ACQUIRED RESISTANCE IN MICE TO THE DOG HOOKWORM, *ANCYLOSTOMA CANINUM*.

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INTRODUCTION

It is well known that resistance is acquired in host animals as a result of previous infection with the animal parasites. The general basic principle for acquired resistance to helminths is essentially the same as that for viruses, bacteria or nonliving materials, though some distinctive aspects of the immune reactions of animal parasites are quite characteristic.

It is also noted that acquired resistance against reinfection induced by parasites in the intestinal lumen is not nearly as potent as that stimulated by parasites in tissues and blood. Intestinal tissue-feeders such as *Trichinella* are highly immunogenic.

Blood sucking nematodes such as hookworms, in the course of larval migration which they pass through the tissues and blood can be expected to induce an immunity both parenteral and local in the host.

Studies on acquired resistance against the dog hookworm developed from previous infections with infective larvae have been reported by various investigators. Herrick (1928), McCoy (1932) and Foster (1935).

The work of Otto and Kerr (1939) and Otto (1941) has demonstrated that dogs can be immunized against subsequent severe challenge by immunizing infection with infective larvae and they concluded that actively acquired immunity operates in the host parasite relationship of the canine host and the hookworm.

Kerr (1935b and 1936) studied the acquired immunity of mice to the larvae of *A. caninum* and pointed out that the use of an abnormal host, such as the mouse is advantageous because the larvae pass through the tissues in the normal manner, although they are unable

to attain maturity in the intestine of this host.

In order to detect immunity in white mice against the larvae of *Ascaris lumbricoides*, Sprent and Chen (1949) have attempted to measure hinderance to the migratory activity in the lung and liver, and proved that the degree of liver retention, i.e. so-called "liver ratio" —derived from the number of larvae recovered from the liver divided by the total number in the liver and lungs was fairly constant and satisfactory criterion of immunity.

The present experiments were designed to follow the course of infections of *Ancylostoma caninum* after peroral inoculation of the infective larvae into mice and to demonstrate experimentally an acquired resistance in mouse host induced by low grade infections with the infective larvae followed by challenging infection, as evaluated by criterion of the number of larvae found in the lungs and liver of the host.

MATERIALS AND METHODS

Host and culture of hookworm larvae

In all these experiments mice weighing around 20g were used which were commercially purchased and selected at random from the same shipment lot. These mice from commercial source, free of natural parasites were seldom found. However, the liver and lungs were free of parasites.

To harvest the large number of the infective larvae, authors applied a filter paper method using instead the conventional test tube as a round wide mouth jar (5cm×13cm) in which a piece of filter paper (12×12cm) creased in a six stripform is inserted.

Feces were daily collected from infected dogs which

have been in our laboratory, and filtrated through a layer of gauze before being smeared on to the above filter paper, leaving about 3cm at the end of the paper. This creased filter paper folded smeared side out in the form like Y letter was inserted in the jar with the unsmeared portion reaching the bottom of the jar. About 20ml of heat sterilized water was added in the jar which was covered top with a piece of polyvenyl sheet and fixed it tightly with a rubber band. This jar was kept at 30°C for 7 days. After removal of the polyvenyl cover and the filter paper, larvae were obtained from water at the bottom of the jar. After several washings with sedimentation the larvae were further cleaned by using a 20 mesh sieve and were counted individually.

Infection of mice with the infective larvae:

Mice were infected under slightly narcotized condition with ether. The animals were starved over night before infection and the doses of larvae were administered orally into stomach by means of the polyethylene tubing (intramedic, Clay-Adams, Inc. RE-90/S12, I. D., 0.034 "O.D., 0.05"). The counted larvae were taken up in 0.2ml of tap water by a finely drawn bulbed pipette connected into the polyethylene tubing. The pipette and tubing were checked microscopically after each feeding.

Autopsy and larval counts:

After killing a mouse by a sharp blow the lungs and liver in which larvae were to be counted were

removed and placed separately in tissue grinder added with 5ml saline and homogenized in homogenizer drive (Thomas) for one to two minutes. Such organ suspensions after several washings with sedimentations were immediately diluted with warmed saline and poured into petri dish and thoroughly examined for the motile larvae.

