

STUDIES ON THE SKIN REACTION IN HUMAN FILARIASIS WITH A PURIFIED ANTIGEN FROM *DIROFILARIA IMMITIS*

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Introduction

Since the application of skin reaction for the diagnosis of human filariasis was first reported by Taliaferro and Hoffman in 1930, many reports have been shown concerning the preparation method of antigens and their antigenic properties. Recently a historical review of the immunodiagnostic methods of human filariasis was shown by Kagan (1963) and the usefulness of skin reaction for the diagnosis of this disease was emphasized. Morisawa *et al.* (1960) reported the potent basic polypeptide isolated from the tubercle bacilli, which showed specific tuberculin activity.

The authors first isolated the antigen from heart-worms of dogs according to Morisawa's method and investigated the antigenic properties in human filariasis. Some of the results were reported preliminarily by the authors (Tada, Kawashima *et al.* 1962a, b; 1963a, b).

In the present paper the authors wish to present the data concerning the isolation, chemical properties, immunological activities and the application of this antigen.

Isolation and purification of antigen

Adult worms of *Dirofilaria immitis* from the dog heart were washed with physiological saline and collected by filtration. The worms were then blended in a small mortar with sufficient volume of cold acetone, filtrated and washed several times with cold acetone. The defatted residue was stored in a desiccator as an acetone-

dried material. The dried powder was extracted with 25 volumes of 0.1 N HCl solution at room temperature for 48 hours under stirring mechanically. After centrifugation at 3,000 rpm for 10 minutes, the supernatant and washings were combined and brought to pH 7.0 by cautious addition of 1.0 N NaOH solution. It was left for half an hour at room temperature and the white precipitate was removed by centrifugation. The clear supernatant was added to an equal volume of saturated picric acid. Twelve hours later, yellow precipitate obtained was collected by centrifugation and extracted for 3 hours with 3% HCl solution in 99.5% ethanol under stirring. After centrifugation at 3,000 rpm for 10 minutes, the supernatant was concentrated to about 1/10 volume until precipitates appear under the reduced pressure below 35°C. The concentrate was added with 10-20 volumes of cold acetone, and the resulting flocculent white precipitate was collected by centrifugation after standing for 30 minutes. The precipitate was washed twice with cold acetone and dried in a desiccator. The product was designated F P T antigen and tested its activity.

Chemical properties of F P T antigen

Nitrogen content of this antigen was determined by the Micro-Kjeldahl method and found to be 10.8%. Heat and structural stability were not examined, but from the procedure of extraction, this substances seemed to be relatively stable. Ninhydrine reaction and Biuret reaction of this antigen were proved positive. The pat-

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tern of ultraviolet absorbancy showed that this antigen mainly consisted of protein (Fig. 1). The absorption maximum was in 275 m μ and the ratio of optical density in 280 m μ and 260 m μ region showed 1.23.

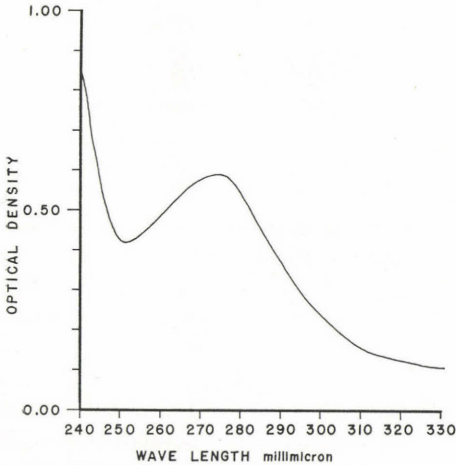


Fig. 1. Ultraviolet absorbancy of F P T antigen (0.1 % solution)

Materials and methods of investigations

Intradermal injections of 10, 8, 4 and 1 μ g of F P T antigen in 0.02, 0.04 and 0.05 ml of phenolized saline solution were made in the volar surface of the forearm of the persons examined. The result of skin reaction was elucidated as the mean size of two diameter of the wheal at right angles. One hundred and eighteen persons were studied as controls in Fukuoka City where no human filariasis had ever been found, and were compared by the skin reaction with 135 microfilaria carriers in some endemic areas such as

Ehime, Nagasaki, Kagoshima and Okinawa. Moreover, 171 inhabitants in Hoino Village, Ehime Prefecture and 149 inhabitants in Iojima Village, Nagasaki Prefecture and 242 soldiers in Kagoshima and Nagasaki were also examined in the endemic areas.

Results

1) Skin reaction in normal controls and microfilaria carriers

Intradermal injections of F P T antigen in several concentrations and volumes were made in both microfilaria carriers and normal persons. In the skin reaction in the former cases, the size of wheal increased, and the erythema appeared in a few minutes after injection, and they attained to their maximum from 15 to 20 minutes later. The appearance continued for a few hours as observed by earlier workers. On the contrary, normal controls showed no or weak responses against the antigenic stimulus. From these observations, the reaction was considered as an immediate type and it was concluded that the measurement of the result should be made in 15 minutes after injection. In the volume of injection, 0.05 ml of antigen solution gave better results than the other volume.

