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Immunohistochemical Localization and the Characteristics of Antigenic Component Inducing IgE and IgG Antibodies in *Spirometra erinacei*

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Abstracts: Antigenic components reacting with IgE and IgG antibodies were localized in muscular layer of adult and of larva, sparganum. But the antigenic components inducing IgG were localized at tegument and parenchyma in addition to muscular layer in adult and sparganum. Also in sparganum, the surface of calcareous corpuscles of parenchyma showed immunoreactivity to IgG antibody. However antigenic components inducing IgE antibody were not localized in tegument and parenchyma, but in adult worm, we observed the immunopositive reaction at the lining of vitelline follicles in mature proglottis and on surface of egg shell within uterus of graved proglottis. By the method of immunogold-labelling, we observed the location of antigenic particles in tegument of sparganum. The density of antigenic particles inducing IgG was higher than that of antigen particles inducing IgE in syncytial tegument, tegument cells. A total of 43 and 36 protein bands were resolved from crude extracts of adult and sparganum, respectively, by SDS-PAGE. 34 bands from crude extracts of adult and larva were migrated to same positions. By EITB, 21 bands of 44 bands in adult were recognized with IgG antibody, and also 21 bands of 36 bands in sparganum. 13 bands of them were common antigenic components both in the adult worm and sparganum. Because 19 bands of 44 bands in adult worm were reacted with IgE antibody, they were IgE antigenic component. In sparganum, 13 bands were IgE antigenic components. 9 bands of them were common antigenic component inducing IgE antibody in both adult and sparganum. 3 bands of antigenic component recognized by IgE and IgG antibody were nonspecific antigen in both adult and sparganum of *Spirometra erinacei.*

Key Words: *Spirometra erinacei*, IgE and IgG antibody, SDS-PAGE, sparganum, EITB, sparganosis, immunogold-labelling assay.

INTRODUCTION

Spirometra erinacei is a parasite which is belong to Diphyllobothriidae. The cats and dogs are its final host, frogs and snakes are intermediate host. When sparganum infects into the paratenic host, it can not develop to adult worm, but live as parasite in the form of larva. If human is infected with sparganum, human gets sparganosis that shows worldwide occurrence, though more common in East Asia[1,3,16,29].

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When hosts, human, and experimental animals are infected with parasites of helminthes, immune system responds to the parasites to elevate the level of IgE antibody and eosinophiliosis[9,13,17,27,28,30]. Although all of the isotypes of antibodies are produced by the stimulation of foreign antigen, IgE antibody is produced in host especially by helminthic infection and allergens. The factor for IgE production depends on the maturation and dosage of antigens, route and schedule of immunization, adjuvant, and genetic background of the animals[26]. Recent intense investigation revealed that cytokine and interleukin play the important role to induce IgE antibody production[4,6,20]. The purpose of present studies is to localize antigens inducing IgE and IgG in *S. erinacei* by immunohistocytochemical technique. Antigenic proteins inducing the IgE and IgG antibody were also investigated in adult and larva of *S. erinacei* by immunoblot.

MATERIALS AND METHODS

Preparation of sparganum and adult worm

Sparganum was collected from subcutaneous tissue and muscles of two species of snakes, *Rhabdophis tigrina lateralis* and *Elaphe rafodorsata*. They were naturally infected and purchased at market in Chinju, Gyeongnam. After removing from host tissue, the worms were washed 3 times in physiologic salt solution. Then several worms of them were used to prepare the sections and the crude extract. Adult worms were recovered from intestine of cat artificially infected with sparganum.

Preparation of the crude extracts from sparganum and adult worm

The crude extract was prepared by the method described by Kim and Yang[10], Kim and Choi[11]. 2 g of lyophilized worms were emulsified in 10 ml of 0.01 M PBS(pH 7.4), and then homogenized with ultrasonicator. After adding 10 ml of 0.01 M PBS, the emulsion was shaken for 1 hour and incubated for overnight at 4℃ to elute the antigenic components. The emulsion was centrifuged at 15,000 rpm for 1 hour and the supernatant was dialysed in 0.01 M PBS solution for overnight. The resulting supernatant was regarded as crude extract and sediment was removed. All procedures were done at 4℃. The protein content of extract was 6.4 mg/ml. The extract was stored at -20℃ until used.