The larvae counted with a dissecting microscope on the warming table and the number of the larvae in whole organs was obtained by this method.

RESULTS

Experiment I : This experiment was designed to determine the distribution of hookworm larvae in the liver and lungs of mice during primary infection.

Fifty four mice weighing 20g were infected with 1,000 larvae and were killed in batches of 6 daily up to 9 days after infection.

In all instances the larvae were alive and active at the time of examination. The numbers of larvae recovered from the lungs and liver in each of batches of mice at various intervals after inoculation are shown in Table 1. The number of larvae recovered in the lungs and liver increased for 48 hours after inoculation. As may be seen in Fig 1, consequent upon the migratory behavior to the other issues, the larvae were found in decreasing number for the following days.

The average number of larvae recovered from the lungs and liver at 48 hours after inoculation was 281.8 and it was 28.2 per cent of the number of larvae

Table 1. Number of larvae of *Ancylostoma caninum* recovered from the lungs and liver at various intervals after peroral inoculation of 1,000 larvae in mice.

Experiment	Number of mice	Dose of larvae	Intervals between inoculation & necropsy	Larvae recovered from (Ave. No./mouse)			The rate of recovery (%)	Lung ratio	Liver ratio
				Lung	Liver	Total			
I	6	1,000	24 hrs	114.3	66.3	180.6	18.0	0.63	0.37
	6	1,000	48	257.5	24.3	281.8	28.2	0.91	0.09
	6	1,000	72	92.7	3.2	95.9	9.6	0.97	0.03
	6	1,000	96	102.2	1.2	103.4	10.3	0.99	0.01
	6	1,000	120	64.8	7.2	72.0	7.2	0.90	0.10
	6	1,000	144	21.2	4.8	26.0	2.6	0.82	0.18
	6	1,000	168	4.5	1.0	5.5	0.6		
	6	1,000	192	2.0	1.0	3.0	0.3		
	6	1,000	216	1.0	0.5	1.5	0.2		

* **Lung ratio** : The number of larvae counted in the lungs divided by the total number of larvae counted both in the lungs and liver.

Liver ratio : The number of larvae counted in the liver divided by the total number of larvae counted both in the lungs and liver.

inoculated.

From the above results, it is indicated that the highest rate of recovery will be expected at 48 hour intervals between inoculation and necropsy of the host.

Experiment II.: This experiment was designed to

Fig. 1. Number of larvae recovered from the lungs and liver of mice during primary infection

get further informations on immunity developed by immunizing infections with the infective larvae followed by challenging infection.

One hundred fifteen mice weighing around 20g were used in this experiment. Eighty mice were given two infections of each 50 infective larvae at 7 day intervals. One to 4 weeks later they were challenged by a testing infection of 1,000 infective larvae and were killed in 4 batches of 20 mice at 48 hours after challenge.

After being infected 4 times with each of 50 infective larvae at 7 day intervals thirty five mice divided three groups 10, 10, 15 mice of each were challenged by a test infection of 1,000 infective larvae to each group at 2, 3 and 4 week intervals respectively and were killed in batches at 48 hours after challenge.

Sixty mice as the controls were given only the challenging infection.

The studies summarized in Table 2 consist of 2 and 4 time immunizing infections with 50 infective larvae followed by challenging infection with 1,000 infective larvae. Results were analyzed by the average total

Table 2. Results of immunizing infections with the infective larvae of the dog hookworm, *Ancylostoma caninum* followed by a challenging infection.

