Immunologic Protection against Canine Heartworm Infection

Motonobu YOSHIDA, Kazuhide NAKAGAKI*, Sadao NOGAMI, Ryô HARASAWA, Ryuichiro MAEDA, Hiromi KATAE, and Yoshihiro HAYASHI

Research Laboratories, Animal Science Division, Dainippon Pharmaceutical Co., Ltd., Ikeda, Osaka 563, 1)Laboratory of Wildlife Medicine, College of Veterinary Medicine, Nippon Veterinary and Animal Science University, Musashino, Tokyo 180, 2)Laboratory of Medical Zoology, College of Bioresource Sciences, Nihon University, Kameino, Fujisawa, Kanagawa 252, 3)Animal Center for Biomedical Research, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113, 4)Department of Parasitology, Teikyo University School of Medicine, Itabashi-ku, Tokyo 173, and 5)Department of Veterinary Anatomy, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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ABSTRACT. This study was conducted to evaluate protective efficiency of three different protocols for vaccination in canine heartworm infection. To evaluate the three protocols of immunization, dogs were separately immunized with living larvae; 1) immunization with gamma-attenuated infective larvae, 2) with 50 µg/kg ivermectin-abbreviation, and 3) with chemical abbreviation plus Freund’s complete adjuvant (FCA). Each group was composed of two dogs. All dogs used for this study were subcutaneously challenged with 100 intact third-stage larvae (L3) various days after the last immunization, and the worms in the pulmonary arteries and the right ventricle of the heart were recovered 17 to 25 weeks post-infection. The numbers and the sexes of the worms were determined. A mean of 38 worms was burdened in the group immunized with irradiated L3, 36 worms in the chemically-abbreviated group, but 15.5 worms in the group with chemical abbreviation plus FCA. The percentages of the protection in the former two groups were nearly 50%, but 72.3% in the group with ivermectin plus FCA. The adjuvant enhanced the protective immunity against L3 challenge. Obvious eosinophilia was observed in both immunized and control dogs except for two dogs. There was no correlation between the suppression of eosinophilia and the protective immunity in the present study. — KEY WORDS: canine, Dirofilaria immitis, immunologic protection, third-stage larvae.


It has been reported that an average of approximately 50% (30.4–68.0%) [1, 7, 12, 13, 17, 19] of worms were recovered from the lung and the heart of experimentally infected dogs. However, there is no report stating that about 100% worms have been recovered; namely, half of inoculated larvae failed to migrate into the lung or the heart, and died. The decreased viability of the larvae during harvesting seemed to be one of the most appropriate reasons for the deaths. It is, however, impossible to explain why most researchers reported the recovery rates of 30.4 to 68.0% in normal animals. There is a possibility that a part of third-stage larvae (L3) that could not reach the lung or the heart were eliminated by the host defense mechanism, especially by an immune mechanism. Indeed, there has been a report stating that macaques treated with an immunosuppressant were susceptible to the infection with Dirofilaria immitis [24].

There is a sufficient time for dogs to raise an immune response to canine heartworm, since it takes 4 months for the larvae to invade the heart and the lung [10, 11]. The host receives an adequate stimulus from the worms during this period and becomes to respond to the antigens of the worms. Actually, dogs produced antibodies to canine heartworm antigens 16 weeks after infection with L3 [8] and the peripheral blood lymphocytes from 4-week-infected dogs proliferated with canine heartworm antigens (unpublished data). Although there is normally a sufficient titer of antibody against the antigen in heartworm infection, naive dogs cannot resist the infection. This escape mechanism of parasites has grounded on a mimicry theory. If parasites can decrease their surface antigens, and the hosts potentiate their immune response, the parasites will be eliminated.

On the basis of this background, studies on immunization of dogs against canine heartworm disease were carried out. It has been reported that immunization with 20-Krad-attenuated L3 resulted in the resistance to the infection with D. immitis [14, 25]. Additionally, immunization with a chemically-abbreviated infection has been reported to make the ferrets and the dogs protect against the challenge infection with L3 [2, 6]. These results suggest the possibility that canine heartworm infection is protected by vaccination.

The immunization described in two previous reports [6, 25] was evaluated with minor modifications and, in addition, the enhanced efficacy of Freund’s complete adjuvant was also confirmed in dogs with chemically abbreviated infection in the present study.

