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Autor: April, M. / D'Antonio, R. / Scott, A.L.
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[1] Division of Veterinary Medical Research, Center for Veterinary Medicine, Food and Drug Administration, Beltsville, Maryland 20705, USA
[2] Department of Immunology and Infectious Diseases, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland 21205, USA

Development of a chemotherapeutic model for microfilaricidal drugs to *Dirofilaria immitis**

M. April[1], R. D'Antonio[2], A. L. Scott[2], A. Malick[2], E. P. Roberts[2], D. A. Levy[2]

Summary

Microfilariae obtained from in vitro culture of adult *Dirofilaria immitis* were inoculated into naive dogs and used to test the in vivo efficacy of the antimicrofilarial drug Dizan. Injection of 33 million microfilariae into a 3-month-old male beagle pup over a 5-day period resulted in a microfilaremia that peaked at 863 microfilariae per ml on day 30. Treatment with Dizan resulted in a rapid clearance of microfilariae from the peripheral circulation. Four additonal male beagle pups that were each given a total of 31 million microfilariae over 100 days maintained an average of 25 microfilariae per ml for 2 months. Microfilaremias were monitored by ELISA, a modified Knott's method and selected blood chemistry tests. It was shown that after residing in the peripheral circulation the microfilariae were able to develop into infective larvae in mosquitos; therefore, the system may be a reasonable model of the natural microfilaremic state.

Key words: microfilaria; in vitro culture; chemotherapeutic in vivo model.

Introduction

Traditionally, a chemotherapeutic trial of a drug's effectiveness against microfilariae (mff) of *Dirofilaria immitis* has required either the generation of

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Correspondence: Dr. Milton April, Food and Drug Administration, Center for Veterinary Medicine, Division of Veterinary Medical Research, Agricultural Research Center, Beltsville, Maryland 20105, USA

an experimental infection or the use of infected random source dogs. The establishment of a patent experimental infection has the disadvantage of a prolonged prepatent period (6–9 months) combined with the maintenance expense incurred during parasite development. The lack of knowledge concerning the genetic, nutritional, physiological, parasitological and immunological status of random source dogs limits their utility as a source of infected animals for experimental drug trials. A model system that would reduce the time necessary to establish a patent infection and employ dogs of defined genetic background and conditioning would facilitate the rapid screening of antimicrofilarial compounds.

The objective of this study was to use in vitro cultured mff to initiate and maintain a microfilaremia in naive experimental dogs and to test the suitability of these dogs as models for the testing of microfilaricidal drugs.

Materials and Methods

Harvesting and in vitro culture of adult worms. Random source dogs with patent *D. immitis* infections were obtained from the Maryland Eastern Shore area. The animals were euthanized with sodium pentobarbital by intravenous administration via the saphenous vein. The chest and abdomen were prepared and surgically opened under sterile conditions. Whole blood was taken by puncturing through the inferior vena cava just caudal to the diaphragm. After exsanguination, the heart and lungs were removed and placed in a sterilized container. Adult worms were removed from the right ventricle and pulmonary artery under sterile conditions. The adults were washed in sterile RPMI 1640 (GIBCO, pH 7.6), containing 3.57 g HEPES (GIBCO)/liter, 2.0 g sodium bicarbonate/liter, and 50 mg gentamicin/ml (modified technique of Weiner et al., 1982, personal communication) and then transferred into roller bottles (Corning) containing RPMI 1640 with 0.07 mg gentamicin/ml. Adults were transferred to a new culture container when the pH of the media was reduced to less than 6.5. The mff were separated by sterile filtration through a 0.45 μm Nalgene filter and maintained in fresh RPMI 1640 (pH 7.6).

Injection of microfilariae. Previous work had established that a minimum microfilaremia count of 700 mff/ml of peripheral blood was desirable so that the effects of drug treatment could be seen clearly.

