[41] Baron RW, Tanner CE. The effect of immunosuppression on secondary *Echinococcus multilocularis* infection in mice. Int J Parasitol 1976;6:37–42.
[42] Bresson-Hadni S, Liance M, Meyer JP, Houin R, Bresson JL, Vuitton DA. Cellular immunity in experimental *Echinococcus multilocularis* infection II. Sequential and comparative phenotypic study of the periparasitic mononuclear cells in resistant and sensitive mice. Clin Exp Immunol 1990;82:378–83.
[43] Kizaki T, Kobayashi S, Ogasawara K, Day NK, Good RA, Onoe K. Immune suppression induced by protoscoleces of *Echinococcus multilocularis* in mice. Evidence for the presence of CD8dull suppressor cells in spleens of mice intraperitoneally infected with *E. multilocularis*. J Immunol 1991;147:1659–66.
[44] Rakha NK, Dixon JB, Carter SD, Craig PS, Jenkins P, Folkard S. *Echinococcus multilocularis* antigens modify accessory cell function of macrophages. Immunology 1991;74:652–6.
[45] Kamiya H. Studies on echinococcosis XXIV. Age difference to infection with *Echinococcus multilocularis* in AKR strain of mouse. Jpn J Vet Res 1972;20:69–76.
[46] Kamiya H. Observations on difference of susceptibility to larval *Echinococcus multilocularis* among uniform strains of the mouse. Jpn J Parasitol 1973;22:294–9 [in Japanese with English summary].
[47] Playford MC, Ooi HK, Oku Y, Kamiya M. Secondary *Echinococcus multilocularis* infection in severe combined immunodeficient (scid) mice: biphasic growth of the larval cyst mass. Int J Parasitol 1992;22:975–82.
[48] Playford MC, Ooi HK, Ito M, Kamiya M. Lymphocyte engraftment conveys immunity and alters parasite development in scid mice infected with *Echinococcus multilocularis*. Parasitol Res 1993;79:261–8.
[49] Kanazawa T, Asahi H, Hata H, Mochida K, Kagei N, Stadecker MJ. Arginine-dependent generation of reactive nitrogen intermediates is instrumental in the in vitro killing of protoscoleces of *Echinococcus multilocularis* by activated macrophages. Parasite Immunol 1993;15:619–23.
[50] Kizaki T, Ishige M, Kobayashi S, Bingyan W, Kumagai M, Day NK, et al. Suppression of T-cell proliferation by CD8+ T cells induced in the presence of protoscolices of *Echinococcus multilocularis* in vitro. Infect Immun 1993;61:525–33.
[51] Schantz PM, Eckert J, Craig PS. Geographic distribution, epidemiology, and control of *Echinococcus multilocularis* and alveolar echinococcosis. In: Uchino J, Sato N, editors. Alveolar echinococcosis strategy for eradication of alveolar echinococcosis of the liver. Sapporo, Japan: Fujishoin; 1996.
[52] Budke CM, Campos-Ponce M, Qian W, Torgerson PR. A canine purgation study and risk factor analysis for echinococcosis in a high endemic region of the Tibetan plateau. Vet Parasitol 2005;127:43–9.
[53] Tang C. Investigation of *Echinococcus multilocularis* in Huluenbeir grassland, Inner Mongolia. Zool Acat Sinica 1988;34:172–9.
[54] Qiu GM. Sichuan pika naturally infected with *Echinococcus multilocularis*. Endemic Dis Bull 1989;4:64–5.
[55] Wang Q, Vuitton DA, Qiu J, Giraudoux P, Xiao Y, Schantz PM, et al. Fenced pasture: a possible risk factor for human alveolar echinococcosis in Tibetan pastoralist communities of Sichuan, China. Acta Trop 2004;90:285–93.
[56] Wang Q, Vuitton DA, Xiao Y, Budke CM, Campos-Ponce M, Schantz PM, et al. Pasture types and *Echinococcus multilocularis*, Tibetan communities. Emerg Infect Dis 2006;12:1008–10.
[57] Shaikenov BS. Distribution and ecology of *Echinococcus multilocularis* in Central Asia. Parasitol Int 2006;55(Suppl):S213–9.
[58] Shaikenov B, Torgerson PR. Changes in the epidemiology of echinococcosis in Kazakhstan. In: Torgerson P, Shaikenov B, editors. Echinococcosis in Central Asia: problems and solution. Zurich Almaty: INTAS Network Project; 2004. p. 3–12.
[59] Shaikenov B. Characteristics of origination of natural breeding ground of alveolar echinococcosus in Western Kazakhstan. Medizinskaya Parazitologia i Parazitarnye Bolezni, N. 3. 1987.
[60] Suzuki K, Uchino J, Sato N, Takahashi H. Epidemiology. In: Uchino J, Sato N, editors. Alveolar echinococcosis of the liver. Sapporo: Fuji Shoin; 1996. p. 213–7.

[61] Minagawa T. Survey of echinococcosis in Hokkaido and measure against it. Hokkaido J Med Sci 1997;72:569–81 [in Japanese].
[62] Oku Y. Expansion of the distribution of *Echinococcus multilocularis* which propagates and spreads in the human body. J Modern Vet Med 2000;48:5–17 [in Japanese].
[63] Doi R, Kanda E, Nihei N, Uchida A. Occurrence of alveolar hydatid disease (multilocular echinococcosis) outside of Hokkaido and a proposal for its prevention. Nippon Koshu Eisei Zasshi 2000;47:111–26.
[64] Eckert J, Conraths FJ, Tackmann K. Echinococcosis: an emerging or re-emerging zoonosis? Int J Parasitol 2000;30:1283–94.
[65] : Yamamoto N, Morishima Y, Kon M, Yamaguchi M, Tanno S, Koyama M, Maeno N, Azuma H, Mizusawa H, Kimura H, Sugiyama H, Arakawa K, Kawanaka M. The first reported case of a dog infected with *Echinococcus multilocularis* in Saitama prefecture, Japan. Jpn J Infect Dis 2006;59:351–2.
[66] Morishima Y, Sugiyama H, Arakawa K, Kawanaka M. *Echinococcus multilocularis* in dogs, Japan. Emerg Infect Dis 2006;12:1292–4.
[67] Kamiya M, Lagapa JT, Ganzorig S, Kobayashi F, Nonaka N, Oku Y. Echinococcosis risk among domestic definitive hosts, Japan. Emerg Infect Dis 2007;13:346–7.
[68] Doi R, Matsuda H, Uchida A, Kanda E, Kamiya H, Konno K, et al. Possibility of invasion of *Echinococcus* into Honshu with pet dogs from Hokkaido and overseas. Nippon Koshu Eisei Zasshi 2003;50:639–49.
[69] Torgerson P, Shaikenov B. Echinococcosis in Central Asia: problems and solutions. INTAS network project: Zurich, Almaty, 2004.
[70] Kamiya M, Ooi HK, Oku Y. Symposium on infectious diseases of animals and quarantine [conference summary]. Emerg Infect Dis [serial on the Internet]. 2005 May [cited 5 December 2006]. Available from 〈http://www.cdc.gov/ncidod/EID/vol11no05/04-1348.htm〉.
[71] Biron DG, Moura H, Marche L, Hughes AL, Thomas F. Towards a new conceptual approach to 'parasitoproteomics'. Trends Parasitol 2005;21:162–8.
[72] Rytkonen MJ. Not all maps are equal: GIS and spatial analysis in epidemiology. Int J Circumpolar Health 2004;63:9–24.
[73] Pleydell DR, Raoul F, Tourneux F, Danson FM, Graham AJ, Craig PS, et al. Modelling the spatial distribution of *Echinococcus multilocularis* infection in foxes. Acta Trop 2004;91:253–65.
[74] Rinaldi L, Musella V, Cringoli G. Disease mapping in veterinary parasitology: an update. Parasitologia 2006;48:85–7.
[75] Thompson RCA. Biology and systematics of *Echinococcus*. In: Thompson RCA, Lymbery AJ, editors. Echinococcus and hydatid disease. Wallingford: CAB International; 1995. p. 1–50.
[76] Eckert J. Predictive values and quality control of techniques for the diagnosis of *Echinococcus multilocularis* in definitive hosts. Acta Trop 2003;85:157–63.
[77] Allan JC, Craig PS. Coproantigens in taeniasis and echinococcosis. Parasitol Int 2006; 55(Suppl):S75–80.
[78] Yagi K, Takahashi K, Hattori K, Kondo N, Oku Y, Ito M. A study of intestinal helminthes of red fox *Vulpes vulpes schrencki* in Nemuro Peninsula. Jpn J Parasitol 1986;35:83–4 [in Japanese].
[79] Deplazes P, Gottstein B, Eckert J, Jenkins DJ, Ewald D, Jimenez-Palacios S. Detection of *Echinococcus coproantigens* by enzyme-linked immunosorbent assay in dogs, dingoes and foxes. Parasitol Res 1992;78:303–8.
[80] Sakashita M, Sakai H, Kohno H, Ooi HK, Oku Y, Yagi K, et al. Detection of *Echinococcus multilocularis* coproantigens in experimentally infected dogs using murine monoclonal antibody against adult worm. Jpn J Parasitol 1995;44:413–20.
[81] Nonaka N, Iida M, Yagi K, Ito T, Ooi HK, Oku Y, et al. Time course of coproantigen excretion in *Echinococcus multilocularis* infections in foxes and an alternative definitive host, golden hamsters. Int J Parasitol 1996;26:1271–8.
[82] Deplazes P, Alther P, Tanner I, Thompson RC, Eckert J. *Echinococcus multilocularis* coproantigen detection by enzyme-linked immunosorbent assay in fox, dog, and cat populations. J Parasitol 1999;85:115–21.