Immunization and preparation of antisera

Albino rat (*Wistar imanichi*, 200～250 g) was immunized with mixture containing equivalent volume of crude extract and Freunds complete adjuvant as described by Freund *et al.*[7], Hosoii[8] and Kim and Choi[11]. The procedure for preparation of primary antisera from immunized rat was presented as Fig. 1.

Immunohistochemical staining

For immunohistochemical staining, the sparganum and adult worm were fixed in 10 % neutral formalin solution and washed 3 times in 0.01 M PBS. The worms were freezed and cut with cryostat sectioner. The preparation of semithin sections was done as previously described[11]. Immunoavidin-biotin complex peroxidase staining was completed as described by Kim and Choi[11] and Takahashi *et al.*[23] By treating the tissue sections in 1 % hydrogenperoxide solution (H_2O_2) for 10 minutes, endogenous peroxidase activity was inhibited. After rinsing in buffer solution(PBS), the sections were incubated in 3 % skim milk buffer solution to block the nonspecific protein. The sections were reacted with primary antibodies in serum dilution of 1:100 in TBS(Tris-buffered saline solution, pH 7.5), and then avidin-biotinylated secondary antibody conjugate peroxidase(serum dilution of 1:800 in TBS) for 30 min. respectively. Antigen-positive sites were identified by peroxidase reaction, as indicated by brown deposits. Finally the immunostained sections were counterstained with

hematoxylin. All procedures were done at room temperature.

Immunogold labelling assay

Immunogold labelling assay was processed by the modified method as described by Bendayan[2], Fayer *et al.*[5] and Kwon *et al.*[14] After fixation in 0.8 % glutaraldehyde and 3 % paraformaldehyde in 0.1 M cacodylate buffer at pH 7.2, 4℃, for 15 min., and 2~5 min. rinses in buffer alone, tissues were dehydrated through a graded series of ethanol to 95 % and then transferred to lowycryl embedding resin for infiltration. Tissues in tightly stoppered capsules were incubated under UV for 12 hr to polymerize the resin. Ultrathin sections were made from worm and lymph node. Sections were stained as follow. First, sections were hydrated in TBS for 10~20 min., and were treated by TBS containing 5 % hydrogen peroxide (H_2O_2) for 5 min. to inhibit the endogenous peroxidase activity, and then with TBS containing 3 % skim milk for 30 min. to block nonspecific antibody binding. They were washed 3 times with TBS-tween-20 solution. Sections were incubated for 90 min. with primary antibody(serum dilution 1:20 in TBS) in humid box, and were washed 3 times as above. Washed sections were covered for 90 min. with secondary antibody, biotinylated anti-rat IgE(secondary antibody dilution 1:50 in TBS) in humid box. Sections were washed 3 times

