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Immunohistochemical localization of 36 and 29 kDa proteins in sparganum*

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Abstract: Antigenic proteins of 36 and 29 kDa were localized in *Spirometra mansoni* plerocercoid (sparganum) immunohistochemically by avidin biotin complex (ABC) staining. When polyclonal antibodies such as BALB/c mouse serum immunized with crude saline extract of sparganum or confirmed sparganosis sera were reacted as primary antibodies, the positive chromogen (3-amino, 9-ethylcarbazole) reactions were recognized at syncytial tegument, tegumental cells, muscle and parenchymal cells and lining cells of excretory canals. A monoclonal antibody(MAb) which was reacting to 36 and 29 kDa proteins in the extract of the worm was localized at the syncytial tegument and tegumental cells. The present results suggested that the potent antigenic proteins of 36 and 29 kDa in sparganum were produced at the tegumental cells and transported to the syncytial tegument.

Key words: *Spirometra mansoni* plerocercoid(sparganum), sparganosis, monoclonal antibody, localization, immunohistochemistry

INTRODUCTION

Sparganosis, a disease caused by tissue invading plerocercoid larva of *Spirometra mansoni* (sparganum), occurs worldwidely though more common in East Asia. Because human sparganosis is diagnosed by surgical removal of the worm in patients of protean manifestations, the recorded incidence is not high so far. Application of enzyme-linked immunosorbent assay (ELISA) has facilitated the diagnosis of human sparganosis. This antibody test has been proved to be sensitive enough to detect specific (IgG) antibody in patient sera or cerebrospinal fluid when crude saline extract of sparganum is used as an antigen (Kim *et al.*, 1984). The crude extract, however, often revealed the cross reactions with sera of other parasitic diseases such as taeniasis, cysticercosis and hydatidosis (Choi *et al.*, 1988; Kim and Yang, 1988). To overcome problems of cross-reactivity, sensitive and specific component proteins in the crude extract have been searched for. By using SDS-polyacrylamide gel electrophoresis(PAGE) and immunoblot, it had been recognized that the proteins of molecular mass of 36 and 29 kilodalton (kDa) are the sensitive protein components in the extract (Choi *et al.*, 1988).

These proteins were purified by affinity chromatography using a MAb (Cho *et al.*, 1990) or gelatin as ligands (Kong *et al.*, 1991). But the nature and biological roles of the 36 and 29 kDa proteins from sparganum are not understood yet. In this study, tissue sources of the proteins were investigated by immunohistochemistry in the larval worm sections using the MAb.

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MATERIALS AND METHODS

1. Sparganum and its preparations

Sparganum was collected from subcutaneous tissue and muscle of naturally infected snakes, *Natrix tigrina lateralis* which had been purchased in Hongchun Gun, Kangwon Do. After removing adhered host tissues, the worms were washed in physiologic saline 3 times. Paraffin blocks of the worm were prepared in a sequence of 10% neutral formalin fixation, dehydration in graded alcohol series and paraffinization.

The crude saline extract was prepared as Cho *et al.* (1990) did. Washed spargana were emulsified with teflon-pestle tissue homogenizer in physiologic saline containing 0.006% (W/V) phenylmethylsulfonyl fluoride (PMSF). The emulsion was shaken for 2 hours and let it stand overnight. It was centrifuged at 10,000 *g* for an hour. The resulting supernatant was regarded as a crude saline extract. All procedures were done at 4°C. The protein content of the crude extract was 6.88 mg/ml when measured by Lowry *et al.* (1951).

2. Preparation of the MAb and its characterization

MAb used in the study was one of those described in Cho *et al.* (1990). Eight-week old female BALB/c mice were immunized with the crude extract. A total of 10^8 spleen cells were hybridized with 2×10^7 SP2/0 plasmacytoma cells. Dispensed cells of hybridization were maintained in 5~10% CO_2 incubator in HT medium. Ten days later, antibody secreting colonies were selected by testing the media for sparganum-specific (IgG) antibody by ELISA (Kim *et al.*, 1984). Antibody secreting colonies were dispensed by limiting dilution and screened the culture media again by ELISA. MAb producing cell lines were expanded in BALB/c mice peritoneum. IgG fraction in mice ascites was purified by Protein A-Sepharose CL-4B affinity chromatography.

