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Observations of the Age Resistance, Eosinophilia, and Larval Behavior in the Helminth-Free Beagles Infected with *Toxocara canis*

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Introduction

The migratory pattern of the larval *Toxocara canis* in dogs has been showed to vary according to the age of dogs. Noda (1957, 58) reported the occurence of tracheal migration when the embryonated eggs were administrated to puppies younger than 30 days of age. Sprent (1958) recognized the somatic route of migration of the larvae when the embryonated eggs were fed to dogs older than 5 weeks of age. On the other hand, Lee (1970) reported the 100% susceptibility of puppies of 1 day to 4 month old to *T. canis*. Webster (1956) already suggested that the trials of feeding a large amount dose of infective eggs of *T. canis* could break the age registance of dogs.

These different results reported in the past might be due to the unavoidable experimental errors in the recoveries of worms by the oral admistration of embrionated eggs.

So far as the cause of age registace, Fernando (1968, 73) tried to explain from the view point of the developemnt of immunity to *T. canis* infection in the aged dogs. On the contrary, Greve (1971) observed the strong age resistance phenomenon in ascarid free beagles which had no previous infection with *T. canis*.

Sprent (1961) succeeded to set up the infection in adult dogs administrating orally the larvae collected from the intestine of prenatally infected puppies between 7 and 17 day after birth, however, the stage of larvae wihch were able to develope in the adult dog's intestine was not determined.

Olson and Schulz (1963) observed the dose-dependent relationship between the number of infective eggs administrated and extent of eosinophilia in guinea pig. Oshima (1976) observed the change of the pattern of eosinophilia by superinfection of mice with infective eggs.

This study was designed to elucidate the nature of age registance, and eosinophilia and the behaviour of larvae in dog by the aid of helminth free beagles.

Materials and Methods

Helmith, *Toxoplasma* and *Coccidium* free beagles from a colony* maintained in a strict closed environment for several generations were used in the present study. Cages, food vessels and drink bottles for dogs were sterilized before use and each dog was kept in a individual cage. To keep the dogs clean, special isolated room was prepared. Daily fecal materials were disposed carefully in order to avoid contamination. Mongrel dogs, prenatally infected with *T. canis* and dewormed by piperazine salt before experiment, were used as the control animals.

Eggs of *T. canis* were collected from the uterus of adult female worms or from the feces of heavily infected mongrel puppies. Feces were washed repeatedly and passed through the nylon sieves of 500 μ, 200 μ, 100 μ and 40 μ mesh sequentially. The last leavings on 40 μ

* This colony has been maintained in Chugai Pharmaceutical Co. Tokyo.

mesh sieve were transferred to zinc sulphate solution of 1.18 specific gravity. After 30 min the floated eggs were transferred to a large amount of water and concentrated in the sediments. The eggs collected from the sediments were decorticated in 2% sodium hypochlorite solution and stored in 0.1 N sulfuric acid at 4 C. Embryonation of eggs was carried out at 26 C in 0.1 N sulfuric acid with gentle shaking and airation continuously for two weeks. Embryonated eggs could be stored at 4 C in 0.1 N sulfuric acid for a year without decrease of infectivity. Hatching of eggs was carried out mechanically without trying any biochemical tricks, such as shown in the report of Hass and Todd (1962).

Decorticated eggs were washed several times in sterile water and stirred at high speed for 30 seconds using tissue homogenizer and centrifuged gently. The sediments were transferred on 40 μ mesh nylon sieve which was placed on 40 C warm water. Active hatched larvae migrated down in the water and the egg shells debris were left on the sieve.

After one hour the active 2nd stage larvae were sedimented to the bottom of the vessel. All the procedure were carried out aseptically. The larvae were stored at 4 C in Hanks medium and used for infection within 2 weeks.

Infection of dogs.

Hatched larvae were suspended in sterile saline and counted the number per ml. Ordinarily desired amount of larvae were inoculated into small saphenous vein of dog. When the portal vein inoculation was tried, the larvae were inocultated into the portal branch of spleen vein by the aid of intravenous teflon catheter.

