Immunological Studies on *Metastrongylus apri* Infection

IV. Influence of Whole Body X-Irradiation or Anti-Guinea Pig Thymocyte Serum Injection on Acquired Immunity in Thymectomized Guinea Pigs

Masataka KOGA*, Minoru YAMADA, Takafumi ONISHI, Makio HORIE, Shusaku NODA and Ryoji NODA

Department of Veterinary Medicine, College of Agriculture,
University of Osaka Prefecture, Sakai-shi, Osaka 591

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Abstract. Two experiments, I and II, were carried out on guinea pigs to elucidate the defense mechanism against *Metastrongylus apri* infection. In Experiment I, to produce the status of scarceness in thymus-derived cells (T-cells) animals were exposed to whole body X-irradiation (300 R) seven days after thymectomy. Immediately after exposure they were vaccinated orally with 1,000 larvae attenuated by 5×10^4 R of X-rays (Group A). On the 25th day after vaccination they were subjected to challenge infection with 500 normal larvae. Two control groups, B and C, were set up. In Group B, animals were not treated, but vaccinated and challenged. In Group C, animals were not treated nor vaccinated, but challenged.

All the guinea pigs were killed on the 21st day after challenge to examine the presence of worms in the lungs. The number of worms recovered from the lungs was 0-77 (mean, 22.8) for Group A, 0-2 (mean, 0.3) for Group B and 1-116 (mean, 43.4) for Group C. There was a significant difference in worm burdens, as determined by Mann-Whitney's "U" test, between Groups A and B, but not between Groups A and C ($P < 0.05$). These results showed that T-cells might play an important role in the identification of larval antigen.

In Experiment II, thymectomized guinea pigs were vaccinated orally with 1,000 larvae attenuated by 5×10^4 R of X-rays. Then 25 days later they were subjected to challenge infection with 500 normal larvae. They were given intraperitoneally with 1.0 ml of antithymocyte serum (ATS) on the day of challenge infection and then with 0.5 ml every two days until 9 injections were made (totalling 5.5 ml each), so that T-cells might be suppressed (Group A). Two control Groups, B and C, were set up. In Group B, animals were not treated, but vaccinated and challenged. In Group C, animals were not treated nor vaccinated, but challenged. On the 21st day after challenge, all the animals were killed to examine the presence of worms in the lungs. The number of worms recovered from the lungs was 4-254 (mean, 44.7), 0-5 (mean, 1.2) and 29-274 (mean, 95.8) for Groups A, B and C, respectively. There was a significant difference in worm burdens, as determined by Wilcoxon's "U" test, between Groups A and B, as well as between Groups A and C ($P < 0.05$). These results showed that T-cells, which had a close relationship to cell-mediated immunity, might be suppressed by thymectomy and ATS injection, suggesting the important role of these cells in the defense mechanism against *M. apri* infection.

* Present address: Department of Parasitology, Faculty of Medicine, Kyushu University, Fukuoka 812

豚肺虫症の免疫に関する研究 IV. X線照射減弱子虫で免疫した除胸腺モルモットの感染防御に対するX線全身照射ならびに抗胸腺細胞血清投与の影響: 古賀正崇・山田 稔・大西堂文・堀江牧夫・野田周作・野田亮二 (大阪府立大学農学部家畜内科学教室)

It has been reported by Onishi [5-7] that guinea pigs vaccinated with infective *Metastrongylus apri* larvae which had been attenuated by X-irradiation developed solid immunity to subsequent infection with normal larvae, and that in the peritoneal cavities of these guinea pigs, the larvae were adhered by peritoneal exudate cells. Later, Shimakoshi [8, 9] did not find the expulsion of worms from guinea pigs which had been given orally normal infective larvae and subsequently received sera from solidly immunized guinea pigs. He also investigated cell adhesion response in the abdominal cavities of these guinea pigs by transmission electron microscopy. He inferred that lymphoid cells might play an important role in this defense response.

In the present work, taking a hypothesis that this immune response might be of cell-mediated type, thymus-derived cells (T-cells) known to participate closely in cell-mediated immunity were suppressed in thymectomized guinea pigs by two procedures, i.e., whole body X-irradiation and antithymocyte serum (ATS) injection. There are a few papers published to report research conducted on helminthic infection along this line, e.g., whole body X-irradiation in *Ascaris suum* [1, 2] and *Nippostrongylus brasiliensis* [3] infections, and ATS injection in *Hymenolepis nana* [4] and *Nippostrongylus brasiliensis* [3] infections.