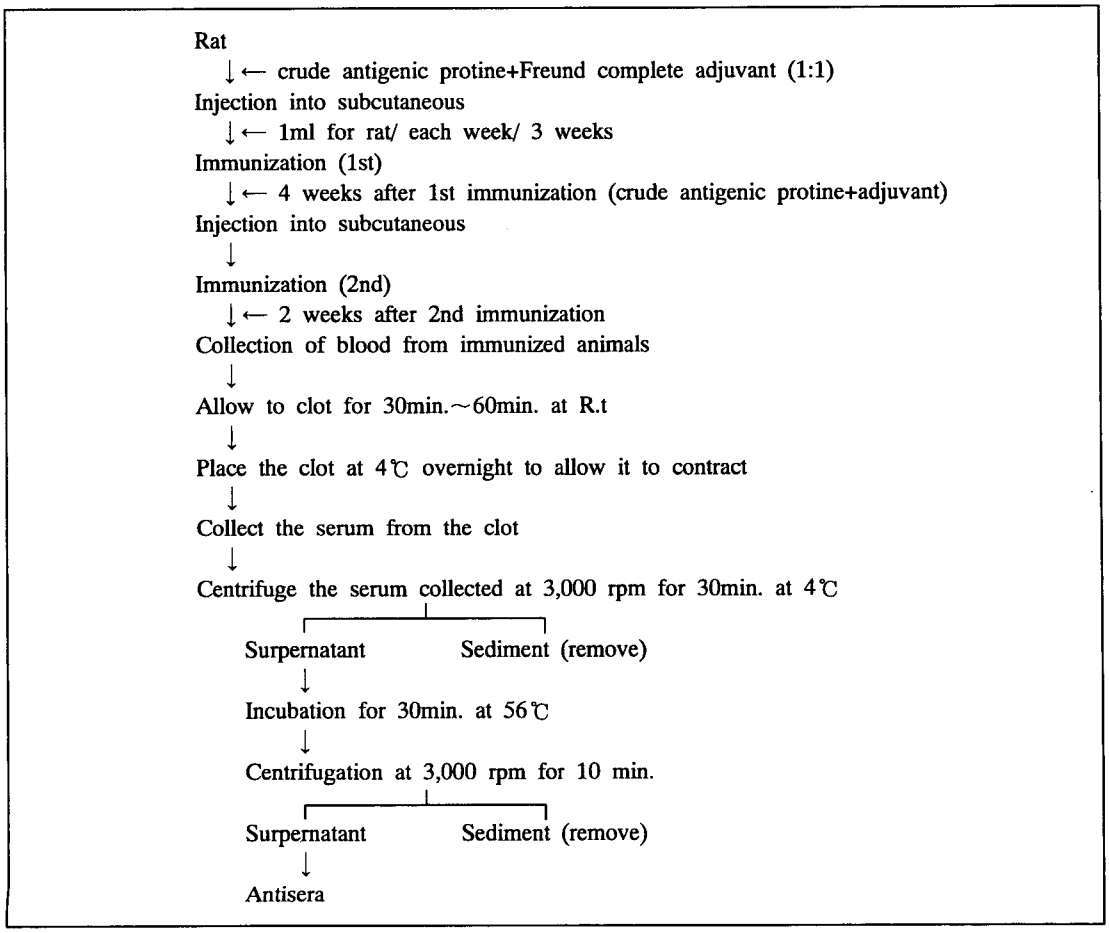

Fig. 1. Procedure of the preparation of primary antiesera from rat.

as above, and then covered for 60 min. with gold-streptavidin 20 nm. Finally sections were stained with uranylacetate(saturated aqueous) for 10 min. to enhance contrast.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) was carried out according to the described method of Laemmli *et al.*[15], Sutton *et al.*[22] and manual procedure of Bio-Rad. Vertical electrophoresis system of mini-protean II (Bio-Rad) was used. Separating gel(12 % polyacrylamide) contained 1 % SDS in 1.5 M Tris buffer(pH 8.4), and then samples were applied to sample wells made in the 5 % stacking gel. After electrophoresis, the gel was stained for 2 hours in 0.1 % Coomassie brilliand blue R-250 containing 4.5 % acetic acid and 25 % methanol. Finally, it was destained with 25 % methanol and 10 % acetic acid for overnight.