In order to characterize the prepared MAb, SDS-PAGE/immunoblot was done. Reducing SDS-PAGE of the crude extract was undertaken as described by Laemmli (1970). Three % stacking gel and 10~15% linear gradient separating gel were used. The crude extract was mixed with the same amount of the sample buffer and electrophoresed at 30 mA constant current. Immunoblot was carried out according to the technique of Tsang *et al.* (1983). The separated proteins in the gel were transferred to a nitrocellulose paper by electrophoresis at 100 V for an hour at 4°C. MAbs were reacted for an hour. Peroxidase-conjugated anti-mouse IgG goat IgG (Cappel, U.S.A.) was reacted at 1 : 1,000 dilution for an hour. Reacted band was stained with diaminobenzidine chromogen.

3. Immunohistochemical staining

General procedures of the ABC staining, described in the bulletin of Biomeda Corp. (U.S.A.) (1990), were followed. Deparaffinized worm sections were equilibrated with PBS (0.1 M, pH 7.6). Endogenous peroxidase activity was inhibited by treating the tissue sections in 2% H_2O_2 for 5 minutes. After rinsing, the slides were reacted with differently diluted primary antibodies and biotinylated secondary antibody for 30 minutes, respectively. Peroxidase complex was reacted for 20 minutes. The reaction was colored by AEC (3-amino, 9-ethylcarbazole) chromogen. Finally the tissue sections were counterstained with water-based hematoxylin. All procedures were done at 36°C.

4. Primary antibodies used in immunohistochemical staining

The following antibodies were used in the immunohistochemical staining:

1) Negative controls: As negative control stainings, PBS (0.1 M, pH 7.6), a 1 : 100 diluted uninfected mouse serum and a healthy human serum were reacted as primary antibodies.

2) Immunized mouse serum: To observe the stainability in each tissue of the worm, an immunized BALB/c mouse serum was reacted in serum dilution of 1 : 100 to 1 : 6,400 in PBS.

3) Human patient sera: To compare the antigenicity of each tissue in the worm, 5 sparganosis patient sera were used. They were all surgically confirmed cases.

4) Monoclonal antibody: To verify the production site of the MAb reacting to 36 and 29 kDa epitopes, MAb in dilution from 1 : 50 to 1 : 1,600 was reacted as a primary antibody in the immunohistochemistry.

RESULTS

1. Monoclonal antibody and immunoblot

As exhibited in Fig. 1, SDS-PAGE/immunoblot using the MAb of SP-53 revealed reactions to 2 bands of 36 and 29 kDa. When the MAb was purified by Protein A Sepharose CL-4B affinity chromatography, a total of 136 mg of MAb was harvested.

2. Stainability of polyclonal antibodies with the worm

Figs. 2 and 3 exhibited a section of sparganum which reacted with PBS and uninfected mouse serum as primary antibodies (negative controls). Structures such as syncytial tegument, tegumental cells, parenchymal tissues and excretory canals were stained with hematoxylin. No tissues in the worm section were stained positively with AEC chromogen. Figs. 4 and 5 were the results of immunohistochemical staining of sparganum which was reacted with immunized BALB/c mouse serum as a primary antibody. Syncytial tegument, tegumental cells, muscle cells, parenchymal tissues and lining cells of excretory canals were stained with the chromogen. Figs. 6 and 7 exhibited the results of ABC staining when infected human sera were used as primary antibodies. Positive reactions were observed at upper layer of syncytial tegument, tegumental cells, parenchymal cells, a part of muscle cells and excretory canals.

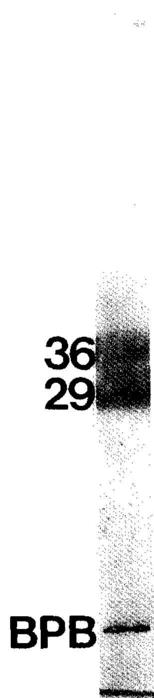

Fig. 1. SDS-PAGE/immunoblot finding of SP-53 monoclonal antibody. In SDS-PAGE, a linear gradient gel of 10~15% was used for protein separation in reducing condition.

3. Localization of 36 and 29 kDa MAb

When the MAb, reacting to 36 and 29 kDa epitopes, was used as a primary antibody, syncytial tegument and tegumental cells were positively stained with AEC chromogen. A part of scattered parenchymal cells in the worm were faintly stained or not at dilution from 1 : 50 to 1 : 200 (Figs. 8 & 9).