MATERIALS AND METHODS

Animals: Animals used in the present study were 12 male beagles purchased from Nihon Nosan Kogyo Inc. (Yokohama, Japan). The dogs were in good health, weighed between 8.5 and 10.2 kg, and their ages ranged from 24 to

* CORRESPONDENCE TO: NAKAGAKI, K., Laboratory of Wildlife Medicine, College of Veterinary Medicine, Nippon Veterinary and Animal Science University, Musashino, Tokyo 180, Japan.
28 weeks. Prior to receiving, all were bred in mosquito-proof indoor and vaccinated against distemper virus, adenovirus type 2, and parvovirus. During the study, they were kept in mosquito-proof individual cages under a constant temperature and an illumination provided by a fluorescent lamp, automatically controlled to give a 12-hr light/dark cycle. They were fed on a standard commercial canine laboratory diet (Nihon Nosan Kogyo Inc.) once a day and offered water *ad libitum* throughout the study.

The present study consisted of three separate trials with the following immunization manners (Fig. 1): immunization with infective larvae attenuated by irradiation with $^{60}$Co at a dose of 20 Krads, that with ivermectin-abbreviation of infection, and that with ivermectin-abbreviation of infection plus Freund’s complete adjuvant. In two trials of ivermectin-abbreviation, control dogs received ivermectin treatment alone. The groups of all trials were each composed of two animals.

**Parasites:** Three microfilaremics, naturally infected with *D. immitis*, were employed as microfilaria donors to mosquitoes, *Aedes togoi*. Mosquitoes were obtained from Department of Parasitology, Teikyo University School of Medicine and maintained in Animal Science Division, Research Laboratories, Dainippon Pharmaceutical Company, Ltd. They were directly fed canine blood for 30 min from a microfilaremic dog anesthetized with sodium pentobarbital (manufactured by Abbott Laboratories, Ltd., North Chicago, IL, U.S.A., and distributed by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Infective larvae were harvested by Baermann’s method from mosquitoes at 14 or 15 days after blood meal. Briefly, the mosquitoes were anesthetized with ether vapor and collected on a gauze. The packed mosquitoes were gently crashed with a roller on a table, placed on a funnel filled up with Ringer’s solution, and incubated for 1 hr at room temperature. Larvae migrating into the outer solution were collected in a fresh Ringer’s solution and washed twice with Waymouth medium (GIBCO BRL, Grand Island, NY, U.S.A.) to remove the component of the crashed mosquitoes. For examining the effect of the attenuated larvae on the protection against the infection with *D. immitis*, the larvae were irradiated with $^{60}$Co at a dose of 20 Krads in Waymouth medium. Dogs were subcutaneously injected with various numbers of the larvae in Waymouth medium. A 5-ml syringe attached with a 18-gauge needle was used for the injection. To make sure of injecting all worms into dogs, injection with saline was repeated twice.

**Schedules of immunization and challenge infection** (Fig. 1):
1) Immunization with attenuated L3 (trial-1)
   The experiment traced that of Wong *et al.* [25]. Briefly, two dogs were immunized three times each with 500 attenuated L3 at intervals of 8 to 10 weeks. Challenge infection of four dogs with 100 intact L3 was performed 8 or 9 weeks after the last immunization.

2) Immunization with chemical abbreviation of infection (trial-2)
   The experiment traced that of Grieve *et al.* [6]. Briefly, two dogs were infected with 500 intact L3, and then given ivermectin (CARDOMEC 136, Produced by Merck, Sharp and Dome Ltd., NJ, U.S.A. and distributed by Dainippon Pharmaceutical Company, Ltd., Osaka, Japan) at an oral dose of 48 to 57 µg/kg 8 or 9 weeks after infection. Two control dogs were also treated with this drug at the same dose and by the same schedule. At an interval of 4 or 5 weeks, this schedule was repeated three times. Challenge infection was given to four dogs with 100 intact L3 at 4 weeks after the last ivermectin administration.

3) Immunization with chemical abbreviation plus Freund’s complete adjuvant (trial-3)
   To shorten the duration of the experiment, the above schedule and manipulation were modified. Briefly, two dogs were infected with 500 intact L3, and simultaneously injected at another site with an emulsion of Freund’s complete adjuvant (FCA; DIFCO Laboratories, Detroit, MI, U.S.A.). FCA was mixed with the same volume of saline, sonicated at a maximal power, and the emulsion was suspended in saline containing 2% Tween 80 with a sonicator. Finally, the adjuvant formed water-in-oil-in water. The preparation in 0.8 ml, equivalent to 0.3 ml of FCA, was injected into the back skin of the immunized dogs in eight divided doses. At an interval of 6 weeks, this regimen was repeated twice. Two weeks after the second regimen, all four dogs were medicated with ivermectin at an oral dose of 58 to 62 µg/kg, then challenge infection was accomplished in the same way as above.

