Time-course of antibody response in mice against oral infection with eggs of *Echinococcus multilocularis*

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(*Received 17 September 1997; revised 3 December 1997; accepted 3 December 1997*)

SUMMARY

The kinetics of serum antibody response against infection with *Echinococcus multilocularis* eggs was evaluated in AKR mice. The animals were infected by oral inoculation with 300 parasite eggs, and necropsied at 1, 2, 4, 6, 9, 12 and 16 weeks post-infection (p.i.), respectively. The parasite formed the laminated layer at 4 weeks p.i., the brood capsule with a massive proliferation of germinal cells at 9 weeks p.i. and protoscoleces at 16 weeks p.i. Serum antibody responses of the mice to antigen preparations from metacestodes of different stages and protoscoleces were evaluated by ELISA, immunoblotting and immunohistochemistry. In ELISA, the antibody responses began to increase at 4 weeks and became more apparent at 9 weeks p.i. and thereafter. Immunoblots using sera collected at 16 weeks p.i. showed some common bands among the 3 different antigen preparations. In addition to this, the germinal cells and brood capsules of mature metacestodes were stained strongly in an immunohistochemical study. From above, it is suggested that some antigen molecules are expressed in the parasite through these stages and stimulated host antibody responses.

Key words: multilocular echinococcosis, *Echinococcus multilocularis*, antibody response, serodiagnosis.

INTRODUCTION

Alveolar echinococcosis caused by the metacestode of *Echinococcus multilocularis* is one of the most harmful helminthic diseases, which is attributed to the tumour-like invasive growth of the parasite located mainly in the liver. As the period between infection and an appearance of clinical manifestation is often more than 10 years apart, serological screenings for early detection and subsequent treatment of the patients are important to reduce mortality.

The host immune responses against larval *E. multilocularis* have been evaluated in human cases (Gottstein *et al*. 1991) and in experimental animal cases undergoing secondary echinococcosis (Gottstein, Wunderlin & Tanner, 1994). These reports revealed that the growth of the metacestode is associated with humoral and cell-mediated immune responses in the host. But experiments based upon infection with the parasite eggs is necessary to clarify the correlation between early stage parasite development and respective host immune responses. According to our knowledge, no publication has been reported so far on the time-course of antibody response after oral infection with the parasite eggs. One reason for that may have been the high risk for the experimenters to handle the parasite eggs, as respective biosafety facilities are not easily available.

In this study, an experimental infection was performed by inoculation of *E. multilocularis* eggs to the susceptible strain (AKR) of mice in a strictly designed safety facility, and the relationship of the host antibody response and the parasite development was evaluated.

MATERIALS AND METHODS

Experimental infection of mice

Fifty male mice of an inbred strain (AKR/N) susceptible to larval *E. multilocularis* were purchased from Japan SLC, Hamamatsu, Japan. Their age at infection was 31 or 32 days old. The parasite eggs were obtained from faeces of the dog, orally infected with *E. multilocularis* protoscoleces (Nemuro strain, maintained at Hokkaido Institute of Public Health) and excreting mature proglottids. One ml of faecal suspension containing 300 eggs was orally inoculated to each mouse. They were handled within a safety facility at Hokkaido Institute of Public Health, specially designed for *E. multilocularis* infection.

Necropsy

Five mice each were necropsied at 1, 2, 4, 6, 9, 12 and 16 weeks p.i. for infected groups and at 0, 9, 16 weeks p.i. for control groups, respectively. At each

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https://doi.org/10.1017/S0031182098002443 Published online by Cambridge University Press