Two pure bred sibling 3-month-old male beagle pups were maintained from birth in mosquito-free housing at the Food and Drug Administration's Division of Veterinary Medical Research at the Agricultural Research Center, Beltsville, Maryland. Microfilariae were harvested from culture, washed and placed in 2 ml of fresh culture media. On days 0 and 5, 2.0×10^7 and 1.3×10^7 mff were injected intravenously into dog 16I (5.5 kg). Dog 18I (6.0 kg) received 2 ml fresh culture media on the same days. The dogs remained in mosquito-free housing until termination of the project.

Modified Knott's (Weiner and Bradley, 1970) tests were performed daily following the first injection of mff. Total blood cell counts, differential white blood cell counts (WBCs) and blood chemistry tests (alkaline phosphatase, LDH, SGOT and SGPT) were conducted every fifth day on each dog. The blood chemistry examinations were made by Litton Bionetics Laboratories, Kensington, Maryland.

To determine if mff obtained from in vitro culture and injected into the peripheral circulation of a dog would undergo normal development in a vector, a local strain of mosquito, *Aedes triseriatus*, was allowed to feed on test dog 16I. Mosquitoes were maintained in an insectary at 25° C throughout the test period and were dissected on days 1, 6, 16 and 21 after the blood meal to determine the degree of larval development.

In a second experiment, four male sibling pure bred beagles were obtained from the Food and

Drug Administration, Division of Veterinary Medical Research, Beltsville, Maryland. Maintained in mosquito-free housing, the four experimental dogs (11I, 12I, 13I and 14I) received in vitro mff according to the following schedule: day 0, 1.0×10^6; day 8, 1.0×10^6; day 22, 2.0×10^7; day 33, 2.8×10^6; day 91, 3.7×10^6 and day 100, 2.5×10^6, giving a total of 3.1×10^7 mff per dog. Three control dogs received RPMI-1640. Modified Knott's tests were conducted every 3–5 days. Blood cell counts, differential WBCs and selected blood chemistry tests were conducted every 14 days, using the procedures outlined above.

Drug treatment. Dog 16I was given 6.6 mg Dizan (dithiazanine iodide, MED TECH)/kg body weight orally for 10 days, beginning 30 days after the initial transfer of mff. Control dog 18I was given 6.6 mg Dizan/kg body weight for the same 10-day period. Modified Knott's tests were conducted daily during the treatment program. Total and differential WBCs and blood chemistry tests were conducted every third day as outlined above.

Enzyme linked immunosorbent assays (ELISA). A trichloracetic acid (TCAS) extract of an adult/mff homogenate was prepared according to Mantovani and Kagen (1967). The TCAS-antigen (TCAS-Ag) was concentrated by lyophilization and reconstituted in distilled water; the protein concentration was determined by the Lowery technique (Lowery et al., 1951).

Wells of polyvinyl chloride microtiter plates (Dynatech, Alexandria, VA) were sensitized with 0.1 μg TCAS-Ag suspended in 50 μl 0.1 M carbonate buffer, pH 9.6. The antigen was dried to the wells at 37° C and the plates were stored at room temperature. Plates were preincubated with 100 μl of a 1:75 dilution of fetal calf serum (GIBCO) in ELISA buffer (EB 10 mM Tris, 1 mM EDTA, 50 mM NaCl, 0.05% Triton X–100 and 0.01% deoxycholic acid, pH 7.2) for 1 h at 37° C. The plates were washed 3 times with EB. Two-fold serial dilutions of dog sera were made in EB. The sera dilutions (100 μl/well) were incubated for 1 h at 37° C, after which the plates were washed 3 times with EB. Goat anti-dog IgG conjugated with peroxidase (Cappel), diluted in EB containing a 1:250 dilution of normal goat serum, was applied at 100 μl/well and allowed to react for 1 h at 37° C. The plates were washed 6 times with EB. Substrate was prepared by dissolving 20 mg *o*-phenylenediamine in 200 μl methanol and diluting to 50 ml with substrate buffer (10 mM Tris, 1 mM EDTA and 50 mM NaCl, pH 3.2) containing 0.01% H_2O_2. The substrate was added (100 μl/well) and allowed to react in the dark at room temperature for 10–20 min. Reactions were stopped with 50 μl 50% H_2SO_4. Plates were read at 492 nm on a Titertek (Flow Laboratories) microtiter plate reader.