To confirm the humoral immune response in all immunized dogs, serum antibody was titrated a few days before the challenge infection by antibody-capture enzyme-linked immunosorbsent assay.

**Recovery of adult worms:** To count the worms migrating into the heart and the pulmonary arteries, all animals were killed with an intravenous administration of sodium pentobarbital at a dose of 60 mg/kg. Immediately after the heart beats had stopped, they were necropsied and the right ventricle of the heart and the lung were examined in detail. Recovered worms were kept in saline until counting and determining the sexes of the worms. Effectiveness of the immunization was calculated with the following formula: 

\[
\text{Protection} = \frac{\text{Mean worm recovery from control dogs} - \text{worm recovery from the immunized dog}}{\text{Mean worm recovery from control dogs}} \times 100
\]

**Eosinophil counts:** At monthly intervals, samples of the venous blood were drawn from every dog. Eosinophils were counted from the percent eosinophils in a differential count multiplied by the total white blood cell count.

**Enzyme-linked immunosorbsent assay:** Antibody-capture enzyme-linked immunosorbsent assay (ELISA) was performed with immunized canine sera. Wells of a microplate (Flow Laboratories, Costa Mesa, CA, U.S.A.) were coated with *D. immitis* antigen in 0.1 M carbonate buffer (pH 9.6). Sera diluted 1:100 were placed in the wells and incubated for 1 hr at 37°C. After three washes, appropriately diluted anti-canine IgG antibody conjugated with peroxidase was added to the wells and incubated again.
in the same way as above. After free conjugated antibody was washed away, 2,2’-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemicals, Saint Louis, MO, U.S.A.) at a concentration of 0.3 mg/ml, and 0.01% peroxide as a substrate were added to the wells. The plate was placed for about 20 min at room temperature and 1% sodium dodecyl sulfate (SDS; Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution was added to the wells to stop the reaction. The absorbance was read with a microplate reader. ELISA values were calculated with the following formula: ELISA value = (Sample absorbance - the absorbance of the negative control) / (the absorbance of the positive control - the absorbance of the negative control) × 100.

Antigen for ELISA: Adult worms were collected from naturally infected dogs and stored at -40°C until processing for the antigen preparation. The worms were lyophilized and crashed with a mortar and a pestle. The minute fragments of the worms were rehydrated in 0.01 M phosphate buffered saline (PBS; pH 7.0) and sonicated on ice until the cuticle was separated from the inner structures. The preparation was washed with PBS, centrifuged for 1 hr at 10,000 × g until the protein in the supernatant disappeared. The sediments were resuspended in 0.05 M Tris-HCl buffer (pH 5.6) containing 1% SDS and 5% beta-mercaptoethanol (Sigma Chemicals) and sonicated for 15 min on ice. The homogenate was extracted overnight at 4°C and centrifuged for 1 hr at 10,000 × g at 4°C. This supernatant was dialyzed against 0.01 M PBS for three days.

Table 1. Parasite recoveries from all immunized and control dogs, and percentages of protection against *Dirofilaria immitis*

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Adult <em>D. immitis</em> Recovered</th>
<th>% Protection a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dog ID</td>
<td>Total</td>
</tr>
<tr>
<td>Trial-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune</td>
<td>A</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>39</td>
</tr>
<tr>
<td>Control</td>
<td>C</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>68</td>
</tr>
<tr>
<td>Trial-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune</td>
<td>E</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>40</td>
</tr>
<tr>
<td>Control</td>
<td>G</td>
<td>75</td>
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<tr>
<td></td>
<td>H</td>
<td>74</td>
</tr>
<tr>
<td>Trial-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune</td>
<td>I</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>14</td>
</tr>
<tr>
<td>Control</td>
<td>K</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>48</td>
</tr>
</tbody>
</table>

a) % Protection based on the formula: [mean worm recovery from control dogs - worm recovery from immunized dogs] / mean worm recovery from control dogs.

