Changes of IgM and IgG antibody levels in experimental rabbit anisakiasis as observed by ELISA and SDS-PAGE/immunoblot

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Abstract: Antibody changes in experimental anisakiasis were observed in 10 rabbits which were infected each with 10 *Anisakis simplex* larvae. The sera were collected before and on the 6th to the 95th day after the infection. Using crude saline extract of *Anisakis* larvae as antigen, specific IgM and IgG antibody levels were observed by ELISA and SDS-polyacrylamide gel electrophoresis/immunoblot. Levels of specific-IgM antibody were elevated from the 6th day, reached their peaks on the 11th day after the infection, and dropped thereafter. Serum levels of IgG antibody increased from the 6th day and reached their peak on the 26th day after the infection, and decreased gradually thereafter. When SDS-PAGE of the crude extract was done, at least forty-one SDS-polypeptide bands were recognized. Of them, IgM antibody reacted mainly to the bands of 168, 95, 74, 64, 51, 47 and 34 kDa while IgG antibody reacted strongly to 168, 92, 85, 64, 58, 52, 42 and 40 kDa bands. The crude extract showed negligible cross reactions with sera of other parasitic diseases and normal control.

Key words: *Anisakis simplex* larvae, experimental anisakiasis, rabbit, antibody, ELISA, SDS-PAGE/immunoblot.

INTRODUCTION

Anisakiasis is an acute or a chronic gastrointestinal disease caused by the invasion of *Anisakis simplex* larvae. Nematode larvae of *Pseudoterranova decipiens* can also infect man (Suzuki *et al.*, 1972; Seo *et al.*, 1984; Miyamoto, 1990). Human infections are acquired when the raw marine fish and squids are ingested. Geographically, human anisakiasis is most endemic in Japan. However, as consumption of raw fish is spread world-widely, anisakiasis becomes now almost a cosmopolitan disease (Sugimachi *et al.*, 1985; Ishikura, 1989; Sakanari, 1990). In Korea, a total of 93 cases of anisakiasis were

recorded since 1971 (Kim *et al.*, 1971). This figure could be the result of underreporting.

When ingested, *Anisakis* larvae actively penetrate the wall of the stomach or small intestine and cause acute gastrointestinal symptoms mimicking acute appendicitis or peptic ulcer (Yokogawa and Yoshimura, 1967; Ohtaki and Ohtaki, 1989). Intestinal obstruction may occur (Fujino *et al.*, 1984; Kim *et al.*, 1991). Occasionally, the omentum, colon, peritoneum, pancreas and liver can be invaded by the larva and resulted a symptomatic or an asymptomatic granulomatosis which are difficult to diagnose. These rare types of anisakiasis are usually diagnosed postoperatively (Kobayashi *et al.*, 1985; Kikuchi *et al.*, 1990).

Due to the application of endoscopy in diagnosing gastric anisakiasis since the 1970s, more cases were diagnosed preoperatively (Ishikura

1989; Yamamoto and Minami, 1990). Nevertheless, intestinal or chronic anisakiasis is still difficult to diagnose correctly because of introducing the gastrofiberscope into the lesion is almost impossible and their clinical manifestations are ambiguous. Serologic tests may be helpful in these conditions. In order to evaluate the diagnostic efficacy of the antibody tests, such as intradermal test, radioallergosorbent assay (RAST) and enzyme-linked immunosorbent assay (ELISA) have been applied (Suzuki et al., 1974; 1975; Desowitz et al., 1985; Hong and Lee, 1987; Tsuji, 1989). Of them, ELISA is now widely used because of its higher sensitivity and specificity. Results of the antibody tests show that positive reactions are closely related with the surviving worms in the patients. Up until now, however, its applicability of the antibody tests is still under investigation to establish the practical value.

The present study is, therefore, designed to observe the changes of Anisakis-specific IgM and IgG antibody levels in experimental rabbit anisakiasis by ELISA and SDS-PAGE/immunoblot.

MATERIALS AND METHODS

1. Collection of Anisakis larvae and experimental infection

Anisakis simplex larvae were collected from the viscera of Astroconger myriaster which were purchased at a fish market in Seoul; after removal of host tissues attached, the worms were washed with saline 3 times and stored at −40°C until use. For experimental infection, live Anisakis larvae were secured the day before the infection, and stored in physiologic saline at 4°C. A batch of 10 rabbits was infected with 10 larvae each by using a gavage tube.

