EVALUATION OF 8-kDa SUBUNITS OF ANTIGEN B FROM *ECHINOCOCCUS MULTILOCULARIS* FOR SERODIAGNOSIS OF ECHINOCOCCOSIS

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Abstract. Antigen B (AgB) is a thermostable polymeric lipoprotein of 160 kDa and an important diagnostic antigen for serodiagnosis of human cystic echinococcosis due to its promising sensitivity and specificity. Now it has been proven that this protein is encoded by a gene family. Our recent studies demonstrated the existence of homologues of these genes in *E. multilocularis*. Five different cDNAs and corresponding genomic DNA clones encoding homologues of *EgAgB* 8-kDa subunits were identified as EmAgB8/1, EmAgB8/2, EmAgB8/3, EmAgB8/4, and EmAgB8/5. These genes appeared to have been expressed in a developmentally regulated manner in the parasite life cycle. Our current work focused on serological evaluation of recombinant proteins of EmAgB 8-kDa subunits for the immunodiagnosis of human echinococcosis and dogs infected with *Echinococcus*. Western blot analysis indicated that rEmAgB8/1 is the most useful antigen for serodiagnosis of human cystic echinococcosis with highest sensitivity and specificity (81.1%) among the five subunits of EmAgB; rEmAgB8/3 can be used as a candidate antigen for establishment of coproantigen test for diagnosis of dogs infected with *Echinococcus*.

INTRODUCTION

Cystic echinococcosis (CE) and alveolar echinococcosis (AE), caused by the larval stage of *Echinococcus granulosus* and *Echinococcus multilocularis*, respectively, are the clinically and epidemiologically most important forms of human echinococcosis (Craig *et al*, 1996; Schantz *et al*, 1999; Pawlowski *et al*, 2001; Ito *et al*, 2003). The metacestode of *E. granulosus* usually develops in patients into a fluid-filled unilocular cyst with relatively thick cyst walls and an additional fibrous outer layer, originating from the host. By contrast, the metacestode of *E. multilocularis* exhibits a multivesicular, tumor-like infiltrating structure that has a poorly defined barrier between parasite and host tissue, usually containing a semisolid matrix rather than fluid (Siles-Lucas and Gottstein, 2001).

Patients infected with either parasite are exposed to a variety of parasite-derived antigenic molecules that may evoke host-immune responses. Antigen B (AgB) was described initially from *E. granulosus* hydatid cyst fluid (Oriol *et al*, 1971); it is a polymeric lipoprotein of 160 kDa. In SDS-PAGE, the EgAgB disassociates to produce several subunits with molecular sizes of 8, 16, 24, and 32 kDa; the higher-sized subunits are composed of the 8-kDa subunit component (Lightowlers *et al*, 1989; González *et al*, 1996). Furthermore, the 8-kDa subunit band itself was also found to be comprised of a protein family (EgAgB, 8-kDa monomers) that is encoded by a multigene family (Fernández *et al*, 1996; Chemale *et al*, 2001; Arend *et al*, 2004). The AgB is a highly immunogenic major component of hydatid cyst fluid; it is a most sensitive and specific parasite antigen among other *Echinococcal* antigens in the serodiagnosis of human CE. Serological studies utilizing EgAgB revealed cross-reactions with antibodies in the sera of patients with AE infections (Lightowlers *et al*, 1989; Maddison *et al*, 1989; Ito *et al*, 1999; Mamuti *et al*, 2002).

This suggests that a molecule closely related

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to EgAgB was also expressed in the metacestode of *E. multilocularis*. Further studies using molecular and serological tools confirmed the existence of AgB in the metacestode of *E. multilocularis*. A partial DNA homologous to the EgAgB8/1 (EmAgB8/1) was isolated from metacestode of *E. multilocularis* (Frosch *et al*, 1994), and its full-length cDNA was isolated and characterized by Mamuti *et al* (2004). Serological studies revealed that the recombinant protein encoded by EmAgB8/1 (rEmAgB8/1) demonstrated almost the same serodiagnostic value as that of the recombinant protein encoded by EgAgB8/1 (rEgAgB8/1). Recently, four additional cDNAs that encode other 8-kDa subunit monomers of EmAgB were isolated from vesicles, protoscoleces, and immature adult worms of *E. multilocularis*. These genes are expressed in a regulated manner at different developmental stages of the parasite (Mamuti *et al*, 2006). However, the serodiagnostic values of the recombinant proteins of other EmAgB 8-kDa subunit monomers are still unknown. This study aimed to evaluate the serodiagnostic properties of the remaining 8-kDa subunit monomers of EmAgB and to select the most sensitive and specific recombinant protein for serodiagnosis of human echinococcosis and dogs infected with *E. granulosus* adult worms.