[83] Machnicka B, Dziemian E, Rocki B, Kolodziej-Sobocinska M. Detection of *Echinococcus multilocularis* antigens in faeces by ELISA. Parasitol Res 2003;91:491–6.
[84] Kamiya M, Sato H. Complete life cycle of the canid tapeworm, *Echinococcus multilocularis*, in laboratory rodents. FASEB J 1990;4:3334–9.
[85] Kohno H, Sakai H, Okamoto M, Ito M, Oku Y, Kamiya M. Development and characterization of murine monoclonal antibodies to *Echinococcus multilocularis* adult worms and its use for the coproantigen detection. Jpn J Parasitol 1995;44:404–12.
[86] Eckert J, Deplazes P. Immunological and molecular techniques for diagnosing the *Echinococcus multilocularis* infection in definitive and intermediate hosts. Acta Parasitol 2001;46:1–7.
[87] Sakai H, Nonaka N, Yagi K, Oku Y, Kamiya M. Coproantigen detection in a survey of *Echinococcus multilocularis* infection among red foxes, *Vulpes vulpes schrencki*, in Hokkaido, Japan. J Vet Med Sci 1998;60:639–41.
[88] Nonaka N, Tsukada H, Abe N, Oku Y, Kamiya M. Monitoring of *Echinococcus multilocularis* infection in red foxes in Shiretoko, Japan, by coproantigen detection. Parasitology 1998;117:193–200.
[89] Yimam AE, Nonaka N, Oku Y, Kamiya M. Prevalence and intensity of *Echinococcus multilocularis* in red foxes (*Vulpes vulpes schrencki*) and raccoon dogs (*Nyctereutes procyonoides albus*) in Otaru City, Hokkaido, Japan. Jpn J Vet Res 2002;49:287–96.
[90] Reiterova K, Miterpakova M, Turcekova L, Antolova D, Dubinsky P. Field evaluation of an intravital diagnostic test of *Echinococcus multilocularis* infection in red foxes. Vet Parasitol 2005;128:65–71.
[91] Bretagne S, Guillou JP, Morand M, Houin R. Detection of *Echinococcus multilocularis* DNA in fox faeces using DNA amplification. Parasitology 1993;106(Part 2):193–9.
[92] Dinkel A, von Nickisch-Rosenegk M, Bilger B, Merli M, Lucius R, Romig T. Detection of *Echinococcus multilocularis* in the definitive host: coprodiagnosis by PCR as an alternative to necropsy. J Clin Microbiol 1998;36:1871–6.
[93] van der Giessen JW, Rombout YB, Franchimont JH, Limper LP, Homan WL. Detection of *Echinococcus multilocularis* in foxes in The Netherlands. Vet Parasitol 1999;82:49–57.
[94] Stieger C, Hegglin D, Schwarzenbach G, Mathis A, Deplazes P. Spatial and temporal aspects of urban transmission of *Echinococcus multilocularis*. Parasitology 2002;124:631–40.
[95] Trachsel D, Deplazes P, Mathis A. Identification of taeniid eggs in the faeces from carnivores based on multiplex PCR using targets in mitochondrial DNA. Parasitology 2007;1–10.
[96] Yoneda M, Maekawa K. Effects of hunting on age structure and survival rates of red fox in eastern Hokkaido. J Wildlife Manage 1982;46:781–6.
[97] Phillips RL. Age ratios of Iowa foxes. J Wildlife Manage 1970;36:237–48.
[98] Lloyd HG. The red fox. London, UK: B.T. Batsford Ltd.; 1980. 320pp.
[99] Baker PJ, Harris S, Webbon CC. Effect of British hunting ban on fox numbers. Nature 2002;419:34.
[100] Eckert J, Deplazes P. Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. Clin Microbiol Rev 2004;17:107–35.
[101] Tsukada H, Hamazaki K, Ganzorig S, Iwaki T, Konno K, Lagapa JT, et al. Potential remedy against *Echinococcus multilocularis* in wild red foxes using baits with anthelmintic distributed around fox breeding dens in Hokkaido, Japan. Parasitology 2002;125:119–29.
[102] Kamiya M, Lagapa JT, Nonaka N, Ooi HK, Oku Y, Kamiya H. Current control strategies targeting sources of echinococcosis in Japan. Rev Sci Tech Off Int Epiz 2006;25:1055–66.
[103] Schelling U, Frank W, Will R, Romig T, Lucius R. Chemotherapy with praziquantel has the potential to reduce the prevalence of *Echinococcus multilocularis* in wild foxes (*Vulpes vulpes*). Ann Trop Med Parasitol 1997;91:179–86.
[104] Hegglin D, Ward PI, Deplazes P. Anthelmintic baiting of foxes against urban contamination with *Echinococcus multilocularis*. Emerg Infect Dis 2003;9:1266–72.
[105] Tackmann K, Loschner U, Mix H, Staubach C, Thulke H, Ziller M, et al. A field study to control *Echinococcus multilocularis*-infections of the red fox (*Vulpes vulpes*) in an endemic focus. Epidemiol Infect 2001;127:577–87.
[106] Hansen F, Tackmann K, Jeltsch F, Wissel C, Thulke HH. Controlling *Echinococcus multilocularis*—ecological implications of field trials. Prev Vet Med 2003;60:91–105.

[107] Ewald D, Eckert J, Gottstein B, Straub M, Nigg H. Parasitological and serological studies on the prevalence of *Echinococcus multilocularis* Leuckart, 1863 in red foxes (*Vulpes vulpes* Linnaeus, 1758) in Switzerland. Rev Sci Tech 1992;11:1057–61.
[108] Hofer S, Gloor S, Muller U, Mathis A, Hegglin D, Deplazes P. High prevalence of *Echinococcus multilocularis* in urban red foxes (*Vulpes vulpes*) and voles (*Arvicola terrestris*) in the city of Zurich, Switzerland. Parasitology 2000;120:135–42.
[109] Takahashi K, Mori C. Host animals and prevalence of *Echinococcus multilocularis* in Hokkaido. Pub Health Hokkaido 2001;27:73–80.
[110] Ito S. Modified Wisconsin sugar centrifugal-floatation technique for nematode eggs in bovine faeces. J Jpn Vet Med Assoc 1980;33:424–9.

FULL PAPER *Parasitology*

Genetic Uniformity of *Echinococcus multilocularis* Collected from Different Intermediate Host Species in Hokkaido, Japan

Munehiro OKAMOTO[1], Yuzaburo OKU[2], Tsutomu KUROSAWA[3] and Masao KAMIYA[2]

[1]*Department of Laboratory Animal Science, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Tottori 680–8553,* [2]*Laboratory of Parasitology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818 and* [3]*Institute of Experimental Animal Science, Graduate School of Medicine, Osaka University, Suita 565–0871, Japan*

(Received 8 August 2006/Accepted 17 November 2006)

ABSTRACT. DNA from several isolates of *Taenia taeniaeformis* and *Echinococcus multilocularis* were digested with restriction enzymes and hybridized with digoxigenated oligonucleotide probe $(CAC)_5$. Within the six wild isolates of *Taenia taeniaeformis* from Norway rats in Hokkaido, although several bands were common among isolates, fingerprinting patterns were specific to each isolate. In the case of *E. multilocularis*, regardless of hosts from which each isolate has been isolated, the five isolates collected from Hokkaido, showed the same fingerprinting pattern. These results indicate that there was very little genetic difference among these isolates. Although the fingerprinting pattern of *E. multilocularis* from St. Lawrence Is. was similar to that of the Hokkaido isolates, some bands were different from those in the Hokkaido isolates. *Echinococcus multilocularis* in Hokkaido seems to be closely-related genetically to that from St. Lawrence Is.

KEY WORDS: different intermediate host species, DNA fingerprinting, *Echinococcus multilocularis*, genetic uniformity, Hokkaido.

J. Vet. Med. Sci. 69(2): 159–163, 2007

Intraspecific variation has been described from different geographic areas or host species in all major groups of parasites by applying a number of differential criteria [25]. Traditionally, the morphology was used as the sole differential criterion. However, more recently several intrinsic or extrinsic characteristics have been used [26]. *Echinococcus granulosus*, which is responsible for cystic hydatid disease in humans and animals, is known to exist as biologically and genetically distinct subspecific variants or strains. However, few intraspecific variations of *E. multilocularis* have been reported [2, 4].

Echinococcus multilocularis, which is responsible for alveolar hydatid disease in humans, is one of the most medically important cestodes in the holarctic region. The natural intermediate hosts of *E. multilocularis* are arvicoline rodents. However, in Hokkaido Japan, natural infections of *E. multilocularis* in swine and horse, which have been scarcely reported in other geographical areas, have been frequently observed [12, 21]. Moreover, natural infection of *E. multilocularis* in a Norway rat, *Rattus norvegicus*, has been found in southern Hokkaido [16]. Although many species of mammals have been reported as possible natural or experimental intermediate hosts of *E. multilocularis*, there are few reports on infection in the Norway rat, swine, or horse outside Hokkaido [15, 20, 23]. Therefore, the possibility that a different strain or population exists in the Hokkaido district from those reported in other geographical areas is considered.

Sequencing of mitochondrial or nuclear genes is a useful tool to infer the phylogenetic relationships of organisms. So we have examined the partial sequences of mitochondrial CO1 gene for Hokkaido isolates of *E. multilocularis*. However, all isolates shared the same sequence of CO1 gene [19].

DNA fingerprinting has proved to be powerful in resolving genetic identity or relationships and is applied in many diverse areas of biological sciences including forensic science, paternity testing, animal breeding and population genetics [6, 8, 9]. One attractive DNA fingerprinting method is the detection of hypervariable simple repetitive DNA by means of oligonucleotide probes, which make it possible to establish highly informative DNA fingerprints for any eucaryotic organisms. Oligonucleotide probe $(CAC)_5$ is multilocus and a very informative probe to identify human individuals [28]. It is also useful for verifying genetic relationships in domestic animals and wild birds [5]. Okamoto *et al.* [18] applied DNA fingerprinting with $(CAC)_5$ to analysis of genetic variation within *Taenia taeniaeformis* and reported that $(CAC)_5$ was a highly resolvable and informative probe for cestodes.

Taenia taeniaeformis is a common parasite of cats in Japan, and its intermediate hosts are Norway rat (*Rattus norvegicus*), small Japanese field mouse (*Apodemus argenteus*) and gray red-backed vole (*Clethrionomys rufocanus bedfordiae*) so far reported. From these intermediate hosts, all isolates of *T. taeniaeformis* from Norway rats shared the same sequences for CO1 gene so far examined [19]. However, DNA fingerprinting patterns of those isolates constructed with the oligonucleotide probe $(CAC)_5$ were different from each other [18]

In this study, we examined the genetic variability of *Echinococcus multilocularis* collected from different intermediate host species using DNA fingerprinting and discussed the genetic features of *E. multilocularis* population in Hokkaido comparing with the case of *T. taeniaeformis*.

Table 1. Hosts and geographical origins of cestodes examined in this study

Species	Isolate	Host	Geographical origin
Echinococcus multilocularis	EmHok	Gray red-backed vole	Higashimokoto, Hokkaido, Japan
	EmTob	Gray red-backed vole	Tobetsu, Hokkaido, Japan
	EmYak	Gray red-backed vole	Yakumo, Hokkaido, Japan
	Empig	Pig	Kitami, Hokkaido, Japan
	Emrat	Norway rat	Kamiiso, Hokkaido, Japan
	EmStL	Tundra vole	St. Lawrence Is., U.S.A.
Taenia taeniaeformis wild isolates	TtSap1	Norway rat	Sapporo, Hokkaido, Japan
	TtSap2	Norway rat	Sapporo, Hokkaido, Japan
	TtSap3	Norway rat	Sapporo, Hokkaido, Japan
	TtTom	Norway rat	Tomikawa, Hokkaido, Japan
	TtKam	Norway rat	Kamiiso, Hokkaido, Japan
	TtEbe	Norway rat	Ebetsu, Hokkaido, Japan
	TtCat	Cat	Sapporo, Hokkaido, Japan

Gray red-backed vole: *Clethrionomys rufocanus bedfordiae*, Norway rat: *Rattus norvegicus*, Tundra vole: *Microtus oeconomus*.

MATERIALS AND METHODS

Parasites: Five Hokkaido isolates and one Alaskan isolate of *E. multilocularis* were examined. Each isolate was passaged by intra-peritoneal injection in Mongolian gerbils (*Meriones unguiculatus*) in Hokkaido University. As for isolates of *Taenia taeniaeformis*, 6 wild isolates were used. These taeniid samples were stored in liquid nitrogen, at –80°C or in 70% ethanol until required for DNA extraction. The sample list and locality map are shown in Table 1 and Fig. 1, respectively.

Preparation of DNA: DNA fingerprinting requires comparably high-molecular weight DNA, so we have prepared the genomic DNA using the extraction with phenol. The details of the method have been given in a previous report [18].

Sequencing for CO1 gene: A partial fragment of CO1 gene was amplified from the total DNA by PCR using the primer pair pr-a and pr-b [19]. Direct sequencing of the PCR amplification product was performed with a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, U.S.A.) using the pr-a and pr-b.