Fig. 1. Procedures of immunization and challenge. These protocols were separated by three immunization manners. The dogs (Dog A and B) in trial-1 were immunized with gamma-irradiated third-stage larvae (#L3: inoculation with 500 L3 attenuated with 60Co at 20 Krads), and the intact dogs (Dog C and D) were used as controls. In trial-2 and -3, the dogs (Dog E and F, and Dog I and J, respectively) were immunized with the abbreviation of the infection using ivermectin (L3: inoculation with 500 intact L3, Rx: oral administration of ivermectin, FCA: Freund’s Complete Adjuvant), and the control dogs (Dog G, H, K and L) were treated with ivermectin alone. All of the dogs were subcutaneously challenged with 100 intact L3 (CHL).
and centrifuged for 1 hr at 10,000 × g at 4°C to remove the precipitate. The supernatant was used for ELISA after determining protein concentration in the supernatant with Bradford’s method.

RESULTS

Recovery of Adult Worms (Table 1):
1) Immunization with attenuated L3 (trial-1)
From the two immunized dogs, 37 and 39 worms were recovered 21 to 22 weeks after the challenge infection. These dogs showed 43.5 and 40.5% protection.
2) Immunization with chemical abbreviation of infection (trial-2)
From the two dogs immunized with the chemical abbreviation of the infection, 32 and 40 worms were found 25 weeks after the challenge infection. These dogs revealed 57.0 and 46.3% protection.
3) Immunization with chemical abbreviation plus Freund’s complete adjuvant (trial-3)
From the two dogs immunized with the chemical abbreviation of the infection and stimulated with FCA, 17 and 14 worms were discovered 17 weeks after the challenge infection. These dogs (69.6 and 75.0% protection) apparently protected against the challenge infection.

Eosinophil Counts (Fig. 2):
1) Immunization with attenuated L3 (trial-1)
Eosinophilia was uncertain during the immunization in all dogs but dog B that showed a slight eosinophilia immediately before challenge infection. Although eosinophilia in dog B disappeared in 8 weeks after the infection, the other three showed a prominent elevation of eosinophil counts in 10 weeks or 17 weeks after the infection. All dogs recovered from eosinophilia in 21 or 22 weeks after the infection.
2) Immunization with chemical abbreviation of infection (trial-2)
Eosinophil counts did not change significantly throughout immunization, but mild eosinophilia appeared in 13 weeks after the challenge infection in three of the four animals.
3) Immunization with chemical abbreviation plus Freund’s complete adjuvant (trial-3)
During immunization, the number of eosinophils did not exceed the upper limit of the normal range. However, severe eosinophilia was recognized in control dogs in 15 weeks after the infection. In 17 weeks, these eosinophil counts dropped but still remained high. In immunized dogs, the counts slightly elevated at 15 weeks. The eosinophil count

![Fig. 2. Eosinophil counts in the peripheral blood throughout the study. Solid lines (— —) indicate the counts of the immunized dogs, and dotted lines (— ——) those of the control dogs. Not only the controls but also the immunized dogs showed severe to mild eosinophilia. One immunized animal (Dog B) undoubtedly suppressed eosinophilia after challenged.](image-url)
DISCUSSION

of a dog (dog J) restored the normal range at 17 weeks after
the infection, but that of the other increased. In immunized
and control dogs, the suppression of eosinophilia was
unrelated to the intensity of the protection from the infection.

ELISA (Table 2): The ELISA values, prior to the
immunization and the challenge infection in the immunized
dogs, are shown in Table 2. Before the first immunization,
ELISA values of five dogs in the immunized groups were
lower than the negative control level. Only dog J had a
value of 1.79. Then, in every dog immunization was
finished; a few days before the challenge infection, their
values were between 26.6 and 99.5. Dog F indicated a
relatively lower titer in the serum than did the others at
this time, but there was no difference in the recovery rate
of the worms among these immunized animals. The ELISA
values do not explain the intensity of the protection, but do
show adequately the immunized dogs responding to the
immunization performed by the authors.

A total of 1,500 L3 worms was used for the immunization
of one dog in trial-1 and -2. This number of worms may be
enough to develop immunity in dogs, since high antibody
titers were demonstrated by ELISA in immunized dog sera
before the challenge infection. Additionally, dog F showed
a relatively lower titer in the serum than did the others at
this time, but there was no difference in the recovery rate
of the worms among these immunized animals. The ELISA
values do not explain the intensity of the protection, but do
show adequately the immunized dogs responding to the
immunization performed by the authors.