Experiment	No. of mice	Dose of Immuniz	Intervals between challeng. & last immuniz.	Dose of challeng- ing	Intervals between challeng & necr- opsy	Larvae recovered from (M±SD/mouse)			The rate of recovery (%)	Lung ratio	
						Lung	Liver	Total			
Two time immunizing infections at one week interval											
a	1	20	50	7 days	1,000	48 hrs.	105.9±15.5	12.3±2.5	117.2±15.6	11.7	0.89
	2	20	50	14	1,000	48 "	105.7±18.4	12.5±6.6	118.2±17.4	11.8	0.89
Control											
1&2	20	—	—		1,000	48 "	294.3±9.8	14.2±3.1	308.5±11.9	30.9	0.95
a	3	20	50	21 days	1,000	48 hrs	138.9±24.9	18.0±5.7	156.9±27.2	15.7	0.88
	4	20	50	28 "	1,000	48 "	157.5±21.2	17.7±5.0	175.1±20.9	17.5	0.90
Control											
3 & 4	20	—	—		1,000	48 "	290.5±11.8	19.2±2.2	309.7±11.0	31.0	0.94
Four time immunizing infection at one week interval											
b	1	10	50	14 days	1,000	48 hrs	129.0±29.8	12.7±1.6	141.7±29.6	14.2	0.91
	2	10	50	21 "	1,000	48 "	142.0±10.5	12.5±3.6	154.5±9.8	15.5	0.92
	3	15	50	28 "	1,000	48 "	149.3±21.4	12.3±3.3	161.5±22.4	16.2	0.93
Control											
1,2&3	10	—	—		1,000	48 hrs	288.0±17.9	16.3±2.5	304.3±14.8	30.4	0.95
	10	—	—		1,000	48 "	265.8±26.6	17.6±4.0	283.4±25.6	28.3	0.94

* Lung ratio: The number of larvae counted in the lungs divided by the total number of larvae counted both in the lungs and liver.

number of larvae recovered from the lungs and liver or the rate of recovery after challenge.

In each of the above experiments control mice of the same source as the experimentals were given no immunizing infection but were subsequently challenged and treated as were the corresponding experimental mice.

As shown in the Table 2, in all experiments the average total number of larvae was significantly less than that in the controls. The rate of recovery of the larvae after challenge was markedly reduced in both the lungs and liver, compared with the controls (Experiment a and b).

In the first experiment(a) the range of these rates of recovery was from 11.7 to 17.5 per cent, these showed distinctively reduced rates compared with the range from 30.9 to 31.0 per cent of the control. These figures were nearly half or one third of those of the controls.

In experiment(b) in which 4 time immunizing infections had been given the range of rates of recovery was from 14.2 to 16.2 per cent. Compared with the rates of 28.3 and 30.4 per cent in the controls, the markedly reduced rates were also shown as well as in experiment(a).

Although the numbers in the liver and lungs were within the fairly wide range of variability, both the lung or liver ratios showed interesting stability.

The rates of recovery to the different intervals between the last immunizing infection and the challenge also showed apparently significant.

This rate remained low for the first 7 day intervals, then began to increase. In other word resistance in mice is gradually reduced due to the longer intervals.

From the above results, it is most probable that the two time immunizing infections were quite enough to confer significant resistance ($P < 0.01$), compared with the controls.

DISCUSSION

Kerr(1938) reported his failure to produce a marked resistance in mice to the dog hookworm, *Ancylostoma caninum*, and in guinea pigs to the pig ascaris, *Ascaris lumbricoides* and he said this may be due either to the size of the test infection or to the type of antigens used.

Kerr (1936) suggested the advantage of using an

abnormal host of mouse in the studies of the immunity of *A. caninum*, because in the development of immunity, the importance of the larval stages, in which the more intimate contact with the host tissues occurs, can be determined selectively. His serial experiments showed conclusively that previous infection by either skin or mouth with the larvae of *A. caninum* can produce a resistance in the host of mice. He noted that mice died in seven days after infection by mouth or skin with the 2,500 larvae, however, that mice given sublethal doses of larvae by the same above methods survived a dose of larvae well above the average lethal number.

Since the adults of this nematodes, hookworm do not develop in the mouse, the resistance is produced entirely through the reaction which is caused by close contact between the host and the parenteral phase of this nematodes.