MATERIALS AND METHODS

Serum samples

Human serum samples of CE and AE were collected from the following groups of patients after obtaining appropriate ethics approvals and patients' informed consent (Table 1). Thirty serum samples were taken from patients with CE confirmed by surgery in China and 60 serum samples from CE patients confirmed by serology using Antigen 5, with or without surgery, in Australia (Lightowlers *et al*, 1989, 1995). These were followed by WB and ELISA with antigen B in hydatid cyst fluid of *E. granulosus* at Asahikawa Medical College (AMC), Japan (Mamuti *et al*, 2002). Fifty serum samples were taken from AE patients diagnosed by surgery at Hokkaido University Hospital and by Em18-serology and at AMC, Japan; and 23 serum samples were taken from AE patients diagnosed by surgery plus serology in France using a commercially available kit (Liance *et al*, 2000; Ito *et al*, 2002). These serum samples were obtained from patients who had been confirmed as CE or AE, and who do not necessarily represent samples taken from patients prior to their commencement of treatment, by either surgery and/or chemotherapy. Fifteen serum samples were collected in Australia from *E. granulosus* infected dogs, confirmed by necropsy after obtaining appropriate ethics approval.

Recombinant antigens and Western blots

The recombinant proteins corresponding to each 8-kDa subunit monomer of EmAgB were expressed in a bacterial expression system and purified as described in a previous report (Mamuti *et al*, 2004, 2006, 2007). SDS-PAGE and Western blots were carried out as described by Ito *et al* (1999), with some modifications. In brief, approximately 2 mg of each recombinant protein that corresponded to a related 8-kDa subunit monomer of EmAgB was separated on a two-dimensional 4-20% polyacrylamide gradient gel (6 cm wide, Tefco Co, Nagano, Japan) and transferred electrophoretically onto a PVDF membrane. Each membrane was cut into fifty strips and probed with diluted human sera (1:50). Bound antibodies were detected using rec-Protein G-peroxidase conjugate (Zymed Laboratories, USA) at 1:1,000 dilution, and 4-chloro-1-naphtol (Nacalai Tesque Inc, Japan) as substrate at a final concentration of 0.05%.

RESULTS

Echinococcosis in humans

Recombinant proteins corresponding to each 8-kDa subunit monomer of EmAgB were successfully expressed and purified from the bacterial lysate. The purified recombinant proteins were applied for subsequent serological tests in Western blots after removal of fusion partners. The results obtained in Western blots are summarized in Table 1. The sensitivity of these recombinant proteins to detect IgG antibodies in serum samples from CE and AE varied with each other. The representative data

are shown in Fig 1. The rEmAgB8/1 showed the highest sensitivity (81.1%) to detect IgG antibodies in serum samples from CE patients than that (41.1%) from AE patients. These results are in agreement with our previous study and the possible reason underlining such phenomenon was discussed in our previous report (Mamuti *et al*, 2004). However, all other recombinant antigens revealed lower reactivities with these serum samples from both CE and AE. Especially, the rEmAgB8/3 showed lowest reactivity with CE serum samples rather than other recombinant antigens. This may be due to the lower immunogenicity of the EmAgB8/3;

Table 1
Summary of serological results obtained in Western blots.

Serum sample sources	No. of samples tested	No. of serum sample positive (%) with indicated antigens			
		rEmAgB8/1	rEmAgB8/2	rEmAgB8/3	rEmAgB8/4
CE patients from:					
Australia	60	48 (80.0)	26 (43.3)	5 (8.3)	29 (48.3)
China	30	25 (83.3)	20 (66.7)	5 (16.7)	16 (53.3)
AE patients from:					
Japan	50	19 (38.0)	7 (14.0)	9 (18.0)	11 (22.0)
France	23	11 (47.8)	7 (30.4)	6 (26.1)	5 (21.7)
Dogs infected with *E. granulosus* from: Australia	15	ND	ND	1 (6.7)	ND

