[Jap. J. Parasit., Vol. 16, No. 6, 415-422 1967]

Experimental anisakiasis in guinea pigs; Factors influencing infection of larvae in the host

KEIZO ASAMI AND YOSHIYUKI INOSHITA

Department of Parasitology, School of Medicine, Keio University, and Section of Parasitology, Kitasato Institutes for Infectious Diseases, Tokyo, Japan

(Received for publication; August 2, 1967)

Since van Thiel *et al.* (1960) reported 11 cases of human anisakiasis in Holland, about one hundred similar cases have been diagnosed in Japan (Asami *et al.* 1965; Otsuru *et al.* 1965; Yokogawa & Yoshimura, 1965). Clinical features of anisakiasis are characterized by acute abdominal symptoms, i. e. severe epigastric pain, nausea, vomiting and sometimes unusual findings in X-ray examination of the stomach. Pathological findings in almost all cases demonstrate a typical granuloma with extensive eosinophilic infiltration in the gastro-intestinal wall. Although there is no doubt that this pathological condition is caused by eating a raw *Anisakis* infected fish, the factors concerning establishment of *Anisakis* larvae in humans and the following manifestation of symptoms are still unknown. The present paper deals with evaluation of some of the factors influencing experimental infection of guinea pigs with *Anisakis* larvae.

Materials and Methods

Anisakis larvae used: The larval parasites used in the experiments were obtained from the abdominal cavity of mackerel, *Scomber japonicus*, which were caught in the Pacific Ocean near Tokyo. The larvae were found on the surface of the viscera either free or encapsulated, and in a few instances, in the muscle of the abdominal wall.

In the preparatory studies, the larvae found in the fish were examined microscopically to identify species based on York and Maplestone's classification, and later macroscopic identification become possible without difficulties, because the characteristic structures, posterior to the oesophagus were distinguishable from those of other species of larvae macroscopically. Only when the larvae were too small, dissecting microscope was used. The larvae used throughout the experiments represented a short and simple ventriculus posterior to the esophagus having neither appendices nor diverticles. According to the esophagoventricular structures described above, the larvae were identified as belonging to genus *Anisakis* (York & Maplestone, 1926). It has been known that two types of anisakid larvae were found in marine fish in Japan (Oshima, 1966). The present larvae belong to *Anisakis* type I by Berland's classification. Nematode larvae other than *Anisakis* were infreqently found from mackerel.

Experimental animals and experimental procedures: In preliminary experiments where several kinds of laboratory animals were used by the present authors, guinea pigs proved to be the most favorable ani-

This work was partially supported by grant from Ministry of Education, Japanese Government.

mals for the experiments in view of their high susceptiblility as well as ease of handling. Therefore conventional guinea pigs of either sex were used throughout the experiments.

A determined number of the larvae were carefully force-fed to guinea pigs under slight ether anaesthesia in order to avoid destruction of the larvae by chewing. The animals were necropsied three to five hours later and examined for the presence of larvae. Hemorrhagic lesions were detected on the serosa of the gastro-intestinal tract of the guinea pigs in which the larvae were penetrating. These hemorrhagic lesions indicate either the localization of the larvae in the submucosal tissue under the lesions or the site through which the larvae migrated into the abdominal cavity. The worms which were found in the gastro-intestinal wall, free in the abdominal cavity or frequently attached on surface of the viscera were regarded as infected parasites, while the worms found in the lumen of the stomach or in the intestine were considered to be uninfected, because in the human cases pereviously reported it has been substantiated that lesions were produced when the worms penetrated into the tissues.

Untreated control animals were infected at the same time as were the experimental animals and from the same batch of worms.

Results

The experiments were performed with a dual purpose; to determine the properties of parasite infectivity, and the factors contributing to host susceptibility.

1. Infectivity of the larvae

Relationships between the size of the larvae and their infectivity were evaluated by grouping the larvae in ranges of 3 mm in length. As shown in Table 1, larvae measuring 2.3 to 2.5 cm in length appeared to be the most infective to the guinea pigs, showing infection of 56% of the worms administered. Infectivity of extremely long or short larvae was not determined because of a shortage of the number of larvae belonging to these groups.