Immunoblotting

Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose filter membranes(Bio-Rad). Blot-transfer was carried out according to the method of kim and Choi[11], Towbin *et al.*[24] and Tsang *et al.*[25] After transfer, the membranes were washed 3 times for 10 minutes each in washing solution (0.01M TBS-0.1 % tween-20). Membranes were blocked by soaking in 3 % skim milk powder-0.01 M TBS(pH 7.5) with 0.1 % Tween-20 for 1 hour. After washing the membrane again in TBS, nitrocellulose membrane was incubated in primary antibody(1:100 dilution) for overnight. The strips were washed 3 times and then incubated for 1 hour with 1:100 and 1:50 dilution of biotinylated anti-rat IgG and IgE, respectively. After 3 more washes, the strips were reacted in avidine-biotinylated peroxidase(1:800 dilution in TBS) and substrate(0.05 % 4-chloro-1-naphthal in 1 part cold methanol to 5 parts 0.05 M Tris/0.25 M NaCl with 0.01 % H_2O_2) for 30 min., and the reaction was stopped by washing 3 times in distilled water.

RESULTS

Localization of antigenic components inducing IgE and IgG antibody in worms of *Spirometra erinacei*

The sections of sparganum and adult worm were stained by ABC peroxidase-linked immunohistochemical techniques. The localization of antigenic components reacting with IgE and IgG was performed (Fig. 2).

Syncytial tegument, tegument cells, muscle cells, parenchymal tissues, and the lining of the calcareous corpuscles in sparganum were immunopositive in immunohistochemical staining using the IgG antibody (Fig. 2-2). When IgE antibody was applied, however, the positive reaction was exhibited only at muscle cells of sparganum (Fig. 2-1). But in the adult worm, the positive reaction was observed not only at muscles cells but at the linining cells of vitelline follicles in mature proglottis and surface of egg shell within uterus of graved proglottis of adult worm (Fig. 2-3, 4). Table 1 presents the relative sensitivity of positive reaction with IgE antibody in the tissues of worm.

Ultralocalization of antigenic component particles

Figure 3 shows the ultralocalization of antigenic components in sparganum. The sections were exposed to secondary antibody of the anti-rat IgE and IgG labelling gold, respectively. In tegument of sparganum, antigenic proteins reacting with IgE and IgG antibody were distributed at syncytial tegument and tegumental cells. The density of particles, however, showed difference between the antigenic components inducing IgG and IgE. Antigen particles inducing IgG were distributed more densely, compared with the distribution of antigen particles inducing IgE in tissues ex-