Recovery of larvae from dog tissues

At the necropsy, 40 g of muscle, 20 g of lung and 10 g of liver were collected and each sample was minced by homogenizer and digested in the acid pepsin solution at 40 C for 3 hours and then the sediments of these digests were examined. In order to elute the larvae from the whole lung, the lung was chopped and minced and placed on the modified Baerman's apparatus at 40 C for several hours.

Identification of the developing stages of *Toxocara canis* larvae was carried out in accordance with the description of Sprent (1958) and Schacher (1957).

Recovery of the adult worms.

The necropsies of dogs were done between 50 to 60 days of infection and the contents of the digestive tracts were carefully examinded to collect the worms. The number of the adult worms discharged in the stools before the necropsy was added to the number of the worms found by the necropsy.

Eosinophile count

Eosinophiles in the peripheral blood were counted by Speier's counting plate using Manner's counting fluid and the number in 1 mm^3 blood was calculated.

Results

Differences of the result of small saphenous and portal vein inoculation of 2nd stage larvae.

Four 72 days old helminth free beagles of the same litter were inoculated 1,600 to 2,000 sterile 2nd stage larvae intravenously. Two

Table 1 Comparison of the recovery of adult worms after inoculation of 2nd stage larvae into small saphena and portal veins of 72-days-old helminth free beagles

Inoculation	No. larvae inoculated	Appearance of eggs in feces	Recovery of worms from intestine (61–63 days)		Recovery rates
			Females	Males	
Portal vein	1,600	40th day	23 (59–137 mm)	7 (40–77 mm)	1.9%
	2,000	40th day	29 (51–112 mm)	16 (42–80 mm)	2.3%
Small saphenous vein	1,600	38th day	11 (38–100 mm)	7 (24–68 mm)	0.9%
	2,000	38th day	42 (50–123 mm)	19 (50–82 mm)	3.1%

of them were inoculted into small saphenous veins and other two were inoculated into portal veins. They were autopsied between 61 to 63 day of infection.

Although the recovery rates were varied greately, it was almost difficult to recognize obvious difference between two different ways of venous inoculation as shown in Table 1. The growth of the worms was not as uniform as the result in the paper of Greve (1971). The onset of the appearence of eggs in feces of dogs were 38 days by small saphenous vein inocultion and 40 days by portal vein incultion.

In the former, the eosinophilia appeared rather earlier than the latter, however, the eosinophile counts reached the highest peaks at the 28th day of infection and then decreased gradually as in Fig. 1.

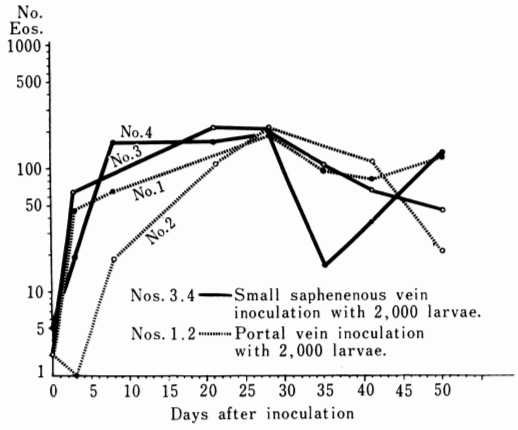

Fig. 1 Eosinophile level of helminth free young beagles (72 days old) after intravenous inoculation of 2,000 *T. canis* larvae. No. cells/ml

Age resistance phenomenon of helminth free beagles and mongrel dogs against T. canis infection.

Ten helminth free beagles of vaious ages from 30 days old to 46 months old were inocultaed 1,000 to 5,000 sterile 2nd stage *T. canis* larvae into small saphenous veins and autopsied from 50 to 68 day of infection.

The results were shown in Table 2 and Fig. 2. Age resistance phenomenon was not under the all or nothing law, but it was intensified as the age of beagle advanced. As the age (in days) of dogs at the day of the inoculation advanced from 30 days to 111 days, the recovery rates of the adult worms from their intestines decreased gradually from 9.1 % to 0.1 %. Table 3 and Fig. 2 showed the results of the same trials on mongrel puppies which were prenatally infected with *T. canis* and dewormed just before experiments. In these cases the recovery rates of the adult worms were much lower than those of the clean beagles and 80 days old puppy showed as low as 0.1%.

