consensus maximum likelihood (ML) tree of ALD inferred by the JTT model taking across-site rate heterogeneity into consideration. The α-value of the Γ shape parameter used in the analysis was 0.85937. Bootstrap proportions (BPs) by the ML method are attached to the internal branches. Branches with less than 50% BP support are unmarked. For the four nodes, BP values determined by the distance matrix (DM) and maximum parsimony (MP) methods are also shown.

common ancestor of diplonemids and kinetoplastids after separation of the euglenoid lineage.

Evidence of Compartmentalization of *D. papillatum* ALD

Trypanosomatid OMPDC is fused with orotate phosphoribosyltransferase (OPRT) as OMPDC-OPRT and possesses a C-terminal PTS (PTS1), whereas *D. papillatum* OMPDC is a stand-alone enzyme lacking PTSs (Makiuchi et al. 2008). Since PTS2 is present in *D. papillatum* ALD, the ALD and OMPDC may be localized differently in diplonemids. To determine their cellular localizations, we performed western blot analysis of the *D. papillatum* subcellular fractions using specific antibodies to *D. papillatum* ALD and OMPDC (Fig. 1B).

We first confirmed the specificity of the antibody raised against *D. papillatum* ALD by western blotting (Fig. 1B, left). Immunoprecipitates of extracts of both *D. papillatum* and *T. cruzi* using anti-DpALD antibody showed bands at 41 kD, as expected, which were confirmed as aldolase by LC-MS/MS analysis (Supplementary Table S1)

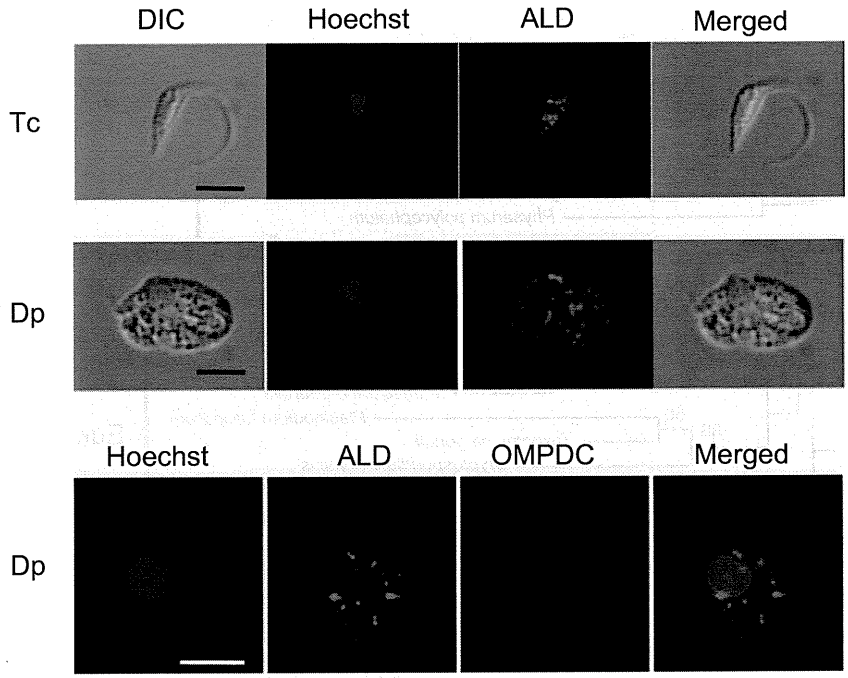

Figure 3. Indirect immunofluorescence analyses of *T. cruzi* and *D. papillatum*. *T. cruzi* (Tc, top) and *D. papillatum* (Dp, middle) were fixed, reacted with anti-*D. papillatum* ALD antibody, conjugated with Alexa Fluor 488, and counterstained with Hoechst 33342. Localization of ALD and OMPDC in *D. papillatum* cells was assessed by confocal microscopy (bottom, counterstained with Hoechst 33258). Neither pre-immune sera nor the antiserum absorbed with the corresponding antigens showed any fluorescent signal. DIC, differential interference contrast image; Merged, merged image. Scale bar = 5 μm.

D. papillatum ALD was detected predominantly in the membrane-rich fraction (105,000 ×*g* sediment, lane 3, middle) but not in the cytosolic fraction (105,000 ×*g* supernatant; lane 2, middle). In contrast, OMPDC was detected predominantly in the cytosolic fraction (105,000 ×*g* supernatant; lane 2, right). These results suggest that, in *D. papillatum,* ALD is membrane-associated and likely to be localized in peroxisome-related organelles, whereas OMPDC is a cytosolic protein.

Different Subcellular Distributions of ALD and OPMDC in *D. papillatum*

We further examined the localization of ALD and OMPDC by indirect immunofluorescence analysis. ALD was detected as "*dots*" in both *D. papillatum* and *T. cruzi* (Fig. 3). Confocal fluorescence microscopy showed that the fluorescence signals of mitochondrial DNA and ALD did not merge (Fig. 3, bottom), suggesting that ALD is not a mitochondrial protein and that ALD is compartmentalized into peroxisome-related organelles in both species. In contrast, *D. papillatum* OMPDC was detected throughout the cells and did not co-localize with ALD, again suggesting that OMPDC is a cytosolic protein. Taken together, these findings suggest that, in *D. papillatum,* ALD and OMPDC are separately localized in peroxisome-related compartments and the cytosol, respectively.

Discussion

This is the first report suggesting that, in diplonemids, a glycolytic enzyme is compartmentalized in peroxisome-related organelles. At present, the biochemical properties of glycolytic enzymes in diplonemids are largely unknown; only partial nucleotide sequences for the fifth enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), have been cloned but their cellular localization is unknown (Qian and Keeling 2001).

The presence of PTS suggested that peroxisomes are most likely to be the ALD-associated organelles. The presence of ALD in other organelles, such as the mitochondrion and endoplasmic reticulum, is unlikely. The mitochondrion

of *D. papillatum* is large and highly branched throughout the cell (Marande et al. 2005). We found, however, that the fluorescence signal of mitochondrial DNA did not co-localize with that of ALD. Moreover, the predicted signal sequences by MitoProt (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) indicated that it was unlikely that *D. papillatum* ALD had been imported into the mitochondrion (probability, 0.1330) or endoplasmic reticulum (probability, 0.000).

Glycosomes contain the first seven of the ten enzymes of glycolysis (Michels et al. 2006). In general, the accumulation or compartmentalization of enzymes involved in a metabolic pathway is advantageous for concerted metabolism. This may occur by the formation of enzyme complexes or the fusion of functionally related enzymes. This, however, does not seem true for glycosomes. The compartmentalization of glycolytic enzymes appears physiologically critical for trypanosomatids, especially because hexokinase and 6-phosphofructokinase, the allosteric enzymes that regulate glycolysis in canonical eukaryotes and catalyze the reactions upstream of ALD, are insensitive to their allosteric inhibitors (Michels et al. 2006). The occurrence of these enzymes in the cytosol may result in the accumulation of toxic glycolytic intermediates and the consumption of cytosolic ATP (Bakker et al. 2000). The compartmentalization of glycolytic enzymes may have resulted in the feedback regulation of hexokinase and 6-phosphofructokinase becoming redundant and finally lost, causing their compartmentalization to be critical.

Notably, OMPDC is localized differently in kinetoplastids and diplonemids. Glycosomes also contain enzymes involved in gluconeogenesis, nucleotide biosynthesis, and sugar-nucleotide biosynthesis (Michels et al. 2006). One sugar-nucleotide, UDP-GlcNAc, has been predicted to be an important metabolite in the synthesis of glycosylphosphatidylinositol (GPI)-anchored glycoproteins, which are essential components in trypanosomatids (Ferguson 1999; Stokes et al. 2008). It is important to know whether the enzymes of non-glycolysis pathways became compartmentalized in a common ancestor of diplonemids/kinetoplastids.

We previously showed that, after separation of the kinetoplastid and diplonemid lineages, a common ancestor of kinetoplastids acquired the OMPDC gene by lateral gene transfer, and that this gene subsequently fused with the OPRT gene (Makiuchi et al. 2008). Thus, glycolytic enzymes likely accumulated in the peroxisomes of a common ancestor of kinetoplastids and diplonemids. After the separation of the kinetoplastid lineage, the compartmentalization of other enzymes, including OMPDC (-OPRT fusion), into peroxisome-derived organelles may have occurred, resulting in the formation of glycosomes.

