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Histopathological and Serological Observations on Experimental Anisakiasis of Rabbits

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INTRODUCTION

Anisakid larvae which are found from human are *Anisakis* sp., *Pseudoterranova decipiens* (synonym *Phocanema* and *Terranova decipiens*) and *Contracaecum* sp. (Beaver *et al.*, 1984). The infection by *Anisakis* sp. is called anisakiasis, whereas that by *Pseudoterranova* is codworm anisakiasis (Oshima, 1987). However, the infection by any species of anisakid larvae is called anisakiasis because the infection by *Anisakis* sp. is most common and morphological differentiation of the larvae is not easy in most infected cases.

Thousands of human anisakiasis cases have been recorded over the world since van Thiel *et al.* (1960) reported the first one in the Netherlands. Most of them were found in Japan where consumption of raw marine fishes is popular, and the remaining cases were also recorded in European, Asian and American countries (Smith and Wootten, 1978; Oshima, 1987).

In Korea, total 15 cases of human anisakiasis have been recorded since Kim *et al.* (1971) reported the first one (Cho *et al.*, 1980; Lee *et al.*, 1981; Jeong and Suk, 1984; Paik *et al.*, 1984; Seo *et al.*, 1984; Lee *et al.*, 1985; Lee *et al.*, 1986). However, real incidence of human anisakiasis is suspected to be higher than the recorded cases. Most of those cases were diagnosed as infection of *Anisakis* sp. or simply as anisakiasis without generic identification of the worm. Only two cases (Seo *et al.*, 1984; Lee *et al.*, 1985) were known to be infected by the larva of *Terranova* type A which is now called *Pseudoterranova decipiens* (Oshima, 1987). The intestinal case of *Terranova* type A larva by Seo *et al.* (1984) is regarded as a rare one because the larva is known to cause mainly gastric infection (Oshima, 1987).

The diagnosis of acute gastric anisakiasis is rather easy since endoscopy or radiological technique can be applied (Oshima, 1987). Meanwhile, preoperational diagnosis of acute intestinal anisakiasis is quite difficult because the case is usually operated in an emergency. Only the case is diagnosed by the worm sections detected from the surgical specimens. The diagnosis of chronic anisakiasis is even more difficult because the worm invades the wall of stomach or intestine to make a mass and finally to degenerate. Such patients are usually operated due to the tumor, but parasitological diagnosis is almost impossible because of unidentifiably degenerated worm.

Serodiagnosis may help such diagnostic difficulties in acute or chronic anisakiasis. Skin test and complement fixation test were tried, but they showed low efficacy (Oshima, 1972). Thereafter, indirect fluorescent antibody test (Suzuki *et al.*, 1974), latex agglutination (Yoshimura *et al.*, 1980), radioallergosorbent antibody test (RAST) (Desowitz *et al.*, 1985) and ELISA (Takahashi *et al.*, 1986) have shown good results. However, any of the serodiagnostic method in

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anisakiasis has limitations for practical use due to cross reaction (Suzuki *et al.*, 1974; Yoshimura *et al.*, 1980). Furthermore, there is few available information at present on the serological reaction by the degeneration course of the worm in anisakiasis, although application of serodiagnosis should be based upon that information.

In this context, it is necessary to understand the chronological pattern of serological reaction in anisakiasis by the duration of infection. This study aimed to observe the relation between the IgG antibody production and histopathological changes during the course of experimental anisakiasis and also to evaluate the feasibility of ELISA as a serodiagnostic tool in anisakiasis.

MATERIALS AND METHODS

1. Collection of the larvae of *Anisakis*: A total of 50 yellow corvinas (*Pseudosciaena manchurica*) and the guts of living congers (*Astroconger myriaster*) were purchased at a fisheries market in Seoul from March to September, 1987. The fish and the viscera were dissected and larval nematodes were collected. The larvae of *Anisakis* type I were isolated under a stereomicroscope and stored in physiologic saline at 4°C until the experimental infection.

2. Experimental infection to rabbits: A total of 24 New Zealand white rabbits (2.5~3.5 kg) was infected with 30 *Anisakis* larvae each into the stomach through a Levin tube.

3. Serum collection: The sera of the infected rabbits were collected from ear vein chronologically as shown in Table 1 and were stored at −20°C until use. The sera obtained before the infection were used as the control.

4. Gross and histopathological observations: The rabbits were killed 13 to 150 days after the infection (Table 1). The viscera, from stomach to sigmoid colon including mesentery, were examined grossly for the presence of the larvae. The foci of larval infection were prepared for histopathological observation.