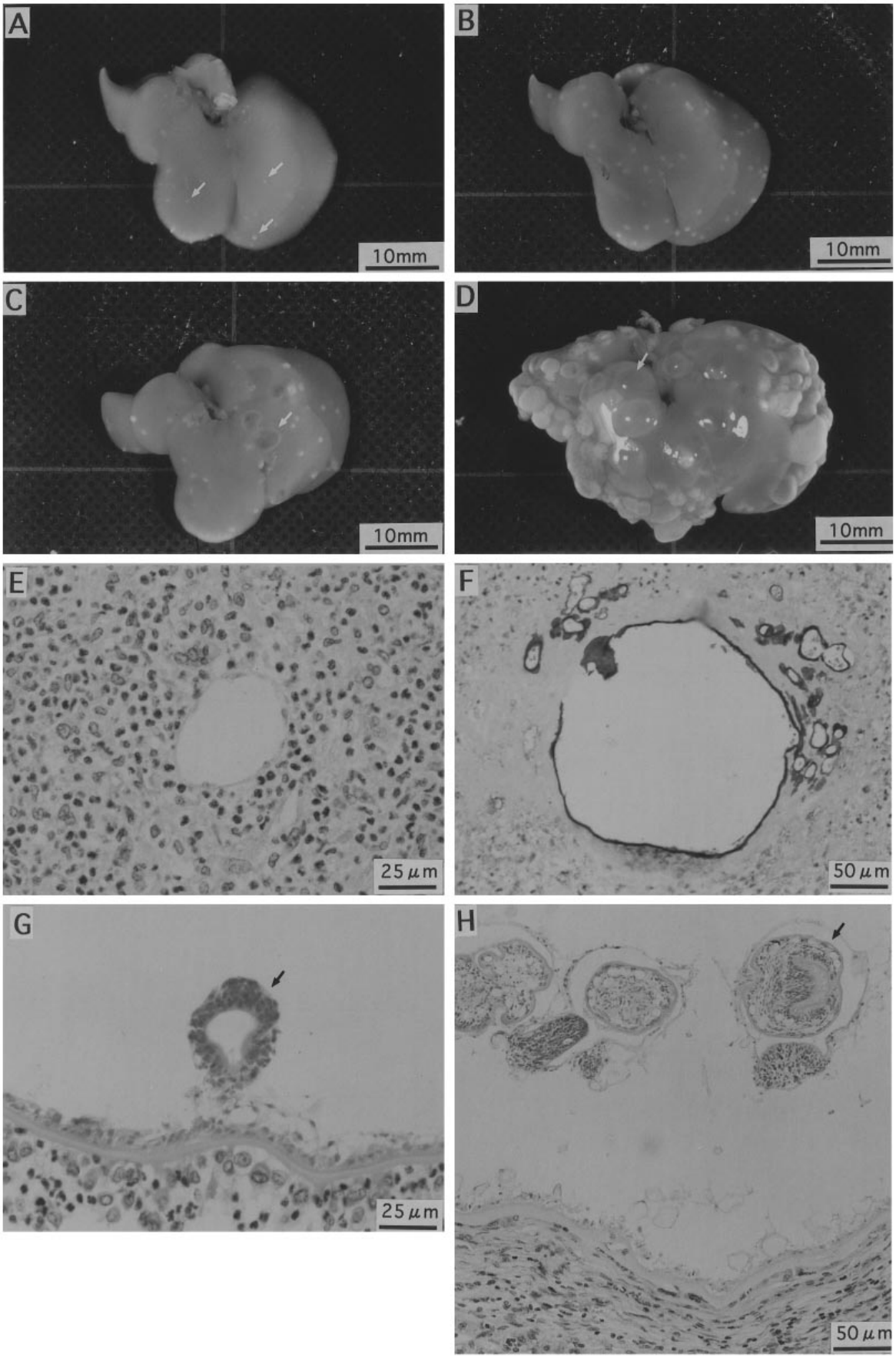

Fig. 1. (A–D) Macroscopical findings of the livers of larval *Echinococcus multilocularis*-infected mice at various weeks post-infection (p.i.). (A) At 1 week p.i., showing small (less than 1 mm) white spots of echinococcal foci on the surface of the liver (arrows). (B) At 6 weeks p.i. some of the foci on the surface were over 1 mm. (C) At nine weeks