As Chandler (1936) indicated in the work of the interrelations between parenteral and intestinal immunity in rats infected with *Nippostrongylus*, a parenterally induced immunity has a marked effect on the growth and reproduction of parasites in the intestine to where it is also communicated. In this connection, McCoy (1931) ascertained a specifically acquired immunity distinct from the immunity acquired with age resulted from the data obtained after feeding the test dose of larvae of *A. caninum* to dogs and cats. These results were evaluated by checking of egg productivity of the experimental animals and the controls.

This evaluation method of acquired immunity to *A. caninum* can not be applied in the experiments using abnormal hosts of mice. However there are some other criteria available for the experimental demonstration of acquired immunity such as histological examination of affected host tissues and reduced rate of recovery of the larvae after challenge in the parenteral phase.

Sprent and Chen (1949) have devised a method for the estimation of immunity against *Ascaris lumbricoides* in an abnormal mouse host and attempted to measure the degree of liver retention of the inoculated *Ascaris* larvae. They found that the "liver ratio" i.e, the number of larvae in the liver divided by the number in the liver and lungs was fairly constant for several days after infection of *Ascaris* larvae. Therefore, it was suggested that this liver ratio may be one of several criteria to

detect immunity in mice against the larvae of *A. lumbricoides* from the pig. However, in the studies of the course of infection of hookworm, the degree of lung retention should be rather considerable than the one of liver retention because of the difference in their peculiar life cycles.

It was noted in these experiments that the lung and liver ratios were also constant.

As may be seen in the second experiment, the average total number of the larvae recovered in each series of experiments showed distinctively significant difference, compared with the controls. Therefore the recovered rate of the larvae after challenge infection in these experiments also indicated the considerable significance. The recovery rates in the control mice were in fact obviously higher than these in the immunized challenged mice in all instances.

From these results, it seems highly probable that a certain degree of protective immunity against development of the hookworm larvae in the parenteral phase was induced in the immunized mice by low grade repeated previous infections with the infective larvae.

Soh (1958) reported that when mice were inoculated with the infective larvae of *A. caninum*, most of the larvae promptly migrated into the muscles and about 10 per cent of them persisted without growth for 66 days. Nichols (1956) also noted that the larvae of *A. caninum* can survive for a year or longer in the abnormal host tissues.

From the foregoing observations on the migratory behavior of the larvae of *A. caninum* in the abnormal host, some of larvae inoculated in the immunized mice in our experiments may also be found in their muscle tissues, even in low grade immunizing infection with the 50 larvae, and they might effect on a homologous challenging infection with the larvae.

Regardless of this fact, it is highly suggested and concluded from our experiments that acquired resistance was induced in the abnormal host of mouse through the low grade immunizing infections with the infective larvae of *A. caninum* and evaluated by the total number of the larvae recovered from the lungs and liver in mice after challenge.

SUMMARY AND CONCLUSION

The present study attempted to induce an acquired

immunity against the dog hookworm, *Ancylostoma caninum*. The experiments were carried out to demonstrate and confirm whether an acquired resistance could be developed by the repeated low grade infections with the infective larvae of *A. caninum* in the abnormal host of mouse.

1. In order to determine the distribution of hookworm larvae in the lungs and liver of mice after inoculation of infective larvae, 54 mice were inoculated with 1,000 larvae and sacrificed in batches daily up to 9 days after infection.

It was found that in all cases the average total number of larvae recovered from the lungs and liver increased at 48 hours after infection, then began to decrease and reached 0 to 1 at the 9th day after inoculation.

2. One hundred fifteen mice were immunized 2 or 4 times at 7 day intervals with 50 infective larvae, followed by challenging infection with 1,000 infective larvae and killed in batches at 48 hours after challenge. The intervals between challenge and last immunization were from one to four weeks for each group. Sixty mice as the controls were given only challenging infection without previous immunizing infections.

Induced resistance was evaluated by the rate of recovery or the average total number of the larvae recovered from the lungs and liver in the challenged mice, compared with the controls.

It was noted that the rates of recovery in the controls were twice or nearly thrice higher than those in the previously infected mice in all instances and these remained low for the first 7 day intervals between the last immunizing infection and the challenge, then gradually increased.