Either free or encapsulated larvae were given to the animals in order to compare their infectivity. As shown in Table 2, 52 out of 100 larvae given were found to be penetrated in cases where encapsulated larvae were administered, while 41 out of 100 larvae were recovered in cases of free larvae administration. Marked differences in the site of infection by encapsulated or free larvae were not recognized. However, the encapsulated larvae showed a tendency to penetrate into the stomach wall easier than the free larvae.

Infectivity of larvae preserved in a refrigerator was studied by giving a determined number of the larvae to the guinea pigs at five days interval. Ten larvae were placed in each of a series of small Petri

Table 1 Relationship between size and infectivity of the larvae

Length of the larvae (cm)	No. of larvae administered	No. of larvae infected	Rate of infection (%)
1.6—1.9	9	5	
2.0—2.2	24	6	25.0
2.3—2.5	34	19	55.0
2.6—2.8	30	6	20.0
2.9—3.0	3	1	

Table 2 Infectivity of free and encapsulated larvae

	No. of guinea pigs		No. of worms in			Total
	exposed	infected	stomach wall	abdominal cavity	intestinal wall	
free larvae	10	10	13	25	3	41
encapsulated larvae	10	9	20	29	3	52

10 larvae were given to each guinea pig

Table 3 Infectivity of the larvae cut in half

	No. of guinea pigs		No. of larvae infected in			Total
	exposed	infected	stomach wall	abdominal cavity	intestinal wall	
Anterior portion of worm cut in half	5	3	5	0	0	5
whole worm (control)	5	4	7	11	1	19
Anterior portion of larvae cut in thirds	6	3	5	0	1	6
whole worm (control)	6	5	7	7	2	16

10 larvae were given to each guinea pig

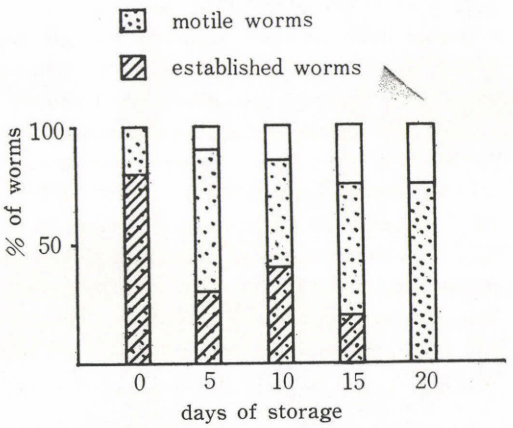

Fig. 1 Infectivty of the larvae stored at 4°C

dishes containing a piece of flesh and stored at 4°C for 20 days. Two dishes were removed every five days, allowed to warm up to room temperature, after which the motility of the larvae was checked. The surviving larvae were administered to the animals to determine larval viability. As shown in Fig. 1, the rate of infectivity of freshly isolated larvae was as high as 80 percent. With storage, however, the number of surviving worms decreased and the infectivity of the larvae gradually fell, reaching zero after 20 days storage, despite 75% of the stored larvae still maintaining motility.

Larvae cut in halves or thirds were examined for their infectivity. Immediately after the larvae were cut with a sharp razor blade, the anterior portions of the larvae were administered to the animals. As shown in Table 3, anterior portions of larvae cut in half still retained their infectivity in some degree. The larvae cut in halves or even in thirds invaded into the submucosa of the stomach just same as the whole worm. The rate of invasion of larvae both cut in halves and in thirds were as low as 10 percent, whereas that in controls receiving whole worms was more than 35 percent. It might be noted that all of the larvae invaded were found in the stomach wall except for one in the intestinal wall.

2. Factors contributing to host susceptibility

As larval anisakiasis in humans is established by invasion of the larvae into the wall of the alimentary canal, it may be thought that physico-chemical conditions of the alimentary organs of the host influence the penetration of the larvae. Experiments were performed to examine the effects of experimentally induced changes of physico-chemical conditions of guinea pigs upon susceptibility to the infection.

The effects of the presence or absence of a full stomach were evaluated using starved and regularly fed animals. In the experimental groups, no food was given for two days prior to the administration of worms, while the control guinea pigs were fed regularly until initiation of the experiments. When autopsied, little or no food material was found in the stomach of the experimental animals. As shown in Table 4, 54 and 41 larvae became infected in the starved and control guinea pigs respectively.