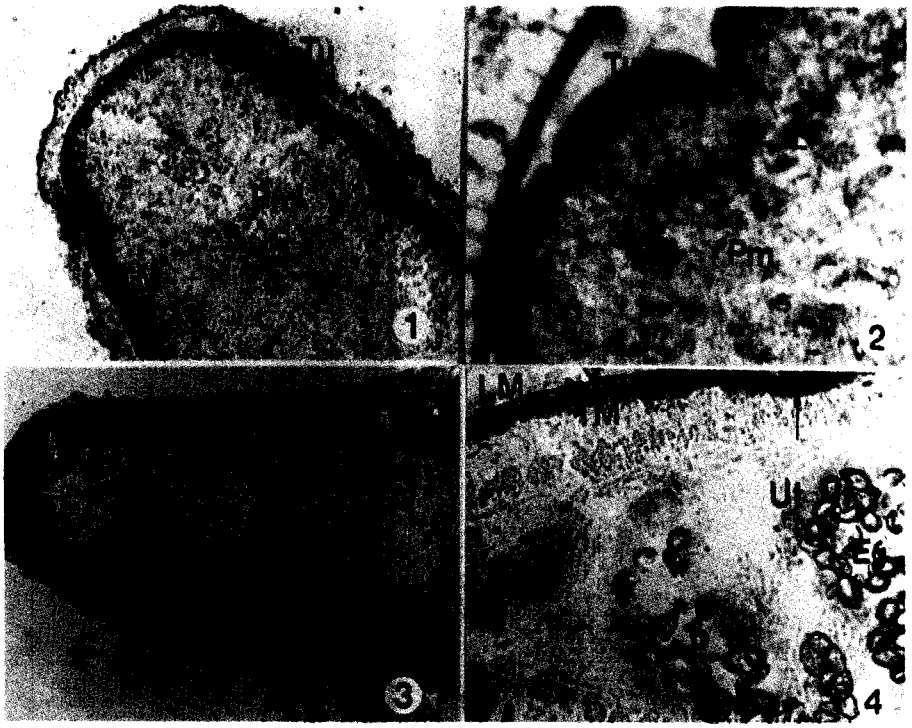

Fig. 2. Localization of IgG and IgE reacting antigenic protein in adult and sparganum(larva: plerocercoid) by ABC immunohistochemical method
1. IgE-antigenic protein in sparganum(×80)
2. IgG-antigenic protein in sparganum(×80)
3. IgE-antigenic protein in mature proglottid of adult(×80)
4. IgE-antigenic protein in gravid proglottid of adult(×80)
arrow →): indicates localization of antigenic protein
Tu: tegument, Pm: parenchyma, TM: transverse muscle, LM: longitudinal muscle, Te: testis, Ov: ovary, Ut: uterus, Vi: vitelline follicles, Eg: egg.

Table 1. Histochemical localization of antigenic portein inducing IgE in *Spirometra erinacei.*

Tissue	Sparganum	Adult	
		Mature proglottid	Gravid proglottid
Tu	—	—	—
Sub-Tu	++	++	++
Mascular	+++	+++	+++
Parenchyma	—	—	—
Egg contained in uterus	—	—	++
Vitelline follicles	—	+	—

Tu: tegument, Sub-Tu: subtegument, relative sensitivity of reaction by IgE: - no reaction, + slightly, ++ moderately, +++ intensively

amined.

The protein bands of crude extract resolved by SDS and EITB

The crude extract from adult worm were separated on 12 % polyacrylamide gels. Coomassie blue staining revealed 43 bands in eluate lane ranging from >200 kDa to 21 kDa with major bands of 106, 95, 92, 87, 74, 68, 59, 55, 50, 48, 42, 40, 35, 33, 30, 28, 27, 23,

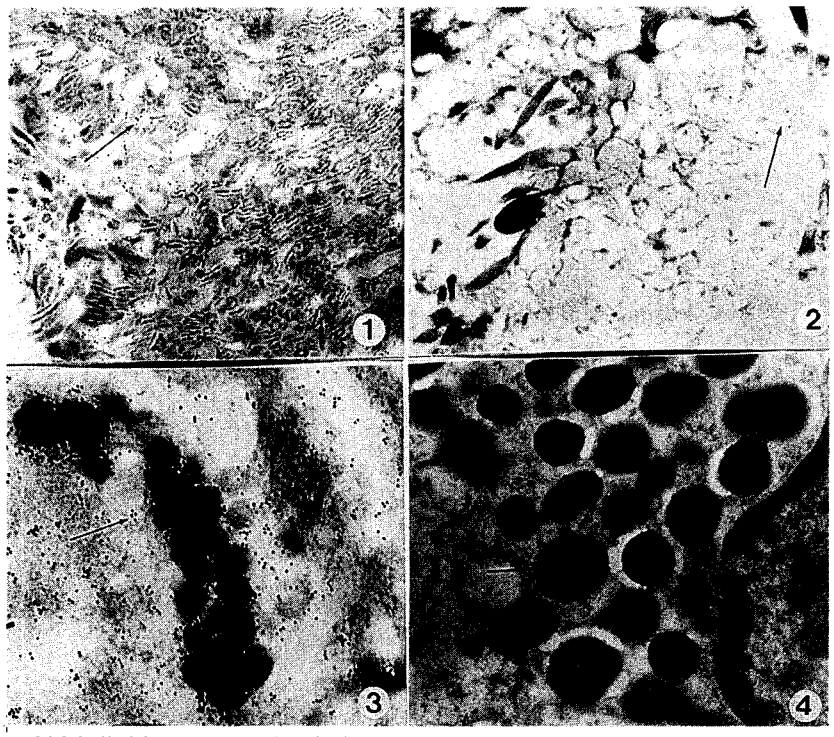

Fig. 3. By gold labelled immunocytochemical method ultralocalization of IgE, IgG antigenic particles in tegument of sparganum.
1. IgG-inducing antigenic particles in syncytial tegument of sparganum(×44,200)
2. IgE-inducing antigenic particles in syncytial tegument of sparganum(×44,200)
3. IgG-inducing antigenic particles in around secretory cell in tegument of sparganum(×78,000)
4. IgE-inducing antigenic particles in around secretory cell in tegument of sparganum(×52,000)
arrow ⟶): indicates localization of antigenic particles