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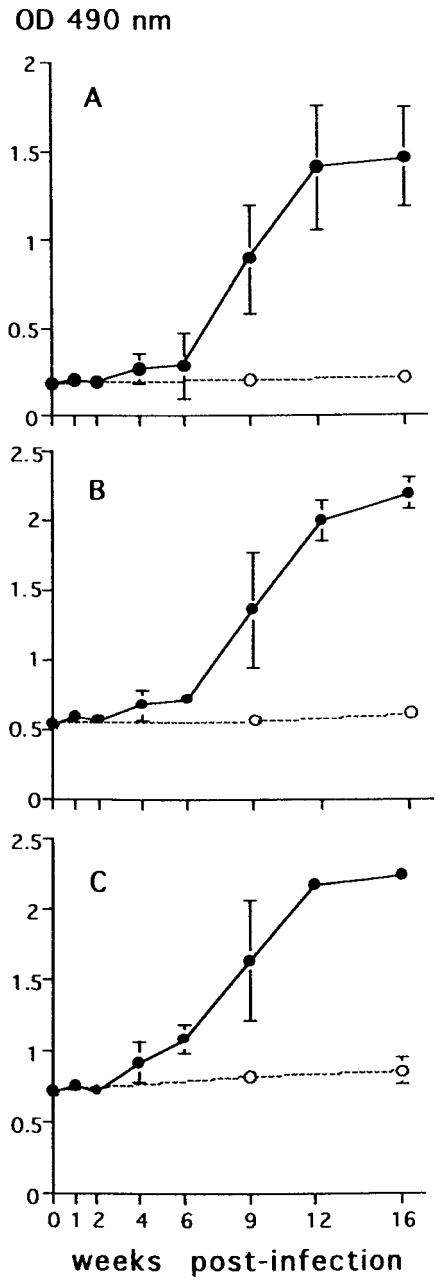

Fig. 2. Antibody response against (A) immature metacestode, (B) mature metacestode and (C) protoscolex antigens in larval *Echinococcus multilocularis*-infected mice during the course of infection (at 0, 1, 2, 4, 6, 9, 12 and 16 weeks post-infection) measured by an indirect ELISA. Error bars represent standard deviation of 5 mice in each group. (—●—) infected groups; (– – ○ – –) normal control groups.

necropsy, blood samples were collected by bleeding under anaesthetization for subsequent serum sampling. The livers harboring the metacestode were fixed in 10% formalin solution for preparing histological sections. After fixation, serial paraffin sections were made and stained with haematoxylin–eosin or with periodic-acid-Schiff (PAS) reaction followed by haematoxylin counter staining.

Mature and immature metacestode antigens

The mature and immature metacestodes, with and without forming protoscoleces, were obtained at 4 and 3 weeks p.i. from experimentally infected cotton rats (*Sigmodon hispidus*), respectively. The minced parasite organism in an equal volume of phosphate-buffered saline (PBS, pH 7·4) containing 1% Triton X-100 was then homogenized with a glass/Teflon homogenizer and sonicated for 3 min on ice. After repeated freezing and thawing, the material was centrifuged at 10000 ***g*** for 30 min at 4 °C. Finally, the supernatant fraction was dialysed for 24 h in PBS and used as metacestode antigen for serological examinations. Finally, protein concentration was measured with BioRad protein assay kit (BioRad, California).

Protoscolex antigen

To collect protoscoleces, the mature metacestode was pushed to pass through a metal mesh with ice-cold PBS, and then washed with ice-cold PBS by decantation. The protoscolex suspension was subsequently passed through a 200 μm nylon mesh, and then washed on a 95 μm nylon mesh to remove impurities (protoscoleces can not pass 95 μm nylon mesh). Finally, pure protoscoleces were recovered by washing off the 95 μm nylon mesh with PBS. From this material, antigen solution was prepared by the same procedure as described in the metacestode antigen preparation.

Enzyme-linked immunosorbent assay (*ELISA*)

Specific serum antibodies to the mature metacestode, the immature metacestode and protoscoleces were detected by an indirect ELISA, respectively.

A microtitre plate (Immulon 600; Greiner, Frickenhausen, Germany) was coated with antigen

p.i. The foci appeared cystic, containing fluid (arrow). (D) At 16 weeks p.i. protoscoleces were observed macroscopically inside the cyst (arrow). (E–H) Microscopical findings of the metacestodes in the livers at various weeks p.i. (E) At 1 week p.i. unilocular vesiculation was observed (haematoxylin-eosin staining). (F) At 6 weeks p.i., showing further progress of multilocular vesiculation and formation of the laminated layer (PAS staining). (G) At 9 weeks p.i., showing the early stage of brood capsule formation (arrow, haematoxylin-eosin staining). (H) At 16 weeks p.i. formation of mature protoscoleces was observed (arrow, haematoxylin-eosin staining).