It is worth noting that glycosomes of the parasitic bodonids, *Trypanoplasma borreli* and *Cryptobia salmositica,* possess catalase activity, an authentic peroxisome marker enzyme (Ardelli et al. 2000; Opperdoes et al. 1988). In conjunction with the notion that trypanosomatids are monophyletic and nested within the bodonid clade, stepwise evolution of peroxisome-related organelles in each lineage may have occurred in association with the acquisition and/or loss of the related genes in a lineage-specific manner.

Our findings not only unite kinetoplastids and diplonemids at the level of peroxisome evolution but provide evidence of the diversification of these organelles in Euglenozoa. Further identification of the enzymes involved in the specialized peroxisomes of both lineages would clarify their physiological and biochemical importance.

Methods

Organisms: An axenic culture of *D. papillatum* (ATCC 50162) was routinely maintained as described (Makiuchi et al. 2008). *T. cruzi* Tulahuen strain was maintained in LIT medium (Annoura et al. 2005).

cDNA cloning of the *ALD* gene from *D. papillatum*: The full-length *D. papillatum* ALD cDNA was obtained by 5'- and 3'-RACEs using the partial sequence of a putative *ALD* gene (GenBank™; EC842358, TBestDB; DPL00001327) registered in the TBest database and the following primer sets: for 5'-RACE, 5'-CGTCGAACTTCTGGGTAAGG-3' (cDNA synthesis), 5'-GCTACAGTTTCTGTACTTTATTG-3' (sense PCR primer, spliced-leader specific), and 5'-ATGACACCGCTGATGTACTG-3' (antisense primer) (Sturm et al. 2001); for 3'-RACE, 5'-AATAAAGCGGCCGCGGATCCAATTTTTTTTTTTTTTTTVN-3' (cDNA synthesis), 5'-GCTGCGACAAGCGCTTCAAG-3' (amplification of the sense strand), 5'-AGACGCTACCGTGCCCTGAT-3' (sense PCR primer), and 5'-AATAAAGCGGCCGCGGATCCAA-3' (antisense primer). Finally, the full-length ALD cDNA was PCR amplified using the primers 5'-CACCATGTCTGCCCGTATCGAAGTCCTGA-3' (sense) and 5'-CTAGTAGGTGTTGCCCTTGATGTAC-3' (antisense) and *D. papillatum* cDNA as a template.

Phylogenetic analysis: All sequence data, with the exception of that of *D. papillatum* ALD cloned by us and first reported here, were collected from public sequence databases by taxonomic and BLAST searches. The sequences reported in this paper appear in the DDBJ/EMBL/GenBank databases under accession number AB550707 for *Diplonema papillatum* ALD mRNA. Multiple alignment of ALD sequences was obtained using CLUSTAL W (Thompson et al. 1994), with the alignment

corrected by manual inspection. Unambiguously aligned positions were selected and used for phylogenetic analyses, which were performed by the maximum likelihood (ML), maximum parsimony (MP), and distance matrix (DM) methods implemented in the PHYLIP3.69 package, and by the ML method in the PAML package (Felsenstein; Yang 1997). With 33 taxa, 310 unambiguously aligned amino acid sites were used for analysis, corresponding to residues 81-105, 107-157, 166-174, 179-225, 240-250, 255-260, 265-281, 290-308 and 317-361 of the *D. papillatum* sequence.

Expression of recombinant *D. papillatum* ALD and OMPDC: The full-length PCR products for the *ALD* and *OMPDC* genes of *D. papillatum* were subcloned in the pET151 expression vector (Invitrogen), which allows N-terminal fusion with His_6-tag, and sequenced. After transformation of *Escherichia coli* BL21-CodonPlus® (DE3)-RP cells (STRATAGENE®, Agilent Technologies, Inc., CA, USA) with the plasmids, expression of ALD and OMPDC was induced by incubation at 25 °C in the presence of IPTG. The recombinant proteins were affinity-purified with His•Bind® resin (Novagen, EMD Biosciences, Inc., Madison, WI, USA), equilibrated with phosphate-buffered saline (PBS, pH 7.2), cleaved with AcTEV™ Protease (Invitrogen) to remove the tag and, finally purified further on a His•Bind® column.

Antibodies: Female BALB/c mice were immunized with 100 μg recombinant ALD, and female Japanese white rabbits were immunized with 200 μg OMPDC, each in Freund's complete adjuvant, followed by booster injections with the same antigens in Freund's incomplete adjuvant. IgG was purified from each antiserum using HiTrap™ Protein G HP (GE Healthcare).

Subcellular fractionation, immunoprecipitation, and Western blotting: *D. papillatum* cells were washed once with artificial seawater and twice with 25 mM Tris-HCl/10 mM KCl/1 mM EDTA/250 mM sucrose (pH 7.4). The cells were homogenized in the latter buffer containing a protease inhibitor cocktail (Complete Mini, Roche Diagnostics K.K., Tokyo, Japan) using a cell disruption bomb (Parr Instrument Company, Moline, IL, USA) at 250 psi for 10 min. The homogenates were centrifuged twice at 1,000 ×*g* for 10 min and the supernatant was centrifuged at 105,000 × *g* for 1 hour. The resulting pellet and supernatant were defined as the membrane-rich and cytosolic fractions, respectively. For immunoprecipitation, the cells were harvested by centrifugation at 1,700 ×*g* for 10 min at 4 °C and washed once with PBS (pH 7.2) by centrifugation at 1,700 ×*g* for 10 min at 4 °C. Using an ultrasonicator, the cells were lysed in 10 mM Tris-HCl/150 mM NaCl/1% TritonX-100/0.5% NP-40 (pH 8.0) supplemented with the protease inhibitor cocktail. The cell lysates, each containing 140 μg protein, were incubated with the antibody and precipitated using Protein G Magnetic Beads (New England Biolabs, Beverly, MA). The resulting precipitates were separated by SDS-PAGE and transferred to PVDF membranes, which were incubated with the corresponding antibody. Antibody reactions were visualized using alkaline phosphatase-conjugated secondary antibody and a chemiluminescent substrate (CSPD, Roche Diagnostics).

Immunofluorescence microscopy: *D. papillatum* cells were allowed to adhere onto poly-L-lysine coated slides for 15 min in a humidity chamber at 4 °C. After removal of unbound cells, the bound cells were fixed for 25 min with 4% paraformaldehyde and permeabilized in PBS containing 0.5% Triton-X 100 for 10 min at 4 °C. The slides were incubated in blocking solution (PBS containing 0.05% Tween 20 and 1% BSA) for 30 min, and subsequently with the specific antibody in blocking solution for 90 min at room temperature. After washing with PBS, the slides were incubated with secondary antibodies and counterstained for 10 min with Hoechst 33342 for immunofluorescence microscopy or Hoechst 33258 for confocal laser microscopy. The localization of each enzyme was assessed by fluorescence microscopy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2010.11.003.

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Sex Hormones Alter Th1 Responses and Enhance Granuloma Formation in the Lung

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Key Words
Granuloma · Sex hormones · Th1/Th2 balance · Bronchoalveolar lavage fluid

Abstract
Background: The lung is one of the sites of granulomatous responses, which are characterized by the recruitment and organization of activated macrophages and lymphocytes. There have been several reports that have shown that some pulmonary granulomatous diseases, such as sarcoidosis and nontuberculous mycobacterial disease, are likely to be characterized by a preponderance in postmenopausal females. Although sex hormones have been shown to play an important role in the regulation of the immune system, the influence of sex hormones on pulmonary granuloma formation is still unclear. ***Objectives:*** The purpose of this study was to assess whether sex hormones are involved in granulomatous inflammation and to evaluate how sex hormones modulate this response in the lung. ***Methods:*** Ovariectomized rats were used as an experimental postmenopausal model in which chronic pulmonary granulomatous inflammation was induced by intravenous injection of complete Freund's adjuvant. ***Results:*** Histological analysis of lung tissues demonstrated enhancement of granuloma formation in the ovariectomized group. Such enhanced granuloma formation was significantly associated with generalized Th1-biased cytokine production in the bronchoalveolar lavage fluid. ***Conclusion:*** These results indicate that sex hormones play an important role in pulmonary granuloma formation by altering the Th1 responses.