and 21 kDa. Also, the crude extract from sparganum revealed 36 bands in elute lane ranging from >200 kDa to 21 kDa with major bands of 122, 104, 79, 74, 59, 55, 44, 43, 42, 33, 32, 30, 27, 25, 23, and 21 kDa. As shown in Fig. 4, 34 bands from both extracts were migrated to the same positions. 3 bands of 122, 86, and 43 kDa were only resolved from the crude extract of sparganum, while 3 bands of 112, 84 and 62 kDa were only resolved from adult worm. The antigenic components resolved from the crude extracts of worms were recognized by immunoblotting using IgE and IgG antibody. Analysis of antigenic components detected by IgE and IgG antibodies was showed as Fig. 5, and were summarized in Table 2. 21 bands of antigenic components were recognized in adult worm and same number of bands were recognized in sparganum by IgG antibody. 13 bands of them were cross reacted, indicating that they were common antigenic components between adult worm and sparganum. 6 bands of 304, 268, 174, 162, 106, and 16 kDa were found only in adult worm, and 7 bands of 412, 356, 252, 170, 146, 64, and 23 kDa only in sparganum. 19 bands of them were recognized in adult worm and 13 bands in sparganum by IgE antibody. 10 bands of them were common antigenic components. 9 bands of 440, 356, 304, 268, 252, 224, 162, 86, and 62 kDa were found only from adult worm, and 3 bands of 204, 152, and 79 kDa from sparganum. 3 bands of 116, 92, and 59 kDa were recognized with IgG and IgE antibody as common antigen components in adult worm and

Table 2. Antigen protein from adult and sparganum of *Spiroemtra erinacei* recognized by antibodies of IgG and IgE

Antigenic protein (kDa)	Reaction of Antibodies			
	IgG		IgE	
	Adult	Sparganum	Adult	Sparganum
440	+++	++	++	—
412	—	++	—	—
394	+++	++	—	—
356	—	++	++	—
326	—	—	+	+
304	+++	—	+	—
286	—	—	+	+
268	+++	—	+	—
252	—	++	+	—
239	+++	++	—	—
223	—	—	+	—
204	+++	++	—	+
182	—	—	—	—
174	+++	—	++	+
170	—	++	—	—
162	++	—	+	—
152	—	—	—	+
146	—	++	—	—
116	+++	+++	+++	+++
106	+	—	++	+
104	+++	+++	—	—
97	++	++	—	—
92	++	++	++	+
86	++	++	+	—
79	—	++	—	+
74	+	—	—	—
72	++	+	+	±
64	—	+	—	—
62	+++	—	++	—
59	++	+	+	±
48	++	++	—	—
43	—	—	±	±
40	—	—	+	±
36	++	±	—	—
32	++	++	—	—
23	—	±	—	—
16	+++	—	—	—

relative sensitivity of reaction by IgG and IgE antibodies: +++ intensively, ++ moderately, + slightly, ± doubetely, - negative

sparganum. In adult worms, 11 bands of antigenic components were recognized as common antigen while, in sparganum, 5 bands of antigen were common antigen component inducing both IgG and IgE antibody. Table 2 presents antigenic components recognized with IgE and IgG antibody in adult worm and in sparganum, respectively.