5. ELISA: Thousands of living anisakid larvae (5.5 g in wet weight) obtained from the yellow corvinas were homogenized at 4°C with a tissue teflon-coated homogenizer. The supernatant from the homogenized solution was frozen at −80°C. Protein content of the antigen was 0.312 mg/ml by Lowry method. The antigen was thawed and used after 1:100 dilution (protein content 3.1 μg/ml) with carbonate buffer (pH 9.6). Antigen coating and further reactions were carried out in the wells of micro-ELISA plates (Titertek). The sera were used under 1:200 dilution after chequerboard titration. Peroxidase conjugated anti-rabbit IgG (H&L) goat serum (Cappel lab., U.S.A) was used at 1:4,000 dilution as conjugate, while *o*-phenylenediamine (4 mg in 10 ml PBS) was used with hydrogen peroxide (H_2O_2, 20 μl in 10 ml PBS) as substrate. The micro-ELISA procedure of McLaren *et al.* (1978) was followed in general. Optical density (O.D.) was read at 492 nm with an ELISA reader.

Table 1. The number of rabbits used by the duration of infection

Days after infection	No. of rabbits for	
	serum collection	histopathology
0*	23	0
3	23	0
13	23	2
20	23	2
30	23	3
60	19	3
90	15	3
150	1	1

*0: Before infection for the control

RESULTS

1. IgG antibody in serum by ELISA

All of the rabbit sera were tested in duplicates and their mean O.D. were used for the analysis. The O.D. of control rabbits ranged from 0.038 to 0.526 (mean 0.165). Mean O.D. increased to 0.257 (range 0.036~0.735) after 3 days, 0.448(0.075~0.912) after 13 days, 0.662(0.124~1.334) after 20 days, 1.113(0.611~1.661)

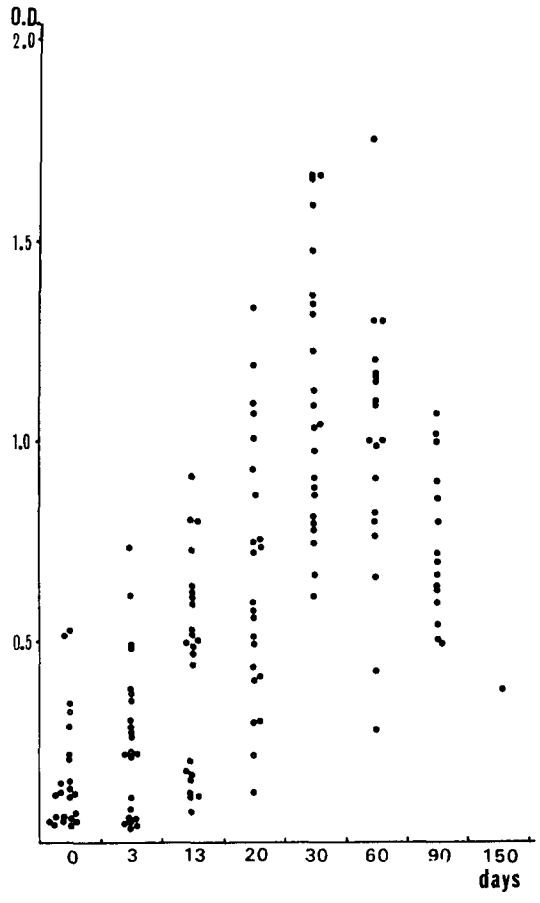

Fig. 1. IgG antibody levels (O.D. at 492nm) by ELISA in sera of experimental rabbits by duration of infection.

after 30 days, 0.993(0.281~1.750) after 60 days, 0.740(0.501~1.066) after 90 days and 0.376 after 150 days of infection. The O.D. of each rabbit were plotted in Fig. 1 and mean O.D.(±S.D.) of each group were summarized in Fig. 2 by the duration of infection.

2. Gross findings of the stomach and the intestine

Many larvae were found in the wall of the stomach, omentum and/or intestine as summarized in Table 2. The worms in subserosal layer of the stomach wall or omentum were easily detectable as in Figs. 3 & 4, while the worms in submucosal or muscle layer were found only as the thickened wall was examined through light. A few larvae were detected from the peritoneum of abdominal wall near the stomach. They were chalky white and hard, and revealed no motility. The worms which were found more than 60 days after the infection were stony hard and mummified. It became difficult to detect the larvae long after infection, and only one larva was found at stomach wall of the rabbit after 150 days.

3. Histopathological findings

1) 13 days after infection: The worms were found mainly in submucosal layer of the stomach. The infected wall was thickened 2 to 3 times by severe infiltration of inflammatory cells around the worms (Fig. 5). Most of the worms preserved their anatomical structures such as intestine, subcuticular muscles and lateral cords(Fig. 6), but a few were under necrosis. The worms were encircled by fibrinous exudate layer intermingled with inflammatory cells. The exudate layer was surrounded by

Table 2. Numbers of recovered worms in experimental anisakiasis of rabbits by location

Days after infection	No. of rabbits	Total No. of infected worms*	No. (%) of worms found at					
			Stomach	Omentum	Intestine	Mesentery	Abdominal wall	Total
13	2	60	15	1	2	5	2	2
20	2	60	2	2	2	2	0	8
30	3	90	8	3	0	3	0	14
60	3	90	7	4	0	0	0	11
90	3	90	12	3	2	1	1	19
150	1	30	1	0	0	0	0	1
Total	14	420	45(10.7)	13(3.1)	6(1.4)	11(2.6)	3(0.7)	78(18.6)

*Each rabbit was orally challenged with 30 larvae of *Anisakis* type I.