Results

Chemotherapeutic model. In the first experiment, dog 16I developed a microfilaremia of 10 mff/ml on day 2 after receiving 20 million mff and reached a high of 863 mff/ml on day 30 after receiving an additional 13 million mff (Fig. 1). Control dog 18I received RPMI only and was amicrofilaremic throughout the experiment. Dog 16I had a significant titer, detected by ELISA, on day 8 (1:3200); as the microfilaremia increased, so did the titer to a maximum of 1:12,800. After the mff counts rose above 200 mff/ml, the mean ELISA titer was 1:6400. Dizan was administered on day 30 when the microfilaremia in dog 16I was 863 mff/ml. Eight days after the beginning of drug treatment no microfilariae could be detected in the peripheral circulation.

From day 4 until termination of the project, dog 16I maintained an average WBC of 14,100/mm^3, with 48% neutrophils, 43% lymphocytes and 9.7% eosinophils. Blood cell counts for dog 18I were within normal limits through day 30. Results of the selected blood chemistry tests were normal for both dogs until Dizan was administered. After the drug was given, both dogs showed

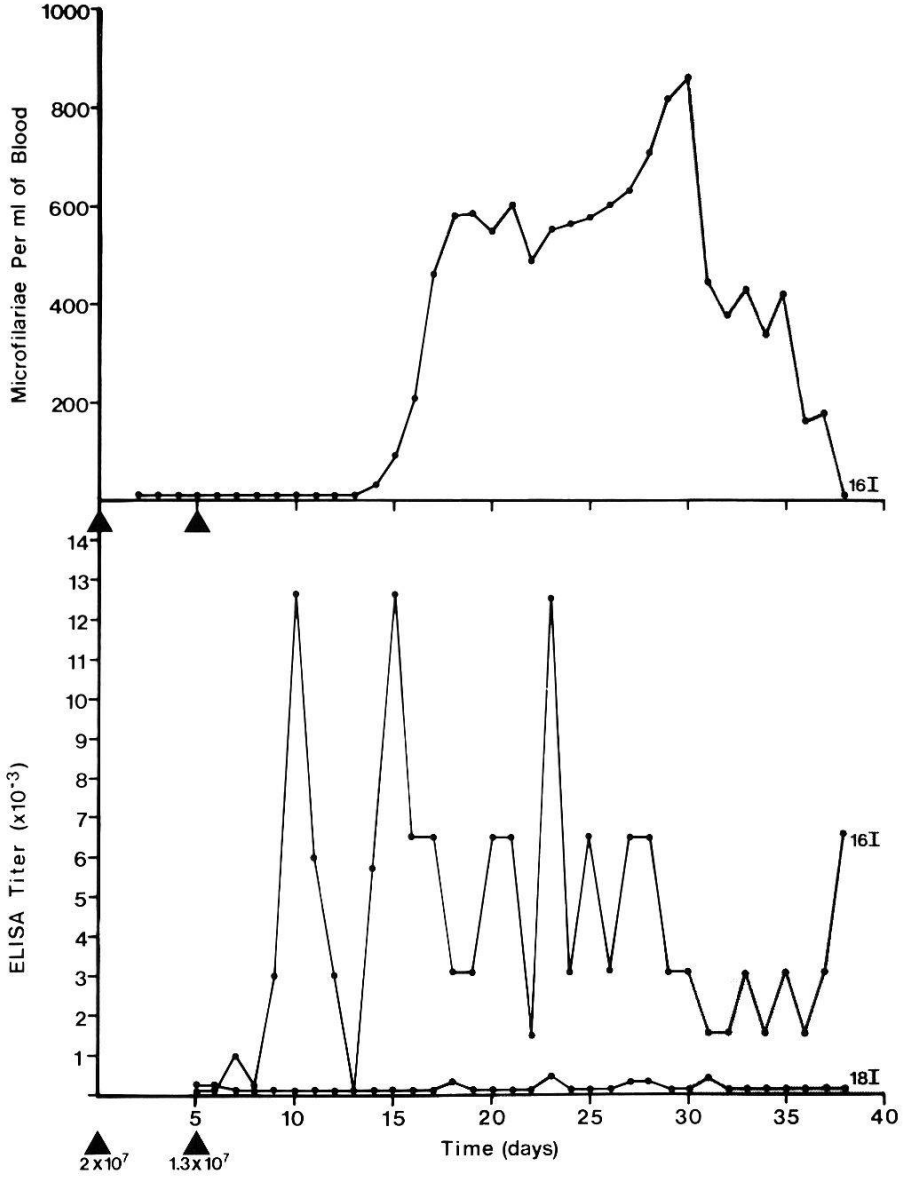

Fig. 1. ELISA titers and level of microfilaremia in dog 16I after infusion of in vitro-cultured microfilariae of *Dirofilaria immitis*. Dog 16I received 2×10^7 and 1.3×10^7 mff on days 1 and 8 (large triangles), respectively. Dog 18I received injections of culture media and was amicrofilaremic throughout the experiment. Treatment with Dizan began on day 30 (shaded area).

elevated levels of alkaline phosphatase, LDH, SGOT and SGPT (data not shown).

In the second experiment 31.2 million mff (Fig. 2) were injected intravenously into each of 4 dogs over 100 days. The mff counts/ml of blood from the 4 dogs ranged from a high of 42 to a low of 3 over the course of the experiment. The reciprocal ELISA titers in the dogs with low levels of microfilaremia ranged from 0 to 3200. A detectable microfilaremia was maintained throughout the course of the experiment.

Microfilarial development in mosquitoes. Of the 29 mosquitoes *(Aedes triseriatus)* which fed on dog 16I, 22 (75.9%) were infected with mff. The mosqui-