Table 4 Effects of full stomach of the host on the susceptibility

	No. of guinea pigs		No. of worms infected in			Total
	exposed	infected	stomach wall	abdominal cavity	intestinal wall	
starved guinea pigs	10	10	21	31	2	54
regularly fed guineapigs	10	9	17	22	2	41

9 to 10 larvae were given to each guinea pig

Table 5 Effects of histamine on the susceptibility of the host

	No. of guinea pigs		No. of worms infected in			Total
	exposed	infected	stomach wall	abdominal cavity	intestinal wall	
Consecutive injection						
histamine-injected	10	10	19	9	0	28
control	10	10	24	10	0	34
8 to 10 larvae were given to each guinea pig						
Single injection						
histamine-injected guinea pigs	15	8	16	5	0	21
control guinea pigs	15	9	19	8	3	32
9 to 10 larvae were given to each guinea pig						

Histamine is known as a strong accelerator of gastric secretion as well as gastric movement in guinea pigs. Histamine hydrochloride solution was injected intramusclarly in doses of 0.001 mg per 100 g of the body weight, and immediately thereafter, the worms were administered. As shown in Table 5, 21 larvae were found in the histamine-injected guinea pigs, while 30 were recovered in the controls.

Since the effective period of a single injection of histamine in the above described dose seemed to be too short to observe its effect against the infection, injections were made repeatedly throughout the experiments. Histamine was injected every 20 minutes from the administration of the larvae until necropsy of the animals. The results obtained in this experiment were similar to those of the former experiment, showing establishment of a smaller number of worms in the histamine-injected groups than in the controls (Table 5).

Prostigmine preparation, Vagostigmine Sionogi, which stimulates peristalsis of the intestine was injected into the experimental animals in a dose of 0.1 mg per 100 g of body weight just before administration of the larvae. As shown in Table 6, no marked difference was recognized between prostigmine injected and control animals.

Carnitin hydrochloride preparation,

Table 6 Effect of prostigmine on susceptibility of the host

	No. of guinea pigs		No. of worms infected in			Total
	exposed	infected	stomach wall	abdominal cavity	intestinal wall	
prostigmine-injected guinea pigs	15	10	19	5	0	24
control guinea pigs	15	8	14	9	1	24

8 to 10 larvae were given to each guinea pig

Table 7 Effect of carnitin preparation on susceptibility of the host

	No. of guinea pigs		No. of worms infected in			Total
	exposed	infected	stomach wall	abdominal cavity	intestinal wall	
carnitin-injected	17	5	10	2	2	14
control	17	9	10	1	2	13

8 to 10 larvae were given to each guinea pig

Table 8-a Effect of iso-propamide on susceptibility of the host

	No. of guinea pigs		No. of worms infected in			Total
	exposed	infected	stomach wall	abdominal cavity	intestinal wall	
iso-propamide administered	10	6	7	3	1	11
control	10	3	5	0	1	6

10 to 13 larvae were given to each guinea pig

Table 8-b Effect of iso-propamide on susceptibility of the host

	No. of guinea pigs		No. of worms infected in			Total
	exposed	infected	stomach wall	abdominal cavity	intestinal wall	
iso-propamide	13	9	17	9	4	30
control	13	10	25	8	0	33

8 to 13 larvae were given to each guinea pig

Monokamin Tanabe, which slightly accelerates both secretion and movement of the alimentary canal by stimulating the parasympathetic nerve, was injected in a dose of 100 mg per 100 g body weight 1.5 to 2.0 hours before administration of the larvae. Differences in susceptibility were not observed between experimental and control animals as it is indicated in Table 7.

Effect of a parasympathetic nerve blocking agent upon susceptibility was evaluated using an isopropamide preoaration, Marigin Sumitomo-Kagaku. Isopropamide was given orally in a dose of 0.9 mg per day for 2 days prior to the administration of the larvae. Experiments were performed using two worm batches which were different in their infectivity to control animals.