Materials and Methods

Animals: Female guinea pigs of the Hartley strain weighing 200-250 g were used in these experiments. In the first experiment 40 guinea pigs were randomly allotted to three groups, A to C. Twenty were thymectomized, whole body-irradiated, vaccinated and challenged (Group A), ten vaccinated and challenged (Group B), and the other ten challenged only (Group C).

In the second experiment 44 guinea pigs were also randomly assigned to three groups, A, B and

C. Twenty-five were thymectomized, vaccinated, challenged, and injected with ATS (Group A), eleven vaccinated and challenged (Group B), and eight challenged only (Group C). All the animals were maintained in the same manner as mentioned in the previous paper [5].

Parasite and parasitological methods: Infective *Metastrongylus apri* larvae were used for oral inoculation. The larval vaccine attenuated by 5×10^4 R irradiation and the normal larval suspension used for challenge infection had been prepared in completely the same manner as mentioned in the previous paper [5]. In both experiments 1,000 attenuated larvae were inoculated into each guinea pig of Groups A and B 7 days after thymectomy (or a few hours after whole-body irradiation in Experiment I). Each guinea pig of all the groups was challenged with 500 normal larvae 25 days after vaccination. Guinea pigs were starved for 24 hours before and after oral inoculation. The inoculation was carried out by the same method as mentioned in the previous paper [5].

Thymectomy: Under mild anesthesia, guinea pigs were thymectomized surgically in Group A in both experiments.

Whole-body irradiation: In a preliminary experiment, 4 groups of thymectomized guinea pigs were irradiated with 200, 300, 400 and 500 R X-rays, respectively. Irradiation was done at 200 KVP and 20 mA, with 0.5 mm filter of Al and 0.5 mm filter of Cu, and at 24 rpm. The animals were examined for the peripheral red and white blood cell counts and clinical changes every day for 30 days. The dose of 300 R was used in the first experiment on the basis of data on the leukocyte count and death rate in irradiated guinea pigs. The two legs of each guinea pig were covered with a leaden board to guard the stem cell.

Preparation and injection of ATS: Antiserum against guinea pig thymocytes for the second experiment was prepared in rabbits by injecting subcutaneously with 10^7 guinea pig thymocytes suspended in Freund's complete adjuvant (Difco) at first and then with only thymocytes three times at weekly intervals. Seven days after the last injection these rabbits were bled to death, and serum samples were collected from them and stored at -20°C . A few weeks later the thawed serum was inactivated by holding at 56°C for 30 minutes, and then absorbed three times with the same volume of whole blood cells of guinea pigs.

A 1:128 dilution of ATS killed approximately 50 percent of thymocytes, as judged by the trypan blue exclusion test, in the presence of fresh guinea pig serum. In a preliminary experiment performed

Table 1. Outline of the experiment I

Group	No. of guinea pigs	Thymectomy	Whole body X-irradiation (300 R)	Vaccination	Challenge
				1,000 X-irradiated (5×10^4 R) larvae	500 normal larvae
A	20	+	+	+	+
B	10	—	—	+	+
C	10	—	—	—	+

Thymectomy	Whole body X-irradiation and vaccination		Challenge	Autopsy
↓	↓		↓	↓
7 days	25 days		21 days	

Table 2. Outline of the experiment II

Group	No. of guinea pigs	Thymectomy	Vaccination	Challenge	ATS injection (i. p.)
			1,000 X-irradiated (5×10^4 R) larvae	500 normal larvae	
A	25	+	+	+	+
B	11	—	+	+	—
C	9	—	—	+	—

Thymectomy	Vaccination		Challenge	Autopsy
↓	↓		↓	↓
7 days	25 days		↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑	↑
			ATS 1.0 ml	ATS 0.5 ml
				21 days
				Every 2 days

to determine the injection dose of ATS, nine thymectomized guinea pigs were divided into two groups, Group α of four and Group β of five. The daily dose of undiluted ATS injected into the peritoneal cavity was 2 ml on the first day and 1 ml on the other days in Group α , and 1 ml on the first day and 0.5 ml on every other day in Group β .

The lymphocyte count was done every day in blood samples collected from the lobe of the ear by puncturing veinlet, as well as clinical observation. It was markedly reduced in both groups. Death occurred to three of the four animals of Group α and three of the five animals of Group β . The other animals of this group lived rather long. Consequently, the dosage of ATS used in Group β was adopted for Experiment II. Each guinea pig of Group A was challenged with 500 normal larvae on the 25th day after vaccination. It was injected intraperitoneally with 1.0 ml of ATS on day 0 and 0.5 ml on days 2, 4, 6, 8, 10, 12, 14, 16 and 18 after challenge infection (the total dose being 5.5 ml).