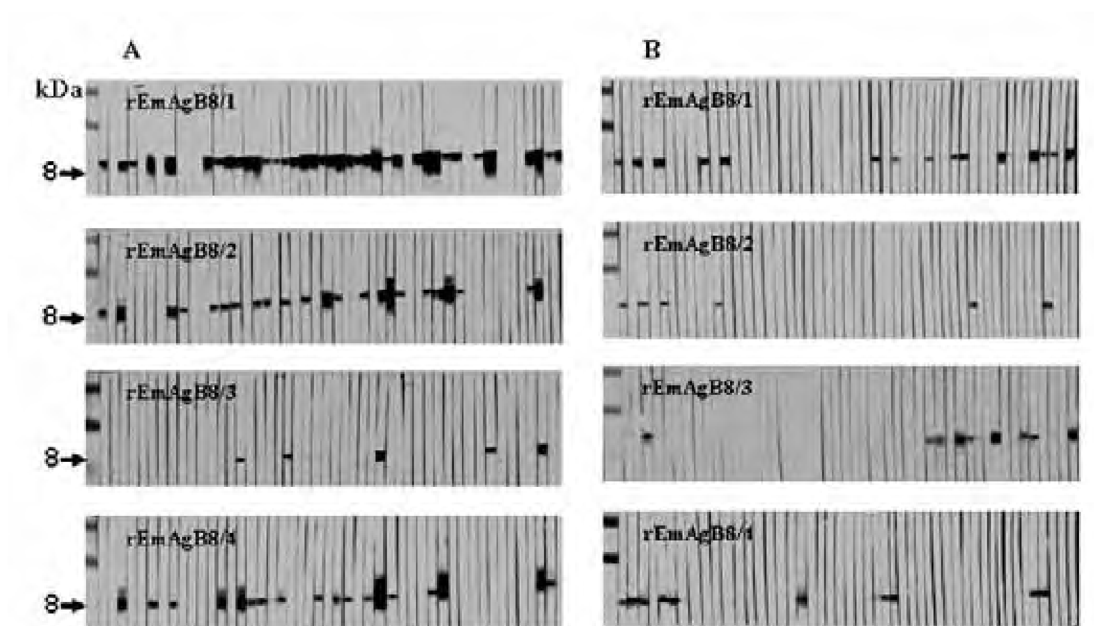

Fig 1- Comparative analysis of recombinant proteins of EmAgB 8-kDA subunit monomers by Western blots. Representative data are shown. The rows 1-4 are the membranes which were transferred with rEmAgB8/1-rEmAgB8/4, respectively. The membrane then probed with serum samples from CE patients (column A) or AE patients (column B). The numbers on the left are molecular sizes (in kilodaltons) of the reacted antigens with IgG antibodies in patients' sera.

even it was expressed in a considerable amount in metacestode stage of the parasite (Mamuti *et al*, 2006). Comparative analysis revealed that rEmAgB8/2, rEmAgB8/4, or rEmAgB8/3 could only be detected by the CE serum sample, which could detect rEmAgB8/1 (Fig 1, column A). However, this was not evident when these antigens were probed with serum samples from AE patients. In these cases, some serum samples were positive with rEmAgB8/3 or rEmAgB8/4, but not positive with the EmAgB8/1. This may be associated with distinctive post-translation, polymerization process, and immunogenic mechanism of the antigen B 8-kDa subunits in these two species.

Echinococcosis in dogs

Additional Western blots were carried out with 15 serum samples from dogs infected with *E. granulosus* adult worm using rEmAgB8/3, because this antigen was expressed in a greater amount in adult worms (Mamuti *et al*, 2004). We found that this recombinant antigen was only detected by one of these 15 AE serum samples (Fig 2). The low reactivity of this antigen with infected dog serum samples may due to the locality of the adult worm; the *E. granulosus* is an intestinal parasite of dog, which may not be able to produce strong serum IgG antibodies to detect the parasite antigen. However, it could be applicable for the establishment of a coproantigen test for the diagnosis of dogs infected with *Echinococcus*, as it is expressed in a greater amount in adult worms of the parasite.

Fig 2- Western blot result of dog serum samples reacted with rEmAgB8/3. Lanes 1-15 were probed with serum samples from 15 dogs infected with *E. granulosus*. The last lane (-) was probed with a serum sample obtained from a parasite free dog as negative control. Lane M, protein maker.

DISCUSSION

The smallest subunit (8-12 kDa) of native AgB in hydatid cyst fluid is recognized to be strongly immunogenic in patients with echinococcal infections (Leggatt et al, 1992; Maddison *et al*, 1989). In a previous study, we have observed that the sensitivity of recombinant EmAgB8/1 is similar to that of EgAgB8/1 to detect the specific total IgG antibodies in serum samples from patient with AE and CE (Mamuti *et al*, 2004). The sensitivity of other EmAgB 8-kDa subunit monomers that we have described here are also not improved for detection of IgG antibodies in serum sample from patients with AE, although they were detected in parasite vesicles and protoscoleces. This may be because the AgB in patients with CE initially accumulated in the fluid of unilocular cysts of metacestode, after being secreted and aggregated into bigger molecules contact with the host immune system. In contrast, the AgB in patients with AE have less chance to accumulate and aggregate into bigger molecules before interacting with the host immune system due to the tumor-like infiltrating structure of the metacestode with no limiting host-tissue barrier. Usually parasite antigens with a bigger molecule size are more immunogenic than the smaller ones to evoke host immune responses. This may be one of the reasons why AgB is more sensitive to detect specific IgG antibodies in serum samples from CE patients than in from AE patients. Rott *et al* (2000) reported that the recombinant protein of EgAB8/2 showed no cross-reactions with serum samples from AE patients in their experiment. This may be due to an insufficient number of serum samples from AE patients. We found the rEmAgB8/2 showed positive reactions with about 20% (14/73) of serum samples from AE patients. In addition, our previous study showed that EmAgB8/2 has more than 94% homology at protein level to that of EgAgB8/2 (Mamuti *et al*, 2006). In conclusion, first, rEmAgB8/1 is the most promising recombinant antigen for serodiagnosis of human echinococcosis among the other 8-kDa subunit monomers of EmAgB. Second, rEgAgB8/3 can be used as a candidate antigen for establishment of a coproantigen test

for diagnosis of dogs infected with *E. granulosus* and *E. multiloculais*.

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