Arterial inoculation of the larvae

Two helminth free beagles of 105 days old were inoculated with 3,500 larvae of *T. canis* intra-vascularly. The one was inoculated into small saphenous vein and the other was inoculated into femoral artery. They were

Table 2 Recovery of adult worms from the intestine of helminth free beagles after intravenous inoculation of the 2nd stage larvae

Sex and Age of dogs	No. larvae inoculated	Days autopsied	No. adult worms from intestine	% Recovery
F. 30 days	1,000	50	81	9.1
M. 72 days	2,000	61	61	3.1
M. 72 days	2,000	61	45	2.3
M. 72 days	1,600	63	30	1.9
F. 72 days	2,000	62	18	0.9
F. 105 days	3,500	57	26	0.7
M. 111 days	5,000	61	9	0.2
F. 111 days	5,000	68	5	0.1
F. 3 y. 10 m.	5,000	no eggs at all in feces		0

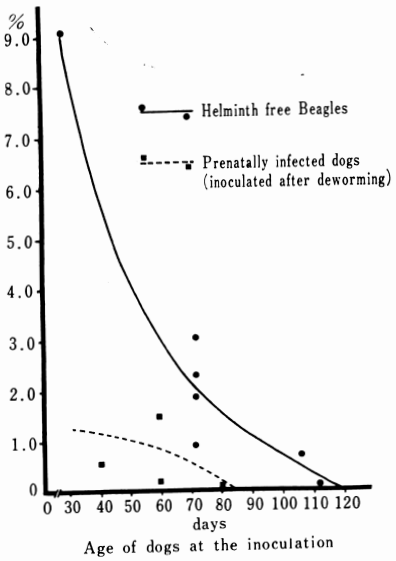

Fig. 2 Comparison of the age resistance of helminth free beagles and previously infected conventional dogs to the infection of *T. canis*. (Percentage shows the recovery rate of the adult worms after 50–60 days of inoculation).

sarcrificed at the 57th day of infections. The recovery rates of the adult worms from their intestines were almost the same. The old haemorrahgic changes were clearly seen in the lungs of both dogs. The size of the adult worms were varied greatly in both cases. (Table 4)

Oral administrations of 3rd and 4th stage larvae and preadults of T. canis

A dewormed mongrel puppy of 40 days old was infected orally with 20,000 embryonated eggs of *T. canis* and sarcrificed 12 days later. By the aid of modified Baerman's technique, 97 3rd stage larvae were collected from the lung. All these larvae were administrated orally to a 152 days old beagle. On the 37th day of infection the beagle was sarcrificed and only one female preadult of 28 mm in length was recovered from the intestine.

A dewormed mongrel puppy of 30 days old was infected orally with 2,000 embryonated eggs of *T. canis* and sarcrificed 30 days later and 3 females (4.2–7.4 mm) and 3 males (3.1–4.2 mm) of the 4th stage larvae were recovered from the intestine. These 4th stage larvae were fed to a helminth free beagle of one year old and the beagle was sacrificed on the 25th day of infection. One female of 70 mm and 1 male of 45 mm were collected from the intestine.

A helminth free beagle of 30 days old was inoculated 1,000 larvae intra-venously and sarcrificed 50 days after. 6 females (55–65 mm), 20 males (35–52 mm) and sex unidentified 11 adults (22–45 mm) were recovered from the intestine. There 37 adults were fed to a helminth free 116 dys old beagle. The dog was sacrificed 62 days after and 7 females (137–200 mm) and 5 males (102–110 mm) were collected from the intestine.

Eosinophilia observed after primary and super infection of T. canis.