Clinical observation: In Experiment I, the red

and white blood cell counts were determined every five days by using a micro-cell counter. Thin dry blood films were prepared to determine the elements of leukocytes, especially the lymphocyte. In Experiment II, lymphocytes were counted by the aid of the Thoma-Zeiss counting cell every five days before and every two days after challenge infection.

Collection of worms: From all the guinea pigs killed on the 21st day after challenge infection lungs were removed and digested with 1% pepsin solution (pH 1) to collect worms quite easily, as described in the previous paper [5]. The outline of Experiment I and II is shown in Tables 1 and 2, respectively.

Results

A. Experiment I

1. Clinical signs: The guinea pigs of Group A began to show depression, anorexia and paleness of the iris about seven

Fig. 1. Mean lymphocyte count in Experiment I

days after irradiation. After that, they recovered to a considerable degree, but continued to exhibit poor appetite, coughing and labored respiration caused by challenge infection up to about 40 days after irradiation. These symptoms were also observed in Group C during the same period, but none of them were recognized in Group B.

2. Lymphocytes (Fig. 1): In Group A the lymphocyte count showed a maximum decrease ($350/\text{mm}^3$) within 24 hours after irradiation. Then it increased to reach about half the level of untreated guinea pigs about 20 days after irradiation and remained at this level thereafter. Temporary lymphocytosis occurred in Group B after vaccination and challenge infection. A marked lymphocyte increase was seen in Group C after challenge infection.

3. Macroscopic findings of lungs: Mild or severe emphysema and atelectasis were found in some parts of the lungs in Group A. They seemed to have been caused by the infection of *M. apri*. They were severer in Group C than in Group A. The lungs collapsed well and showed few or no lesions in Group B.

4. Worms recovered (Table 3): Guinea

pigs which died by the 15th day after challenge infection were excluded from the experiment. The number of worms recovered from both lungs was 0–77 (mean, 22.8) with a female to male ratio of 1 : 4.56 for Group A (10 animals), 0–2 (mean, 0.3) with a ratio of 1 : 2 for Group B (10 animals), and 1–116 (mean, 43.4) with a ratio of 1 : 2.14 for Group C (10 animals).

B. Experiment II

1. Clinical signs: The guinea pigs of Group A showed a marked depression, ruffled hair, coolness of all the legs and inappetence due to ATS injection. Some of them died and others tended to recover gradually. About 14 days after challenge infection they showed again poor appetite, coughing and labored respiration which seemed to have been caused by challenge infection. About 14 days after challenge infection these symptoms were also seen in Group C, but no symptoms were recognized in Group B.

2. Lymphocytes (Fig. 2): The lymphocyte count increased a little after vaccination, and then decreased to a normal level. After challenge infection it decreased greatly (to about 8% of the level before injection) due to ATS injection in Group A.

Table 3. Number of worms recovered from the lungs of guinea pigs in Experiment I

Group	Worm burdens on 21st day after challenge				Group mean
	Individual count				
	Male	Female	Undetermined	Total	
A	0	0	0	0	22.8
	1	1	0	2	
	0	3	0	3	
	0	5	0	5	
	1	4	0	5	
	2	7	0	9	
	1	10	0	11	
	15	42	0	57 (20)	
	13	45	1	59	
7	69	1	77		
B	0	0	0	0	0.3
	0	0	0	0	
	0	0	0	0	
	0	0	0	0	
	0	0	0	0	
	0	0	0	0	
	0	0	0	0	
	0	0	0	0	
	0	1	0	1	
	1	1	0	2	
C	0	1	0	1	43.4
	3	1	0	4	
	2	3	0	5	
	6	7	0	13	
	4	27	0	31	
	13	31	2	46	
	12	49	0	61 (17)	
	16	57	1	74	
	25	57	1	83	
	56	60	0	116 (19)	

Remarks.

In parentheses is shown the interval of time in days from challenge to death. There is a significant difference between Groups A and B, but not between Groups A and C when Mann-Whitneys "U" test was used ($P < 0.05$).

Then it increased gradually in about 6 days. It began to decrease again slowly in about 10 days. It increased temporarily in Group B after vaccination. It increased remarkably in Group C after challenge infection.

3. Macroscopic changes in lungs: Mild or severe emphysema and atelectasis which had been caused by infection with worms

were found in some parts of both lungs in Group A. These changes were moderate or severe in Group C. Each lung had few or no lesions and collapsed completely in Group B.