DISCUSSION

Antigenic proteins of 36 and 29 kDa of sparganum were localized at tegumental cells and syncytial tegument of the worm when observed by immunohistochemistry using the MAb reacting to these proteins. On the contrary, polyclonal antibodies in immunized mouse serum or patient sera were reacting to almost all structures of sparganum such as muscle, parenchymal cells and lining cells of excretory canal. Syncytial tegument and tegumental cells also reacted with these polyclonal antibodies. Only a part of calcium corpuscles were antigenic though not shown in the figures. Interestingly, however, syncytial tegument and tegumental cells were weakly stained whereas parenchymal cells were darkly stained in a patient serum. Different stainability of sera from surgically confirmed sparganosis patients might represent the different

stages of the infection.

In this connection, Ohnishi *et al.* (1986) undertook an experimental rabbit infection of sparganum and observed the antibody changes during the infection period using differently prepared sparganum antigens. When observed with indirect fluorescent antibody technique, specific IgM antibody in infected sera reacted to syncytial tegument, tegumental cells and a part of parenchymal cells whereas specific IgG antibody reacted to reticular cells in the parenchyme. Kim and Choi (1991) also observed the antigen producing tissue of sparganum using infected mice and rat sera by ABC stainings. They reported that the parenchymal cells near to surface of the larval worm and calcium corpuscles showed the highest stainability while syncytial tegument was the most reactive in the later stage. These somewhat contradictory results between the two experiments were hardly confirmed in the present study.

The positive immunohistochemical findings of MAb bindings suggested strongly that these antigenic proteins were produced at the tegumental cells and transported to the syncytial tegument via connecting tubules or pore canals (Fig. 9). The positive reactions in ABC staining to the antigenic proteins were especially strong at the distal margin of syncytial tegument where microtriches are present. This histologic finding suggested also that the proteins of 36 and 29 kDa might be released outside of the worm. They might be surface associated enzymes of the parasite, such as acid and alkaline phosphatase (Kwak and Kim, 1988) or other kinds of secreted enzymes. On the point, cysteine protease of sparganum was known to have molecular mass of 38-19 kDa (Fukase *et al.*, 1985 & 1987) or 28 kDa in the excretory-secretory product (Song and Choi, 1990). The protective mechanism of the protease has been suggested strongly in *Schistosoma mansoni* (Chappell and Dresden, 1987). Functional aspects of 36 and 29 kDa proteins need further studies to elucidate the roles in the host-parasite interactions.

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REFERENCES

Biomeda Corp. (1990) Instructions in AutoProbe III

LEGENDS FOR FIGURES

Fig. 2. Negative control in ABC staining(×100). A 1 : 1000 diluted healthy human serum was used as a primary antibody. Positive reactions were not observed in any tissue.

Fig. 3. Another negative control(×100). Uninfected mouse serum was reacted at dilution of 1 : 100 as a primary antibody instead of human serum. No structures were stained with AEC chromogen.

Fig. 4. Positive control(×100). A hyperimmune BALB/c mouse serum was reacted as a primary antibody. Syncytial tegument, tegumental cells, parenchymal cells, excretory canals and muscle cells were stained positively.

Fig. 5. Another positive control of immunized BALB/c mouse serum(×200). Tegument, parenchymal cells and excretory canals were stained with AEC chromogen.

Fig. 6. When a 1 : 200 diluted patient serum was reacted, strong positive reactions were observed at surface of syncytial tegument, tegumental cells, a part of muscle cells, parenchymal cells and excretory canals(×100).

Fig. 7. Staining with another patient serum(×200). Stronger reactions were shown at surface of syncytial tegument and tegumental cells.

Fig. 8. MAb reacting to 36 and 29 kDa was used as a primary antibody (×100). Surface of syncytial tegument and tegumental cells showed strong positive reactions.

Fig. 9. Another example of ABC staining treated with 36 and 29 kDa MAb (×400). Strong positive reactions at syncytial tegument and tegumental cells were observed.

