FULL PAPER *Parasitology*

Immunological Analysis of Agglutination in *Dirofilaria immitis* Microfilariae

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ABSTRACT. The mechanism of agglutination phenomenon of *Dirofilaria immitis* microfilariae was analyzed. Circulating microfilariae were collected from a *D. immitis*-infected microfilaremic dog and cultured in the several kinds of sera from dogs and animals. The agglutination of *D. immitis* microfilariae is a specific phenomenon due to some immune complexes formed with the anti-microfilarial antibody, heat-instable factor(s) and excretory-secretory products of microfilariae. Only live microfilariae were agglutinated and the agglutinated microfilariae remained alive as long as 27 days in culture *in vitro*.

KEY WORDS: agglutination, *Dirofilaria immitis*, immune complex, microfilaria.

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A filarial infection, in which a host is infected with adult filariae of both sexes but no microfilariae (amicrofilaremia) appear in the blood circulation, is referred to as "occult infection". It is known that microfilariae obtained from a microfilaremic dog show agglutination or "Medusa-head formation", as is called from their shape, when cultured *in vitro* in the serum taken from another dog with occult infection [5, 10, 11]. The agglutination of microfilariae, however, is not observed when they are cultured in the serum of a microfilaremic dog. The agglutination occurs only in live microfilariae and has some similarity to the Sarles' phenomenon, which is characterized by some precipitates formed around parasite eggs or in the regions of excretory/anal pores of larvae [10]. Besides, a possibility has been suggested that the agglutination of microfilariae would work as a lethal process of host in coordination with the adhesion of leukocytes onto the parasite [4, 10]. However, a few reports are available for reconfirming this subject and the mechanism is not fully understood yet. The purpose of this study is to analyze the mechanism of microfilarial agglutination by using *Dirofilaria immitis* (canine heartworm) microfilariae.

MATERIALS AND METHODS

Microfilariae: One part of blood taken from a microfilaremic dog naturally infected with *D. immitis* was mixed with 2 parts of an autoclaved solution of 0.5% saponin in phosphate buffered saline (PBS, pH 7.2) in a sterilized disposable test tube and the mixture was mildly shaken in a water bath at 37°C for 5 min, followed by centrifugation at 55 × g for 10 min. The sediment obtained was washed twice in PBS(pH7.2) and suspended in Dulbecco's minimum essential medium(DMEM). The experiment was carried out at room temperature under sterile conditions within 1 hr. All the microfilariae recovered was alive.

Sera of microfilaremic and amicrofilaremic dogs: Sera were obtained from a naturally infected dog with microfilaremia and from the dog experimentally infected by the method of Hayasaki [3] which showed occult infection. The sera used were divided into 2 groups: the untreated and the inactivated by heating at 56°C for 30 min.

Assay on microfilarial agglutination: The microfilariae were cultured with the 2 groups of dog sera, those of other animal species, rabbits and hamsters, physiological saline and DMEM, on 96-well or 24-well disposable micro-culture plates to check their agglutination and survival. Wells of the culture plates were each sealed off with sealing film and the plates were maintained at 37°C in a 5% CO_2 incubator in order to avoid any change in the concentration and/or pH of the culture medium. The microfilariae were examined for agglutination after 24 hr under an inverted microscope.

Count of Microfilariae: The microfilariae were examined for their survival 24 hr after cultivation. The microfilariae showing motility and the transparent body were judged alive, while those showing no or faint motility and the opaque body having the visible cells inside their body were regarded as dead.

Anti-D. immitis mouse monoclonal antibodies: According to the standard method of cell-fusion technique, the hybridoma cells were produced from both the spleen cells of BALB/c mice immunized by i.p. injection of the mixture of 0.5 m*l* of PBS-extract (300 μg protein/mouse) of *D. immitis* and 0.5 m*l* of Complete Freund's Adjuvant once a week for 4 weeks, and the mouse myeloma cells (SP_2O-Ag14). The hybridoma cells were i.p. injected to normal BALB/c at a dose of 1–2 × 10^6 cells/mouse to produce ascites in them. The specificity of the monoclonal antibody to *D. immitis* circulating antigen was verified to the sera of other experimentally infected microfilaremic dogs (data not shown), by the enzyme-linked immunosorbent assay (ELISA) with the IgG fraction of monoclonal antibody separated from the ascites. One of the monoclonal antibodies with high specificity to *D. immitis* circulating antigen was used in the indirect immunofluorescence.