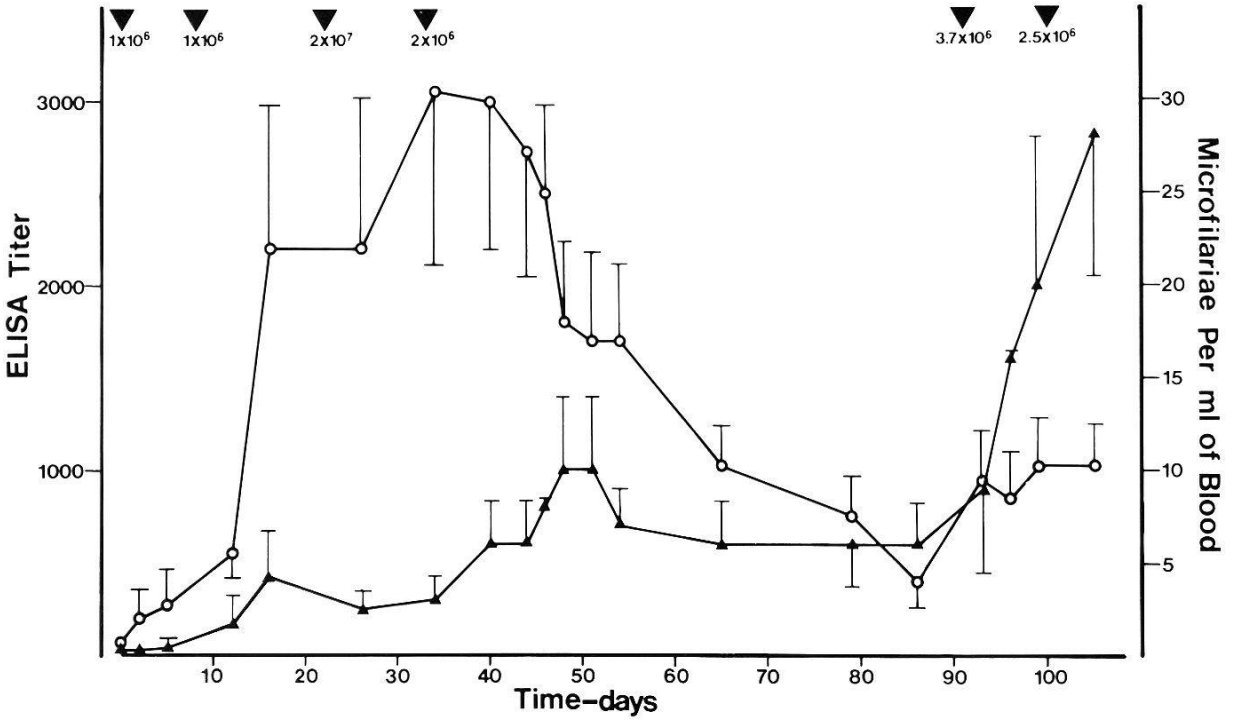

Fig. 2. Temporal sequence of ELISA titers (▲) and levels of microfilaremia (o) of four experimental dogs (11I, 12I, 13I, 14I) that received intravenous injections of in vitro-cultured mff of *Dirofilaria immitis*. Each point represents mean of response of four experimental animals ± 1 SD. Large triangles indicate dates on which dogs received mff. Control animals were amicrofilaremic throughout the experiment and showed no significant antibody response.

toes were fed on day 29 when the microfilariae count was 740 mff/ml. Normal larval development occurred in all infected mosquitoes. On day 21 the infected mosquitoes averaged 3.2 infective larvae in the head/proboscis area. At 25° C complete larval development occurred in about 19 days.

Discussion

The in vitro culture system provided a large number of *D. immitis* mff, which were used in an in vivo model for testing microfilaricidal drugs. Adult worms produced actively motile mff (5,000–10,000 mff/day/female) in this culture system for 2.5 months. The ability of these mff to develop to the infective stage in the mosquito vector after a 29-day residence in the dog host indicated that the system may be a reasonable model of the natural microfilaremic state.

Despite the large number of mff injected into the experimental dogs, only about 1.5% were present in the peripheral blood. These findings are similar to those of others (Hawking, 1967: Wong, 1964) in which 5–20% of the total number of mff infused into a host were detectable in the peripheral blood and consistent with the hypothesis proposed by Pacheco (1974) that mff are preferentially sequestered in the peripheral capillary beds and are not randomly distrib-

uted throughout the macro- and microcirculations. Thus the mff detected in the peripheral blood are only a small portion of the total viable mff population.

There was a 12–15 day lag between the initial injection of mff and the detection of a significant microfilaremia (Figs. 1 and 2). In dog 16I the number of mff in the peripheral blood rose rapidly and remained elevated until the initiation of drug treatment. Dogs 11I, 12I, 13I and 14I showed a similar rise in microfilaremia; however, microfilaremia peaked at a lower level, and after day 40, detectable mff progressively declined. Attempts to establish a higher level of microfilaremia on days 91 and 100 by infusion of an additional 6.2×10^6 mff into each dog were unsuccessful.