DNA fingerprinting with oligonucleotide probe $(CAC)_5$: Approximately 1 μg of taeniid DNA was digested with 30–50 units of restriction endonuclease *Pst* I or *Pvu* II (Nippon Gene), using the buffer and reaction conditions as recommended by the manufacturer of the respective enzymes. Reaction was stopped by the addition of 1/10 volume of 50% glycerol, 10 mM $NaHPO_4$, 100 mM EDTA and 0.4% bromophenol blue. Digested DNA was loaded on 0.8% agarose gel and run in TBE buffer (89 mM boric acid, 89 mM Tris, 2 mM EDTA).

After electrophoresis, DNA was denatured by soaking the gel for 30 min in 0.5 M NaOH, 1.5 M NaCl with constant gentle agitation and then neutralized by soaking for 30 min in 0.5 M Tris-HCl (pH 7.8), 1.5 M NaCl. Thereafter, DNA was blotted with 20 × SSC(3 M NaCl, 0.3 M Nacitrate, pH7.0) on to positively charged nylon membranes (Hybond N+, Amersham, U.S.A.) for 6 hr.

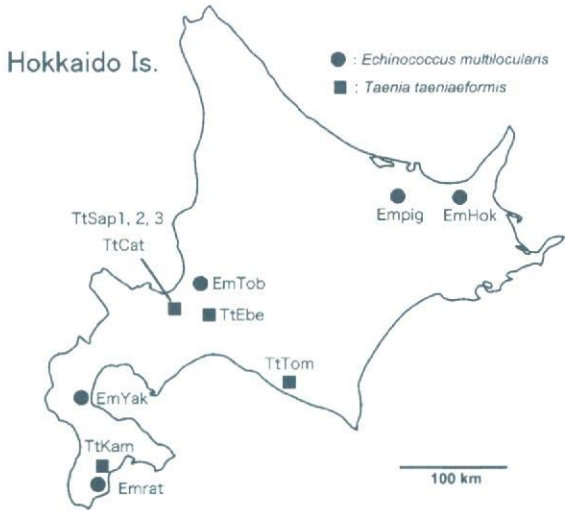

Fig. 1. Collection localities of *Echinococcus multilocularis* and *Taenia taeniaeformis* in Hokkaido Island. For explanations of abbreviations of sample names, see Table 1. Four isolates of *T. taeniaeformis* from Sapporo were collected from distant localities in the same city.

The oligonucleotide $(CAC)_5$ was chemically synthesized and purified by reversed phase HPLC. Labeling with digoxigenated dUTP was done in terminal deoxynucleotidyl transferase using a DNA Tailing Kit (Roche Diagnostics, Germany). Membranes were baked at 80°C for 2 hr. Hybridization with digoxigenated probe and immunological detection were performed by using a DIG Nucleic Acid Detection Kit (Roche Diagnostics). Prehybridization and hybridization were done at 42°C for 1 hr and for at least 10 hr respectively. Filters were then washed twice in 2 × SSC containing 0.1% SDS at room temperature for 5 min and for 15 min, respectively. These were washed twice in 2 × SSC containing 0.1% SDS at 45°C for 30 min. Subsequent immunological detections were performed according to the manufacturer's instructions.

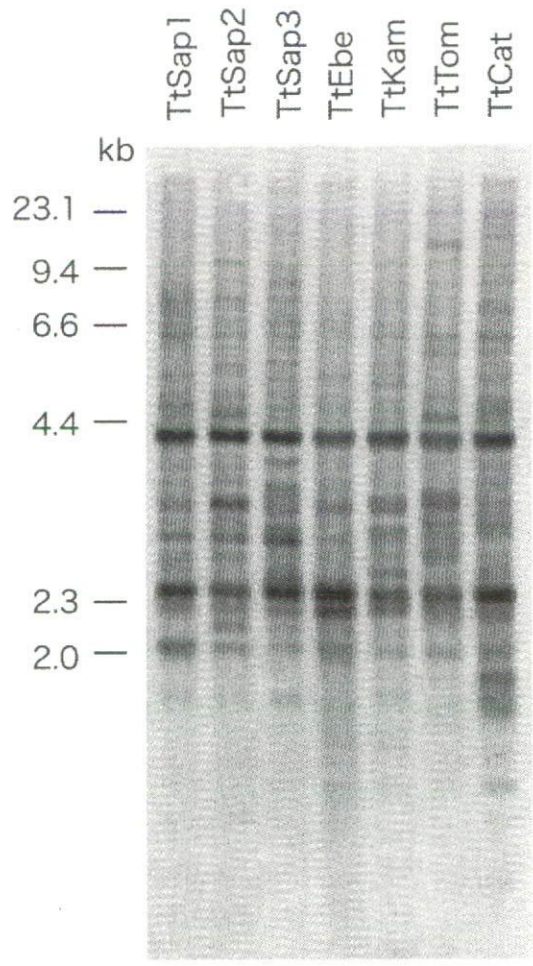

Fig. 2. DNA fingerprinting of wild isolates of *T. taeniaeformis* with digoxigenated oligonucleotide probe $(CAC)_5$. DNA was digested with *Pst* I and electrophoresed in 0.8% agarose gel at 30 V 15 hr. Molecular weight markers are given on the left in kilobases.

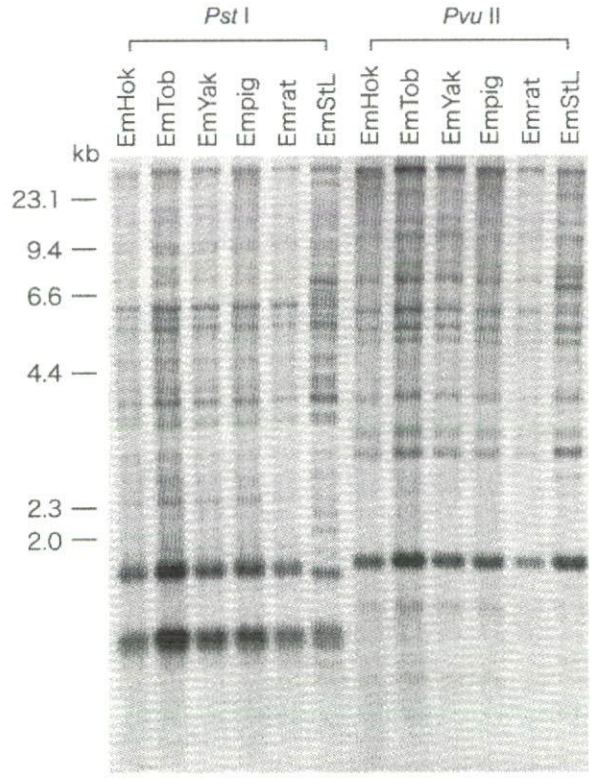

Fig. 3. DNA fingerprinting of six isolates of *Echinococcus multilocularis* with digoxigenated oligonucleotide probe $(CAC)_5$. DNA was digested with *Pst* I or *Pvu* II and electrophoresed in 0.8% agarose gel at 20 V 24 hr. Molecular weight markers are given on the left in kilobases.

RESULTS

Sequencing of the CO1 gene: Partial sequences of the mitochondrial CO1 gene from some samples had been examined [19]. In this study, therefore, all wild isolates of *T. taeniaeformis* except TtTom were examined. All wild isolates of *T. taeniaeformis*, including TtCat, shared the same sequence as that from TtSRN (GenBank/EMBL/DDBJ accession no. AB221484) [19]. No sequence variation was observed in *E. multilocularis*, regardless of the hosts or geographical areas from which each metacestode had been isolated [19]. Phylogenetic relationships of taeniid cestodes, including some isolates examined in this study, inferred from the CO1 gene have been reported [19].

DNA fingerprinting: DNA from *T. taeniaeformis* were digested with *Pst* I and hybridized with digoxigenated oligonucleotide probe $(CAC)_5$. All isolates showed clear multibanding patterns, which were characteristic of multilocus DNA fingerprinting (Fig. 2). Fingerprinting patterns of wild isolates of *T. taeniaeformis* resembled each other. However, several bands were specific to some isolates, so isolate was easily distinguishable each other by fingerprinting patterns.

Fingerprinting patterns of 6 isolates of *E. multilocularis* when digested with *Pst* I and *Pvu* II are shown in Fig. 3. Regardless of hosts from which each isolate had been isolated, the five isolates from Hokkaido showed completely the same fingerprinting pattern. Fingerprinting pattern of EmStL was similar to that of the Hokkaido isolates. However, some bands seen in EmStL were either different in molecular size, or missing in Hokkaido isolates. Moreover additional bands were seen in EmStL.

DISCUSSION

The DNA fingerprinting pattern often is specific to an individual, except in extreme case of inbreeding or in monozygotic twins, or clones. It was reported that $(CAC)_5$ represented the informative fingerprints for genetic analysis

of *T. taeniaeformis*, when digested with *Hinf* I [18]. In the case of *E. multilocularis*, digestion with either *Pst* I or *Pvu* II was effective in preliminary study (data not shown). In this study, therefore, the digestion with *Pst* I was applied.

One of the major problems with applying multilocus DNA fingerprinting to population analysis is that it is impossible to identify which bands are derived from the same locus, especially between distant organisms. Although wild populations usually are highly polymorphic, with many alleles at a single locus, bands that appear to be shared by individuals are not always identical alleles at the same locus [3]. On the contrary, among closely-related, especially blood-related organisms, many identical alleles are derived from the same locus, and the fingerprinting patterns resemble each other. In the present study, it seemed that six wild isolates of *T. taeniaeformis* were genetically related to each other. However, no isolate had a fingerprinting pattern that was same as that of any other isolate.

As contrasted with *T. taeniaeformis*, five isolates of *E. multilocularis* from Hokkaido showed the identical fingerprinting pattern. The main intermediate host of *E. multilocularis* in Hokkaido is a gray red-backed vole. However, several reports have been published on natural infections of *E. multilocularis* in uncommon intermediate hosts in Hokkaido Island, namely, swine [21], horses [12] and Norway rats [16]. There have been few reports of natural infection of *E. multilocularis* in these intermediate hosts in other endemic areas. In the present study, although we compared isolates derived from gray red-backed voles, swine and Norway rats, no difference was detected in their fingerprints. These results indicate that there was very little genetic difference among these isolates. It seemed that infections with *E. multilocularis* to the unusual animals in Hokkaido were not responsible for the variation in *E. multilocularis*.

Generally, wild populations are highly polymorphic, with many alleles at a single locus. It is very rare that the same fingerprinting pattern is obtained from two individuals, except in the case of monozygotic twins. Nevertheless, why were fingerprints from all Hokkaido isolates of *E. multilocularis* identical?

Although all isolates from Hokkaido Island showed an identical fingerprinting pattern, this did not mean that all isolates were as uniform as clones. Actually, Nakao *et al.* [13] reported that polymorphism of microsatellite DNA was detected in Hokkaido's population of *E. multilocularis*. Because the multilocus fingerprint is the technique used to detect RFLPs of genome DNA, sometimes it cannot detect a slight difference in DNA such as several base indels in microsatellites. Although it is known that self-insemination occurs in *Echinococcus* [10, 24], the heterozygosity of microsatellite alleles indicates that cross-fertilization also occurs in *E. multilocularis*. However, the heterozygosity observed was low in Hokkaido's population of *E. multilocularis* [13].

Since asexual proliferation occurs in the larval stage of *Echinococcus*, a large number of clonal protoscoleces are produced in intermediate hosts. When foxes prey on the rodent infected, the majority of adult worms, therefore, are clonal. An increase in homozygosity within a population of *Echinococcus* is well explained by self-fertilization [13], which can be achieved by a sperm of the same individual (autogamy) or of another clonal individual (geitonogamy) [11].