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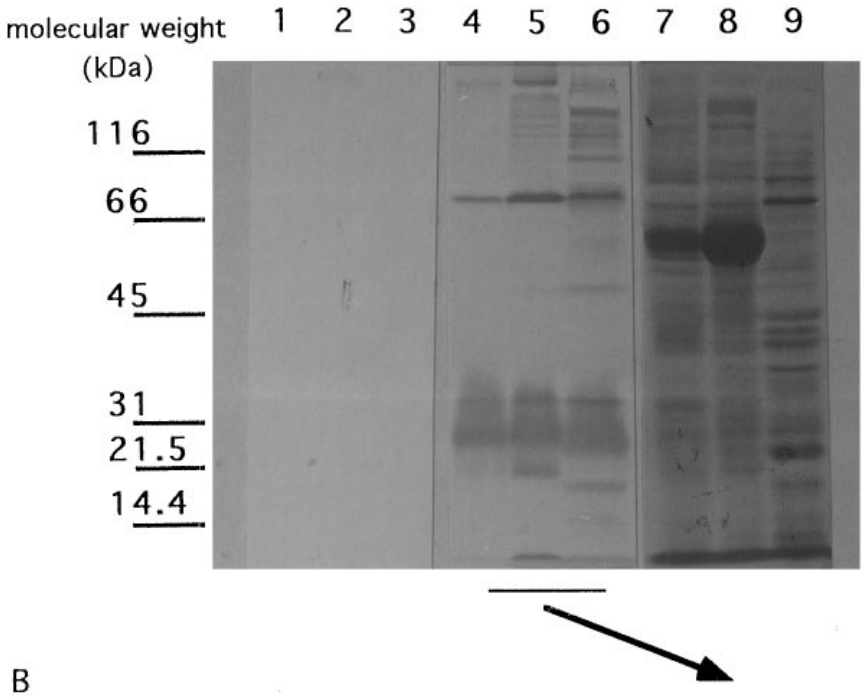
A



B

band	molecular weight (kDa)
a	126
b	121
c	115
d	102
e	85
f	70
g	56
h	33
i	27
j	24
k	21
l	80-90
m	25-35