Introduction

Granuloma formation, which is characterized by the recruitment and organization of activated macrophages and lymphocytes in discrete lesions, is a consequence of chronic antigenic stimulation and is one of the important defense mechanisms against infectious microbes such as fungi and mycobacteria [1]. Nontuberculous mycobacte-

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rial disease is one of the infectious granulomatous diseases in the lung [2–5]. It is characterized by the formation of various degrees of granulomas. It is important to note that patients with nontuberculous mycobacterial disease are predominately female and postmenopausal [4, 6, 7].

The lung is also a site of noninfectious granulomatous responses, such as sarcoidosis, which affects the pulmonary and lymphatic systems [8]. This disease shows a consistent predilection for adults less than 40 years of age, peaking in subjects of 20–29 years. Interestingly, for males, there is no second peak of disease incidence, but there is a second peak of disease incidence in females older than 50 years of age in Scandinavian countries and Japan [9–12]. This suggests that age-related changes in females, such as the hormonal dysregulation associated with menopause, may contribute to sarcoidosis. Although significant heterogeneity in disease presentation and disease severity occurs among different ethnic and racial groups [13–16], both of these pulmonary granulomatous diseases, sarcoidosis and nontuberculous mycobacterial disease, are likely to be characterized by a preponderance in postmenopausal females. Therefore, it is likely that fluctuations of sex hormones may be associated with enhanced granuloma formation in the lung.

Analyses of the experimental animal models of granulomatous diseases have suggested that humoral mediators, such as IL-4, IL-12, IFN-γ, TNF-α and CXC3 ligand, play crucial roles [17–19]. In addition, there is ample evidence that sex hormones can act as immunomodulators [20–22]. Indeed, a variety of immune cells, including T cells, B cells, monocytes and macrophages, have been shown to possess functional sex hormone receptors [23–26]. However, the role of sex hormones in pulmonary granuloma formation is still unclear. Moreover, there are no studies showing the effect of sex hormones on pulmonary granuloma formation by histological findings.

In the present study, we established an experimental postmenopausal model in which female rats were ovariectomized (OV) and treated by intravenous injection of complete Freund's adjuvant (CFA) to induce chronic pulmonary granulomatous inflammation. To our knowledge, the present study is the first report to reveal that granuloma formation in rats treated by ovariectomy was significantly greater than that in sham-operated rats on the basis of histological findings. We also show that sudden withdrawal of sex hormones enhances pulmonary granuloma formation and increases the local Th1 cytokine responses.

Materials and Methods

Animals

Female, specific pathogen-free DA rats weighing 100–135 g (8 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan). These rats were maintained in a limited access barrier and housed in a humidity- (55 ± 10%) and temperature- (24 ± 2°C) controlled room under a 12-hour light/dark cycle. The study protocol was reviewed and approved by the Juntendo University School of Medicine Committee on Animal Care and complies with National Institutes of Health guidelines for animal care.

The Rat Treatment Regimen

To initiate the studies, 62.5 μl/100 g body weight CFA (Sigma, St. Louis, Mo., USA) was injected into the dorsal tail vein under anesthetization by isoflurane (Abbott, Abbott Park, Ill., USA). Seven days later, one group of rats (n = 12) was OV. An aseptic surgical procedure was employed for all animals. The rats were anesthetized using isoflurane, then the dorsal part of the lumbar region was shaved, and the site was cleaned with 75% ethanol. A 2-cm incision was made in the skin through the musculature and peritoneum and the ovaries were retracted and removed along with the oviduct. The wound was then closed using a 4-0 sterile suture. The other group of rats (n = 12) underwent sham operation (SHAM). The SHAM group underwent the same surgical procedure except that the ovaries were exposed but not removed. At 36 days after injection, the rats were euthanized with an overdose of pentobarbital sodium. Then, bronchoalveolar lavage was performed as described in the next paragraph, and the lungs were prepared for histological evaluation.

Immunoassays of Bronchoalveolar Lavage Fluid and Serum

Rats were euthanized with an overdose of pentobarbital sodium, followed by the collection of blood and bronchoalveolar lavage fluid (BALF). After opening the chest, an incision was made in the neck, and the trachea was cannulated with a plastic catheter secured in place with silk suture. Lavage was performed with calcium- and magnesium-free PBS at a volume of 6 ml. The procedure was performed a total of 3 times. Total cell counts and differential cell counts were performed by Diff-Quick staining. Cytokine levels were measured using ELISA. Prior to the ELISA, the BALF samples were concentrated 12.5 times by Amicon Ultra (Millipore, Billerica, Mass., USA). Rat IFN-γ, IL-12 and IL-13 ELISA kits were purchased from Biosource (Invitrogen Corporation, Calif., USA).

Quantification of Pulmonary Granuloma Formation

After lavage, the lungs were fixed by intratracheal infusion of 10% formalin buffered with PBS at a constant hydrostatic infusion pressure (20 cm H_2O). After 1 h, the lungs were removed from the thoracic cavity and immersed in the fixative for an additional 24 h. The lungs were embedded in paraffin and sliced into 4 transverse sections (apical, upper, middle and lower). Histological sections were prepared at a thickness of 2.5 μm and stained with hematoxylin and eosin. For measurement of the granulomatous areas in the lung parenchyma, a KS 400 image analysis system (Zeiss, Germany) was used. For each section, the whole lung area with 6–27 microscopic fields was examined at a magnification of ×20. The total area of granuloma and lung parenchyma in each rat was calculated by summing all of these measured fields in each section.

Statistical Analysis

Before cytokine analyses were preformed, the Smirnov-Grubbs test was used to eliminate outliers. Statistical analysis was performed with an unpaired t test using SPSS statistical package version 15.0F (SPSS Inc., Chicago, Ill., USA).

Results

Decreased Serum Levels of Sex Hormones in OV Rats

In the present study, we used OV rats as a postmenopausal model. To confirm whether ovariectomy mimics the postmenopausal state, which is characterized by decreased levels of sex hormones and increased body weight, we measured and compared the serum levels of estrogen and body weight between OV and SHAM rats 29 days after operation. Serum levels of estrogen were significantly lower in OV than in SHAM rats (fig. 1). Similarly, the body weight of OV rats was significantly higher than that of SHAM rats (data not shown). We conclude that the OV rat is a useful model for investigating immunological reactions in the postmenopausal state.

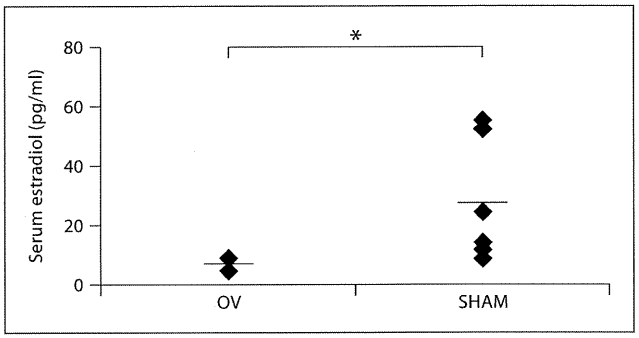

Fig. 1. Effect of ovariectomy on serum levels of estradiol. Serum levels of estradiol were measured by ELISA 29 days after operation. The bars indicate the means (n = 3 for OV, n = 6 for SHAM). * $p < 0.05$.

Enhancement of Pulmonary Granulomatous Inflammation by Sex Hormone Withdrawal

CFA is a well-known adjuvant and itself can induce granuloma formation in the lung when emulsified CFA is administered intravenously [27, 28]. Intravenous injection of CFA induced granulomas in the lungs of both OV and SHAM rats (fig. 2a–d). The granulomas were composed of a central core of macrophages, epithelioid cells and giant cells surrounded by lymphocytes, consistent with the findings in sarcoidosis (fig. 2e, f).

There were no differences in the area of total lung parenchyma between the OV and SHAM groups (fig. 3a). In contrast, the size of the granulomas was significantly larger in the OV group than in the SHAM group (fig. 3b). Likewise, the relative size of the granuloma areas in the whole lung parenchyma in the OV group was significantly larger than that in the SHAM group (fig. 3c). These findings suggest that sex hormone withdrawal favors a Th1-polarized granulomatous response in OV rats.