In the first experiment, as shown in Table 8-a, the infectivity of the larvae in the control guinea pigs was as low as 6 percent. In isopropamide treated guinea pigs, rate of the infection increased considerably, resulting in establish mentof 11% of the larvae administered.

In the second experiment, infectivity of the larvae used was high, as indicated in Table 8-b. In this case, marked differences of susceptibility were not recognized between experimental and control groups. The results obtained here includes some discrepancies with those of the former experiment. Judging from the results obtained in these two experiments, the establishment of the infection appears to depend more on the infectivity of the parasite than on the susceptibility of the host.

Discussion

Larval anisakiasis in humans is becoming a new, important problem in the fields of medicine, surgery, parasitology and public health in Japan. According to the extensive surveys which have been performed by Japanese parasitologists, *Anisakis* larve are

found in many kinds of marine fish which Japanese people usually eat (Otsuru *et al.*, 1965). Although it is obvious that anisakiasis among the Japanese is caused by the widespread custom of eating raw marine fish meat, it seems to be true that the incidence of proved cases of anisakiasis is very low compared with frequency of eating marine fish. It is reasonable to suppose that almost all of the cases have been misdiagnosed or cured by symptomatic treatment under wrong diagnosis, because the symptoms of anisakiasis are quite similar to those of many diseases which manifest acute abdominal symptoms, and in addition, there is no specific diagnostic method for anisakiasis at the present time.

Since human anisakiasis is an infectious disease in which larvae of *Anisakis* penetrate the tissue of the alimentary canal, factors related to the infection involve both the larvae and conditions of the host. Evaluation of the factors concerning the infection of *Anisakis* has not been made except for one comment by van Thiel *et al.* (1960) who supposed, based on analysis of their clinical cases, that an achlorhydric condition in the patient might be favorable to the infection. Some papers regarding experimental infection with the larvae in laboratory animals have appeard previously (van Thiel *et al.* 1960; Meyers, 1963; Otsuru *et al.* 1965). van Thiel *et al.*, Otsuru *et al.*, Ashby *et al.* (1964), and Ishikura (1965) suggested an allergic mechanism in developing the pathological findings in this infection. In the present paper, immunological aspects of the disease have not been evaluated. However, pathological changes which were caused by primary invasion of the larvae into the the tissues appeared to be strong enough to bring about severe symptoms in the host. Pathology and immunology of experimental anisakiasis will be reported by the present authors in later papers.

Regarding the classification of the larvae used in this study, a detailed investigation was not made to determine their species. Therefore it is possible that the larvae used in these experiments include several species, rather than a single species of larval *Anisakis*. Oshima (1966) found two different types of *Anisakis* larvae in marine fish caught in the offshore of the Japan Islands. It is reasonable to assume that any of several species of *Anisakis* larvae could be the causative agent of human anisakiasis. Morphological differences have been found among the worms found in the histological preparations of diagnosed cases (Asami *et al.*, 1965). In addition to the morphological differences, the site of infection is also different in countries or districts from where the cases were reported. For instance, in the Netherlands, the site of infection is the intestinal wall in most of the cases reported, whereas in Japan more than 60 percent of the cases showed presence of the worm in the stomach wall. However, in Iwanai, Hokkaido, Japan, the worms were found in the ileum wall in almost all of the cases (Ishikura, 1966). These facts could be explained as being due to the difference of species of the larvae which cause human anisakiasis in various areas in the world. In the present experiments, the worms 2.3 to 2.5 cm in length showed highest infectivity to guinea pigs. This fact may indicate possibility of presence of species which has a higher infectivity for guinea pigs.

Storage of worms reduced their infectivity. It is expected that different results will be attained by various conditions of storage. However there is evidence that almost all of the larvae found in refrigerated fish sold in markets have already been killed or have lost their infectivity even if they still survived. Therefore, it can be concluded that storage of fish at temperatures 0°C for 20 days is one of the effective procedures to prevent the infection.

The experimental evidence that larvae cut in half still retained their ability to invade into the stomach wall indicates

surprising infectivity of the larvae. Contrary to expectation, the worm cut in half completely invaded into the submucosal tissue of the stomach as early as four hours after administration. Because the observation was performed shortly after the administration of worms, it is not known whether or not the worms persist in the tissue long enough to cause pathological changes as the whole worms do. From the practical point of view, this evidence seems to be important ; sliced raw fish, "sashimi", is one of the most favorite dishes of the Japanes people, and the larvae adhering to the flesh still retain the ability to penetrate the stomach tissue of the host even if they are cut in half by a fish knife.