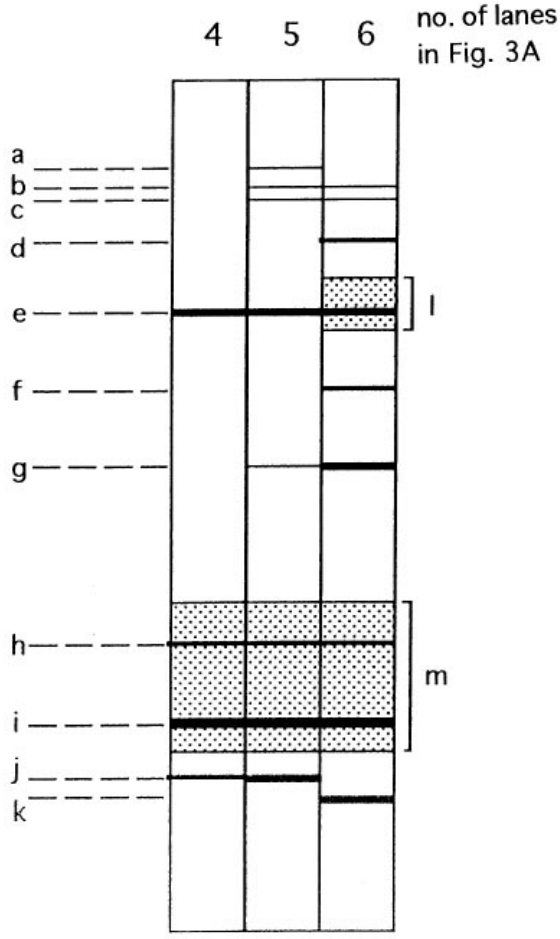


Fig. 3. (A) Immunoblot using immature metacestode (lanes 1, 4, 7), mature metacestode (lanes 2, 5, 8) and protoscolex antigens (lanes 3, 6, 9) with the serum of a normal control mouse (lanes 1–3) and the *Echinococcus multilocularis*-infected mouse at 16 weeks p.i. (lanes 4–6). Lanes 7–9 are amide black-stained strips. Molecular weights (kDa) are indicated by lines on the left. (B) The schema of the immunoblot using the 3 antigens with the *E. multilocularis*-infected mouse serum at 16 weeks p.i. (lanes 4–6 in A).

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(1650 ng protein/well) in 0·05 M carbonate buffer (pH 9·6) and left overnight at 4 °C. Each well was washed with PBS containing 0·05% Tween 20 (PBS/Tween) and blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature (RT). After washing, the sample sera, diluted 1:100 with dilution buffer (0·5% BSA, 0·5% casein and 0·05% Tween 20 in PBS), were reacted for 1 h at RT. After next washing, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin (G+A+M) (Zymed Laboratories, USA) at a dilution of 1:1000 in dilution buffer was reacted for 1 h at RT. After the final washing, substrate solution containing 0·04% *o*-phenylenediamine (Wako, Japan) and 0·1% H_2O_2 in 100 mM citrate phosphate (pH 5·0) was added to each well. Finally, the plate was incubated for 15 min at 37 °C and the optical density (OD) read at 490 nm.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and Immunoblotting

SDS–PAGE was performed using a 10% polyacrylamide gel according to the method of Laemmli (1970). After SDS–PAGE and equilibration, parasite antigen in the gel was transferred electrophoretically to a polyvinylidene difluoride microporous membrane (Immobilon™ PVDF, Millipore, Bedford, USA) at 4 °C overnight (100 mA constant current). The membrane was cut into strips and blocked with 2% BSA and PBS for 2 h at RT. After washing with PBS–Tween, the strips were incubated with the sample sera diluted 1:100 with dilution buffer for 1 h at RT, then washed again and subsequently incubated with HRP-conjugated rabbit anti-mouse immunoglobulin (G+A+M) at a dilution of 1:1000 in dilution buffer for 1 h at RT. After washing, the strips were incubated with substrate solution containing 0·06% 3,3′-diaminobenzidine (DAB) and 0·1% H_2O_2 in Tris–HCl buffer (pH 7·6) for 15 min for colour development.

Immunohistochemistry

The paraffin sections of the metacestode were treated basically as described by Nakane & Pierce (1967). Briefly, the deparaffinized sections were treated with methanol containing 1% H_2O_2 for 20 min in order to inactivate endogenous peroxidase, followed by washing with PBS. After blocking with 2% normal rabbit serum in PBS the sample sera, diluted 1:200, were applied and incubated for 1 h at RT. After washing, HRP-conjugated rabbit anti-mouse immunoglobulin (G+A+M) diluted to 1:200 was reacted for 1 h at RT. The sections were washed and then treated with substrate solution containing 0·02% DAB, 0·02% H_2O_2 in Tris–HCl buffer (pH 7·6) for 10 min. The sections were washed with tap water to stop the reaction, counterstained with haematoxylin and mounted.

RESULTS

Development of the parasite

All animals of infected groups had echinococcal foci in their livers. The recovery rate of echinococcal foci (against 300 eggs inoculated) was 31·4±14·6% (mean±standard deviation) at 4 weeks p.i. The following are the typical macro- or microscopical findings of the metacestode development on each necropsy day. One week p.i.: echinococcal foci were already found in the liver and unilocularly vesiculated (Fig. 1A and E). Two weeks p.i.: the parasite vesiculated multilocularly. Four weeks p.i.: the vesiculation progressed and the PAS reaction-positive laminated layer was formed. Six weeks p.i.: the vesiculation further progressed (Fig. 1B and F). Nine weeks p.i.: large vesicles also developed (Fig. 1C). Germinal cells accumulated inside the cyst wall and began to form the brood capsule. A small number of calcareous corpuscles were found (Fig. 1G). Twelve weeks p.i.: the cell accumulations transformed into a sucker-like form and immature protoscoleces were observed inside them. Many calcareous corpuscles were found. Sixteen weeks p.i.: mature protoscoleces, together with immature ones, were observed inside the brood capsule (Fig. 1D and H).