The lower maximum level of microfilaremia and the gradual decrease in numbers of mff found in the second experiment may have been due to the difference in the injection schedules along with an increase in the dogs' capacity to accommodate more mff in the peripheral vascular beds. Although the animals in both experiments received approximately 3×10^7 mff, dog 16I received them over an 8-day period, whereas they were received over a 33-day period in the second experiment. The difference in the rate of exposure may have influenced the expression of microfilaremia. In addition, the 3-fold increase in body weight (from 2.7 to 8.3 kg) and attendant expansion in vascular capacity increased the ability of the animals to sequester mff from the peripheral circulation.

From these data the relationship between the amount of circulating antibody against *D. immitis* and the level of microfilaremia in the blood remains obscure. Intravenous injection of mff either transiently lowered or had no effect on the antibody level. To eliminate the possibility that we were missing a significant portion of the antibody response directed against mff by using an antigen derived from an adult/mff homogenate (TCAS-Ag), we also screened the sera against a crude extract of *D. immitis* mff. There was no significant difference in the ELISA results (data not shown).

Although data were collected from a limited number of animals, the use of in vitro cultured mff to establish an in vivo microfilaremia appears to be a functional model for screening antimicrofilarial drugs, particularly since mff developed to the infective stage after residence in an experimental canine host. Compared with the alternative drug testing systems which use random soucre animals or experimentally patent infections, the model outlined in this study is potentially a more rapid and cost-effective procedure for the screening of microfilaricidal compounds.

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