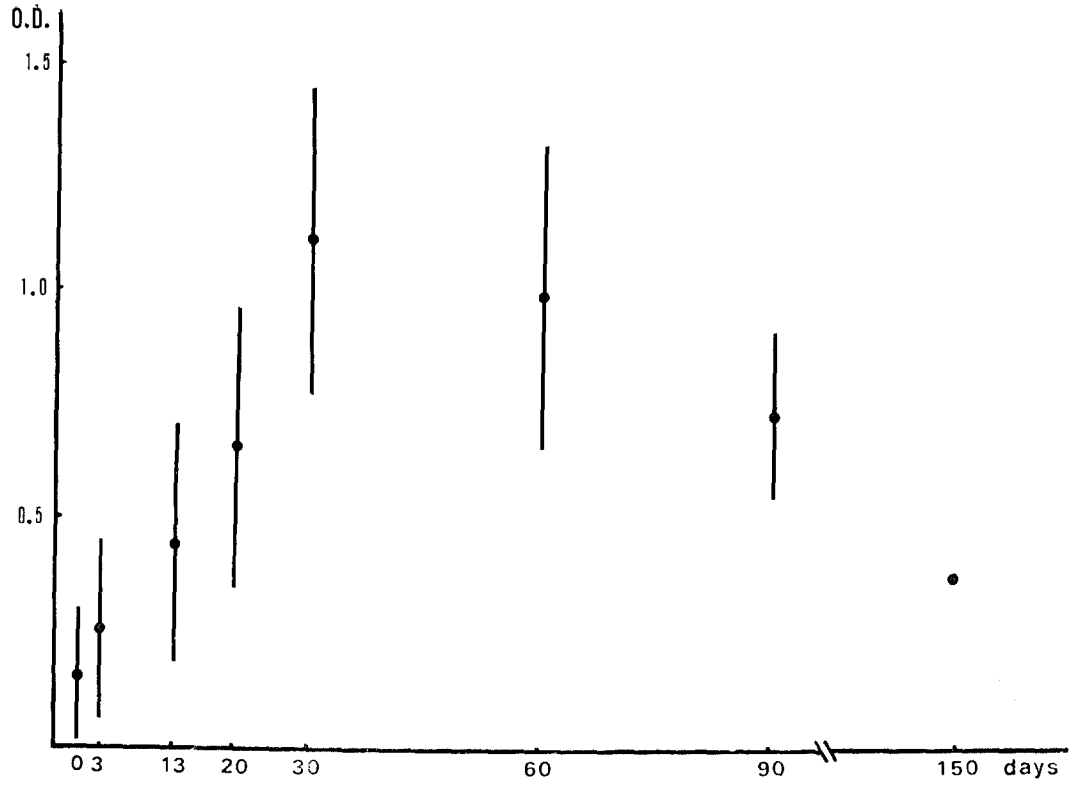

Fig. 2. Mean (±standard deviation) of the optical densities by ELISA during the course of experimental anisakiasis.

numerous histiocytes, and again neutrophils, eosinophils, lymphocytes and fibroblasts were aggregated in the submucosal layer to make a mass (Figs. 7 & 8). The inflammatory cells were infiltrated also at the base of mucosal layer and in the muscle layer. The foci of fibrinous exudate without worms were scattered at center of inflammatory cells in submucosal layer. The intestine involved by the worm showed also the submucosal mass which consisted of inflammatory cells around the worms. There was a focus of transmural invasion of inflammatory cells adjacent to the worm. The worms in the omentum were found necrotized in mass of inflammatory cells (Figs. 9 & 10).

2) 20 days after infection: A few worms were found in the stomach, intestine and mesentery. Less fibrinous exudate was found. The necrotized worm in subserosal layer of intestine was surrounded also by histiocytes, inflammatory cells and fibrous tissue (Fig. 11). Size of the mass became smaller compared with that of 13 days after infection. At mesentery, the mass size decreased more and the worm was surrounded by granulation and fibrous tissue. The necrotized worm shrank and its cuticle stippled (Fig. 12).

3) 30 days after infection: One of the worms was calcified, but others were necrotized and collapsed. Foamy epithelioid histiocytes encircled tightly the necrotic worm material, and histiocytes and eosinophils invaded the worms. Numerous inflammatory cells with fibrotic tissue surrounded the outer layer (Fig. 13). Pyknosis and nuclear debris were noted around the worm (Fig. 14).

4) 60 days after infection: The size of inflammatory cell mass diminished in submucosal layer of the stomach. More inflammatory cells invaded the worms and fibrosis progressed more (Fig. 15).

5) 90 days after infection: Almost all of the worms were calcified. Inflammatory cell

infiltration and fibrosis around the worms were observed persistently (Fig. 16). One calcified worm was found in muscle layer of the stomach, which was surrounded by less inflammatory cells and fibrous tissue than in other layers of the stomach (Fig. 17).