4. Worms recovered (Table 4): Guinea pigs which died up to the 14th day after challenge infection were excluded from the experiment. The number of worms

Fig. 2. Mean lymphocyte count in Experiment II

recovered from both lungs was 4–254 (mean, 44.7) with a female to male ratio of 1 : 2.6 for Group A (16 animals), 0–5 (mean, 1.2) with a ratio of 1 : 2.5 for Group B (6 animals), and 29–274 (mean, 95.8) with a ratio of 1 : 1.6 for Group C (8 animals).

Discussion

An experiment was carried out by Casarosa [1] and Casarosa et al. [2]. In it, two groups of guinea pigs were vaccinated orally with 500 and 1,000 embryonated *Ascaris suum* eggs on day 0 and 15, respectively. They were exposed totally to X-irradiation (400 to 450 R as a single dose) 15 days later. Four days later the guinea pigs were challenged with 2,000, 15,000 or 20,000 embryonated *A. suum* eggs. As a result, the X-irradiation greatly reduced the protective activity afforded by vaccination. Meanwhile, Okamoto and Koizumi [4] injected mice intraperitoneally with 0.1 ml of ATS on days –1, 0, 1, 2 and 3. They immunized mice with 500 eggs of *Hymenolepis nana* and challenged with

2,000 eggs 10 days after the immunizing inoculation. As a result, the mice injected with ATS showed a complete depression of acquired immunity when challenged. Keller and Keist [3] exposed rats to X-irradiation (400 R) and found that the expulsion of 300 *Nippostrongylus brasiliensis* worms damaged with antiserum was significantly delayed in the rats, as compared with that in controls. Neonatally thymectomized adult rats showed a more remarkable delay in the expulsion of damaged worms than controls when injected with ATS by the intraperitoneal route.

In Experiment I, worm burdens in the lungs of guinea pigs were considerably great in Group A and much greater in Group C than in Group B. There was a significant difference in worm count, as determined by Mann-Whitney's "U" test, between Groups A and B, but not between Groups A and C ($P < 0.05$). Thus, the immunized guinea pigs of Group A were involved in *M. apri* infection as a result of challenge. These results may show

Table 4. Number of worms recovered from the lungs of guinea pigs in Experiment II

Group	Worm burdens on 21st day after challenge				Group mean
	Individual count				
	Male	Female	Undetermined	Total	
A	2	2	0	4	44.7
	0	4	0	4	
	2	4	0	6	
	5	2	0	7	
	3	6	0	9	
	2	12	0	14	
	4	16	0	20	
	9	13	0	22	
	2	20	0	22	
	16	16	0	32	
	17	28	1	46 (17)	
	5	44	0	49	
	12	37	0	51	
	22	39	0	59	
	16	99	1	116	
	80	174	0	254 (19)	
B	0	0	0	0	1.2
	0	0	0	0	
	0	0	0	0	
	0	1	0	1	
	0	1	0	1	
	2	3	0	5	
C	11	18	0	29	95.8
	11	13	0	34	
	18	37	0	55	
	20	36	2	58	
	30	49	0	79	
	33	56	0	99 (17)	
	63	75	0	138 (15)	
	101	171	2	274 (20)	

Remarks.

In parentheses is shown the interval of time in days from challenge to death. There is a significant difference between Groups A and B, and between Groups A and C when Wilcoxon's "U" test was used ($p < 0.05$).

that T-cells, which are said to constitute the majority of lymphocytes in the peripheral blood stream, were suppressed to a considerable degree by thymectomy and whole-body X-irradiation. The larval antigen used for vaccination was recognized to be insufficient. The activity of the protective immune mechanism, was proved to be incomplete.

In Experiment II, worm burdens in the lungs of guinea pigs were fairly large in Group A and much larger in Group C than in Group B, while few or no worms were found in Group B. There was a significant difference in worm count, as determined by Wilcoxon's "U" test, not only between Groups A and B but also between Groups A and C ($P < 0.05$). The macro-

scopic findings of the lungs and clinical signs also reflected the levels of worm burdens. Thus, the immunized guinea pigs of Group A were involved in *M. apri* infection as a result of challenge. These results suggest that the protective immune response of the host to challenge infection may have been suppressed by thymectomy and ATS administration.

It is concluded from the results of the two experiments that T-cells which participate in the formation of cell-mediated immunity may be necessary for the defense mechanism against *M. apri* infection in guinea pigs.

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