Alteration of Immune Cell Populations in the Lung by Ovariectomy

To investigate the populations of infiltrated immune cells in the lungs, BALF was collected and the cell populations were compared between the OV and SHAM groups. The total number of immune cells recovered in BALF was comparable between the groups (data not shown). The cells recovered from the BALF of the control rats that received saline were mostly alveolar macrophages (97–99%; data not shown). In contrast, the BALF of the OV and SHAM rats, both of which received CFA, showed increased numbers of lymphocytes (fig. 4). The relative population of macrophages in the OV group was significantly lower than that in the SHAM group. Conversely, the relative population of lymphocytes was significantly higher in the OV rats than that in the SHAM rats. There was no difference in the relative population of neutrophils between the OV and SHAM groups.

Th1 Shift of the Cytokine Profiles in the Serum and BALF by Ovariectomy

To investigate the mechanisms responsible for modulation of the granulomatous responses and immune cell populations in the lungs of OV rats, we compared the cytokine levels in the serum and BALF between the OV and SHAM groups. The Th1-type cytokines IFN-γ and IL-12 and the Th2-type cytokine IL-13 were assayed. There was no difference in the serum levels of these cytokines between the OV and SHAM groups, suggesting that ovariectomy does not alter the systemic cytokine balance (fig. 5).

On the other hand, the levels of IFN-γ and IL-12 in the BALF in the OV group were significantly higher than those in the SHAM group (fig. 6a, b), suggesting the occurrence of a local Th1 shift in the lungs of OV rats. The levels of IL-13 in the BALF of OV rats were not significantly different from those of the SHAM rats (fig. 6c). These findings suggest that a Th1-polarized environment in the lung may be contributing to the altered granulomatous response in OV rats.

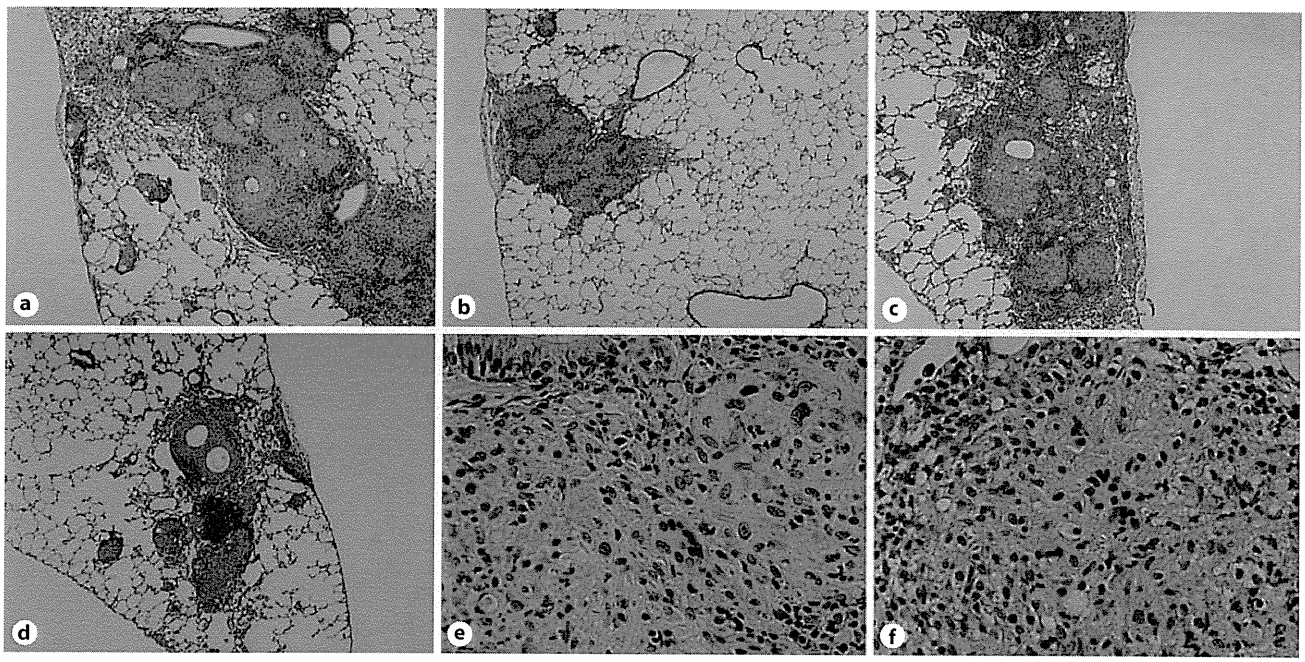

Fig. 2. Histological findings of pulmonary granulomas in lungs of rats. **a–d** Representative photomicrographs of pulmonary granulomas in the lungs of OV rats (**a**, **c**) and SHAM rats (**b**, **d**). Hematoxylin and eosin. Original magnification: ×40. **e**, **f** Higher magnification reveals that granulomas in lungs of OV rats (**e**) and SHAM rats (**f**) contain epithelioid cells and numerous lymphocytes. Original magnification: ×400.

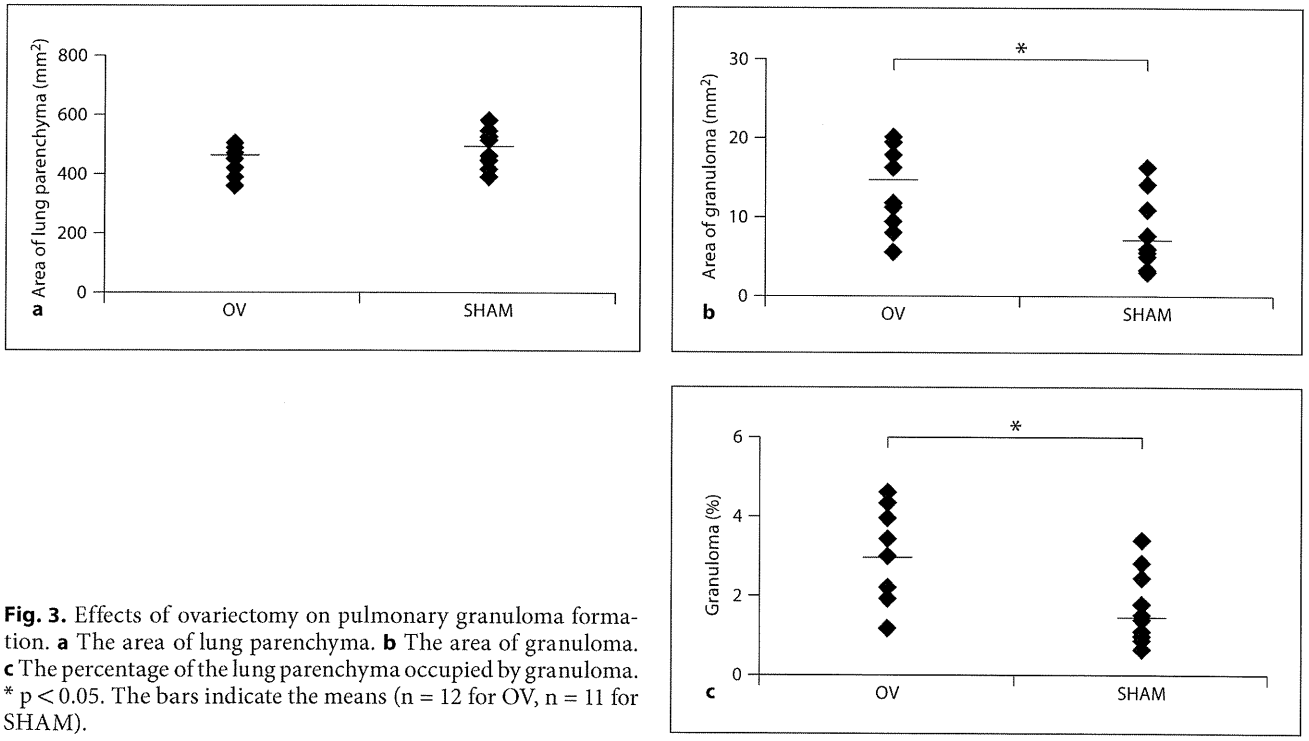

Fig. 3. Effects of ovariectomy on pulmonary granuloma formation. **a** The area of lung parenchyma. **b** The area of granuloma. **c** The percentage of the lung parenchyma occupied by granuloma. * $p < 0.05$. The bars indicate the means (n = 12 for OV, n = 11 for SHAM).