Immunofluorescence: Agglutinated microfilariae were transferred from the micro-culture plate onto a microscopic slide using a capillary pipette, left to dry, fixed with methanol and stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-dog IgG antiserum (Cappel, Malvern, PA,

U.S.A.) and FITC-conjugated goat anti-dog C_3 antiserum (Cappel, Malvern, PA, U.S.A.). The indirect immunofluorescence was also performed using the anti-*D. immitis* mouse monoclonal antibody specific to *D. immitis*, as mentioned above, and FITC-conjugated goat anti-mouse IgG antiserum (Cappel, Malvern, PA, U.S.A.).

Cultivation of microfilariae: Microfilariae were cultured to determine their survival time in a mixture of 0.5 m*l* serum from the dog with occult infection and 0.5 m*l* DMEM, therefore the resultant serum concentration being 50%, and kept in a 5% CO_2 incubator at 37°C, after sealing off the culture plates. All the tests were carried out under sterile conditions to avoid contamination.

RESULTS

Agglutination of microfilariae in the sera from microfilaremic and amicrofilaremic dogs: Various concentrations of the untreated and inactivated sera taken from the dogs with microfilaremia or amicrofilaremia were compared in terms of agglutination of microfilariae (Table 1). No agglutination was observed in any of the concentrations ranging from 25% to 75% in either the untreated or inactivated serum of the microfilaremic dog. In the serum of the dog with occult infection, on the other hand, microfilariae were agglutinated in all the concentrations of the untreated serum but no agglutination was seen in any concentration of the inactivated serum. Thus, the microfilariae was not agglutinated when cultured in the untreated or inactivated serum of the microfilaremic dog or in the inactivated serum of the occult-infection dog. When the untreated serum of the occult-infection dog was then added to the above sera, all of them did again agglutinate after another 24 hr (Table 2). These results suggest that the heat-instable factor(s) will be involved in the phenomenon.

Survival and agglutination of microfilariae in various sera: Different kinds of sera were compared in terms of the survival of microfilariae (Table 3). Almost the same survival rate was observed in the sera of both occult-infection and non-infected healthy dogs. The microfilariae cultured in the serum of the microfilaremic dog showed a relatively low survival rate and a few of them were even agglutinated. Not so many microfilariae survived in DMEM and especially in the physiological saline probably because these solutions did not provide them with all the nutrients necessary for their survival. Especially in the saline, which has no buffer action, all the microfilariae were killed, assumedly, because its pH value had been lowered by the acid excretory-secretory products of microfilariae. Agglutination and viability of *D. immitis* microfilariae in the serum of some animals other than dogs were studied as well. A high percentage and about a half of the microfilariae were alive in the rabbit and hamster sera, respectively, without agglutination.

Immunofluorescence: The precipitation in the mass of agglutinated microfilariae, which contained a large amount of excretory-secretory products of the microfilariae, was stained well with the anti-*D. immitis* mouse monoclonal (Figs. 1a and 1b). Further, the test with anti-dog IgG antiserum proved that a large quantity of IgG existed in both agglutinated microfilariae and culture medium (Figs. 1c and 1d). The anti-dog C_3 antiserum also indicated the existence of the dog C_3 in the agglutinated microfilariae (Figs. 1e and 1f).

Survival time of microfilariae in vitro: After 24 hr of cul-

Table 1. Agglutination of *D. immitis* microfilariae in the serum of a dog with microfilaremia (Mf+) or with occult infection (occult)

Well No.	Mf suspension(μl)[c]	Dulbecco's MEM(μl)[d]	Mf+ serum(μl)[a]		Occult serum(μl)[b]		Concentration of serum(%)	Mf agglutination[f]
			untreated	inactivated[e]	untreated	inactivated[e]		
1	50[g]	0	150	0	0	0	75	negative
2	50	50	100	0	0	0	50	negative
3	50	100	50	0	0	0	25	negative
4	50	0	0	150	0	0	75	negative
5	50	50	0	100	0	0	50	negative
6	50	100	0	50	0	0	25	negative
7	50	150	0	0	0	0	0	negative
8	50	0	0	0	150	0	75	positive
9	50	50	0	0	100	0	50	positive
10	50	100	0	0	50	0	25	positive
11	50	0	0	0	0	150	75	negative
12	50	50	0	0	0	100	50	negative
13	50	100	0	0	0	50	25	negative
14	50	150	0	0	0	0	0	negative