Even though the population of *Echinococcus* has such properties, if *E. multilocularis* is native to Hokkaido and has inhabited that area for a long time, geographical variation in fingerprinting should be detected, because the mutation rate of fingerprinting is very high. In humans, the spontaneous mutation rate of fingerprinting with $(CAC)_5$ has been estimated to be approximately 0.001 per DNA fragment and gamete [14]. *Echinococcus multilocularis* in Hokkaido seems to be a recent population that invaded from another endemic area. Actually, endemic areas of *E. multilocularis* on Hokkaido Island were restricted to its eastern part before 1975 [7]. In addition, if the *E. multilocularis* that invaded Hokkaido had been polymorphic genetically, variation in its fingerprinting should still have remained. The origin of *E. multilocularis* endemic in Hokkaido seems to be a single or very uniform population.

Yamashita [27] assumed that the *E. multilocularis* prevalent in Hokkaido Island was introduced from St. Lawrence Island via Komandorskie and Kuril Islands. It was reported that a sequence of CO1 gene of *E. multilocularis* from Kunashiri Island, which was the southern Island of Kuril Islands, was same as that from Hokkaido [22]. The fingerprinting pattern of EmStL resembled that from Hokkaido isolates with slight differences in several bands. Comparing with the case of *Taenia taeniaeformis*, it appears that Hokkaido's *E. multilocularis* is not identical to EmStL, but is closely-related genetically.

A partial sequence of the mitochondrial CO1 gene of EmStL examined is shared with that from Hokkaido's isolates. However, it was recently revealed that *E. multilocularis*, which had a different type of the mitochondrial CO1 gene, also inhabits St. Lawrence Island (data not shown). Thus, *E. multilocularis* endemic to St. Lawrence Island may be polymorphic. In the present study, only one isolate from St. Lawrence Island was examined. Therefore, it is possible that population, which is identical to *E. multilocularis* in Hokkaido, may inhabit St. Lawrence Island. In order to determine the origin of *E. multilocularis* in Hokkaido, additional investigations are needed, including isolates from St. Lawrence Island, Komandorskie and Kuril Islands.

ACKNOWLEDGEMENTS. We would like to thank members of the Department of Parasitology, Faculty of Veterinary Medicine, Hokkaido University, for assistance in collection and rearing of the animals. This study was supported in part by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science to M. Okamoto (12680811, 16500277, 18406008)

REFERENCES

1 Azuma, H., Okamoto, M., Oku, Y. and Kamiya, M. 1995. Intraspecific variation of isoenzymes in *Taenia taeniaeformis*. *Parasitol. Res.* **81**: 103–108.

2 Bowles, J., Blair, D. and McManus, D. P. 1992. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Mol. Biochem. Parasitol.* **54**: 165–174.

3 Bruford, M. W., Hanotte, O., Brookfield, J. F. Y. and Burke, T. 1992. Single-locus and multilocus DNA fingerprinting. pp. 225–269. *In*: Molecular Genetic Analysis of Populations (Hoelzel, A. R. ed), Oxford University Press, New York.

4 Bowles, J. and McManus, D. P. 1993. Molecular variation in *Echinococcus*. *Acta Tropica* **53**: 291–305.

5 Buitkamp, J., Ammer, H. and Geldermann, H. 1991. DNA fingerprinting in domestic animals. *Electrophoresis* **12**: 169–174.

6 Gill, P., Jeffreys, A. J. and Werrett, D. J. 1985. Forensic application of DNA 'fingerprints'. *Nature (Lond.)* **318**: 577–579.

7 Ito, A., Romig, T. and Takahashi, K. 2003. Perspective on control options for *Echinococcus multilocularis* with particular reference to Japan. *Parasitology* **127**: S159–S172.

8 Jeffreys, A. J., Wilson, V. and Thein, S. L. 1985. Hypervariable 'minisatellite' regions in human DNA. *Nature (Lond.)* **314**: 67–73.

9 Jeffreys, A. J. and Brookfield, J. F. Y. 1985. Semeonoff R. Positive identification of an immigration test-case using DNA fingerprints. *Nature (Lond.)* **317**: 818–819.

10 Kamiya, M. and Sato, H. 1990. Survival, strobilation and sexual maturation of *Echinococcus multilocularis* in the small intestine of golden hamsters. *Parasitology* **100**: 125–130.

11 Lymbery, A. J., Constantine, C. C. and Thompson, R. C. A. 1997. Self-firtilization without genomic or population structuring in a parasitic tapeworm. *Evolution* **51**: 289–294.

12 Miyauchi, T., Sakui, M., Ishige, M., Fukumoto, S., Ueda, A., Ito, M. and Ohbayashi, M. 1984. A case of multilocular echinococcosis in a horse. *Jpn. J. Parasitol.* **32**: 171–173.

13 Nakao, M., Sako, Y. and Ito, A. 2003. Isolation of polymorphic microsatellite loci from the tapeworm *Echinococcus multilocularis*. *Inf. Genet. Evol.* **3**: 159–163.

14 Nürnberg, P., Roewer, L., Neitzel, H., Sperling, K., Pöpperl, A., Hundrieser, J., Pöche, H., Epplen, C., Zischler, H. and Epplen, J. T. 1989. DNA fingerprinting with the oligonucleotide probe $(CAC)_5/(GTG)_5$: somatic stability and germline mutations. *Hum. Genet.* **84**: 75–78.

15 Ohbayashi, M. 1975. *Echinocuccus* (in Japanese). *Hokujukaishi* **19**: 126–257.

16 Okamoto, M., Fujita, O., Arikawa, J., Kurosawa, T., Oku, Y. and Kamiya, M. 1992. Natural *Echinococcus multilocularis* infection in a Norway rat, *Rattus norvegicus*, in southern Hokkaido, Japan. *Int. J. Parasitol.* **22**: 681–684.

17 Okamoto, M., Ito, A., Kurosawa, T., Oku, Y., Kamiya, M. and Agatsuma, T. 1995. Intraspecific variation of isoenzymes in *Taenia taeniaeformis*. *Int. J. Parasitol.* **25**: 221–228.

18 Okamoto, M., Ueda, H., Hayashi, M., Oku, Y., Kurosawa, T. and Kamiya, M. 1995. Application of DNA fingerprinting with digoxigenated oligonucleotide probe $(CAC)_5$ to analysis of the genetic variation within *Taenia taeniaeformis*. *J. Vet. Med. Sci.* **57**: 267–272.

19 Okamoto, M., Bessho, Y., Kamiya, M., Kurosawa, T. and Horii, T. 1995. Phylogenetic relationships within *Taenia taeniaeformis* variants and other taeniid cestodes inferred from the nucleotide sequence of the cytochrome *c* oxidase subunit I gene. *Parasitol. Res.* **81**: 451–458.

20 Rausch, R. L. 1986. Life-cycle patterns and geographic distribution of *Echinococcus* species. pp. 44–80. *In*: The Biology of *Echinococcus* and Hydatid disease (Thompson, R. C. A. ed.), George Allen & Unwin, London.

21 Sakui, M., Ishige, M., Fukumoto, S., Ueda, A. and Ohbayashi, M. 1984. Spontaneous *Echinococcus multilocularis* infection in swine in North-eastern Hokkaido, Japan. *Jpn. J. Parasitol.* **33**: 291–296.

22 Satho, M., Nakaya, K., Nakao, M., Xiao, N., Yamasaki, H., Sako, Y., Naitoh, Y., Kondo, S., Kobayashi, M., Ohtaishi, N. and Ito, A. 2005. *Echinococcus multilocularis* confirmed on Kunashiri Island, 15 kilometers from the eastern part of Hokkaido, Japan. *Am. J. Trop. Med. Hyg.* **72**: 284–288.

23 Smyth, J. D. 1964. The biology of the hydatid organisms. *Adv. Parasitol.* **2**: 169–219.

24 Smyth, J. D. and Smyth, M. M. 1964. Self insemination in *Echinococcus granulosus in vivo*. *J. Helminthol.* **43**: 383–387.

25 Thompson, R. C. A. 1988. Intraspecific variation and epidemiology. pp. 391–411. *In*: Parasitology in Focus (Melhorn, H. ed.). Springer, Berlin.

26 Thompson, R. C. A. and Lymbery, A. J. 1988. The nature, extent and significance of variation within the genus *Echinococcus*. *Adv. Parasitol.* **27**: 209–258.

27 Yamashita, J. 1988. *Echinococcus* and echinococcosis. pp. 65–123. *In*: Progress in Medical Parasitology, Japan (Morishita, K., Kamiya, Y. and Matsubayashi, H. eds.). Vol.5, Megro Parasitological Museum, Tokyo.

28 Zischler, H., Hinkkanen, A. and Studer, R. 1991. Oligonucleotide fingerprinting with $(CAC)_5$: Nonradioactive in-gel hybridization and isolation of individual hypervariable loci. *Electrophoresis* **12**: 141–146.

Mycopathologia (2007) 163:117–128
DOI 10.1007/s11046-007-0095-4

Protection induced in BALB/c mice by the high-molecular-mass (hMM) fraction of *Paracoccidioides brasiliensis*

Wander R. Pavanelli[1], Mari S. Kaminami[2], Juliana R. Geres[3], Ayako Sano[4], Mario A. Ono[2], I. C. C. Camargo[5] & Eiko N. Itano[2]

[1]*Fundação Araucária, Curitiba, PR, Brazil;* [2]*Department of Pathology Science, CCB, State University of Londrina, 86051-990, Londrina, PR, Brazil;* [3]*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasilia, Brazil;* [4]*Research Center of Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan;* [5]*Department of Biology Science, Paulista State University, Assis, SP, Brazil*

Received 30 October 2006; accepted in revised form 29 January 2007

Abstract

Paracoccidioidomycosis (PCM) is a granulomatous disease caused by a dimorphic fungus, *Paracoccidioides brasiliensis*. The present study investigated the protective activity of the *P. brasiliensis* high-molecular-mass (hMM) fraction (~380 kDa) in experimental murine PCM. In the first step, lymphocyte proliferation and production of IFNγ (but not IL-4) were observed in "in vitro" spleen cells (from female BALB/c mice infected (i.v.) with *P. brasiliensis*) that were stimulated with hMM fractions. In the second step, female BALB/c mice were previously immunized (s.c.) with hMM fraction (25 μg/protein = F-25 and 50 μg/protein = F-50), and the colony-forming units (CFU) of the lung and spleen, the histopathological characteristics of the granulomatous lesions, and plasmatic gp43 soluble antigens and anti-hMM IgG levels were analyzed at 28 and 56 days after infection. The lung and liver CFU were lower in mice previously immunized with the hMM fraction ($P < 0.05$). The granulomatous lesions revealed a greater degree of compaction and organization, with no dissemination of the fungus to other organs. Lower soluble antigen levels ($P < 0.05$) and higher IgG anti-hMM fraction ($P < 0.05$) were observed in immunized groups. The results for CFU, histopathology and antigenemia suggest that the hMM fraction has a protective effect in experimental paracoccidioidomycosis in BALB/c mice.