Serological studies

As shown in Fig. 2A–C, the kinetics of antibody response measured by ELISA using the 3 different antigens from immature, mature metacestodes and protoscoleces showed approximately the same pattern; the increase of OD value was initially detected at four weeks p.i. and was more apparent at nine weeks p.i. and thereafter.

Immunoblotting showed 13 major bands of different molecular weights (Fig. 3A andB). Bands e, h, i and m (85, 33, 27 and 25–35 kDa) were commonly observed in the 3 different antigens. Bands d, f and k (102, 70 and 21 kDa) were specific to the protoscolex antigen. Bands l and m were recognized as smear-like broad bands.

In immunohistochemical studies, only limited parts of the parasite were stained heavily (Fig. 4A–F). Namely, the germinal cell of unilocularly vesiculated cysts (Fig. 4A), germinal cells inside the cyst wall and inner or outer surface of the developing brood capsule of immature metacestodes (Fig. 4C) and germinal cells of the brood capsules or the cyst wall, and concaved surface of the unevaginated protoscoleces in mature metacestodes (Fig. 4E).

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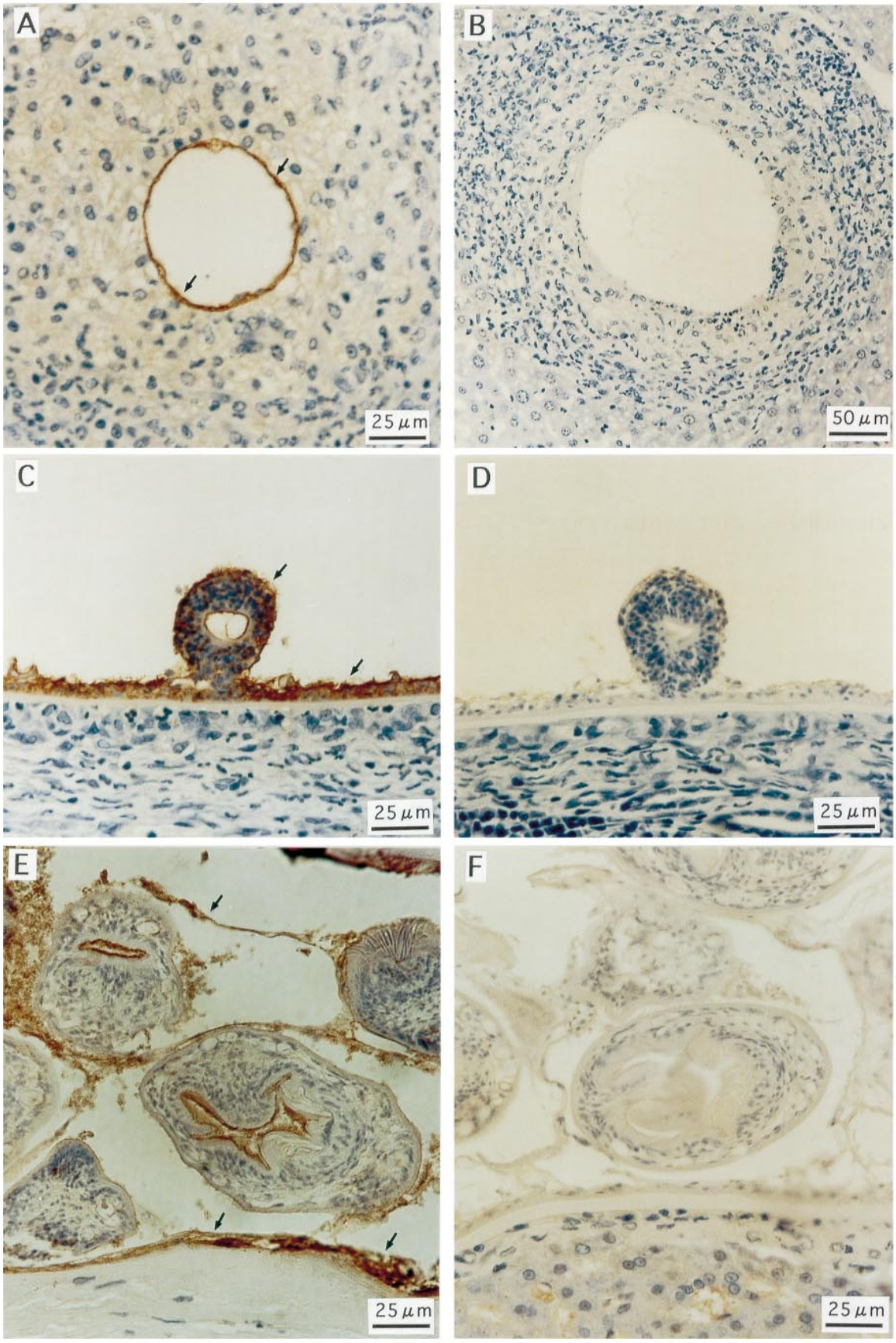