6) 150 days after infection: A few sections of a tiny calcified worm were detected in submucosal layer of the stomach (Fig. 18). The worm was encircled by fibrous tissue which was infiltrated by a few inflammatory cells. The stomach wall with the lesion was not so thick.

DISCUSSION

Within a few hours after oral ingestion, *Anisakis* larvae invade the wall of stomach or intestine in humans or experimental animals. The larvae are usually arrested at the wall of stomach or intestine, but often penetrate the wall to reach peritoneal cavity, omentum or mesentery.

The stomach is known as a predilection site in human or rabbits, but no such tendeny was found in dogs or albino rats (Oshima, 1987; Oyanagi, 1967; Choi and Kim, 1984). The worms in the present study were recovered mainly from the stomach as previous records.

Seven days after infection, a worm was found moving in the stomach wall (Choi and Kim, 1984). After then, the worm should die off very rapidly. All of the worms recovered later than 13 days after infection were found immotile and in the process of necrosis. Practically, the distribution of recovered worms by the organ was same throughout whole period of infection from 5 to 90 days. Therefore, it seems that *Anisakis* larvae are able to migrate the digestive tract in mammals only within the first week of infection.

The histopathological finding of the stomach and the intestine in the present study was abscess type 13 or 20 days after infection and abscess-granulomatous type later than 30 days (Oshima, 1972). When observed on 13 days, the lesion consisted of 3 zones surrounding the necrotizing worm. Fibrinous exudate zone surrounded the worm, then histiocytes linked together forming a band-like zone around exudate, and the large zone of inflammatory cells filled remaining lesion. These histological finding meant chronic nature compared with the earliest lesion (Oyanagi, 1967; Choi and Kim, 1984), which was characterized by bleeding, edema and infiltration of inflammatory cells.

After 30 days, the histopathology became more chronic; almost all exudate zone disappeared and histiocytes attached the worm. A calcified worm was found also from 30 days after the infection. From this period, the dead worms began to be dissolved by host cells or calcified. Most of the worms were calcified, and fibrosis around the worm was the major finding 90 days after the infection. On 150 days, only one calcified worm was detected. These pathological process indicates that the dead worms are resolved almost completely by granulation tissue until 90 days if they have not been calcified. However, the calcified worms remain longer than 150 days in the tissues.

Serological reaction to *Anisakis* larvae was observed to increase significantly after the infection. O.D. by ELISA increased continuously up to 30 days to make a plateau until 90 days, and then decreased slowly until 150 days (Fig. 2). After 150 days, the O.D. decreased below that of 13 days after the infection. In paragonimiasis of cats, it is known that the maximum O.D. by ELISA is found after 140-180 days from the metacercarial infection (Choi *et al.*, 1986).

Such a fluctuating pattern of antibody production in anisakiasis should be interpreted with the course of worm degeneration. The larvae die within a week and undergo necrosis and absorption slowly until 90 days after the infection. Especially the present study showed that the worms began to be resolved or calcified around 30 days after infection. At this period, serum Ig G antibody was found at its maximum level. These two corresponding findings suggested that the degenerating *Anisakis* larvae released the

greatest antigenic material for the first 30 days after infection.

ELISA is now the most commonly used serodiagnostic tool for various parasitic diseases. This method could be applied to human anisakiasis if the cross reaction, which is well-known between *Anisakis* and its taxonomic cousins such as *Ascaris* and *Toxocara* (Suzuki *et al.*, 1974; Yoshimura *et al.*, 1980), is minimized by antigen purification. Especially it will be valuable for confirmative diagnosis in cases of chronic anisakiasis manifested by eosinophilic granuloma with only unidentifiably necrotized worm or even without any evidence of parasites. Takahashi *et al.* (1986) applied ELISA to 7 suspected human cases of anisakiasis and all were found to have increased level of serum Ig (GAM). Serological diagnosis by ELISA would be helpful for confirming anisakiasis in a certain period, especially in the first several months after the infection.

SUMMARY

This study was performed to observe histopathological changes and serological reactions in chronic anisakiasis of rabbits. Each rabbit was infected per os with 30 larvae of *Anisakis* type I. Their sera were collected chronologically and the rabbits were killed for histopathological examination, 3, 13, 20, 30, 60, 90 and 150 days after the infection. The results were summarized as below.

1. Most of the larvae were recovered from the stomach, but a few from the omentum, intestine, mesentery and abdominal wall. The recovery rates and distribution of worms by organ were not differed by duration of infection.

2. Histologically the lesion was abscess type on 13 days, i.e., the dead worms were surrounded by fibrinous exudate, histiocytes and thick zone of numerous inflammatory cells. After 30 days, histiocytes were found to invade the worms and the lesion was changing into abscess-granulomatous type. Also a calcified worm was found on the 30th day. After then the worms were observed to be dissolved slowly until 90 days. On 150 day, only one calcified worm was observed.

3. The levels of serum IgG antibody by ELISA reached their maximum 30 days after the infection. After then, it decreased slowly until 150 days after the infection.