Sterilized 96-well micro-culture plates were employed for the assay.
a) A spontaneously infected dog with microfilaremia.
b) A dog with occult infection which was experimentally infected with 115 infective larvae.
c) Microfilairae were suspended in Dalbecco's MEM at a rate of about 100 microfilariae in 10 μl of medium.
d) Contains a fetal calf serum at 10%.
e) Heated at 56°C for 30 min.
f) Examined after 24 H.
g) Unit in μl.

Table 2. Participation of the heat-instable factor(s) in *D. immitis* microfilaria agglutination, evaluated in the serum of a dog with microfilaremia (Mf+) or with occult infection (occult)

Well No.	Mf suspension[c]	Dulbecco's MEM[d]	Mf+ serum[a]		Occult serum[b]		Addition of untreated occult serum	Mf agglutination[f]
			untreated	inactivated[e]	untreated	inactivated[e]		
1	50[g]	50	100	0	0	0	0	negative
2	50	50	0	100	0	0	0	negative
3	50	50	0	0	100	0	0	positive
4	50	50	0	0	0	100	0	negative
5	50	50	100	0	0	0	100	positive
6	50	50	0	100	0	0	100	positive
7	50	50	0	0	0	100	100	positive
8	50	50	0	0	0	0	0	negative

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c) Microfilairae were suspended in Dalbecco's MEM at a rate of about 100 microfilariae in 10 μl of medium.
d) Contained a fetal calf serum at 10%.
e) Heated at 56°C for 30 min.
f) Examined after 24 H.
g) Unit in μl.

Table 3. Viability of *D. immitis* microfilariae (Mf) cultured in various sera

Sera and medium	Number of Mf well-1 Alive	well-1 Dead	well-1 Survival (%)	well-2 Alive	well-2 Dead	well-2 Survival (%)	Average survival±SD	Mf agglutination
Dog with occult infection[a]	116	4	96.6	113	8	93.4	95 ± 1.6	++
Dog with microfilaremia[b]	126	46	73.2		Not done		73	+
Normal young dog	122	0	100		Not done		100	-
Normal rabbit	58	14	80.5	82	12	87.2	83 ± 3.3	-
Normal hamster	40	49	44.9	54	51	49.5	47 ± 2.3	-
Physiologic saline	0	138	0	0	119	0	0	-
Dulbecco's MEM	76	33	69.7	46	62	42.6	56 ± 13.5	-

The assay was performed in duplication by using 24-well culture plates containing 0.3 m*l* of serum and one or two drops of Mf suspension (about 60–70 Mf/drop) in each well. The plates were sealed with film and maintained at 37°C for 24 hr before the viability was evaluated.
a) An experimentally infected dog with occult infection.
b) A spontaneously infected dog with microfilaremia.

ture, agglutination was observed in many microfilariae with a variety of patterns such as 2 to more than 10 microfilariae joined together or even 1 microfilaria stuck to itself to form a ring. The precipitates were found not only on the agglutinated microfilariae but also floating freely in the culture medium. Incidentally, the agglutinated larvae showed high motility. Up to Day 18 of culture, all the microfilariae were alive. On Day 23, some of the agglutinated microfilariae reduced their motility and the survival rate decreased to 25%. On Day 27, most of them died with a survival rate of 3%. The microfilariae thus remained alive and highly motile for quite a long period of time even though they were agglutinated (Fig. 2).