Key words: antigenemia, CFU, granuloma, *Paracoccidioides brasiliensis*, paracoccidioidomycosis, protection

Introduction

Paracoccidioidomycosis (PCM) is an infectious granulomatous disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*. The pathogen grows as a yeast form at a temperature of 35–37°C, or as a mycelium at 25 °C. It is restricted to Latin America, with extensive endemic areas in Brazil, Colombia, Venezuela and Argentina [1]. It is believed that about 10 million people are infected with the fungus; 2% of them may develop the disease [2]. The disease primarily involves the lungs, and then disseminates to other organs and systems. Two main clinical forms are recognized: acute (AF) and chronic (CF). The CF is more frequent in men over the age of 40, and has multiple forms, ranging from benign and localized (unifocal) to severe and disseminated (multifocal) disease [3].

Infection by *P. brasiliensis* is characterized by long latency periods. These periods are especially evident in imported cases, in which the disease

only appears decades after a patient visited an endemic region [4]. This means that during this period these patients have been able to control the fungal dissemination. Resistance to *P. brasiliensis* is linked to a cellular immune response associated with IFN-gamma production, as shown by clinical and experimental PCM data [5–7].

It has grown the investigations of fungal components that induce cellular immune response and host protection as alternative immuno-therapeutic method for treatment of PCM considering the toxicity of the current antifungal agents derived from azole and amphotericin-B [8, 9]. The most investigated component of *P. brasiliensis* is the gp43. Immunization with gp43 or 15-amino-acid peptide (P10), the immunodominant T cell epitope or even the gp43 gene protects against experimental infection in mice [10, 11]. Also the chromatography F0 and FII fractions from *P. brasiliensis* soluble antigens [12] or monoclonal antibodies to gp70 induce protection in murine PCM [13].

The yeast forms of *P. brasiliensis* also produce high-mass molecular antigens [14] as soluble forms [14]. In a previous study using a fraction, which was determined as ~380 kDa (hMM), we observed differences in IgG and IgE levels between the acute and chronic PCM forms [15].

In the current study, we evaluated the ability of the hMM fraction to induce a protective effect in experimental paracoccidioidomycosis in BALBc mice.

Material and methods

Cell-free antigen (CFA) and exoantigen (ExoAg)

CFA from *P. brasiliensis* (Pb18) was obtained according to Camargo et al. [16], modified by the addition of phenylmethylsulfonyl fluoride (PMSF) at 2.5 mM to the supernatant, which was subsequently frozen at -80 °C. A lyophilized exoAg was prepared from a yeast-phase culture of *P. brasiliensis* strain B-339 according to Camargo et al. [17].

Sephadex G-200 chromatography

Two milliliters of *P. brasiliensis* (Pb18) CFA (1 mg/ml) were applied to a Sephadex G-200 column (2 × 48 cm) buffered with 0.15 M PBS, pH 7.2. Fractions of 1 ml each were collected in an automatic fraction collector, and read in a spectrophotometer (Ultrospec-2000 Pharmacia Biotech UV/Visible) at 280 nm. The first peak fraction 17, considered as hMM, was characterized as ~380 kDa by Márquez et al. [15]. The hMM fraction correspondent to F17 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7% gel and silver stained. Prestained SDS-PAGE standards from BIO-RAD 161-0318 was used as protein standards.

Gp43 purification

Purified gp43 was obtained by affinity chromatography of the CFA of *P. brasiliensis* (Pb18) on Affigel-10 (Bio-Rad Laboratories, Hercules, CA, USA) with anti-gp43 Mab as a specific ligand. The gp43 was eluted with 0.1 M glycine–HCl, pH 2.8, and immediately neutralized with 2 M Tris, pH 9.0. Fractions (1.0 ml) were collected and read in a spectrophotometer at 280 nm. The fractions with the highest absorbance were mixed. The resulting pool was dialysed against 0.15 M PBS, and the protein concentration was determined.

Proliferation assay

Spleen cells from infected (28 days, 1×10^5 yeast cells) and normal mice were removed aseptically and teased. The erythrocytes were lysed with *tris*-ammonium chloride, and the cell suspension was washed three times in RPMI medium. For proliferative assays, 100 µl of the cells were cultured in triplicate wells at a concentration of 1×10^6 cells/ml in RPMI 1640 containing L-glutamine, 10% fetal calf serum in 96-well flat-bottom culture plates. About 100 µl of Ag (hMM, gp43, exoAg) was then added to each well at concentrations of 50 µg/ml. The cells were cultured for 4 days at 37 °C with 5% CO_2 and were pulsed with 1 µCi of [^{3}H] thymidine 18 h before harvesting on glass filter strips. The radioactivity was determined by liquid scintillation (Beckman LS 6.800). The Stimulation Index was calculated as the triplicate of stimulated cells (cells + Ag) divided by the cell control (cell + RPMI). For culture supernatant preparations, cells were cultured in the same conditions as for the proliferation assay for three days. Culture supernatants were collected and stored at –80°C.

Cytokine assays

IFNγ and IL-4 were determined by commercial ELISA kits: IFNγ (BD OptEIA), IL-4 (R&D System), according to the manufacturer's instructions. The reaction was revealed with peroxidase-conjugated streptavidin (Sigma) followed by the substrate mixture containing hydrogen peroxide and ABTS (Sigma) as a chromogen. The absolute cytokine level was calculated based on a standard curve provided by the manufacturer.

Immunization and experimental infection

Groups of 5–7 BALB/c mice (6–8-week-old females) were immunized by subcutaneous injection of 25 μg protein (F-25) (group a) and 50 μg protein (F-50) (group b) of hMM with Freund's adjuvant (three times at 2-week intervals, first with complete and second and third with incomplete Freund's adjuvant) and 45 days after infected i.v. with 1×10^5 yeast cells. The infected mice group, without immunization (group c) and normal mice inoculated with sterile PBS (group d) were used as controls. The animals were killed at days 28 and 56 post-infection.

Organ colony-forming units (CFUs)

The lung and liver fractions were removed, weighed, homogenized, and washed three times in phosphate-buffered was plated on brain–heart infusion (BHI) agar supplemented with 4% fetal serum and 5% spent culture medium from *P. brasiliensis* as a growth factor. Gentamycin and cloramphenicol were added at 60 and 100 mg/ml, respectively. The plates were incubated at 35°C and read after 8 days. The results were expressed as the number of viable *P. brasiliensis* CFUs per mg of tissue per mouse.

Histopathology

The lung and liver fractions were excised, fixed in 10% buffered formalin, and embedded in paraffin for sectioning. Sections were stained with haematoxylin-eosin (HE) and examined microscopically.

Detection of soluble gp43 in plasma

Immunoplates (TPP, Switzerland) were coated with rabbit IgG anti-gp43 (12 μg ml^{-1}), blocked with PBS-T- 5% skim milk for 1 h, and incubated with plasma samples (1/10) at 37 °C for 1 h. After washing, 30 μg ml^{-1} IgG anti-gp43 (monoclonal antibodies obtained by fusion of P3U1 cells and spleen cells from BALB/c mice immunized with gp43) was incubated for 1 h at 37 °C, and then incubated with 100 μl of goat anti-mouse IgG labeled with peroxidase (1:4000), according to Miura et al. [18] with some modifications. The reaction with OPD solution was stopped with 50 μl of 4 N H_2SO_4 and the absorbance read at 492 nm in a Multiskan EX reader (Labsystems).

Detection of anti-hMM fraction IgG

Immunoplates were coated with sephadex hMM fraction (25 μg ml^{-1}), blocked with PBS-T- 5% skim milk for 1 h, and incubated with plasma samples (1/10) at 37 °C for 1 h. They were then washed again and incubated with goat anti-mouse IgG labelled with peroxidase (A2554; Sigma-Aldrich), diluted 1:4000, at 37 °C for 1 h. Next, 100 μl of substrate solution was added (5 mg ortho-phenylenediamine, 10 ml of 0.1 M citrate buffer, pH 4.5 and 10 μl H_2O_2 After 15 min, the reaction was stopped with 50 μl 4 N H_2SO_4, and the absorbance was read in a Multiskan EX reader at 492 nm, according to Miura et al. [18] with some modifications.

Statistical analysis

Statistical comparisons were made by analysis of variance (ANOVA) and by Tukey's test. All values are reported as the mean ± SD of the mean, with significance assumed in the range of $P < 0.05$.

Results

Sephadex G–200 chromatography

Samples of *P. brasiliensis* CFA were applied to a Sephadex G-200 column balanced with 0.15 M PBS, pH 7.2. Fractions were collected in an

automatic fraction collector, and read in a spectrophotometer (Ultrospec-2000 Pharmacia Biotech UV/Visible) at 280 nm (Figure 1A). Fraction 17 considered as hMM fraction was submitted to SDS-PAGE 7% and stained with silver stain (Figure 1B). The approximate molecular mass was calculated based on protein standards molecular masses.

Proliferation assay

The spleen cells from mice at 28 days post-infection were stimulated "in vitro" with *P. brasiliensis* antigens (exoAg, gp43 and hMM fraction) for evaluation of cell immune response. A higher proliferative response was found in the spleenocytes stimulated (5 days) with hMM and exoAg than gp43 and those from the controls mice inoculated with PBS. Mean values ± SD of the proliferation index (cpm of test well/cpm of control without antigens) of triplicate cultures are shown in Figure 2A.

Cytokine levels

The spleen cells from mice at 28 days post-infection were stimulated "in vitro" with *P. brasiliensis* antigens (hMM fraction, gp43 and exoAg) for cytokines detection in culture supernatants (IFNγ and IL-4 from the 3 days culture supernatants). A higher IFNγ level was detected in the supernatant of stimulated spleenocytes with hMM than gp43 and exoAg in infected mice by ELISA (Figure 2B). No difference was observed with IL-4 levels between those groups (Figure 2C).

Analysis of the protection using the CFU technique

The lung and liver fractions from 28 and 56 days post-infection or immunized and infected (immunized previously with 25 μg protein and 50 μg protein) were homogenized, plated on BHI agar culture medium and the results expressed as CFUs per mg of tissue. The lung CFU results for mice previously immunized with the hMM fraction (F-25 and F-50) showed fewer colonies compared to the group of infected mice, for both time periods (28 and 56 days), with $P < 0.001$ for the 28-day period and $P < 0.05$ for the 56-day period (Figure 3A). The same result was observed for the liver CFU, with significant values in relation to the infected animals, with variations of $P < 0.01$ and $P < 0.05$ for both groups F-25 and F-50, and both periods (Figure 3B).

Histopathological analysis

At 28 days post-infection, mice immunized with F-25 of the hMM fraction showed the formation of exudative lesions with compact granulomas, with some cells of *P. brasiliensis* observed in the lung and liver but with no dispersion to the spleen. At 56 days, small granulomas with only a few fungal cells were observed. The animals immunized with

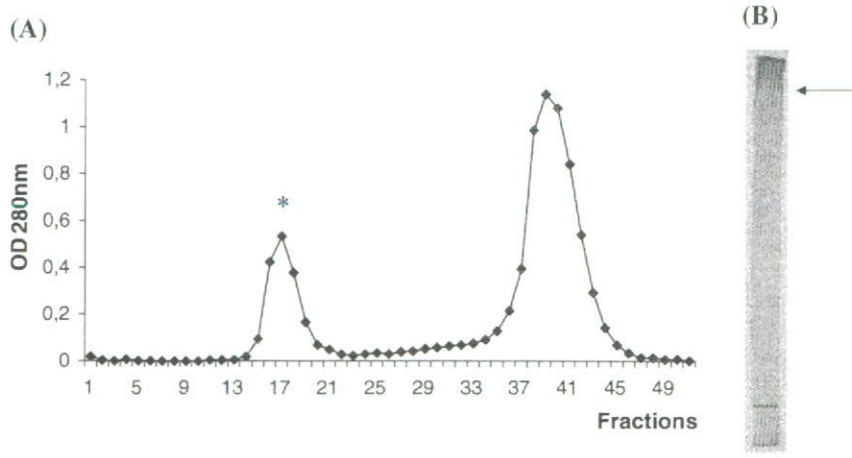

Figure 1. (A) Spectrophotometric profile at 280 nm of CFA fractions chromatography in Sephadex G-200. The fractions collected with an automatic fraction collector were read in a spectrophotometer at 280 nm and the fraction 17 (*) considered as hMM fraction. (B) The hMM fraction correspondent to F17 analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7% gel and silver stained (← ~380 kDa).