Above serological and histopathological findings indicated that antigenic stimulation from degenerating *Anisakis* larvae was the greatest during the first 30 days after infection. This period was corresponding with the beginning of worm resolution or calcification. Serologic test by ELISA would be a valuable tool for confirming chronic anisakiasis.

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=국문초록=

실험적 토끼 아니사키스증에서의 조직학적 병변과 혈청 항체가의 경시적 변동 양상

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아니사키스증에서 조직병리학적 병변과 혈청학적 반응의 시간 경과에 따른 변동 양상을 관찰하고자 이 실험을 실시하였다. 조기와 아나고 내장에서 구한 *Anisakis* type I 유충을 체중 3kg 내외의 집토끼에 30마리씩 경구 투여하고, 감염 전과 감염 후 3, 13, 20, 30, 60, 90, 150일에 채혈하여 혈청을 냉동 보관 하였다. 동시에 13, 20, 30, 60, 90, 150일에 1~3마리 씩 도살하여 위부터 하행결장까지 육안으로 검사하여, 아니사키스 충체의 유무를 조사하고, 충체가 감염된 부위를 선택하여 조직학적으로 관찰하였다. 또한 냉동 보관한 혈청을 이용한 micro-ELISA를 시행하여 항체가의 변동을 관찰하였다.

그 결과를 요약하면 다음과 같다.

1. 육안 검사에서 아니사키스 유충은 위에서 57.7%가 검출되고, 그 이외에 대망(omentum), 장, 장간막, 복막등에서도 검출되었다. 감염기간에 따른 충체의 검출율이나 검출부위의 차이는 인정할 수 없었다. 전체 충체 검출율은 18.6%이고 살아서 움직이는 충체는 하나도 없었다.

2. 조직병리학적으로 관찰한 바, 감염 후 13일에는 대부분의 충체가 내부구조를 온전하게 유지하고 있으면서, 충체 주위에는 농양이 형성되어 있어 감염된 위장관벽이 2~3배 두꺼워져 있었다. 충체 주변에 섬유성 삼출액이 염증세포와 혼합되어 있고 그 주위를 조직세포(histiocytes)가 층을 이루며 둘러싸고 다시 주위의 병소 대부분을 수많은 염증세포(중성구, 호산구, 림프구등)와 섬유아세포가 차지하고 있었다. 감염 후 20일과 30일에는 삼출액이 차츰 줄고, 섬유소가 증식하는 육아조직으로 전환되었다. 충체는 20일 관찰된 것부터 괴사에 빠지고, 30일에는 조직구가 직접 충체를 둘러싸며 숙주세포가 충체를 침윤하는 것이 뚜렷이 관찰되었다. 또 30일부터 칼슘이 침착된 충체가 관찰되었다. 감염 후 90일과 150일에는 칼슘화된 충체만이 관찰되며 충체주위는 섬유조직으로 대체되고 소수의 염증세포 만이 모여 있었다.

3. ELISA를 이용하여 혈청내 Ig G 항체가의 측정결과 감염 후 기간에 따른 각 군별 흡광도(O.D.)의 평균(±표준편차)치가 대조군 0.165(±0.144), 3일 0.257(±0.194), 13일 0.448(±0.257), 20일 0.662(±0.311), 30일 1.113(±0.336), 60일 0.993(±0.331), 90일 0.740(±0.185), 150일에 0.376이었다.

이상의 결과로 미루어 토끼에 감염된 아니사키스는 감염 1주 경에 죽고, 2주 정도 까지는 형태학적 구조를 유지하지만, 20일 이후에는 괴사되며, 30일 이후에는 숙주 세포에 의하여 파괴 흡수되거나 석회화되는 과정을 겪고, 90일 이후에는 석회화된 것 이외에는 거의 흡수되어 관찰되지 않는다고 할 수 있겠다. 충체가 감염된 후 죽어서 흡수되기 시작하는 30일 정도의 기간동안 집중적으로 충체의 항원이 방출되어 항체가가 위와 같은 변화양상을 보인다고 판단된다. 감염 150일에는 항체가가 뚜렷하게 감소한 상태이므로 감염 후 20~90일에는 ELISA가 유용한 혈청학적 진단법이 될 수 있을 것으로 보인다. 실제로 진단에 적용하기 위해서는 교차 반응등에 관하여 더 연구가 진행되어야 할 것이다.

EXPLANATIONS FOR FIGURES

Fig. 3. Two *Anisakis* larvae (arrow heads) on the surface of the stomach, 30 days after infection.

Fig. 4. A calcified, chalky white and stony hard larva (arrow head) was found in omentum, 60 days after infection.

Fig. 5. A section of *Anisakis* larva within abscess at submucosa of stomach, 13 days after infection, HE stained, ×40.

Fig. 6. A sectioned larva which preserved its structures was surrounded by histiocytes, fibroblasts and inflammatory cells in submucosa of stomach, 13 days after infection, HE stained, ×100.

Fig. 7. Sections of a necrotizing worm in fibrinous exudate, which is surrounded by histiocytes and by numerous inflammatory cells in stomach, 13 days after infection, HE stained, ×40.