DISCUSSION

The localization of the antigenic components inducing IgE and IgG antibodies was investigated. The result was presented in Fig. 2. In both sparganum and adult worm, common antigenic components inducing IgE and IgG antibodies were detected at the muscle cells of muscular layer. The antigenic components in-

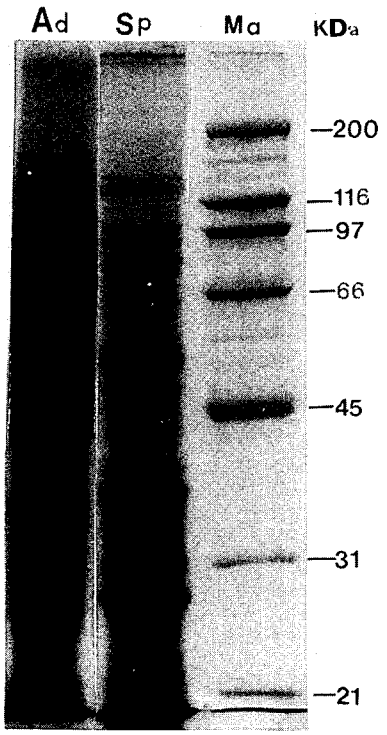

Fig. 4. SDS-PAGE of crude extract from adult and plerocercoid(sparganum) of *Spirometra erinacei*. Ma: marker, Ad: adult, Sp: sparganum.

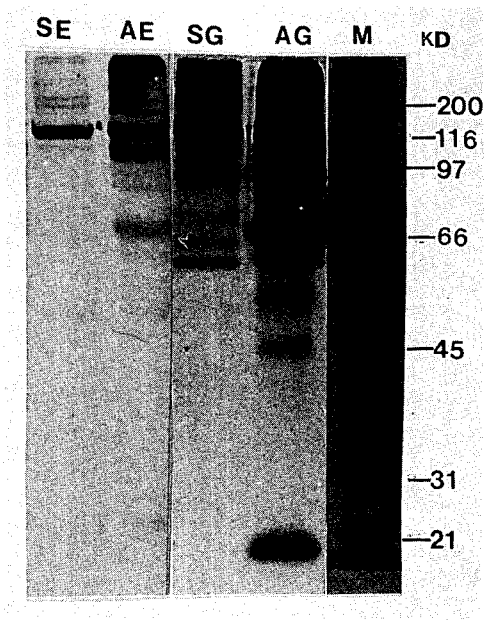

Fig. 5. The reactive fraction of IgG and IgE inducing antigenic protein with antisera immunized with crude extract from *Spirometra erinacei* by EITB.
M: marker, AG: IgG in crude extract from adult
SG: IgG in crude extract from sparganum
AE: IgE in crude extract from adult
SE: IgE in crude extract from sparganum.

ducing IgG were observed in tegument and in parenchyma, while antigenic components inducing IgE were not observed in both tissues. These areas of immunopositive staining by IgG antibody were corresponded to the distribution of IgG antigen in sparganum by IFA (immunofluorescent antibody assay) and by ABC complex peroxidase linked immunoassay, respectively[11,12]. Kim *et al.*[12] reported that the antigenic proteins of 36 and 26 kDa were specifically reacted with sera from sparganosis patients. These antigens were found in the upper layer of tegument, and a part of parenchyma by monoclonal IgG antibody, whereas immunoreactive areas by polyclonal antiserum were the upper layer of tegument, parenchyma, excretory sac, muscular layer, and calcareous corpuscle. This report was consistent with our results showing the localization of IgG antigenic component in sparganum and in adult of *Spirometra* by polyclonal antisera from rat.

The antigenic components inducing IgE in adult worm were mainly distributed in the muscular layer as in sparganum, but immunopositive reaction was also found in vitelline follicles of mature proglottis and the surface of egg shell within uterus of graved proglottis. Especially, the immunoreactivity of egg in *Spirometra* supports other results that soluble components from eggs of *Schistosoma mansoni* and *S. japonicum* might be functional antigen[18,19]. Thus further studies is necessary to elucidate the antigenic components of eggs.