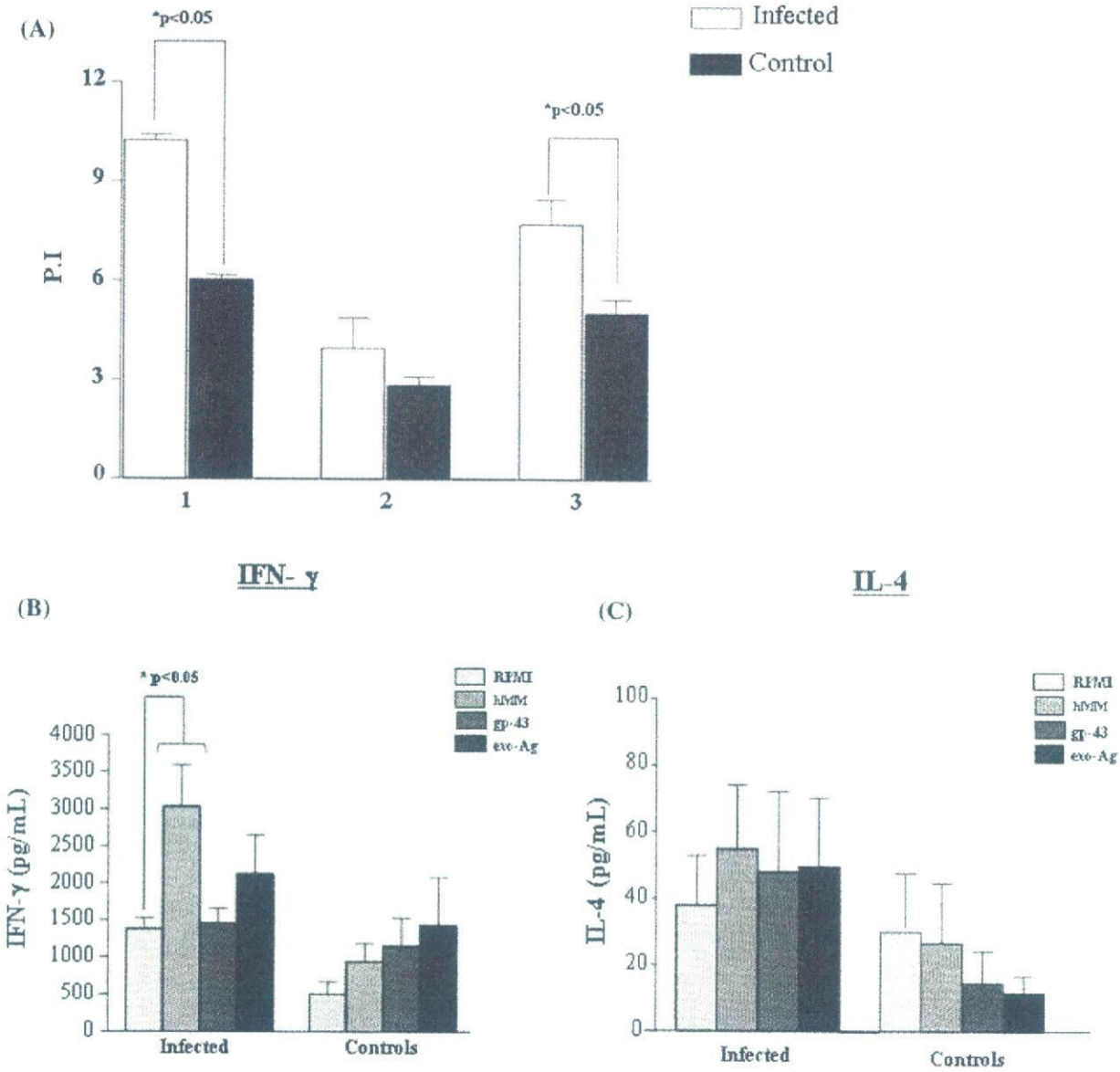

Figure 2. (A) Lymphoproliferative response. Spleenocytes stimulated during 5 days with hMM fraction (1), gp43 (2) and exoAg (3) (50 μg/ml) (spleenocytes from infected BALB/c mice, 1×10^5 Pb18, 28 days post-infection or not infected). Mean values ± SD of the proliferation index (cpm of test well/cpm of control) of triplicate cultures. (B) IFNγ in culture supernatants. Spleens cells from infected BALB/c mice (1×10^5 Pb18) or not infected (control) were cultured for 48 h with hMM fraction, gp43 and exoAg (50 μg/ml) or without stimulus (RPMI). IFNγ was analyzed by capture ELISA (BD OptEIA). (C) IL-4 in culture supernatants. Spleens cells from infected BALB/c mice (1×10^5 Pb18) or not infected (control) were cultured for 48 h with hMM fraction, gp43 and exoAg (50 μg/ml) or without stimulus (RPMI). IL-4 was analyzed by capture ELISA (R&D System).

F-50, at both 28 and 56 days showed small compact granules, with few or no cells present in the liver and spleen. For the infected and non-immunized animals, at both periods we observed giant granules with innumerable viable cells of *P. brasiliensis* in the lung and liver, and extensive tissue destruction with exudative epithelioid granulomas (Figures 4, 5 and Table 1).

Level of soluble antigen gp43 (Antigenemia) in the plasma

Immunoplates coated with rabbit IgG anti-gp43, incubated with plasma samples (1/10), IgG monoclonal anti-gp43, goat anti-mouse IgG labeled with peroxidase were used for antigenemia analysis. Soluble gp43 levels, in OD at

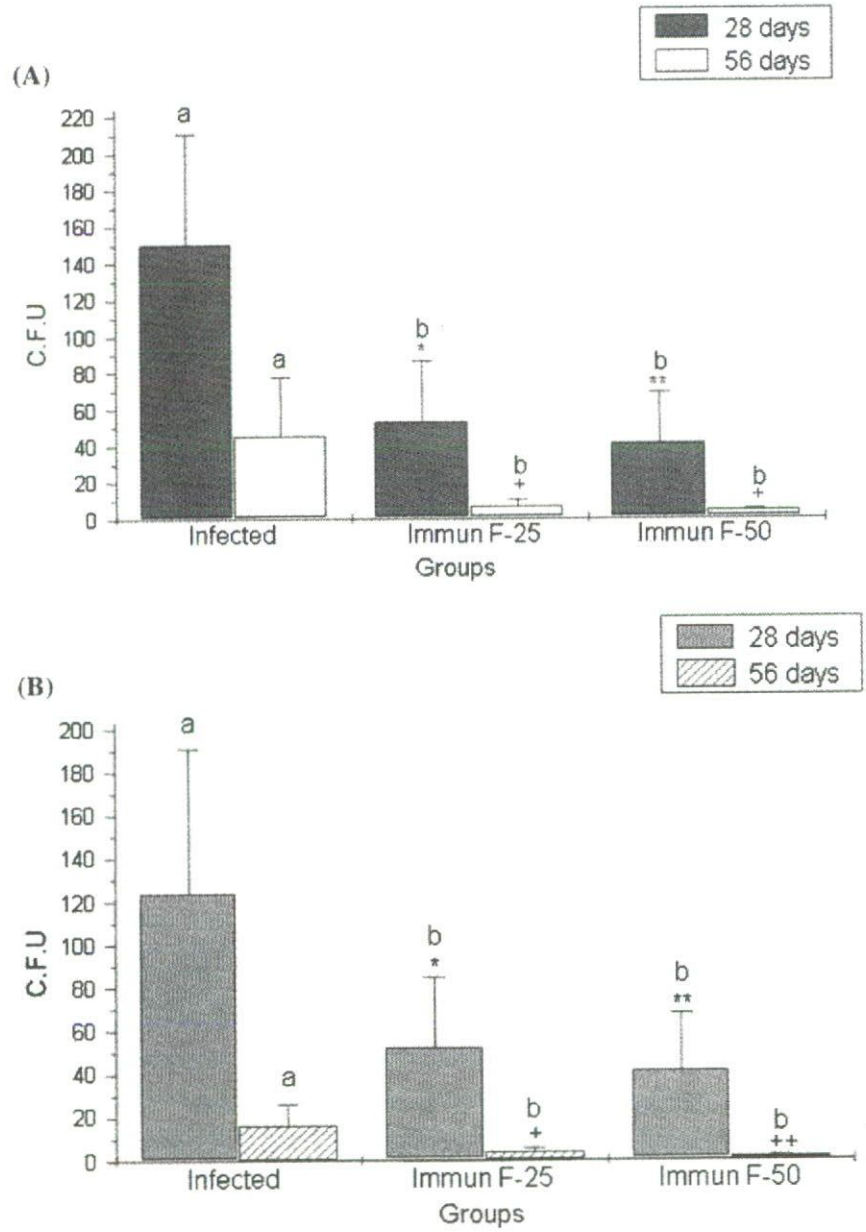

Figure 3. (A) Lung colony-forming units (CFU). Median number of *P. brasiliensis* CFU in the lungs of mice immunized with hMM fraction. The results are expressed as the number of viable *P. brasiliensis* CFUs per mg of tissue per mouse after 8 days in BHI agar medium in groups: Infected = infected with 10^5 *P. brasiliensis* during 28 days or 56 days, Immuni F-25 = immunized with 25 μg of hMM fraction and infected with 10^5 *P. brasiliensis*, Immuni F-50 = immunized with 50 μg of hMM fraction and infected with 10^5 *P. brasiliensis*, during 28 days or 56 days. (n = 08). Infected × Immuni F-25 or F-50 $^{**}P < 0.01$, $^{*}P < 0.05$ for 28 days and $^{+}P < 0.05$ for 56 days. a and b inside of group for period of infection. (B) Liver colony-forming units (CFU). Median number of *P. brasiliensis* CFU in the livers of mice immunized with hMM fraction. The results are expressed as the number of viable *P. brasiliensis* CFUs per mg of tissue per mouse after 8 days in BHI agar medium in groups: Infected = infected with 10^5 *P. brasiliensis* during 28 days or 56 days, Immuni F-25 = immunized with 25 μg of hMM fraction and infected with 10^5 *P. brasiliensis*, Immuni F-50 = immunized with 50 μg of hMM fraction and infected with 10^5 *P. brasiliensis*, during 28 days or 56 days. (n = 08). Infected × Immuni F25 or F50 $^{**}P < 0,01$, $^{*}P < 0,05$ for 28 days and $^{++}P < 0,01$, $^{+}P < 0,05$ for 56 days. a and b inside of group for period of infection.