Although several kinds of examinations were performed to evaluate the factors contributing to the susceptibility of the host to the larvae, results in all of the cases proved to be non-significant by statistical estimations. However, there was a tendency that increased and decreased gastric secretion as well as peristalsis affected inhibitory and acceleratively on penetration of the worm into the gastro-intestinal tissue respectively. This tendency seems to agree with some clinical findings. According to the clinico-pathological analysis of about 100 cases of anisakiasis reported previously in Japan, Yoshimura (1966) found hypo- or achlorhydria in 5 out of 8 cases whose stomach acidity was examined prior to operation. As cited above, among 11 cases reported by van Thiel *et al.* (1960), 2 cases were found to be achlorhydric which resulted from previous gastrectomy.

Although these results seem to show possibility that susceptibility of the host is modified by conditions of the gastric secretion, it is concluded that penetration of the larvae depends more on the condition of the larvae themselves than that of a host.

Summary

Factors related to the infectivity of *Anisakis* larvae and the susceptivility of a host to the larvae were evaluated using guinea pigs as an experimental host. *Anisakis* larvae obtained from the abdominal cavity of mackerel were force fed to guinea pig, and penetration of the larvae into the viscera of the guinea pigs were checked by necropsy of the animals 3 to 5 hours after administration of the larvae. Larvae ranging from 2.3 to 2.5 cm in length were found to possess maximum infectivity. Larval worms cut in half still retained some degree of infectivity. Storage of the worm in a refrigerator at 0°C reduced infectivity, resulting in complete loss of infectivity by 20 days storage. In the experiments to find out some factors contributing to susceptibility of the host to the larvae, significant influence was not recognized so far as the experimental conditions concerned. Tendency that accelerated or suppressed gastric secretion influence on penetration of the larvae into wall of the alimentary canals was recognized.

In view of the above findings, it is concluded that whether or not the parasite can establish depends more on the infectivity of the larvae themselves, rather than on the susceptibility of the host.

Acknowledgement

The authors wish to express their appreciations to Dr. Hisakichi Matsubayashi, Keio University, for his advices and interests throughout this study.

Read by K. Asami at the 11th Pacific Seience Congress in Tokyo, 25 August 1966.

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モルモットにおける実験的アニサキス症：特に感染の成立に関係する因子の検討

浅 見 敬 三
（慶応義塾大学医学部寄生虫学教室）
井 下 好 幸
（北里研究所寄生虫部）

サバの腹腔からとり出した Anisakis 型幼線虫をモルモットに経口投与して感染の成立に関与する虫体側及び宿主側の因子の幾つかについて検討を加えた．用いた幼線虫体は York & Maplestone の分類に従って，食道下部に単純な胃をもち，大島の分類の I 型に属するものである．虫体投与後 3～5 時間でモルモットを剖検し，消化管壁に侵入しているもの及び腹腔に脱出している虫体を感染虫体とみなして感染の度合を観察した．魚体からの虫体の体長は 1.6～3.0 cm の範囲内であったが，体長 2.3～2.5 cm のものが最も高い感染性を示した．魚体内での被嚢幼虫と遊離幼虫は感染性には差がない．1/2 断或は 1/3 断した虫体の体前部も全虫体の場合と同様に粘膜下組織に侵入する能力があることが分った．4°C の冷蔵庫に魚肉とともに保存した虫体は，生存はしていても感染能力は急速に低下し，20日間の保存で感染虫体は全く見られなくなった．

宿主側の条件として，種々の薬剤を用いてモルモットの消化器の分泌，運動に変化を与えてみたが，胃の分泌運動の昂進か感染を低下させ，分泌の抑制が感染を促進させる傾向が軽度にうかがわれた．

以上の結果から，アニサキス幼虫のモルモットへの感染の成立は虫体側の因子によって大きく左右されるが，宿主側には影響する因子が少ないと結論された．