Fig. 4. The result of immunohistochemical study. Sections of larval *Echinococcus multilocularis* at various weeks post-infection (p.i.) with the serum of the parasite-infected mouse at 16 weeks p.i. (A, C, E) or of a normal control mouse (B, D, F). (A and B) The unilocularly vesiculated-cyst at 2 weeks p.i. The monolayer of germinal cells was strongly stained (arrows). (C and D) Immature metacestodes at 9 weeks p.i. The edge of developing brood capsules and germinal layers inside the cyst wall were stained heavily (arrows). (E and F) The mature metacestodes at 16 weeks p.i. Germinal cells and brood capsules were strongly stained (arrows).

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DISCUSSION

Susceptibility of mice to *E. multilocularis* larvae varies between strains, and the AKR mouse is reported to be susceptible (Yamashita *et al*. 1958). The development of metacestodes in this study followed the previous report, i.e. mature protoscoleces were formed after egg-inoculation (at 16 weeks p.i.).

In this study following oral inoculation of the parasite eggs to AKR mice, the antibody response measured by ELISA showed similar kinetics despite using 3 different antigens from immature, mature metacestodes and protoscoleces. Immunoblot analysis using the different antigens showed several common bands (bands e, h, i and m (85, 33, 27 and 25–35 kDa, respectively) and demonstrated a conserved antigenic pattern.

Although results are not shown, we performed immunoblotting and immunohistochemical studies using the sera collected at various weeks p.i. to detect early stage-specific antigenic activities. The immunoblot showed all of the main bands began to appear weakly at 4 or 9 weeks p.i. and became more apparent thereafter, and we could detect no early stage-specific band. In the immunohistochemical study, also, we could not find early stage-specific antigenic activities, which would be informative for early detection of the disease. It is probable that some of the antigenic potential was destroyed during our procedure. In fact, by using unfixed materials, Deplazes & Gottstein (1991) detected polypeptide antigen expressed in laminated layers of metacestodes, in which we could find no reaction in the immunohistochemical study. We should have chosen other protocols (e.g. using cryo-sections) to assess natural antigenic activities, especially of infant metacestodes. In addition, the early stage-specific antibody response itself may have been very weak.

These results of serological studies indicate that common antigenic molecules exist in the immature, mature metacestodes and protoscoleces and that the molecules would be useful to detect alveolar echinococcosis of somewhat later but not early stages.

Further consideration is necessary in the future study to improve diagnostic techniques especially for early detection. Nevertheless, the results of this study would provide basic information for further studies on the host antibody response to larval *E. multilocularis*. Concerning serodiagnosis of the disease in humans, several bands of various molecular weights in immunblotting have already been reported to be informative (Furuya *et al*. 1989; Ito *et al*. 1993; Sato & Furuya, 1994; Wen & Craig, 1994). If they correspond to the bands detected in this study, AKR mice could be used as a good model to analyse the immunoblot pattern of patients with alveolar echinococcosis.

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