Fig. 8. High power view of a larva surrounded by fibrinous exudate, histiocytes and inflammatory cells in stomach, 13 days after infection, HE stained, ×100.

Fig. 9. Sections of a larva in omentum surrounded by exudate, and inflammatory cells made a mass, 13 days after infection, HE stained, ×40.

Fig. 10. High power view of Fig. 9. showing neighboring fibrinous exudate and the cells (neutrophils, eosinophils, histiocytes and fibroblasts), HE stained, ×100.

Fig. 11. A necrotizing worm in peritoneum, 20 days after infection, with layers of histiocytes, inflammatory cells and fibrosis, HE stained, ×100.

Fig. 12. A shrunk larva surrounded by fibrosis in mesentery, 20 days after infection. The bulk of inflammatory cells diminished, HE stained, ×100.

Fig. 13. A larva was attached by host cells in stomach wall, 30 days after infection, HE stained, ×100.

Fig. 14. High power view of Fig. 13, host cells attaching the surface of worm. Foamy histiocytes and pyknotic debris were seen nearby, HE staineed, ×400.

Fig. 15. Worm debris scavenged by histiocytes in stomach wall, 60 days after infection, HE stained, ×200.

Fig. 16. Sections of a calcified worm surrounded by fibrosis. Inflammatory cells were much less, 90 days after infection, HE stained, ×100.

Fig. 17. A calcified worm in the muscle layer of stomach enclosed by thin fibrous tissue, 90 days after infection, HE stained, ×40.

Fig. 18. A calcified worm in the stomach wall with surrounding fibrosis, 150 days after infection, HE stained, ×40.


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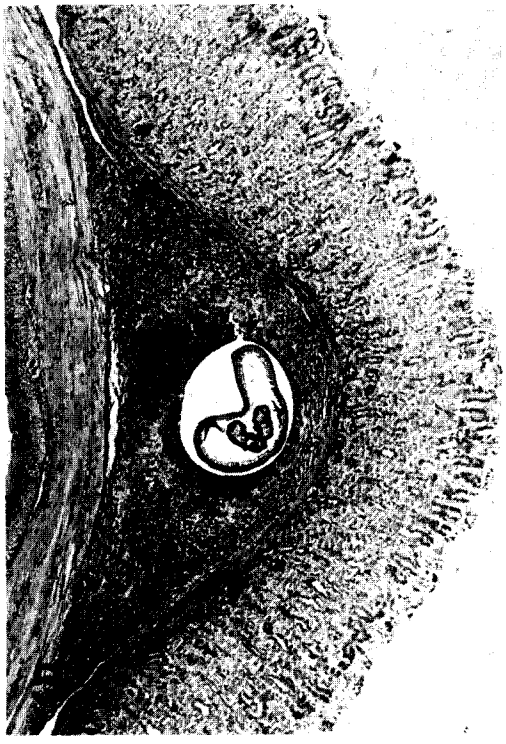