3. From the above observations it is highly suggested that an acquired resistance can be produced by repeated previous infections with the larvae of *A. caninum*, even in low grade, in the abnormal host of mouse, and evaluated by the rate of recovery of the larvae after challenge.

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＝抄 録＝

犬鉤虫(*Ancylostoma caninum*)에 대한 마우스의 獲得免疫에 관한 研究

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著者는 犬鉤虫(*Ancylostoma caninum*)의 非正常 宿主인 마우스를 使用하여 犬鉤虫感染幼虫을 經口 投與한後 마우스 體內移行時期幼虫을 肺 및 肝에서 檢出하고 感染經過에 따라 犬鉤虫幼虫의 非正常宿主內 肺 및 肝通過樣相을 把握하였고 一方 感染幼虫을 마우스에 反復感染시킨 後 各各 一定 時日 間隔을 두고 一定數의 感染幼虫으로 이들 前感染 마우스에 試驗感染 (Challenging infection)을 하여 肺 및 肝에서 檢出된 幼虫數 또는 檢出率을 前感染이 없었던 對照群과 比較함으로써 마우스體內에 獲得免疫이 發展될 수 있는 가를 實驗하였다.

本究研에 使用된 마우스는 全部 體重 20g 前後였고 雌雄區別없이 商人으로부터 購入하였다. 蟯虫 (Syphacia) 등의 自然感染있는 것을 避하기는 거의 不可能하였으나 肺 및 肝에서는 蟯虫類寄生을 發見할 수 없었다.

實驗에 使用된 犬鉤虫幼虫은 研究室에 實驗 感染後 飼育保存中인 犬의 糞便에서 培養한 것으로 單一虫株(strain)였다.

大量感染幼虫을 얻기 爲하여 濾紙培養法을 改良하고 試驗管 대신 80g 드리콘瓶을 使用하였다.

感染幼虫은 마우스를 ether로 輕하게 麻酔시킨後 Intramedic polyethylene tubing (I.D., 034'')으로 마우스 胃內에 正確히 注入하였다.

마우스肺 및 肝內 幼虫數의 檢出은 各臟器를 Homogenizer Drive에서 輕하게 處理한 後 分離된 活

激히運動하는 幼虫을 Warming Table에서 解剖顯微鏡下에 計算하였다.

I) 54마리 마우스에 大鉤虫 感染幼虫 1,000 마리 씩을 輕口 投與한後 6 마리 씩 9 群으로 나누고 各群을 每日 屠殺하여 肺 및 肝內檢出幼虫數를 比較하였다.

肺 및 肝內 平均 檢出 幼虫數는 感染後 48時間까지 增加하나 以後 漸次 減少하였고 感染 第9日 後에서는 發見하기 어려웠다. 各群에 있어 所謂 Lung ratio 및 Liver ratio는 比較的 一定한 數値를 表示하였다.

II) 175 마리 마우스를 a, b 2 群으로 나누고 各群에 對照 마우스 各各 40 및 20 마리를 除外하고 a 群 80마리 및 b 群 35마리에 各各 50 마리 씩 感染幼虫을 每週間隔으로 a 群에 2回 b 群에 4回前感染시킨 後 各各 最終 前感染 後 第1週 乃至 4週 사이에 前感染이 없었던 對照群과 함께 175 마리 마우스 全部에게 各各 感染幼虫 1,000마리로 試驗感染(Challenge)하고 48時間後 屠殺하여 肺 및 肝內檢出幼虫數 또는 檢出率을 對照群과 比較하였다.

對照群에 있어서 檢出率은 28.0~31.0% 사이에 있었으나 前感染群의 檢出率範圍는 11.7~17.5% 사이에 있어 對照群에 比하여 全部 顯著히 低下되어 있었다. 以上の 結果로 미루어 볼 때 마우스에 大鉤虫 感染幼虫으로 試驗感染한 後 肺 및 肺 幼虫 檢出率로 判定한바 感染幼虫의 反復前感染으로 마우스體內에 幼虫移行을 阻害하는 獲得免疫이 發展되었음을 알 수 있었다.