As showed in Figure 3, young helminth free beagles showed sharp rises of eosinophile counts in their peripheral blood in the second to early third week of infection by venous inoculation of 5,000 2nd stage larvae. They reached to the highest counts on the 18th

Table 3 Recovery of adult worm from the intestines of mongrel dogs* after intravenous inoculation of 2nd stage larvae

Age of dog	No. larvae inoculated	Days autopsied	No. adult worms from intestine	% Recovery
40 days	2,000	87	11	0.6
60 days	2,000	35	4	0.2
60 days	2,000	42	30	1.5
80 days	2,000	90	2	0.1
120 days	2,000	no eggs at all in feces		0

(* Prenatally infected with *T. canis* and dewormed before inoculation)

Table 4 Comparison of the recoveries of adult worms from the intestine of helminth free 105 days old beagles after inoculation of 2nd stage larvae of *T. canis* by venous and arterial routes.

Inoculation	No. larvae inoculated	Days autopsied	Worms recovered from intestine	% Recovery
Femoral artery	3,500	57	Male 15 (24- 65 mm) Female 18 (30-125 mm)	0.9
Small saphenous vein	3,500	57	Male 18 (30- 58 mm) Female 8 (36- 65 mm)	0.7

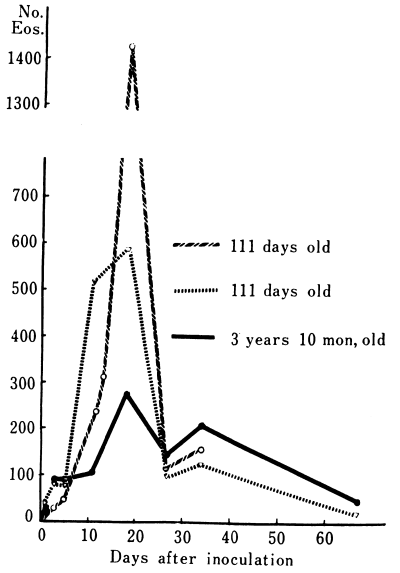

Fig. 3 No. eosinophiles/ml of blood by the primary infection of helminth free beagles with 5,000 *T. canis* larvae

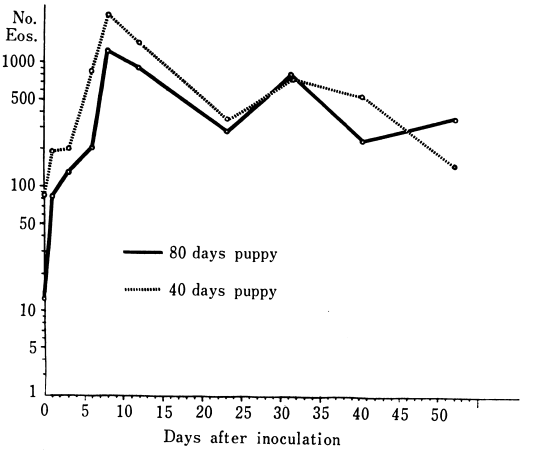

Fig. 4 No. eosinophiles/ml of blood by the superinfection of prenatally infected puppies with *T. canis* larvae

day and decreased abruptly to the former level within several days.

On the other hand, in the cases of previously infected and dewormed mongrel puppies, the eosinophile counts begun to rise as early as 3rd day of venous inoculation of 2,000 2nd stage larvae and reached to the highest counts on the 8th day and then persisted at the high level for a long period as shown in Figure 4.

In the case of old dog (3 years and 10 months old helminth free beagle), the rise of eosinophile count after primary intravenous inoculation of 5,000 larvae was moderate and not as distinct as the cases of young dogs (Figure 3).

Eosinophilia observed during the intestinal phase of development of T. canis

During the course of the infection of feeding 37 adult worms to a 116 days old clean beagle, eosinophile counts were continuously as low as 3 to 13/mm^3, from the 3rd to the 62 days of infection.

Discussion

The advantage of intra-vascular inoculation of sterile 2nd stage *T. canis* larvae was to eliminate the indefinite factors of egg hatching, penetration into intestinal wall and passing through the liver sinusoid without being trapped, and let the larvae

reach the lung directly. By this new inocultation method it became possible to observe the post lung migration behaviour of larvae more accurately.