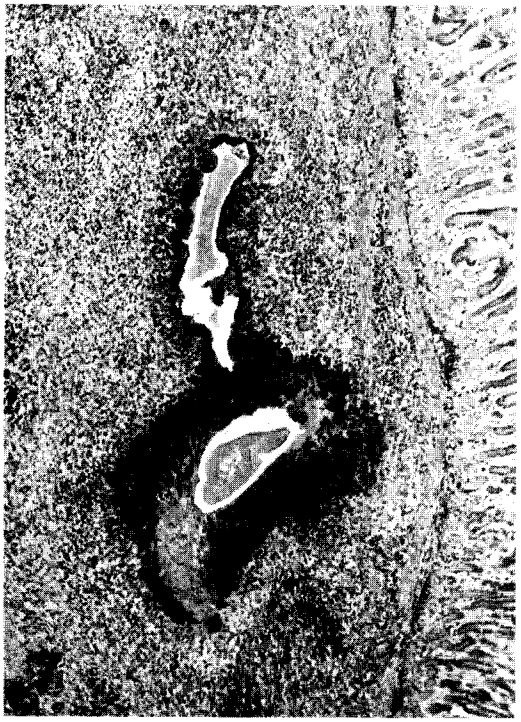

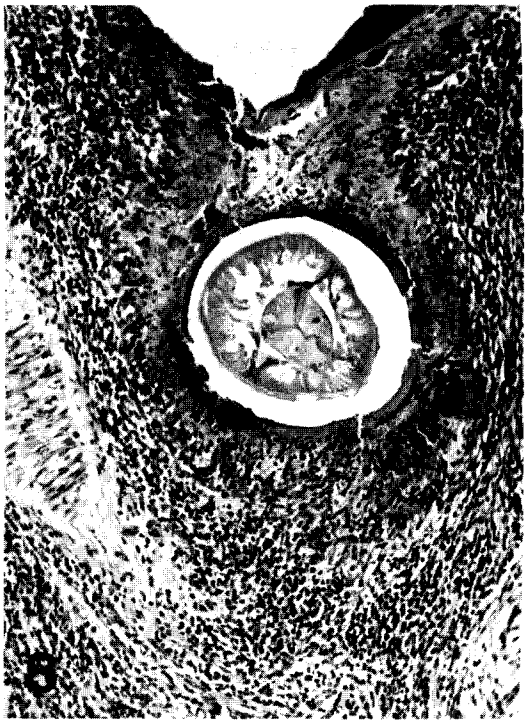

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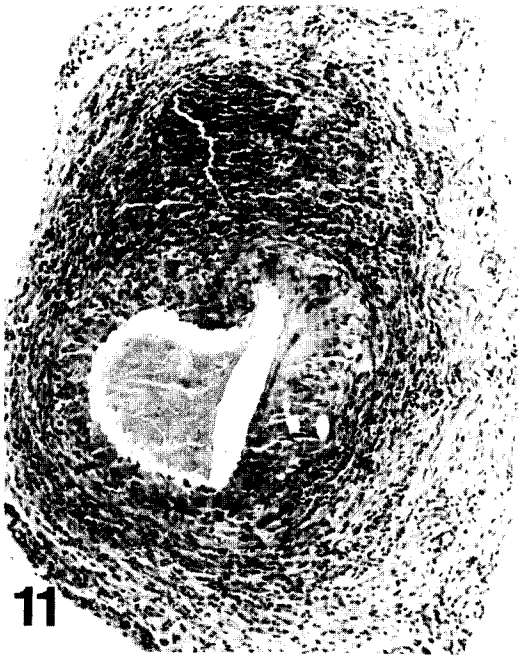

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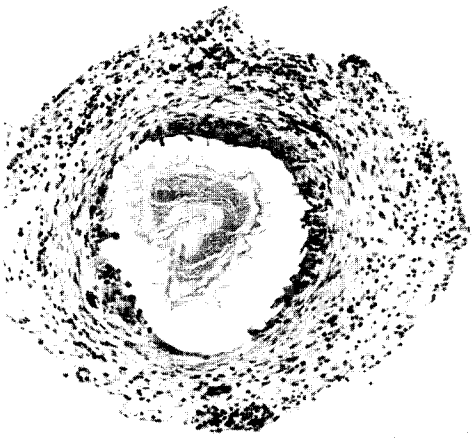

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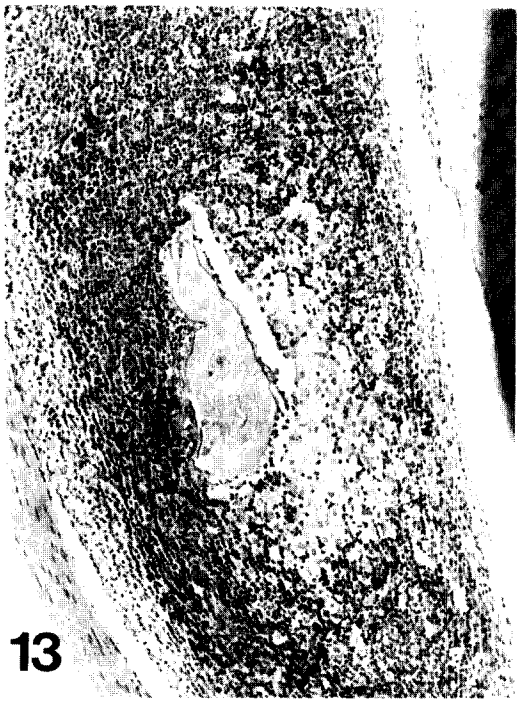

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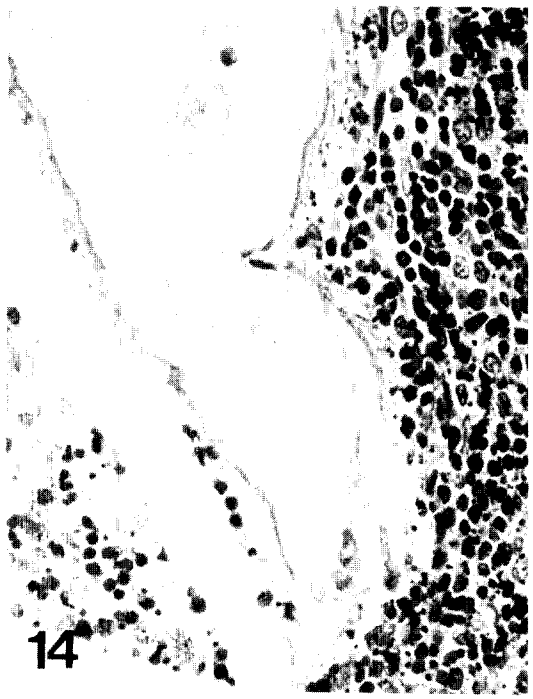