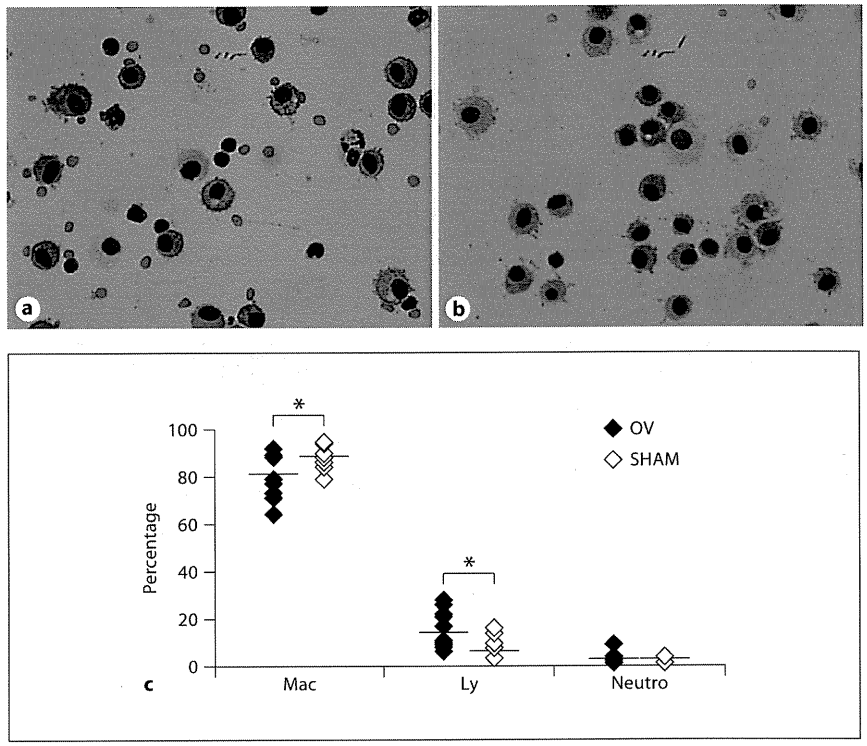

Fig. 4. Effect of ovariectomy on cell populations of BALF. BALF was collected from the lungs of the OV and SHAM rats. Cell populations were identified on air-dried cytocentrifuged smears (800 rpm for 5 min) before staining with Diff-Quick stain. **a**, **b** Representative photomicrographs of air-dried cytocentrifuged smears from BALF of OV rats (**a**) and SHAM rats (**b**). Diff-Quick stain. Original magnification: ×400. **c** Differential cell counts were performed by counting at least 300 cells to identify macrophages (Mac), lymphocytes (Ly) and neutrophils (Neutro). The bars indicate the means. * $p < 0.05$.

Discussion

In the present study, we established an experimental postmenopausal model using OV rats, which can mimic the postmenopausal state. In fact, OV rats present decreased levels of serum estrogen and increased body weight similar to those in postmenopausal women. Using this animal model, we examined the pathogenesis of pulmonary granulomatous inflammation, which is observed in postmenopausal women. We demonstrated that the size of the granulomatous areas in the lungs of OV rats was significantly greater than that in SHAM rats. These results indicate that enhancement of pulmonary granulomatous inflammation is associated with decreased levels of sex hormones.

Menopause, which is accompanied by a decline in the production of sex hormones, affects granuloma formation [3, 4, 6, 9, 29, 30]. However, the histopathological status of the granulomatous lesions found in those studies is unclear. Our histopathological examination revealed enhancement of pulmonary granulomatous lesions induced by CFA in OV rats. The lesions evoked in this experimental model are pathologically similar to those observed in sarcoidosis, suggesting that CFA injection followed by OV in rats may be a good experimental model for sarcoidosis. We would anticipate that ovarian dysfunction in humans is likely to accelerate the granulomatous response in the lung.

We demonstrated that pulmonary infiltration with lymphocytes was significantly increased in the BALF of the OV rats, consistent with observations in OV rats treated with heat-killed bacillus Calmette-Guérin [29]. It is worth noting that the lymphocyte population varies between blood and BALF when examined in healthy rats; in the lung, lymphocytes marginate to the vascular endothelium (marginal pool) and enter the interstitial tissue (interstitial pool), where they can be recovered in BALF [31]. Therefore, it is likely that a decrease in sex hormones provokes local inflammatory responses, resulting in the recruitment of lymphocytes.

The levels of Th1-type cytokines in the BALF of OV rats were significantly higher than those in SHAM rats, while the levels of Th2-type cytokines in both BALF and serum were comparable between OV and SHAM rats. These findings suggest that a decline in the levels of sex hormones does not alter the systemic immune status but

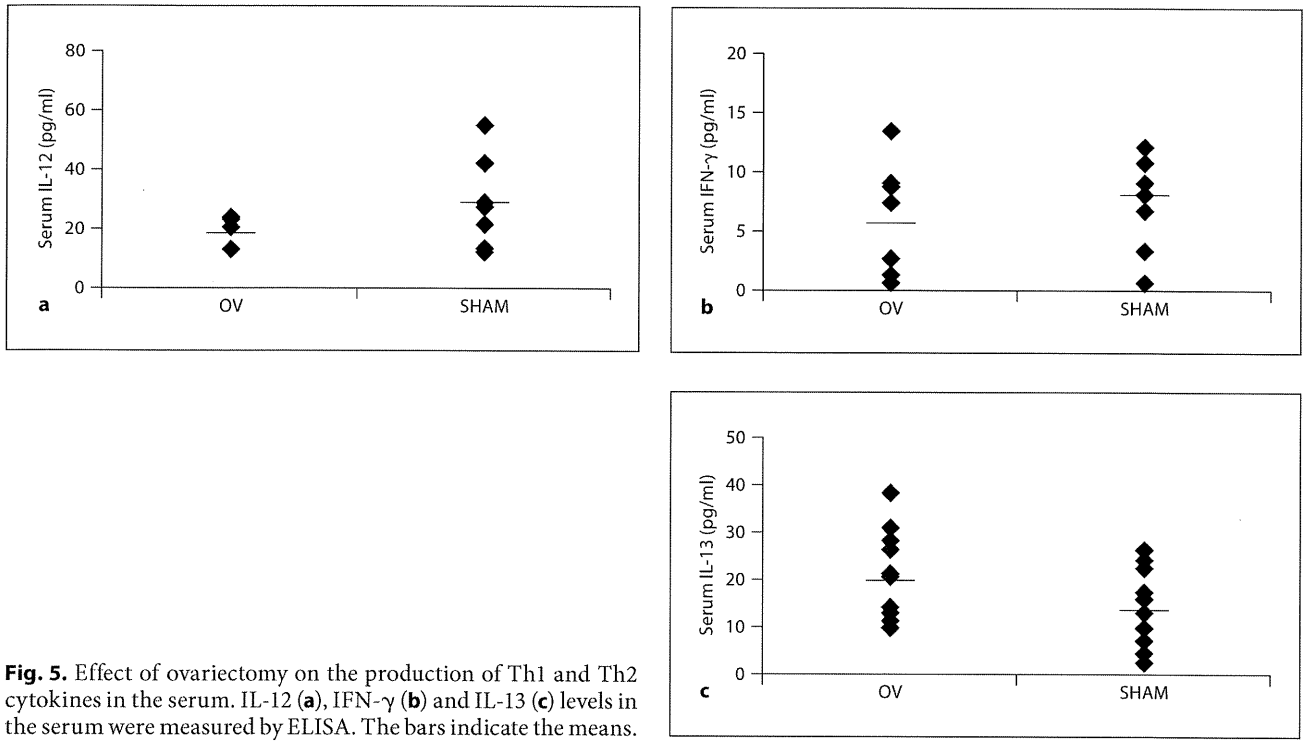

Fig. 5. Effect of ovariectomy on the production of Th1 and Th2 cytokines in the serum. IL-12 (**a**), IFN-γ (**b**) and IL-13 (**c**) levels in the serum were measured by ELISA. The bars indicate the means.