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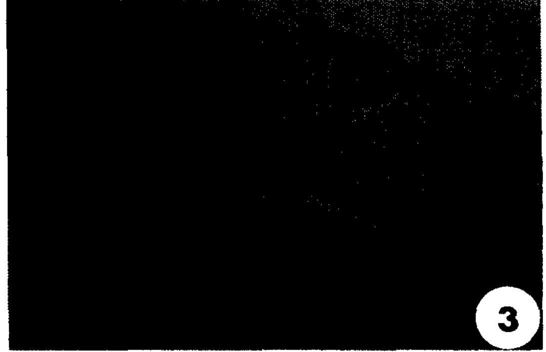
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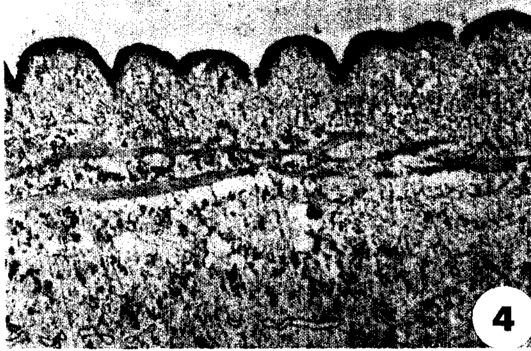
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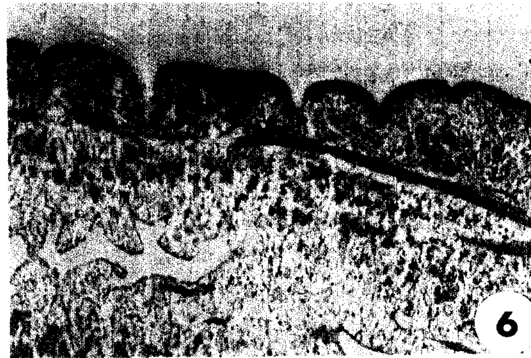
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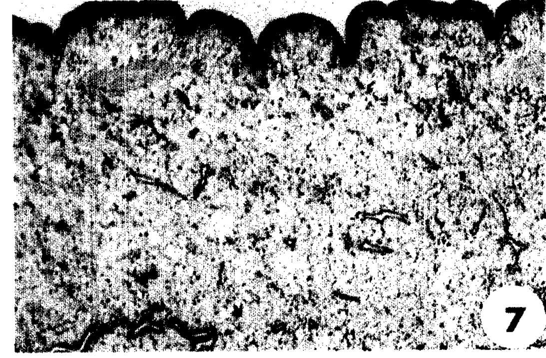
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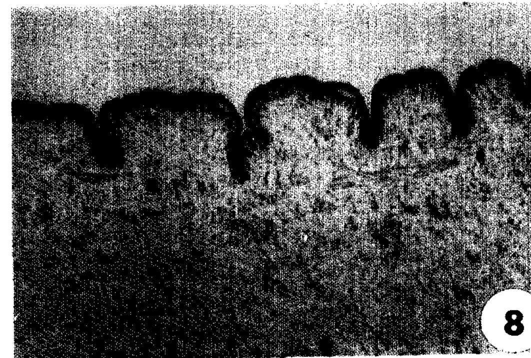
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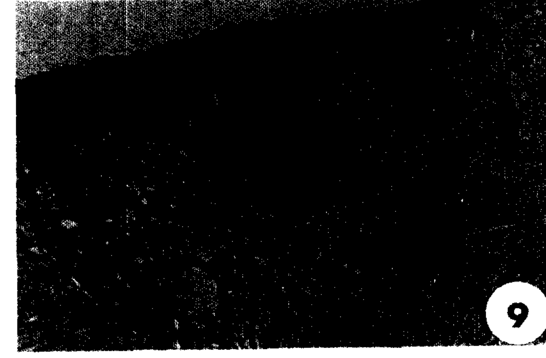
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(3rd ed.). Foster, USA.

Chappell, C.L. and Dresden, M.H. (1987) Purification of cysteine proteinase from adult *Schistosoma mansoni*. *Arch. Bichem. Biophy.*, **256**:560-568.

Cho, S.Y., Kang, S.Y. and Kong, Y. (1990) Purification of antigenic protein of sparganum by immunoaffinity chromatography using a monoclonal antibody. *Korean J. Parasit.*, **28**:135-142.

Choi, S.H., Kang, S.Y., Kong, Y. and Cho, S.Y. (1988) Antigenic protein fractions reacting with sera of sparganosis patients. *Korean J. Parasit.*, **26**:163-167.

Fukase, T., Matsuda, Y., Akihama, S. and Itagaki, H. (1985) Purification and some properties of cysteine protease of *Spirometra erinacei* plerocercoid (Cestoda: Diphyllobothriidae). *Jpn. J. Parasitol.*, **34**:351-360(in Japanese with English abstract).

Fukase, T., Matsuda, Y., Akihama, S. and Itagaki, H. (1986) Distribution of the cysteine protease in *Spirometra erinacei* plerocercoid (Cestoda: Diphyllobothriidae). *Jpn. J. Parasitol.*, **35**:141-144 (in Japanese with English abstract).