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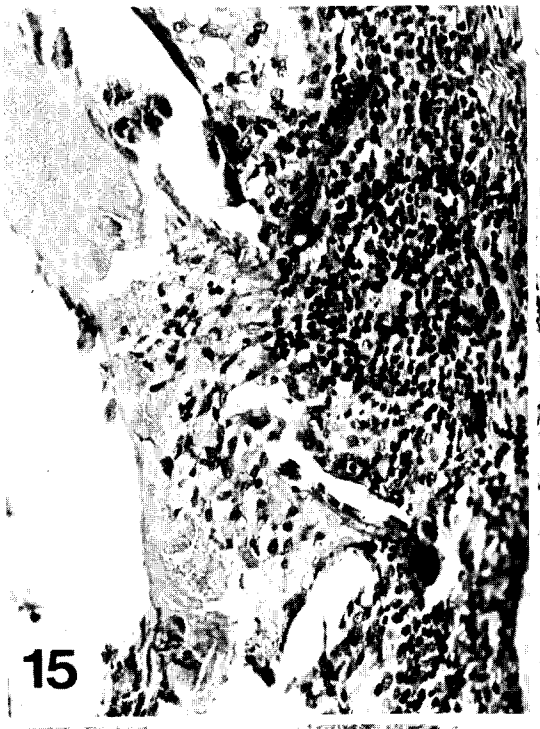

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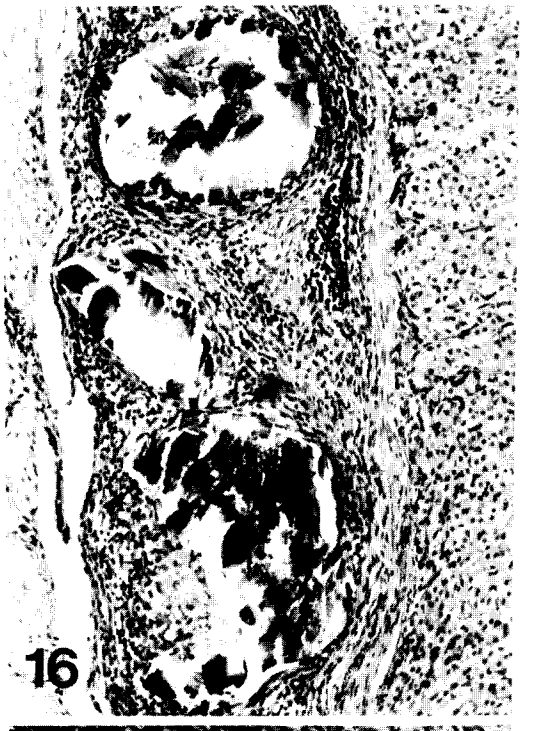

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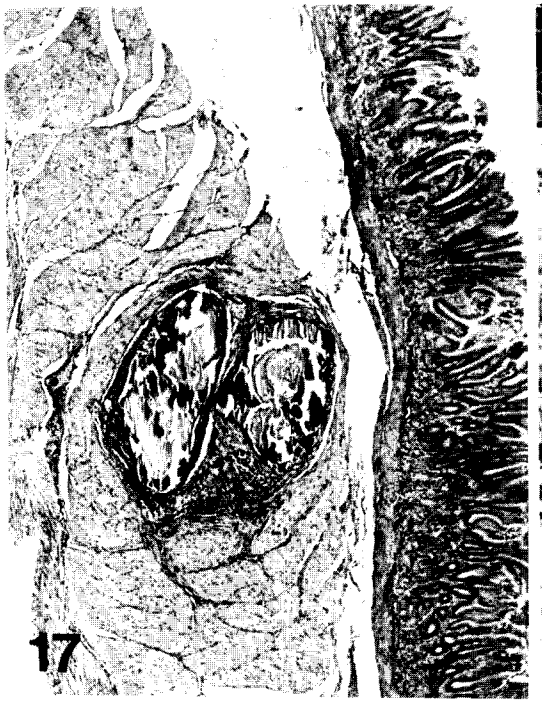

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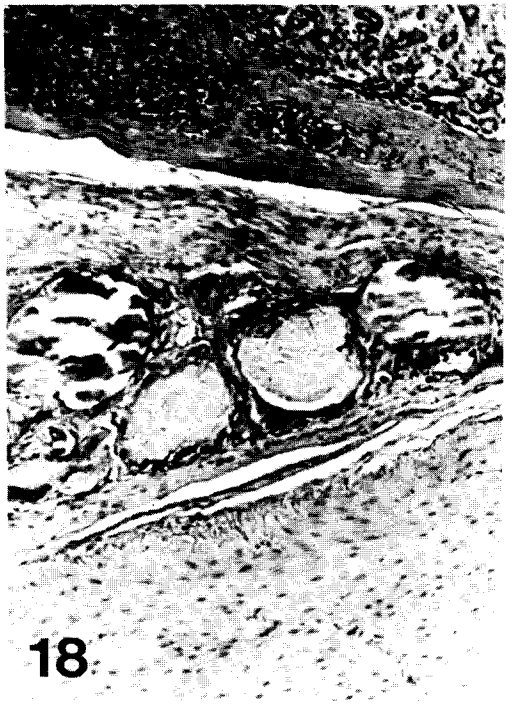

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