2. Collection of infected rabbit sera

The sera of infected rabbits were collected from the ear vein on the day of experimental infection, the 6th, 11th, 19th, 26th, 35th, 45th, 56th, 68th and 95th postinfection days. The sera were stored at −40°C until use. Preinfected sera were used as the control.

3. Other sera of parasitic infections

Nematodiasis: The sera of mice experimentally infected with Trichinella spiralis were obtained by heart puncture on the 60th days after the infection with 500 muscle larvae. Two patients of filarial elephantiasis were also used.

Trematodiasis: Ten patient sera each of paragonimiasis and clonorchiasis which were confirmed by the egg were used in the study.

Cestodiasis: Ten patient sera each of sparganosis, cysticercosis and T. saginata infected sera file0 kept at randomly selected from the serum were Department of Parasitology, Chung-Ang University. They showed strong positive reaction to homologous antigens by ELISA.

4. Crude saline extract of Anisakis larvae

A total of 967 Anisakis larvae (3.03 g in wet weight) were homogenized with a teflon-pestle tissue homogenizer in physiologic saline containing 0.006% (W/V) phenylmethylsulfonyl fluoride (PMSF). The emulsion was shaken for 2 hours and kept overnight. It was centrifuged by 10,000 g for an hour. The supernatant was regarded as a crude saline extract of Anisakis larvae. All procedures were done at 4°C. The protein content was 6.88 mg/ml when measured by the method of Lowry et al. (1951).

5. Enzyme-linked immunosorbent assay (ELISA)

The method of Voller et al. (1976) was adopted. After checkerboard titrations, 5 µg/ml of protein in Anisakis extract in carbonate buffer (pH 9.6) and 1:50 diluted rabbit sera in phosphate buffered saline containing 0.05 % Tween 20 (PBS/T, pH 7.4) were used for the detection of IgM antibody. Peroxidase conjugated anti-rabbit IgM (µ-chain specific, Cappel, USA) was diluted at 1:500. In IgG antibody test, 2.5 µg/ml of protein in the extract and 1:100 diluted sera were used. Anti-rabbit IgG (heavy- and light-chain specific, Cappel, USA) was diluted at 1:1,000 in PBS/T. The sera and conjugate reacted for 2 hours at 36°C respectively. The color was developed by 0.01% OPD chromogen containing 0.03% H_2O_2. The absorbance was

read by using ELISA reader (Bio-Rad, M 3550) at 490 nm.

6. SDS-PAGE/immunoblot

The method of Laemmli (1970) was followed in SDS-PAGE and that of Tsang *et al.* (1983) was adopted in immunoblot. Stacking gel of 3% and separating gel of 7.5~15% linear gradient gel were used. Constant current of 30 mA was supplied. After the electrophoresis, the separated protein bands were transferred onto nitrocellulose (NC) paper by electrophoresis at 1 mA for 2 hours at 4°C. To detect IgM antibody reacting bands, rabbit sera were diluted at 1:25 and peroxidase conjugated anti-rabbit IgM was diluted at 1:500 in PBS/T. For IgG antibody reacting bands, 1:50 diluted sera and 1:1,000 diluted conjugate were reacted for an hour, respectively. The reactions were colored by diaminobenzidine and stopped by washing the strips in running tap water and distilled water.

RESULTS

1. Serum levels of *Anisakis*-specific IgM and IgG antibodies by ELISA

Fig. 1 showed the individual absorbance of the *Anisakis*-specific IgM antibody in infected rabbit sera. Preinfected control sera showed low absorbance from 0.00 to 0.11 (0.08±0.043). Six day after the infection, elevation of IgM antibody was recognized. The absorbance reached their peaks on the 11th day after the infection. Mean and standard deviation of absorbance was 0.25±0.163. Then, the IgM antibody levels in serum decreased. The serum levels of IgM antibody returned to pre-infection levels on the 35th day after the infection. Even on the days of 11th and 19th day after the infection, 4 rabbits showed negligible elevation of absorbance for IgM antibody (in the ranges of 0.01 and 0.08).