DISCUSSION

Agglutination of microfilariae is known in the parasitism of many different filarial species including *D. immitis* [8, 10], *Loa loa* [6,7], *Litomosoides carinii* [9], *Wuchereria bancrofti* [5, 11] and *Brugia pahangi* [10]. This phenomenon is utilized for a diagnostic examination of a host suspected of filarial infection [8, 10]. However, the mechanism of it or its lethal action on microfilariae is not fully understood yet. It is believed that the agglutination of microfilariae has a mechanism similar to that of the Sarles' phenomenon, which is featured by a large amount of precipitation found in the regions of excretory/anal pores of live parasite larvae or around egg shells [10]. However, this argument is open to question because the agglutination of microfilariae is not always accompanied by such an excessive precipitation as observed in the Sarles' phenomenon and it occurs even in the serum inactivated by heating at 56°C for 30 min [4].

In this study, microfilariae were strongly agglutinated in

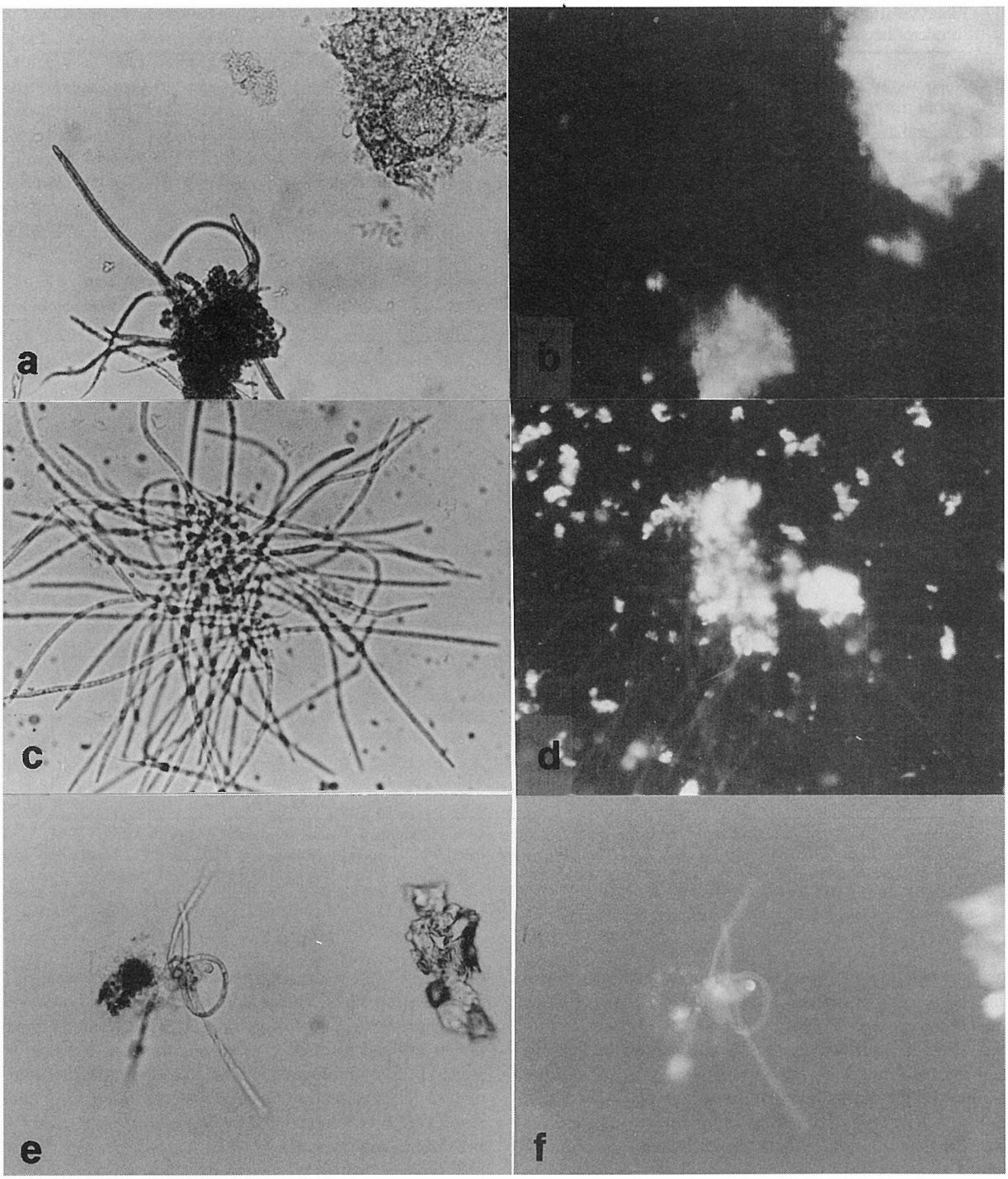

Fig. 1 (a-f). Immunofluorescent images showing the immune complex in the precipitation in the mass of agglutinated microfilariae and the culture medium. Photographs of a and b, c and d, and e and f are in pairs, respectively. Each pair of photographs consists of those by both light (1a, 1c and 1e) and fluorescent (1b, 1d and 1f) microscopies of the same image of agglutinated microfilaria preparation. 1a and 1b: A positive indirect immunofluorescence using a *D. immitis* specific mouse monoclonal antibody and anti-mouse IgG antiserum. 1c and 1d: A positive direct immunofluorescence using anti-dog IgG antiserum. 1e and 1f: A positive direct immunofluorescence using anti-dog C_3 antiserum.

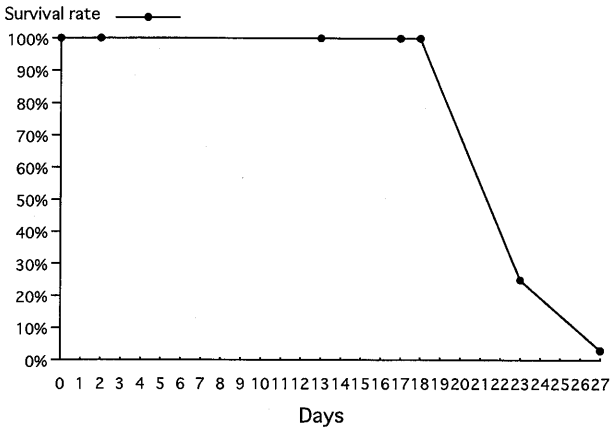

Fig. 2. Change in the survival rates of agglutinated micofilariae of *D. immitis*.

the untreated serum of amicrofilaremic dog (Table 1). Furthermore, agglutination of microfilariae occurred even in the above-mentioned three non-agglutinative sera when the untreated serum of the occult-infection dog was added to them (Table 2). These results suggest that the heat-instable factor(s), such as a complement, may plays an important role in the agglutination of microfilariae. However, in the experiment shown in Table 3, a few