Comparison of the results of the inoculation into portal vein and small saphenous vein indicated that the passing of larvae through the liver had no advantageous effect on the development of larvae, although Kondo(1970) reported the slight development of the 2nd stage larvae in the liver of puppy.

Some of the 2nd stage larvae inoculated into the blood vessel of helminth free beagles developed to adult in the intestines, if the beagle was yonger than 111 days.

By the same trial on mongrel puppies, which were prenatally infected by *T. canis* and dewormed the adult worms appeared in their intestines, if the dog was younger than 80 days.

These age resistance phenomenon were not under all or nothing low, however, the recovery rates of the adult worms continuously decreased as the age advanced. So if a quite big amount of embryonated eggs or larvae were used for infection to resistant aged dogs, the very few adult worms would be able to appear in their intestine.

But ordinary if the recovery rate of adult worms to the number of inoculated 2nd stage larvae was less than 0.1%, it could be safely said that the dog was quite resistant to *T. canis* infection.

In the cases of prenatally infected mongrel puppies, the recovery rates were very low and age resistance appeared much earlier. Unfortunately prenatally infected beagle puppies were not available and it was difficult to compare the results in the strict sense, however, the difference of sensitivity to *T. canis* infection between beagles and mongrel dogs might be negligible.

So it was able to draw the following conclusion regarding the age resistance phenomenon of dog against *T. canis* infection.

Primary cause of age resistance was still unknonwn, however, it might be related to the physiological and biochemical changes of the inner environment of growing puppy. The previous infection of dog with *T. canis* was not the main cause of the age resistance phenomenon, however, it stimulated it.

The factors responsible for the changing of the route of migration of the larvae from tracheal to somatic way as the age of the dog advanced, were closely connected with the inhibition of the development of the larvae from the 2nd stage to the 3rd or 4th stage in their lung.

The 4th stage larvae could develop to the adults even in strongly age registant dogs. Age registance phenomenon was observed only during the period of development of the 2nd stage larvae to the 4th stage larvae.

In the cases of young helminth free beagles, the recovery rates of adult worms from their intestines were almost the same whether the larvae were incoulated into vein or artery. This result suggested that if the larvae primarily inoculted into artery, they could return to the heart without being trapped by the peripheral capillaries.

The maximum diameter of 2nd stage *T. canis* is 16–20 μ and those of peripheral capillaries is 8–20 μ. It seemed difficult for those larvae to pass through the peripheral capillary as in the case of microfilaria. They might break the capillaries and onto escaped into the tissue and migrated back into the moderate size venous capillaries again.

Greve (1971) infcted puppies administrating the 2nd stage larvae subcutaneously and succeeded to get the adult worms from the intestines. The 2nd stage larvae were definitely able to migrate into vein from the subcutaneous tissue. Perhaps, after several recycle of the 2nd stage larvae in the host blood circulation, the larvae might be trapped in the tissue due to the intensified immune responses of the host.

Eosinophilia of peripheral blood of the dog during the course of *T. canis* infection was triggered by the invasion of 2nd stage larvae to the lung. Passing and staying of larvae in the liver seemed to play little roll in enhancing eosinophile in the blood. When

the larvae went out of lung, eosinophile counts begun to decrease.

The development of worms in the intestine of beagles had no influence on their peripheral eosinophilia. Though the beagles still maintained a lot of adult worms in their intestine, the eosinophilia subsided after 2 months of infection. The abrupt rise of eosinophile counts during the second to early third week of infection was not so obvious in the case of old helminth free beagle as younger beagles. Aged beagle seemed to be less sensitive in eosinophile response by *T. canis* infection than younger one. The pattern of eosinophile responses of dogs by the super infection of *T. canis* was quite different from the primary infection. Marked peripheral eosinophilia appeared as early as the 8th day and then persisted for long time.

These phenomenon strongly suggested that the eosinophilia of dog induced by the *T. canis* infection was the indicative of allergic reaction of the hosts as Olson and Shulz (1963) observed by the experimental infection of guinea pigs with *T. canis*.