Kim, C.H. and Yang, J. (1988) Immunological characterization of antigens from *Cysticercus* and sparganum and their application to immunodiagnosis I. Immunological characteristics of crude antigenic components from *Cysticercus cellulosae*. *Korean J. Parasit.*, **26**:245-254(in Korean with English abstract).

Kim, C.H. and Choi, W.S. (1991) Immunohistochemical observation on the antigens inducing IgG antibodies and IgM against sparganum. *Korean J. Parasit.*, **29**:339-353(in Korean with English abstract).

Kim, H., Kim, S.I. and Cho, S.Y. (1984) Serological diagnosis of human sparganosis by means of micro-ELISA. *Korean J. Parasit.*, **22**:222-228.

Kong, Y., Kang, S.Y. and Cho, S.Y. (1991) Single step purification of potent antigenic protein from sparganum by gelatin-affinity chromatography. *Korean J. Parasit.*, **29**:1-7.

Kwak, K.H. and Kim, C.H. (1988) Enzyme-histochemical studies of esterase and phosphatase on developing *Spirometra erinacei*. *Korean J. Zool.*, **31**:225-235 (in Korean with English abstract).

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of the bacteriophage T_4. *Nature*, **222**:681-685.

Lowry, O.H., Rosenbrough, N., Lewis-Farr, A. and Randall, R.J. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.*, **193**:265-275.

Ohnishi, Y., Takakura, Y., Yoshimura, H. and Tsubota, N. (1986) Antibody production in experimental sparganosis of rabbits: Agglutination and indirect fluorescent antibody techniques. *Jpn. J. Parasitol.*, **35**:25-33. (in Japanese with English abstract).

Song, C.Y. and Choi, D.H. (1990) Studies on the cysteine proteinase of excretory secretory product in sparganum. *Proc. Mol. Biol. Genet.*, **5**:141-145.

Tsang, V.C.M., Perralta, J.M. and Simons, A.R. (1983) Enzyme linked immunoelectrotransfer blot techniques (EITB) for studying specificities of antigens and antibodies separated by gel electrophoresis. *Methods in Enzymol.*, **92**:377-391.

=국문요약=

면역조직염색법으로 관찰한 스파르가눔 충체에서의 36, 29 kDa 항원 단백질의 생성위치

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스파르가눔증을 혈청학적으로 진단할 경우, 환자 혈청과 민감하고 특이하게 반응하는 단백질이 36, 29 kDa 임은 SDS-PAGE/immunoblot을 이용하여 이미 밝혀졌다. 이 연구는 이 단백질이 스파르가눔 충체의 어느 부위에서 생성되는 것인지를 알아보기 위하여 실시하였다. 스파르가눔 충체의 조직표본을 만들고 36 kDa와 29 kDa 단백질에 반응하는 단세포군항체를 1차항체로 사용하였다. 그리고 avidin-biotinylated conjugate와 AEC (3-amino, 9-ethylcarbazole) 를 이용하여 면역조직화학염색법을 실시하였다. 아울러 스파르가눔증 환자 혈청, 스파르가눔 생리식염수추출액으로 면역시킨 마우스 항혈청을 사용하여 반응양상을 비교하였다. 대조 염색으로는 PBS, 정상 마우스 혈청 및 정상인(正常人) 혈청을 각각 사용하였다.

1. PBS, 정상 마우스와 정상인 혈청으로 처리한 군은 어느 조직에서도 반응이 없었다.
2. 마우스 면역혈청을 사용하였을 때에는 표피상층, 표피, 표피세포, 유조직(柔組織), 배설강에 반응이 있었고 근육, 칼슘소체의 일부에도 반응을 보였다.
3. 환자혈청은 표피상층이 표피세포보다 강한 양성반응을 보였고 유조직에서도 반응이 있었다. 배설강, 칼슘소체의 일부에 반응을 나타냈으나 표피와 표피세포에서는 반응이 약한 환자혈청도 있었다.
4. 단세포군항체로 처리한 경우, 표피상층과 표피세포에 강하게 반응하였고 유조직의 일부에서 약한 반응을 보였다. 그러나 표피, 칼슘소체, 배설강 등에는 전혀 반응이 없었다.

이상의 결과는 스파르가눔에 존재하는 36, 29 kDa 단백질이 표피세포에서 생성되어 표피상층으로 이동함을 시사하고 있었다.

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