492 nm, were significantly lower in the animals immunized with the hMM fraction (F-25 and F-50) compared to the control infected group at 28 days ($P < 0.05$). After 56 days, only the group of mice immunized with hMM fraction F-50 showed significantly lower levels

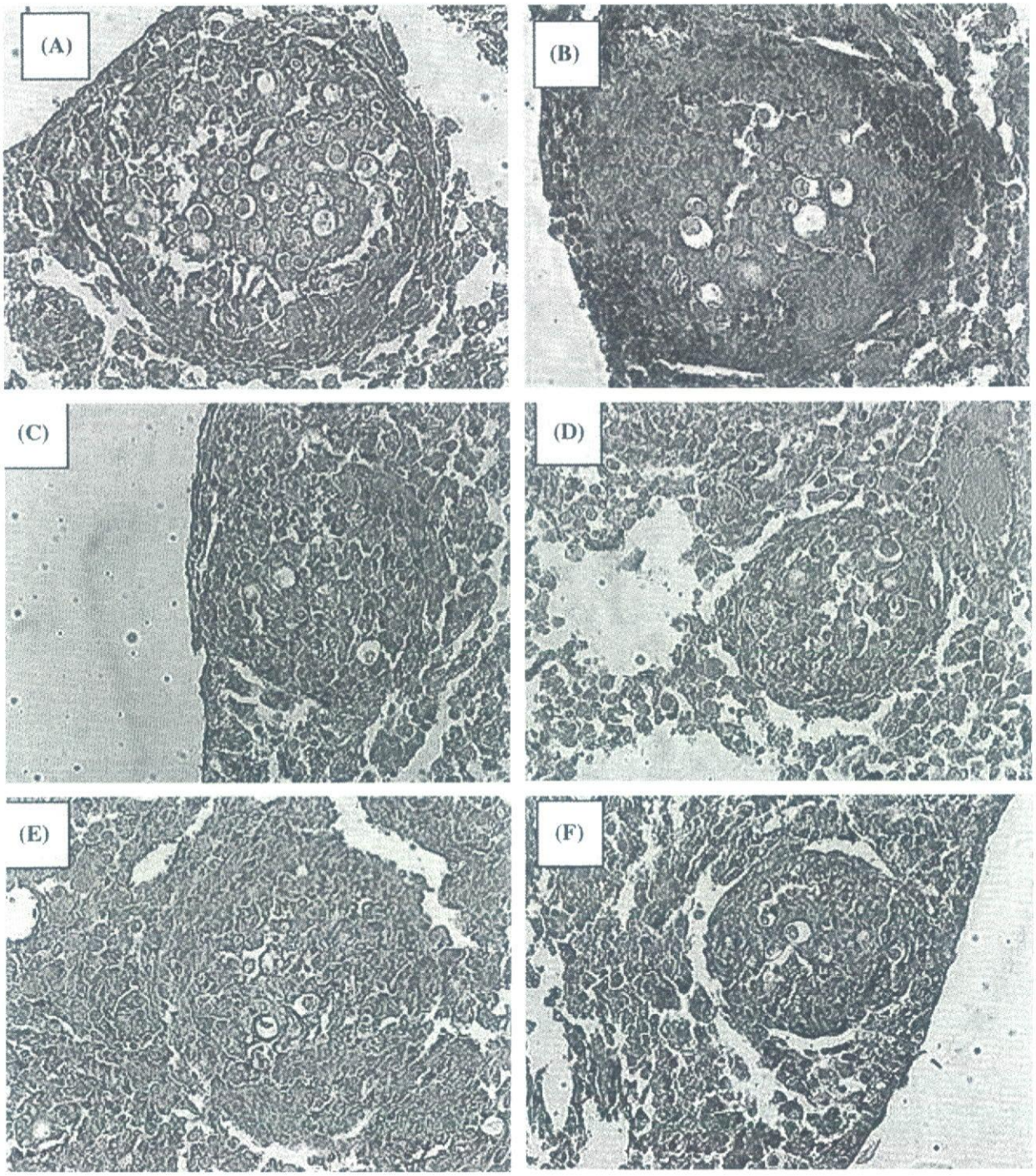

Figure 4. Lung histopathology in immunized mice with hMM fraction A and B: Infected with 10^5 *P. brasiliensis*, C and D: immunized with 50 μg of hMM fraction and infected with 10^5 *P. brasiliensis*, E and F: immunized with 25 μg of hMM fraction and infected with 10^5 *P. brasiliensis*. A, C and E: 28 days post-infection and B, D and F: 56 days post-infection.

in relation to the infected group ($P < 0.05$). This demonstrated a dose-dependent relationship during the course of the infection (Figure 6A).

Level of IgG anti-hMM fraction

For evaluation of humoral immune response to hMM fraction, immunoplates coated with hMM

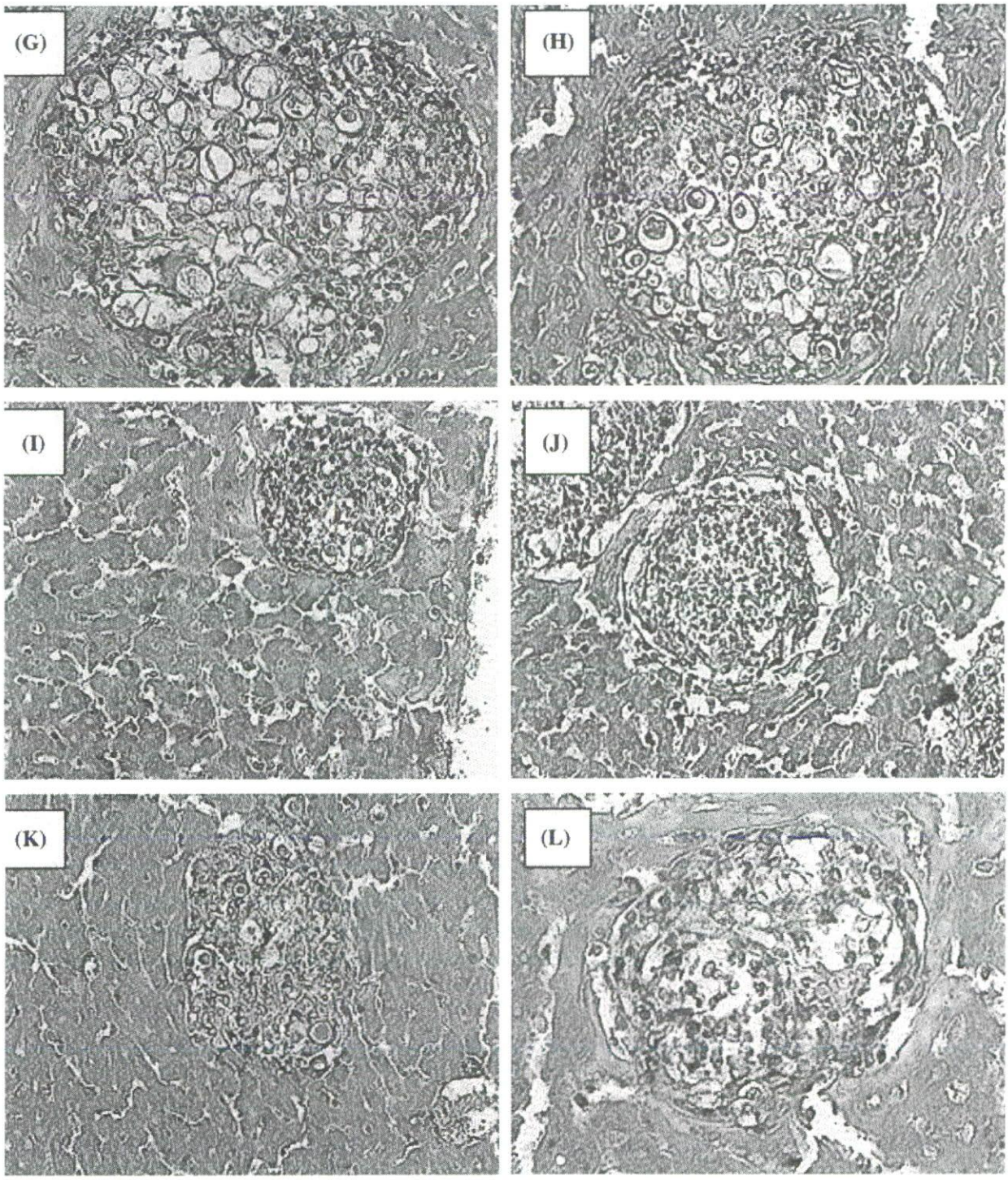

Figure 5. Liver histopathology in immunized mice with hMM fraction. G and H: Infected with 10^5 *P.* brasiliensis, I and J: immunized with 50 μg of hMM fraction and infected with 10^5 *P. brasiliensis*, K and L: immunizated with 25 μg of hMM fraction and infected with 10^5 *P. brasiliensis*. G, I and K: 28 days post-infection and H, J and L: 56 days post-infection.

fraction incubated with plasma samples (1/10), goat anti-mouse IgG labelled with peroxidase were used. The results of ELISA, expressed as OD at 492 nm, were higher in the groups of mice immunized with the hMM fraction (F-25 and F-50) after 28 days, compared to the infected mice. However,

Table 1. Number of intragranuloma fungics cells in immunized mice with hMM fraction

	Organs/Period				
	Lung/28 days	Liver/28 days	Lung/56 days	Liver/56 days	
Number of fungi	8***	6.8***	2.7***	1.3***	F-50
	15.3**	9**	4***	3.3***	F-25
	34.2	23.2	19.6	19.5	Infected

Infected = infected with 10^5 *P. brasiliensis*, F-25 = immunized with 25 μg of hMM fraction and infected with 10^5 *P. brasiliensis*, F-50 = immunized with 50 μg of hMM fraction and infected with 10^5 *P. brasiliensis*. ($n = 6$).
Infected × immunized ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

after 56 days, only the mice immunized with hMM fraction F-50 induced a significant increase in IgG levels compared to the infected group (Figure 6B).

Discussion

Because of the toxicity of the antifungal agents in current use [8, 9], alternative treatment strategies such as immunotherapeutic procedures become interesting for the treatment of PCM, a disease that affects millions of people in Central and South America [2] and the eighth most common cause of death from predominantly chronic or recurrent types of infectious and parasitic diseases in Brazil [19].

Most investigations concerning protection against *P. brasiliensis* have focused on gp43 [10, 11]. Although this is promising, other components that have not yet been investigated, such as hMM components, may be important as alternative candidates for immunotherapeutic procedures or to improve understanding of the host–parasite interaction.

In the present work, "in vitro" spleenocytes proliferation and IFNγ production were observed when spleenocytes from mice infected with *P. brasiliensis* were cultured with the hMM fraction, suggesting that hMM antigens could evoke a cell-mediated immune response, that is the most relevant defense mechanism in PCM [5, 6,]. Then, the results stimulated us to investigate the possibility that these fractions can confer protection.

The results demonstrated a lower number of viable fungi in the lung and liver by CFU, and also the reduced number of fungal cells in the granulomas in immunized groups in both periods (28 and 56 days post-infection) by histological analysis, suggesting the protection activity of hMM fractions. Moreover, the granulomatous lesions formed were fewer, smaller, and more compact and organized in the immunized animals.

The morphology of the granulomatous lesions is related to the host's cellular immune response: the formation of compact granules with few fungus cells is observed in patients with localized chronic disease, whereas in patients with the acute form the lesions are generally disorganized. The same occurs in immunosuppressed animals with low levels of IFNγ, production of Th-2 cytokines and progressive disease, with the presence of many disorganized granulomatous lesions [20–22].