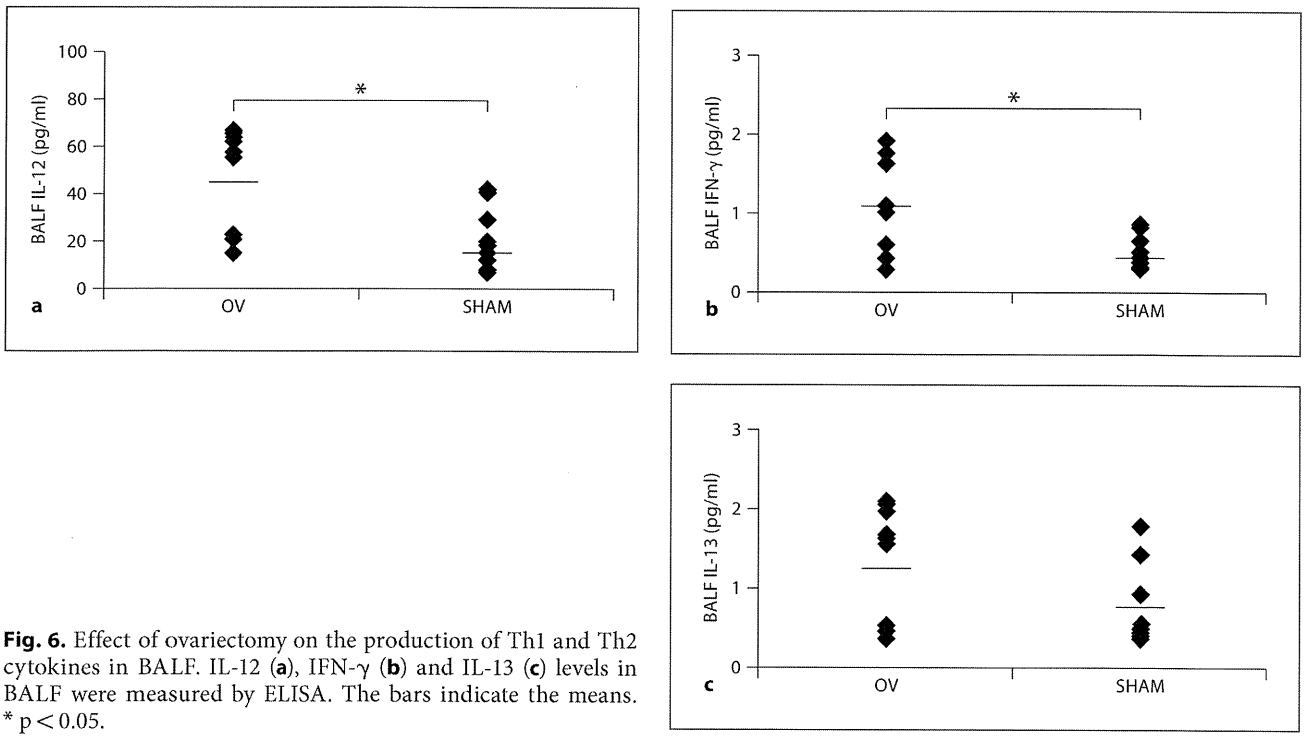

Fig. 6. Effect of ovariectomy on the production of Th1 and Th2 cytokines in BALF. IL-12 (**a**), IFN-γ (**b**) and IL-13 (**c**) levels in BALF were measured by ELISA. The bars indicate the means. * $p < 0.05$.

does affect the local immune responses at the site of inflammation in a Th1-polarized manner. In agreement with this, Th1-polarized local immune responses accompanied by infiltration of lymphocytes have been highlighted not only in sarcoidosis but also in a number of other granulomatous diseases [32, 33].

There is ample evidence suggesting that the sex hormones modulate the Th1/Th2 balance [34–38]. Kamada et al. [39] demonstrated that the levels of Th1 cytokines are augmented in women after menopause, and hormone replacement therapy prevents this augmentation, thereby improving the aberration of the Th1/Th2 balance. Recently, sex hormones were shown to modulate expression of the transcription factors T-bet and GATA3, which are likely associated with Th1 and Th2 responses, respectively [40, 41]. Although further analyses are necessary to identify the molecular clues responsible for the cytokine shift provoked by modulation of sex hormones, our experimental animal model provides potential for understanding the roles of sex hormones in the pathogenesis of menopause-associated granulomatous diseases, such as sarcoidosis.

Financial Disclosure and Conflicts of Interest

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Short Communication

Prevalence of *Schistosoma haematobium* Infection among Schoolchildren in Remote Areas Devoid of Sanitation in Northwestern Swaziland, Southern Africa

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SUMMARY: A parasitological survey of *Schistosoma haematobium* infection among primary schoolchildren in the remote areas of Hhohho and Manzini Provinces in northwestern Swaziland was undertaken. Presence of infection in subjects was confirmed on detection of *S. haematobium* ova in urine or the presence of hematuria. The intensity of the infection was estimated by calculating the total number of *S. haematobium* ova present in a 10-ml urine specimen and was expressed in terms of geometric mean intensity (GMI). The prevalence of *S. haematobium* infection in these populations was 5.3% (21/395) with a GMI of 46.5. Boys had higher prevalence (7.1%, 13/182) and GMI (50.4) than girls (3.8%, 8/213; 40.0) did ($P > 0.05$). Geographically, the prevalence in Manzini schoolchildren (14.6%, 12/82) was significantly higher than that in Hhohho schoolchildren (2.9%, 9/313; $P < 0.001$); however, Hhohho schoolchildren had a higher GMI (70.2) than that observed in Manzini schoolchildren (21.9). Children from schools located in Lowveld had a significantly higher prevalence (11.4%, 19/166) than that in children from schools located in Highveld (0.6%, 1/162) ($P < 0.0001$).

Schistosomiasis transmitted by snails is a major health problem in tropical and subtropical countries, and the World Health Organization (WHO) estimates that there are more than 200 million cases of this disease, 93% of which occur in sub-Saharan Africa (SSA) (1). There are 2 major forms of schistosomiasis found in SSA. Approximately two-thirds of the schistosomiasis cases are a result of *Schistosoma haematobium* infection. Possible consequences of *S. haematobium* infection include hematuria, dysuria, nutritional deficiencies, lesions of the bladder, kidney failure, and an elevated risk of bladder cancer (1).

Primary schoolchildren are particularly vulnerable to schistosomiasis because of their habit of playing in water, where they may contract the infection. As such, they are the ideal target group to investigate the prevalence of schistosomiasis, and the data collected from this age group can be used to assess not only whether schistosomiasis threatens the health of schoolchildren, but can also be used as a reference for evaluating the need for community intervention (2-4).

S. haematobium has been endemic in the Kingdom of Swaziland (KS) for several decades, particularly in areas situated in the eastern Lowveld districts of the country (5). In previous surveys of *S. haematobium* infection among schoolchildren, Logan showed that the average infection rate was 61.2% between 1958 and 1964 (5). In KS, there are 3 main sources of information on schistosomiasis: records obtained from patients' self-referral to the National Bilharzia Control Programme (NBCP), the school reporting system, and the routine health information system. Data from only the NBCP and primary schools in endemic areas are systematically collected and reported on a regular basis. However, the magnitude and impact of *S. haematobium* infection have not been well documented to date. Recently, we carried out a parasitological survey of *S. haematobium* infection among the inhabitants of remote districts devoid of sanitation in Lowveld Swaziland, and the overall prevalence of *S. haematobium* infection was 6.1% (18/295), with a prevalence of 6.4% (5/78) found in schoolchildren between the ages of 6 and 12 years (6). Nevertheless, the prevalence of *S. haematobium* infection among inhabitants in some of the remote districts located of northwestern KS, which are not covered by NBCP, remains unclear.