The recovery rates in control dogs must be reflected by
the viability of infective larvae used for the challenge
infection. Their recovery rates in nonimmunized dogs were
22–54 and 31–40% described by Wong et al. [25]. These rates were distinctly lower
than those of the authors. In the experimental infection, the
authors usually eliminated immotile larvae, but were not
able to separate mildly damaged worms from those used for
the challenge infection. The cryptic damage of the larvae
may affect the molt coming soon after injection and their
survival in immunized dogs, and the damaged worms must
be easily eliminated during the migration without regard to
the intensity of immunity of dogs. Thus, the low viability
of the larvae may produce higher percentage of the
protection than the truth. On the other hand, adult worm
recovery from immunized dogs with gamma-attenuated L3
fluctuated between 34% and 97% [14]. The wide fluctuation
indicates that the worms did not uniformly respond to the
challenge in accordance with the immunization protocol.
Because, the authors used out-bred beagles for the present
study, and there was no information of genetic backgrounds
of the dogs related to immune response. These
immunization protocols may have a problem in the
reproducibility of inducing the protective immunity.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dog ID</th>
<th>Prior to Immunization</th>
<th>Prior to Challenge Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune with attenuated</td>
<td>A</td>
<td>- 0.64</td>
<td>79.1</td>
</tr>
<tr>
<td>third-stage larvae</td>
<td>B</td>
<td>- 0.06</td>
<td>99.5</td>
</tr>
<tr>
<td>Immune with IVM (abbreviation)</td>
<td>E</td>
<td>- 1.02</td>
<td>91.2</td>
</tr>
<tr>
<td>Immune with IVM abbreviation</td>
<td>F</td>
<td>- 0.61</td>
<td>26.6</td>
</tr>
<tr>
<td>Immune with IVM</td>
<td>I</td>
<td>- 1.91</td>
<td>93.3</td>
</tr>
<tr>
<td>abbreviation plus FCA</td>
<td>J</td>
<td>1.79</td>
<td>88.5</td>
</tr>
</tbody>
</table>

All dogs were immunized with 500 third-stage larvae.

a) ELISA was performed with adult cuticle antigen and the titers are represented
as ELISA value calculated with the following formula. ELISA value = (sample OD
- negative control OD)/(positive control OD - negative control OD) ⨉ 100
b) IVM: About 50 µg/kg ivermectin was administered.
c) Freund’s Complete Adjuvant.
The experiments in trial-1 and -2 took 45 and 64 weeks from the first immunizations to necropsies, respectively. The authors considered an immunologic enhancement to shorten the period of experiment and to improve the protection. To stimulate further the immune system with excretory and secretory antigens, molt-related antigens, and dead worms, Freund’s complete adjuvant (FCA) was simultaneously administered with L3 injection for immunization in trial-3 (Table 1). Reduced recovery of adult worms was observed by adding FCA administration to the protocol of trial-2. FCA has widely been used to enhance immune responses. The complete adjuvant that includes *Mycobacterium tuberculosis* potentiates the activation of macrophages and consequently enhances both antibody production and cell-mediated immunity [3]. It is important to activate the effectors even if the larvae are eliminated by the antibody-dependent cell-mediated immunity as the microfilariae [16, 23]. There are reports mentioning the correlation between the presence of anti-cuticular antibody and impairment of L3 growth and motility in the system of *Dipetalonema viteae* and rodents [4, 20, 21]. Resistance to the infective larvae of *Brugia pahangi* has occurred by the T cell-dependent mechanism [18, 22]. The authors have proved that cell-mediated immunity rather than antibody [9], especially macrophages, is important for mice to protect against L3 of *Brugia malayi*. Macrophages are activated also by T helper 1 (Th1) cytokines [5, 15] produced by T lymphocytes and itself. In the present study, adding FCA is thought to stimulate Th1, even though those dogs showed eosinophilia that is induced by T helper 2 cytokine such as interleukin 5. The authors did not recognize any correlation between the intensity of the protection and the suppression of eosinophilia.

It has been reported that microfilariae of *D. immitis* were eliminated from the peripheral blood with antibody against their surfaces in occult dogs [26]. Conversely, in L3 infection, antibody against *D. immitis* may not play an important role in protection of dogs; because, even though high antibody titer was kept in the peripheral blood during the tissue migration of *D. immitis* in its host [27], nearly 50% of the worms experimentally infected have been recovered from the heart and the pulmonary arteries. However, the mechanism of the protection has not yet been explained.

REFERENCES

3. Freund, J. 1956. The mode of action of immunologic adju-