Also in concordance, the lower level of soluble antigen was observed in sera of mice immunized with the hMM fraction, mainly in the group immunized with 50 μg of the hMM fraction. The efficiency of this larger dose could also be seen in the results of the lung and liver CFU at 28 and 56 days, with higher significance for F-50 than F-25. Studies with higher doses may provide better evidence of this effect. The adjuvant used may not have contributed to the protection, considering that no difference in the severity of the disease was observed between the group with adjuvant and the group without adjuvant (data not shown).

In the present work, we also observed a high level of IgG to hMM fraction, suggesting that the hMM fraction contains antigens with T cell as well as B cell epitopes. The potential efficacy of humoral immunity in protecting the host against pathogenic fungi, such as antibodies to *Cryptococcus neoformans* polysaccharides or to *Candida albicans* cell wall components [23] and also anti-gp70 MAbs [13] in experimental PCM, has been described. On the other hand, the humoral immune response is considered not protective in PCM, because a strong humoral response is correlated with severity of the disease [24]. Then if the IgG to hMM fraction participate in protection or pathogenesis in PCM requires further studies.

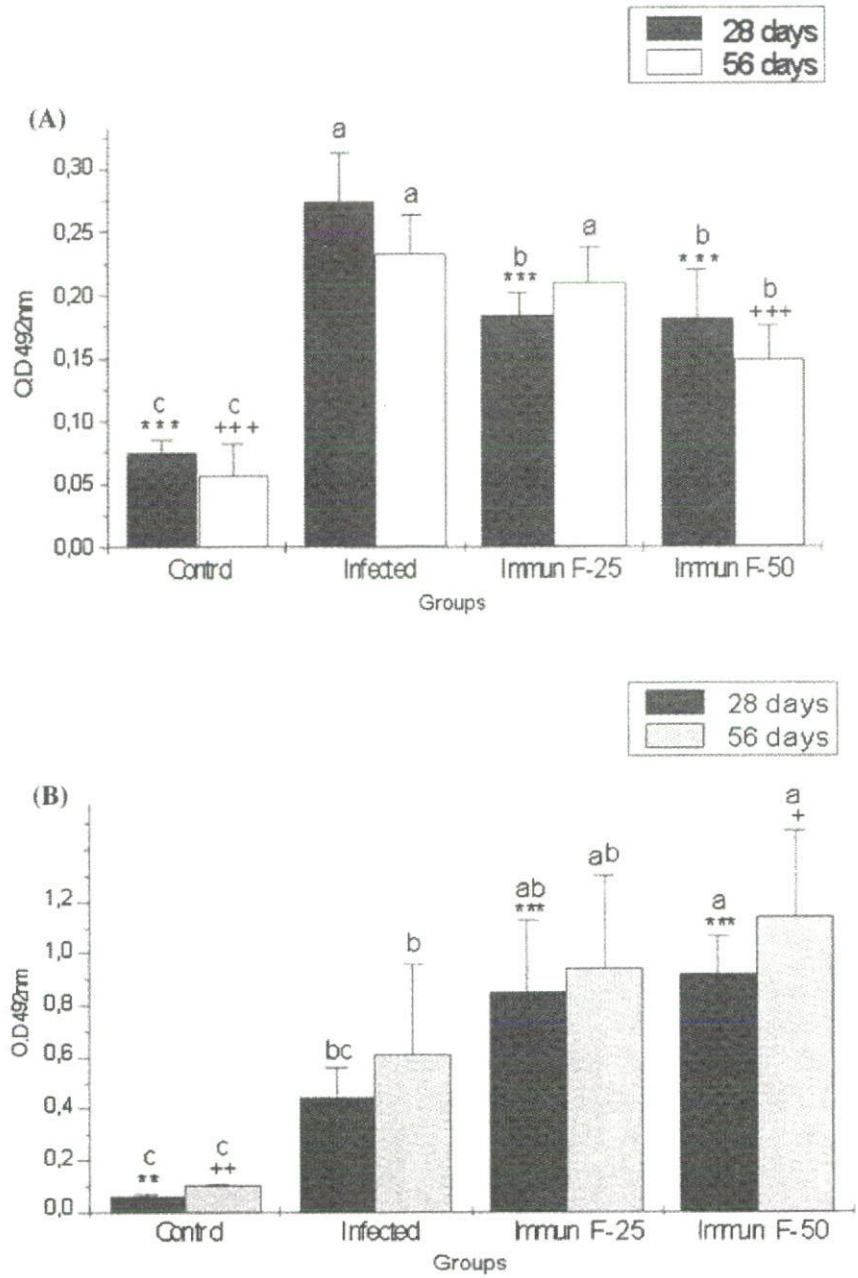

Figure 6. (A) Level of soluble antigen gp-43 (Antigenemia) in the plasma: ELISA plates coated with rabbit IgG anti-gp43 incubated with diluted plasma (1/10), IgG (mouse Mab) anti-gp43 and with peroxidase-conjugated anti-mouse IgG. Results are expressed in optical densities (OD) at 492 nm as mean values and SD. Control = not infected, Infected = infected with 10^5 *P. brasiliensis*, Immuni F-25 = immunized with 25 µg of hMM fraction and infected with 10^5 *P. brasiliensis*, Immuni F-50 = immunized with 50 µg of hMM fraction and infected with 10^5 *P. brasiliensis*. Control or infected × Immuni F-25 or F-50. $***P < 0.001$ for 28 days and $+++P < 0.001$ for 56 days. a, b and c inside of group for period of infection. (B) Level of IgG anti-hMM fraction: The results of ELISA expressed as optical densities (OD) were higher in the groups of mice immunized with the hMM fraction (F-25 and F-50) after 28 days, compared to the infected mice. However, after 56 days, only the mice immunized with hMM fraction F-50 induced a significant increase in IgG levels compared to the infected group.

The low proliferation of spleenocytes observed with gp43 is in accordance with literature data [25]. Apoptosis may be one of the mechanisms leading to hyporesponsiveness [26].

By western blotting Márquez et al. [15] observed one band (~386 kDa) in hMM fraction. In this study we observed two high-molecular-mass bands in hMM fraction by SDS-PAGE. The hMM fraction is partially purified and may contain other components in addition to protein, which could also contribute to protection. This possibility will be the object of future investigations.

Based on the results of CFU, histopathology and antigenemia, we conclude that the hMM fraction shows a protective effect in experimental PCM in BALB/c mice. However, additional studies with more extensive time-course observations associated with mouse strains and fungal isolates are necessary. Also in order to contribute to knowledge of protection mechanisms, determination of the Igs isotypes and subtypes, and further investigation of cytokines are required.

Acknowledgments

This work was supported by FINEP 0.1.04.0215.00, CAPES and Fundação Araucária,PR. We thank Nilson de J. Carlos for technical assistance and Dr. Janet W. Reid for her careful correction of the English text.

Reference

1. Brummer E, Castañeda E, Restrepo A. Paracoccidioidomycosis an update. Clin Microbiol Rev 1993; 6: 89–117.
2. McEwen JG, Garcia AM, Ortiz BL, Botero S, Restrepo A. In search of the natural habitat of *Paracoccidioides brasiliensis*. Arch Med Res 1995; 26: 305–306.
3. Franco MF, Mendes RP, Rezkallah-Iwasso M, Montenegro MR. Paracoccidioidomycosis. Baillières Clin Trop Med Commun Dis 1989; 4: 185–219.
4. Wanke B, Londero AT. Epidemiology and paracoccidioidomycosis infection. In: Franco M, Lacaz CS, Restrepo-Moreno A, Del-Negro G, eds. *Paracoccidioidomycosis*, 1st ed., CRC Press, Inc, Boca Raton, Florida, 1994: 109–120.
5. Oliveira SJ, Mamoni RL, Musatti CC, Papaiordanou PMO, Blotta MHSL. Cytokines and lymphocyte proliferation in juvenile and adult forms of paracoccidioidomycosis: comparison with infected and non-infected controls. Microbes Infect 2002; 4: 139–144.
6. Kashino SS, Fazioli RA, Cafalli-Favati C, Meloni-Bruneri LH, Vaz CA, Burger E, Singer LM, Calich VL. Resistance to *Paracoccidioides brasiliensis* infection is linked to a preferential Th1 immune response, whereas susceptibility is associated with absence of IFN-gamma production. J Interferon Cytokine Res 2000; 20: 89–97.
7. Calich VLG, Kashino SS. Cytokines produced by susceptible and resistant mice in the course of *Paracoccidioides brasiliensis* infection. Braz J Med Biol Res 1998; 31: 615–623.
8. Graybill JR, Tollemar J, Torres-Rodríguez JM, Walsh TJ, Roilides E, Farmaki E. Antifungal compounds: Controversies, queries and conclusions. Med Mycol 2000; 38(supplement I): 323–333.
9. Gubbins PO, Amsden JR. Drug–drug interactions of antifungal agents and implications for patient care. Expert Opin Pharmacother 2005; 6(13): 2231–2243.
10. Taborda CP, Juliano MA, Puccia R, Franco MV, Travassos LR. Mapping of the T-cell epitope in the major 43-kilodalton glycoprotein of *Paracoccidioides brasiliensis* which induces a Th-1 response protective against fungal infection in Balb/c mice. Infect Immun 1998; 6: 786–793.
11. Pinto AR, Puccia R, Diniz SN, Franco MF, Travassos LR. DNA-based vaccination against murine paracoccidioidomycosis using the gp43 gene from *Paracoccidioides brasiliensi*. Vaccine 2000; 18: 3050–3058.
12. Diniz SN, Reis BS, Zouain CS, Leite MF, Goes AM. Protective immunity induced in mice by F0 and FII antigens purified from *Paracoccidioides brasiliensis*. Vaccine 2004; 22: 485–492.
13. Grosso DM, Almeida SR, Mariano M, Lopes JD. Characterization of gp70 and anti-gp70 monoclonal antibodies in *Paracoccidioides brasiliensis* pathogenesis. Infect Immun 2003; 71: 6534–6542.
14. Puccia R, Schenkman S, Gorin PAJ, Travassos LR. Exocellular components of *Paracoccidioides brasiliensis*: Identification of a specific antigen. Infect Immun 1986; 53: 199–206.
15. Márquez AS, Vicentini P, Ono MA, Watanabe MAE, Camargo ZP, Itano EN. Reactivity of antibodies from patients with acute and chronic paracoccidioidomycosis to a high-mass molecular antigen from *Paracoccidioides brasiliensis*. J Clin Labor Anal 2005; 19: 199–204.
16. Camargo ZP, Taborda CP, Rodrigues EG, Travassos LR. The use of cell-free antigens of *Paracoccidioides brasiliensis* in serological tests. J Med Vet Mycol 1991; 29: 31–38.
17. Camargo ZP, Unterkircher C, Campoy SP, Travassos LR. Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. J Clin Microbiol 1988; 26: 2147–2151.
18. Miura CSN, Estevão D, Lopes JD, Itano EN. Levels of specific antigen (gp43), specific antibodies, and antigen-antibody complexes in saliva and serum of paracoccidioidomycosis patients. Med Mycol 2001; 39(5): 423–428.
19. Coutinho ZF, Silva D, Lazéra M, Petri V, Oliveira RM, Sabroza PC, Wanke B. Paracoccidiodomycosis mortality in Brazil (1980–1995). Cad Saúde Pública, Rio de Janeiro 2002; 18(5): 1441–1454.
20. Defavrei J, Rez J, Rezkallah-Iwasso MT, Franco MF. Experimental pulmonary paracoccidioidomycosis in mice: Morphology and correlation of lesions with humoral and