The present study aimed to investigate the prevalence

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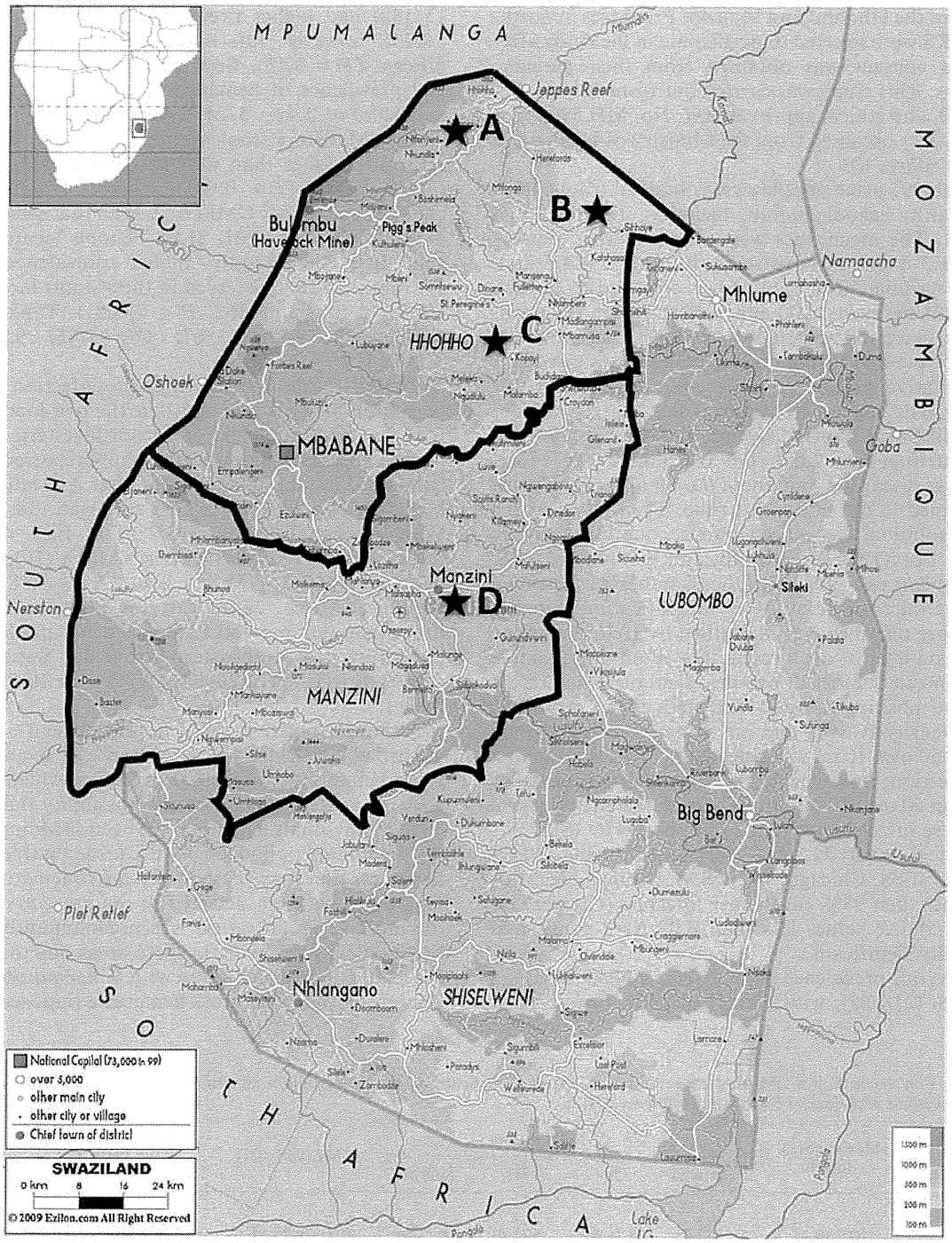

Fig. 1. Geographical locations of primary schools studied in the Kingdom of Swaziland, Southern Africa. Reprinted with permission from Ezilon Company.

of *S. haematobium* infection among primary schoolchildren in remote districts in northwestern KS, which are not covered by NBCP, to help the KS in evaluating whether a deworming program should be introduced in those districts.

Geographically, the KS is a highland country that can be divided from west to east into the following 3 regions on the basis of altitude: Highveld (~1,200 m), Middleveld (~600 m), and Lowveld (~250 m). According to recommendations of the NBCP, 4 primary schools situated in remote districts in northwestern KS were selected in the present study, including 3 schools located in the High- (school A), Middle- (school B), and Lowveld areas (school C), of the Hhohho Province as well as 1 school (school D) located in the Lowveld area of the Manzini Province (Fig. 1). These schools were selected on the basis that the areas in which they were located had poor sanitation, an unsafe water supply, and the absence of a deworming progam. Primary schoolchildren (mean age ± standard deviation: 11.0 ± 0.5 years)

residing in the Hhohho and Manzini Provinces in northwestern KS were selected to participate in the study after informed consent was obtained from their parents/guardians. This study was carried out from July to August 2010, and ethical approval (Ref. No. MH 599B) for the study was obtained from the Ministry of Health and Social Welfare, KS.

In total, 395 urine samples were obtained from apparently healthy schoolchildren, including 182 boys and 213 girls. The mean ages were similar across genders (boys: 11.3 ± 0.4 years versus girls: 10.7 ± 0.4 years). A single terminal urine sample was collected from each participant between 1,000 and 1,400 h, when the maximum ova excretion reportedly occurs (7). Ten milliliters of each of the well-mixed urine samples was poured into a quantitative centrifuge tube used specifically for counting cells or parasites in urine (cat. no. ParaQ 6b; High Skill Co., Chupei, Taiwan), and the samples were then centrifuged at 2,000 rpm for 3 min. The supernatant was discarded, but about 0.6 ml of residual urine was retained in the bottom of tube. Then, 50 μl of the urinary solution was placed into a counting chamber (cat. no. ParaQ 5b; High Skill), and the number of *S. haematobium* ova present in the chamber was counted under the microscope at 100× magnification. Finally, the number obtained was multiplied by 12 to determine the total number of ova present in a 10-ml urine sample (6). The mean number of ova per 10 ml of urine present in all the positive samples was defined as geometric mean intensity (GMI), and any sample that contained less than 50 ova/10 ml was considered an indication of a mild infection; however, if the figure was equal to or more than 50 ova/10 ml, it was considered an indication of a severe infection as defined by the WHO (8).

Statistical analysis was performed using SPSS software (SPSS, Chicago, Ill., USA). Crude odds ratios (ORs) with their 95% confidence intervals (CI) were estimated, and P values less than 0.05 were considered statistically significant.

The overall prevalence of *S. haematobium* infection was 5.3% (21/395), with a GMI of 46.5. Boys (7.1%, 13/182) had higher prevalence than girls did (3.8%, 8/213); however, this trend was not statistically significant ($P = 0.13$). Boys also had higher GMI (50.4) and prevalence of hematuria (4.4%, 8/182) than girls deis (40.0 or 2.4%, 5/213). Geographically, the prevalence in Manzini schoolchildren (14.6%, 12/82) was significantly higher than that in Hhohho schoolchildren (2.9%, 9/313) ($P < 0.001$), whereas Hhohho schoolchildren had higher GMI (70.2) than that seen in Manzini schoolchildren GMI, 21.9). Schools located in Lowveld had significantly higher prevalence (11.4%, 19/166) than that observed in the schools located in the Highveld (0.6%, 1/162: $P < 0.0001$) (Table 1) or the Middleveld (1.5%, 1/67; OR = 8.5; 95% CI = 1.1-65.1; $P = 0.01$).

Present results indicate that the infection rate of *S. haematobium* was low and that the ova load was light for primary schoolchildren in northwestern KS. These numbers were much lower than those reported in studies conducted in schoolchildren in other African countries, including Nigeria (15.1%) (9) and Mali (38.3%) (7) in West Africa; Tanzania (13.2%) in East Africa (3); Sudan (80.6%) in Northeastern Africa (10), and Zimbabwean (60.0%) (11) and South Africa (68%) (12) in Southern Africa. Moreover, the prevalence was also lower than that previously reported in schoolchildren in the northern or southeastern Lowveld of Swaziland conducted in 1983 (5) or 2009 (6) (19.5% or 10.2%, respectively).

Although positive cases in the present study were identified confirmed by the presence of ova in urine or gross hematuria, the actual overall prevalence may be underestimated because of the possibility that some infected individuals may not have been shedding eggs during the limited period when their urine samples were obtained, or they may present with microhematuria, which may not have been detected in the present study. Furthermore, early infection was predominantly ovserved in schoolchildren living in these areas due to the higher percentile of schoolchildren with *S. haematobium* infection presenting with gross hematuria opposed to ova in

Table 1. Prevalence with a GMI and crude ORs with 95% CI for *Schistosoma haematobium* infection among primary schoolchildren in Northwestern Kingdom of Swaziland, southern Africa