As shown in Fig. 2, specific-IgG antibody levels in preinfected rabbit sera exhibited mean absorbance of 0.09±0.022. Specific-IgG antibody was elevated to 0.14±0.042 on the 6th day after the infection. IgG antibody reached their peak absorbance of 0.46±0.274 on the 19-
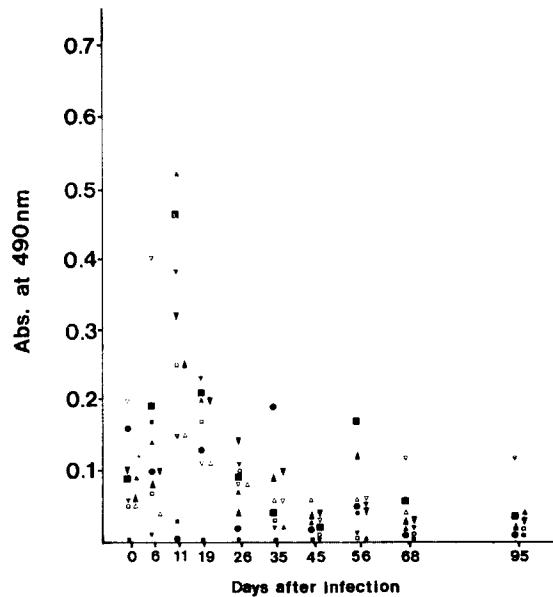

Fig. 1. Changes of the individual absorbance of *Anisakis*-specific IgM antibody in experimental rabbit anisakiasis.
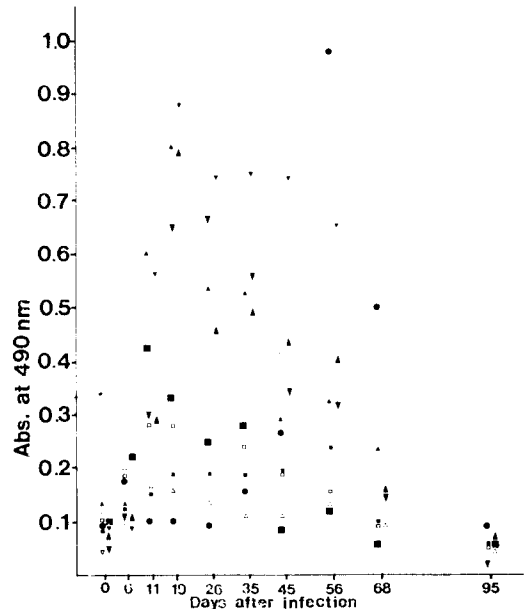

Fig. 2. *Anisakis*-specific IgG antibody levels in rabbit sera of experimental anisakiasis.

day after the infection. Thereafter, the antibody levels decreased slowly until the 98 days after the infection. During the observation period, the serum levels of IgG antibody were not elevated
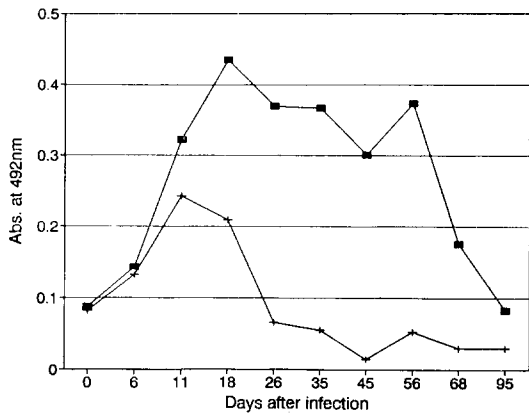
Fig. 3. Changes of mean absorbance of *Anisakis* specific-IgM(+) and -IgG(■) antibody levels (mean±S.D.) in infected rabbit sera.

in 2 rabbits. The changes of mean absorbances of IgM and IgG in experimental anisakiasis are shown in Fig. 3.

2. Protein bands reacting with antibodies in immunoblot

As shown in lane C of Fig. 4, at least forty-one protein bands were recognized in the crude saline extract. Of them, bands of 195, 145, 110, 104, 73, 69, 49, 42.5, 40, 34, 30, 24, 20, 16 and 14 kDa were stained darkly.

A total of 11 bands reacted to IgM antibody (Fig. 4). Of them, protein bands of 168, 95, 74, 64, 51, 47 and 34 kDa were the major reacting bands. The protein band of 34 kDa showed strong reactions from the 6th to the 45th days after the infection. Band of 168 kDa also showed strong reactions from the 6th to the 11th days after the infection. Protein bands of 74 and 64 kDa reacted from the 26th to the 45th days after the infection.
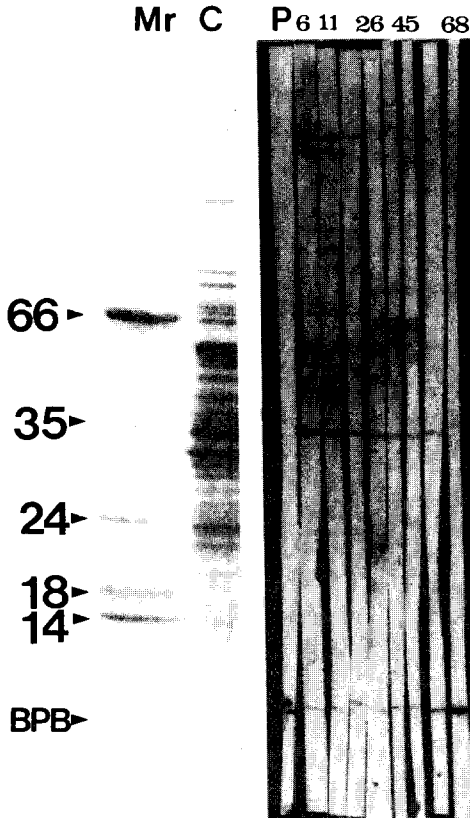