High and persistent eosinophilia frequently observed in the cases of human visceral larva migrance, must be due to the frequent latent infection of *T. canis* in the past history of the patients.

Summary

A new technique of infecting animals with *Toxocara canis*, inoculating the sterile 2nd stage larvae directly into blood vessel, was devised in order to minimize the experimental errors in the recovery of worms.

The beagle dogs from a colony, free of all helminthes and some protozoa (*Toxoplasma* and *Coccidium*), were used to study the migration behaviour and developement of the *Toxocara* larvae in the dog. In the cases of clean beagles the adult worms appeared in their intestines if the 2nd stage larvae were inoculated into the vein of the dogs younger than 111 days of age. On the other hand, in the cases of mongrel dogs which had acquired infection already, the worms appeared in the intestine if they were inoculated before the age of 88 days. The recovery rates of the adults worm were much higher in the clean beagles than the mongrel dogs. The age registance phenomenon was not all or nothing in nature, but the gradual decrease of the adult's recovery rates as the age of dog advanced. The 2nd stage larvae inoculted into artery could return to vein at the primary infection. For the development of 2nd stage larvae, to stay in or pass through the liver had no promoting effect.

Oral administration of the 3rd stage larvae failed to recover the adult worms from the intestine and those of 4th stage larvae could succeed to recover the adult worms even in the case of aged dog. No age resistance of the host could be observed on the development of the larvae after 4th stage in the intestine.

The age resistance phenomenon had some relation to the difficulty of the development of the larvae from the 2nd stage to the 3rd stage in the lung of the aged dog.

It's primary cause was not the acquired resistance of the dog to *T. canis* infection, but some changes occurred in the internal development of the growing puppies.

The primary infection of the helminth free beagles with *T. canis* resulted in the prompt increase of eosinophile counts in peripheral blood showing the maximum peak at the 18th day and subsided gradually. This eosinophilia seemed to be caused by the invasion of larvae in the lung and the post lung development of the larvae and adult worms in the intestine had no stimulating influences on peripheral eosinophilia.

In the case of superinfection, the maximum peak of eosinophilia appeared on the 8th day and lasted for long time at high level. Long lasting eosinophilia observed in human visceral larva migrance cases must be due to the repeated infection with *T. canis*.

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無寄生虫ビーグル犬を用いた犬蛔虫感染実験
—感染に対する年齢抵抗性と免疫，幼虫の体内移動と発育，好酸球増多に関する観察—

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従来の成熟卵経口投与による犬蛔虫感染実験は幼虫が肺に到達するまで不定の障害があり，感染を量的に感察するのに大標本の実験動物を必要としたが，新たに無菌的に第二期幼虫を集め，血管内に直接注入する感染方法を開発し，小標本による感染実験の観察を可能にした．年齢抵抗と既感染の関係を解明するため，全く寄生蠕虫およびトキソプラスマとコクシジウムに感染していないコロニーのビーグル犬を実験に用い，犬蛔虫既感染の通常犬との同様の感染実験と比較した．

ビーグル犬では生後30日で9％にのぼる成虫回収率を示すが，発育につれて感染抵抗性を増し生後111日で0.1％しか成虫になれない．一方既感染通常犬では成虫の発育率も低く，生後80日で0.1％の成虫回収率しかなかつた．犬蛔虫既感染がなくとも生後4カ月に強い感染発育に対する抵抗を示すが，既感染があれば，より強く，早く抵抗性が出現する．

この発育に対する抵抗性は主として肺における幼虫の第2期幼虫以後の発育を阻止するために現われ，第4期以後の腸管内での発育の犬の年齢に関係なく可能であつた．

第2期幼虫は動脈に注入されても，肺に戻ることができるが，既感染による免疫が増強すると末梢毛細管を通過する際，組織に捕捉されると思われる．好酸球増多は幼虫の肺侵入と滞在によつてひきおこされ，腸管内の虫体の発育には無関係であり，再感染でより早く，強く，また永続的に見られる．人体症例の高度持続性の好酸球増多は頻回の感染の結果と思われる．