microfilariae agglutinated in the serum from a microfilaremic dog. It may be conceivable, therefore, that there is a weak agglutination effect even in the microfilaremic serum. For this, further analysis is necessary for reconfirming the hypothesis whether a heat-labile factor(s) is produced specifically in occult-infection dogs or commonly exist among microfilaremic dogs even though it is often very weak.

In the 24 hr culture study, there was no substantial significant difference in the survival rate of microfilariae among the sera of the dog with occult infection, microfilaremic dogs and the non-infected healthy dogs, while no and small percentage of microfilariae survived in the physiological saline and tissue culture medium, respectively. It was shown that *D. immitis* microfilariae could survive with a high and relatively high percentage in the serum of rabbit and hamster, respectively, used as other animal species than dogs.

According to a hypothesis on the production of amicrofilaremia, which is supported by many researchers, both the agglutination of microfilariae and leukocyte adhesion phenomenon would work in the body of a host as well and the parasite larvae would be killed by some coordinated actions of these two phenomena [1, 2, 6, 10]. However, our experiment of microfilarial cultivation revealed that some agglutinated microfilariae remained alive as long as 27 days (Fig. 2). The present study was performed, in strict sense, under the comtamination with a small number of leukocytes because of the difficulty to avoid the contamination during the process of microfilaria collection. In this concern, some investigators indicated that the leukocyte adhesion phenomenon *in vitro* was one of the host's immune attacks on microfilariae [1, 2, 10]. However, it is still unknown whether this phenomenon is an essential agent of host to kill microfilariae or not. Therefore, a further experiment is necessary to verify it. Additionally, the fact that they were able to survive as long as 27days *in vitro* dose not suggest that they were killed by any mortal agents, but rather shows that they died out from an environmental deterioration due to an increase in pH value of the culture medium (or the serum) and/or shortage of their nutritive resources.

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