Variable	Mean age (y) (S.D)	No. (%) of subjects				GMI (95% CI)	ORs (95% CI)	P value
		Examined	Ova	GH	Subtotal			
Gender								
Girls	10.7 (0.4)	213	3 (1.4)	5 (2.4)	8 (3.8)	40.0 (0-77.8)	referent[1)]	
Boys	11.3 (0.4)	182	5 (2.8)	8 (4.4)	13 (7.1)	50.4 (0-73.6)	2.0 (0.8-4.9)	0.13
Province								
Hhohho	11.0 (0.6)	313	3 (1.0)	6 (1.9)	9 (2.9)	70.2 (26.5-113.8)	referent[2)]	
Manzini	10.9 (2.4)	82	5 (6.1)	7 (8.5)	12 (14.6)	21.9 (1.3-42.4)	5.8 (2.4-14.3)	<0.001
Altitude								
Highveld	11.3 (0.6)	162	0 (0.0)	1 (0.6)	1 (0.6)	0 (ND)	referent[3)]	
Middleveld	10.9 (2.8)	67	1 (1.5)	0 (0.0)	1 (1.5)	48 (ND)	2.4 (0.2-40.0)	0.52
Lowveld	10.9 (0.1)	166	7 (4.2)	12 (7.2)	19 (11.4)	32.2 (3.0-61.4)	20.8 (2.8-157.4)	<0.0001
All subjects	11.0 (0.5)	395	8 (2.0)	13 (3.3)	21 (5.3)	46.5 (8.5-59.2)	ND	ND

[1)]: Compared with the prevalence among the girls.
[2)]: Compared with the prevalence in Hhohho Province.
[3)]: Compared with the prevalence in Highveld.
GH, gross hematuria; ND, not determined.

urine. It has been indicated that in endemic areas, hematuria is a common early sign of *S. haematobium* infection in non-treated populations exposed to *S. haematobium*; microhaematuria has been found in 41–100% of the infected children, whereas gross hematuria was observed in 0–97% (1).

Interestingly, boys had higher infection rate than girls did as detected by both a greater number of ova excreted in urine and gross hematuria. This finding may be explained by an increased predisposition in boys to behaviors that expose them to the risk of infection, e.g. swimming or bathing (1). In addition, boys were found to have heavier infection loads (GMI 50.4). As a result, investigation into whether boys in these regions are more susceptible to impaired mental performance than girls are is urgently needed since substantial amounts of evidence have indicated that severe *S. haematobium* infection may lead to impaired growth and cognitive performance (13).

Unfortunately, we did not examine clinical syndromes of the genital-urinary system among the girls infected with *S. haematobium*. A possible association between schistosomiasis and HIV/AIDS is currently receiving increased attention, and substantial evidence has indicated that female genital schistosomiasis (FGS) caused by *S. haematobium* infection could be an important risk factor for the bidirectional transmission of HIV, on the basis of the unique clinical and immunological features that characterize the egg granuloma: frequent chronic lesions in the vulva, vagina, and cervix of afflicted women (14). Furthermore, in a study undertaken in a rural Zimbabwean community, Kjetland et al. found that women with FGS had an almost 3-fold increased risk of having HIV infection (15). Since like many SSA countries, KS is severely affected by the HIV and AIDS pandemic (16), thorough systematic epidemiological studies are urgently required to detect and treat early FGS among Swazi girls with *S. haematobium* infection, with the hope that it might help to decrease the transmission of HIV due to FGS in KS.

We also found that a geographical difference in *S. haematobium* infection in schoolchildren exists between Lowveld and Highveld regions in northwestern KS. This discrepancy may be explained by the timing of the study: urine samples were collected during early winter in northwestern KS, which may have led to a decrease in the shedding efficiency of cercaria from the snail host or possibly the temperature was too low for the parasite to fully develop into snails in Highveld districts. Pitchford has already indicated that although *Bulinus globosus*, the snail host for *S. haematobium*, can be found in slow-moving or still water in Middleveld and Lowveld regions of the KS, this snail host was barely detectable in Highveld, and in addition, *S. haematobium* was not transmitted in the Highveld, particularly in winter when the average temperature (~8.2°C) markedly reduced the development rate of the sporocysts in the snail. On the other hand, the Kamati and Mzimphdu Rivers, which flow through Middleveld and Lowveld, have temperatures that are much more suitable for parasite development (20.4°C) (17). Thus, the geographical discrepancies may be explained by the possibility that children in Lowveld Hhohho and Manzini are particularly highly susceptible to *S. haematobium* infection through contact with water contaminated by cercaria, thus leading to increased prevalence of newly or repeatedly acquired *S. haematobium* infection.

Individuals with urinary schistosomiasis may develop severe pathological defects, including carcinoma of the bladder, periportal thickening, portal enlargement, glomerulonephritis, pulmonary hypertension (18), and transmission of HIV (15). We found that most of the schoolchildren had only a mild infection of *S. haematobium* with the GMI being less than 50; however, if left untreated, such mild infections can lead to severe consequences. However, despite the fact that the early treatment of these infected schoolchildren may prevent advanced pathological consequences, regrettably, the medical resources in Hhohho Province are quite limited. The nearest large hospital is located in the city of Piggs Peak, a 2-h drive from a remote village in Hhohho Province. Add to this the poor economic status of these families and it becomes very difficult for the families of this village to get the required medical assistance. However, in the Manzini Province, the conditions seem better than that in Hhohho Province. There are some large public hospitals and private clinics in Manzini City, and it is easier for people living in the Manzin Province to avail of these facilities, which at a driving distance of 0.5–1 h. Nevertheless, undertaking a routine deworming program for schoolchildren living in remote districts is urgently needed.

In summary, the present report provides useful information for planning an integrated schistosomiasis control program in neglected remote districts that are not covered by NBCP in northwestern KS. It is also recommended that even in districts with low transmission in northwestern KS, each person should be administered praziquantel, irrespective of age. Furthermore, water control and sanitation as well the elimination of the snail hosts by using molluscicides are urgently needed in Middleveld and Lowveld regions in northwestern KS.

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Conflict of interest None to declare.

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Isolated Intradural-Extramedullary Spinal Cysticercosis: A Case Report

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Spinal cysticercosis is an uncommon manifestation of neurocysticercosis (NCC). We present a case of isolated lumbar intradural-extramedullary NCC. The patient was treated successfully with the surgical removal of the cyst. Spinal NCC should be considered in the differential diagnosis in high-risk populations with new symptoms suggestive of a spinal mass lesion.

Case Report

A 59-year-old Asian American female presented in January 2009 with a 1-month history of progressive bilateral leg pain, numbness, and weakness. The patient also developed urinary retention 2 days prior to presentation. The patient had immigrated from Laos to the United States in 1987 and used to return periodically to Laos, every 1 to 2 years. She had traveled to Pakse, Laos, and then crossed the border to Ubon Ratchathani, Thailand, in late 2008. Altogether she was in Laos, September to December 2008, she spent her time there in villages and cities, visiting family and friends. She used bottled water for drinking but ate the traditional fare, which included rare/uncooked beef and pork purchased at local outdoor markets. In the United States, she also sometimes ate uncooked beef and pork. She has a history of adult-onset diabetes mellitus, controlled with oral medication, and is otherwise healthy. Before her recent trip, she had back pain progressing over several months, with some increased weakness and decreased sensation in the lower extremities. The symptoms became suddenly worse, however, the day after returning from her trip to Laos and progressed over the month before her admission to our hospital. Neurological examination revealed normal higher mental functions, optic fundi, cranial nerves, and deep tendon reflexes. She had mild weakness of both legs and the motor power was 4/5 in both hip and knee flexions. There was hypoesthesia in the left lower extremity in L1 to S3 distribution. The sensation of the right lower extremity was intact. The upper extremity examination was normal. She underwent a magnetic resonance imaging (MRI) examination of the lumbar spine, which disclosed the presence of two separate teardrop-shaped cystic structures beginning at level L1 and extending down to L4 with the displacement of the nerve roots peripherally. Post-contrast images showed there was peripherally an avid ring of enhancement along the cysts. There was also an irregular rim with effacement of the roots along the peripheral aspect, and likely there was enhancement of the roots in this location as well (Figure 1). Hematological evaluation and biochemical parameters were normal. The clinical diagnosis was arachnoid cyst or arachnoiditis or possibly spinal tumors, and surgery was believed to be warranted because of the patient's progressive neurological symptoms. A lumbar laminectomy L1 to L4 was performed and the underlying dura mater was opened. Beneath were grossly abnormally thickened arachnoid and a round thick fluid-filled sac that was directly compressing the conus medullaris and the cauda equina. This was carefully removed and sent for pathology. Histological examination was compatible with neurocysticercosis (NCC; Figure 2). The serum was positive for anticysticercus antibodies by enzyme-linked immunosorbent assay (ELISA), using glycoproteins purified from *Taenia solium* cyst fluid as antigens. Examination of stools was negative for the presence of parasites, proglottids, and ova. The patient underwent full craniospinal axis MRI evaluation, which demonstrated no evidence of other cysticercosis lesions.

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