Fig. 4. SDS-PAGE/immunoblot findings of IgM reacting bands. In SDS-PAGE, 7.5~15% gradient separating gels were used. Numericals at top of the figure indicated the postinfection days. M$_r$: Molecular mass in kDa, C: Amidoblack B stained NC slip of the crude extract, P: Preinfection.
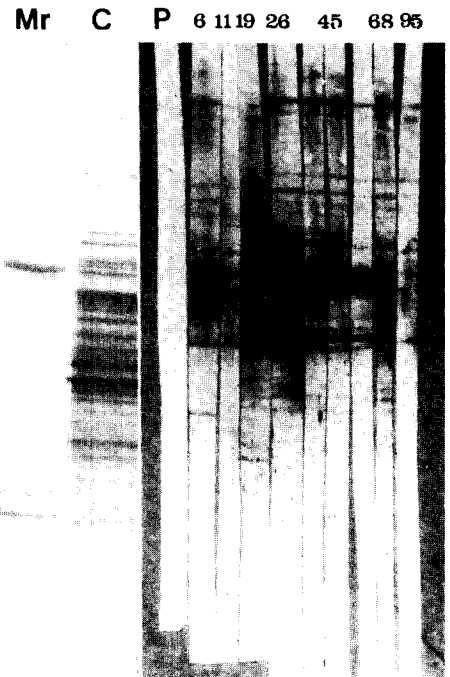
Fig. 5. Findings of IgG reacting bands in SDS-PAGE/immunoblot. In SDS-PAGE, 7.5~15% gradient separating gels were used. Numericals at top of the figure indicated the postinfection days. M$_r$: Molecular mass in kDa, C: Amidoblack B stained NC slip of the crude extract, P: Preinfection.

Table 1. Serum levels of *Anisakis*-specific IgG antibody in other parasitic infections and control

Category	No. of cases	Mean absorbance for antigen of *Anisakis*
Human sera of		
filariasis	2	0.12±0.073
paragonimiasis	10	0.10±0.082
clonorchiasis	10	0.06±0.051
sparganosis	10	0.02±0.032
cysticercosis	10	0.05±0.042
taeniasis	5	0.07±0.054
normal control	10	0.06±0.043
mouse trichinellosis	10	0.04±0.045

Fig. 5 showed the protein bands reacted to IgG antibody. More than 13 bands were recognized as antigenic bands. But bands of 168, 92, 85, 64, 58, 52, 42 and 40 kDa were strongly reacted to infected rabbit sera throughout the observation period. Bands of 195, 100, 74, 68, 48, 35, 27, 23, 18 and 17 kDa reacted to sera collected from the 26th day to the 68th day after infection. But these reactions with the IgG antibody disappeared after the 68th day after infection.

3. Cross reaction to other parasite infected sera

As shown in Table 1, when 57 sera of other parasitic infections and 10 control sera were tested by ELISA against the crude saline extract of *A. simplex* larvae, no sera exhibited absorbance above 0.13 (mean±S.D.; 0.06±0.033).

DISCUSSION

Because the patency of the infection was not examined in this study, we could not observe the sensitivity of the antibody test. Concerning the low antibody levels in some experimental rabbits, in terms of their specific IgM and IgG antibody, some variations in patent infection can be speculated.

Significant elevation of *Anisakis*-specific IgM antibody levels in infected rabbit sera was observed in short period from the 6th day to the 19th day after the infection, whereas serum levels of IgG antibody increased from the 6th

day, reached the peak on the 26th day and remained until the 56th day after the infection. Absorbance of specific IgM and IgG antibodies can not be compared directly each other, but the present results showed clearly that the elevation of *Anisakis* specific-IgM antibody was far lower and the duration of antibody elevation was shorter than those of IgG. Relative quick decrease of specific IgG antibody in experimental anisakiasis may be due to rapid degeneration and calcification of the infected larvae in a month (Hong and Lee, 1987).