To find difference in localization of antigenic components inducing IgG and IgE in the integument, we applied the immunogold labelling technique to the same tissue. In tegument of *Spirometra*, the intensity of immunogold labelling by IgG antibody was higher than that by IgE antibody (Fig. 3). Using

monoclonal IgG and IgM conjugated-gold labelling assay, Speer *et al.*[21] reported that antigenic components inducing IgG and IgM showed different distribution according to the developmental stages of *Eimera*. In studies on the distribution of antigenic component inducing IgG from the different developmental stages of *Paragonimus westermani*, Kim *et al.*[12] reported that 4 week-old larva showed high antigenicity at syncytial tegument, while 12 week-old worm revealed weak antigenicity at tegument with positive reaction at vitelline follicle, secretory granules of parenchyma, and excretory vesicles. In this studies, the localization of antigen-inducing IgE was not recognized at tegument by immunohistochemical technique but weak immunolabelling was observed at same tissue by immunogold-labelling technique. Whereas high density of IgG antigenic particles was observed at the tegument using same technique.

As shown in Fig. 5, 13 protein bands recognized with IgG antibody were the common antigenic components between sparganum and adult of *Spirometra*. Of protein bands recognized with IgE antibody, 19 bands were antigenic components in adult worm, and 13 bands in sparganum. Also, 10 bands of them were common antigen. These results suggest that adult worm might have more antigenic components than those in sparganum. The possible explanation for this result is that some specific organs like vitelline follicle and eggs are differentiated during adult stage. Antigenic proteins of 116, 92, and 59 kDa showed cross reactivity by IgE and IgG antibody, indicating they were nonspecific antigen. Kim *et al.*[12] described that the 36 kDa and 29 kDa bands from sparganum extracts were antigenic components recognized with monoclonal IgE antibody. In our studies, 36 kDa antigenic component was immunoreactive to IgG antibody whereas 29 kDa antigenic component showed immunonegative staining to same antibody.

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=국문초록=

*Spirometra erinacei*에서 IgE와 IgG 항체를 유도하는 항원성분의 면역조직화학적 위치와 특성

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*Spirometra erinacei*의 유충인 sparganum(metacercoid)에 감염되었을 때 호산성백혈구의 증가와 IgE 항체역가가 증가된다는 보고가 있다. 이 기생충에 감염되었을 때 IgG 항체와 IgE 항체를 유도하는 충체의 항원성분의 소재를 면역세포조직화학적 방법으로 충체의 성체와 유충에서 비교하였고, 또한 충체의 추출물을 SDS-PAGE 와 EITB 를 이용하여 IgG 와 IgE 항체 유도 항원성분의 면역적 특성도 추구하였다. IgG 와 IgE 항체 유도성분은 성체와 유충의 근층에서 공통적으로 분포되어 있었고, IgG 항원성분은 근층 뿐만아니라 외피층과 유조직층에서도 반응이 나타났으며, 성체의 수태편절에서는 자궁 내에 있는 충난의 표면에서도 반응이 나타났다. 충체의 외피층에서 항원성분을 면역황금표지법으로 관찰한 결과, 충체의 외피층(tegument)에서 IgG 항원성분의 분포밀도가 IgE 항원성분의 밀도보다 컸다. 충체의 추출물 중 IgG, IgE 유도 항원성 단백질의 면역학적 특성을 비교하였다. 성체의 추출물의 43개 분획 중 21개 분획이 IgG 항원성분으로서 반응하였고, IgE 항원성분으로는 21개 분획에서 반응하였다. 이들 중 11개 분획(410, 304, 268, 174, 162, 116, 92, 86, 72, 62, and 59 kDa)에서 IgG 와 IgE 가 교차반응하였으며, 유충의 추출물의 36개 분획 중 IgG 항원성분으로 22개의 분획에서 반응이 나타났고, IgE 항원성분으로는 13개의 분획에서 반응하였으며, 이들 중 5개 분획(204, 116, 92, 79, and 59 kDa)에서 IgG 와 IgE 가 서로 교차반응하였다.

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