Hong and Lee (1987) and Tsuji (1989) reported that significant elevation of specific IgG antibody was observed from the 10th to the 20th day after the experimental infection. In this study, elevation of IgG antibody was firstly recognized earlier on the 6th day in 4 out of 10 experimental rabbits. But antibody test in anisakiasis has still less value than gastrofiberscopy in diagnosing the invading stage of anisakiasis.

By SDS-PAGE/immunoblot using the crude extract as antigen and rabbit sera, specific IgM antibody reacted to the bands of 168, 95, 74, 64, 51, 47 and 34 kDa. All bands except 34 kDa stopped to react with IgM antibody on the 45th day after the infection. Reacting bands with specific IgG antibody were bands of 168, 92, 85, 64, 58, 52, 42 and 40 kDa. These proteins were reacting to the infected rabbit sera throughout the observation period except 85 kDa, 42 kDa and proteins of molecular mass below 34 kDa especially 56 days after the experimental infection. Similar studies were done by Lee *et al.* (1990). They reported that 46 kDa band protein was specifically reacting to only human anisakiasis serum.

Using monoclonal antibodies, Takahashi *et al.* (1986 & 1989) purified 2 different proteins from *Anisakis* larval extract. Of them, 34 kDa (An1) was produced in the renette cell of which function was known as excretion (Ishii *et al.*, 1989). The other proteins they purified were 40 and 42 kDa which were localized in the digestive tract and muscle cells (An2). In this study,

protein bands of 34 kDa and 40 kDa also showed strong reactions to infected rabbit sera. Of them, 34 kDa band was strongly reactive when the IgM antibody was reacted, on the other hand 40 kDa was reactive with the specific IgG antibody. Because the secretory-excretory components of the invading larval worm are important antigens in anisakiasis, more attention should be paid to the value of the secretory-excretory component as a diagnostic antigen.

Cross reactions between crude extracts of *Anisakis* larvae and the sera of other parasitic diseases or normal control were not found in this study. However, there were many reports which showed cross reactions to other parasitic diseases such as toxocariasis, ascariasis, hookworm disease and *Angiostrongylus* infections (Oshima, 1972; Tsuji, 1989; 1990; Lee *et al.*, 1990). To evaluate the cross reactivity of *Anisakis* antigen, further screening of the sera from cases of various nematodiases should be carried out.

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=국문초록=

ELISA와 SDS-PAGE/immunoblot을 이용한 실험적 토끼 아니사키스증에서 혈청 항체가의 변화

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인체아니사키스증은 우리나라에서도 드물지 않게 발생하나 과거에는 수술에 의한 충체의 확인만이 진단이자 치료이어서 진단에 많은 어려움을 겪었다. 그러나 위내시경이 진단과 치료에 이용되면서 많은 증례의 발견을 가져오게 되었다. 그러나 증상이 심하지 않을 경우나 위내시경의 삽입이 어려운 장(腸) 아니사키증 또는 만성 아니사키스증을 진단하기 위해서는 혈청학적 방법이 필요하다. 이 연구는 아니사키스증의 혈청학적 진단연구를 위해 붕장어에서 채집한 유충을 토끼에 10마리씩 감염시키고 IgM, IgG 항체의 출현시기와 변화양상을 ELISA와 SDS-PAGE/immunoblot으로 관찰하였다. 그 결과는 다음과 같다.

1. ELISA에 의하여 측정한 IgM 항체는 감염 6일부터 증가하여 11일째 최고치에 도달한 후 35일 이후에는 감염 전의 수준으로 저하하였고 IgG 항체가는 감염 6일부터 증가하여 26일째 최고치에 도달한 후 95일까지 감소하는 양상을 보였다.

2. 7.5~15% SDS-PAGE에서 아니사키스추출액은 최소 41개 이상의 단백분획을 나타냈으며 195, 145, 110, 104, 73, 69, 49, 42.5, 40, 34, 30, 24, 20, 16, 14 kDa가 주 분획이었다.

3. Immunoblot에 의해 IgM 항체에 반응하는 주 band는 168, 95, 74, 64, 51, 47, 34 kDa의 5개 band이었다.

4. IgG 항체와 반응하는 항원 분획은 11개 이상 관찰되었고 그 중 168, 92, 85, 64, 58, 52, 42, 40 kDa가 강한 반응을 보였다.

5. 아니사키스추출액 항원과 다른 기생충성 질환자 혈청과의 교차반응은 관찰할 수 없었다.