Injection of 1-4 μ g of antigen on normal controls resulted slight or no responses, but that of 10 μ g showed in a half of them as strong responses as those of microfilaria carriers. Frequency distribution curves among normal controls and microfilaria carriers tested with 1 μ g of antigen were shown in Fig. 2. Ninety seven percent of normal persons examined

Table 1. Skin reactions in several concentrations on normal controls and microfilaria carriers

F P T antigen used in each individual (μ g/ml of phenolized saline)	Normal controls		Microfilaria carriers	
	Number of cases tested	Number of cases over or equal to 9.0 mm in diameter, (%)	Number of cases tested	Number of cases over or equal to 9.0 mm in diameter, (%)
10/0.05	18	11 (61.1)	27	26 (96.3)
8/0.04	/	/	6	6 (100.0)
4/0.02	18	0 (0.0)	6	6 (100.0)
1/0.05	100	3 (3.0)	123	112 (91.0)

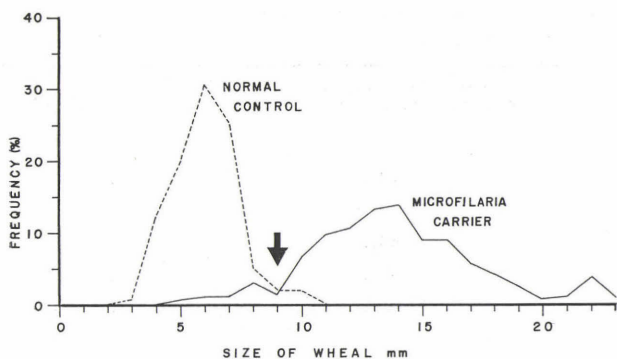


Fig. 2. Frequency distribution curves shown among microfilaria carriers and normal controls examined with $1 \mu\text{g}$ of antigen.

showed 8.5 mm or less in size of wheal, while most of microfilaria carriers (91.0 %) showed 9.0 mm or more. From the analytical observation of frequency distribution curves, the first group - 8.5 mm or less in diameter - might be considered as negative for filarial infections. The second group - 9.0 mm or more - seemed to be composed of the persons suffered from filarial infections.

In relation to the criterion, it may be considered that the reaction is positive when the wheal measures 9.0 mm or more, and negative when it is 8.5 mm or less, in 15 minutes after injection of $1 \mu\text{g}$ of antigen in 0.05 ml solution. These results were summarized in Table 1.

2) Relationship between the size of wheal and the detectability of microfilaria carriers

Blood smears from 171 inhabitants of Hoino were examined and the skin reactions were also tested at the same time. Relations between the size of wheal and the detectability of microfilariae in blood smears were considered. The result was shown in Table 2. Out of 171 inhabitants examined 129 persons showing 8.0 mm or more in size of wheal were divided into 8 groups according to the size of wheal, and the rate of microfilaria carriers in each group was computed.

Positive correlation between the size of wheal and the rate of detectability of microfilariae was observed in the inhabitants of this village, a known endemic area of bancroftiasis. Correlation coefficient between the two was computed

as : $r=0.82$. From this observation, increase in detectability of microfilariae might be expected in accordance with the increase in size of wheal.

3) Effect of treatment on the skin reaction

Sixty cases which had been proved as microfilaria carriers and then had been administered with 1-diethylcarbaryl-4-methylpiperazine citrate (Supatonin) were classified into 5 groups according to the length of period that microfilariae had disappeared by the medication. The rate of positive, who showed 9.0 mm or more in size of wheal and median value of size in each group, were shown in Table 3. In two years after disappearance of microfilariae, 83.3 % of the subjects examined remained yet positive and the median value of size in diameter was 11.7 mm. Four year later, however, only 22.2 % were proved positive and the median value of the wheal size was 9.5 mm.

Table 2. Relations between the size of wheal and the detectability of microfilaria.
Correlation coefficient ; $r=0.82$

Size of wheal in diameter (mm)	Number of cases examined	Number of microfilaria carrier, (%)
8.0- 9.5	19	0(0.0)
10.0-11.5	17	2(11.7)
12.0-13.5	20	5(25.0)
14.0-15.5	26	5(19.2)
16.0-17.5	23	7(30.4)
18.0-19.5	14	5(35.7)
20.0-21.5	6	2(33.3)
22.0-	4	2(50.0)

From this observation, a tendency was shown that the rate of positive in reactions had diminished gradually corresponding to the time of lapsed after disappearance of microfilaria by medication.

4) Relations between the count of microfilaria and the skin reactivity

Total volume of 30 cmm of peripheral blood

Table 3. Effect of treatment on the skin reaction among the subjects administered with Supatonin

Period after the disappearance of microfilaria with medication (year)	Number of cases examined	Number of positives in skin reaction (%)	Median value in size of wheal (mm)
0	28*	28(100.0)	16.5
1	1	1(100.0)	14.0
2	6	5(83.3)	11.7
3	19	8(42.1)	10.5
4	9	2(22.2)	9.5

* under treatment at present.

from microfilaria carriers in Hoino Village was examined individually. Blood smears were made at 9.00 p.m. in each case. Relations between the microfilarial counts per 30 cmm of blood and the size of wheal caused by skin reaction among 27 carriers were shown in Fig. 3. Most of them contained less than 50 microfilariae in each blood sample examined. No relations between the microfilarial counts and the size of wheal was observed.

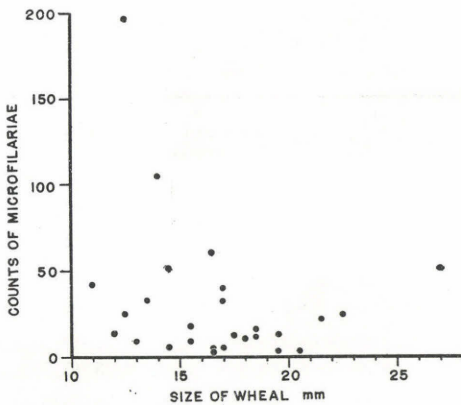


Fig. 3. Relations between the counts of microfilaria and the size of wheal in 27 microfilaria carriers in Hoino, Ehime Pref.

5) Nonspecific and cross reactions

Six cases of asthma, 9 cases of urticaria, 5 cases of Quincke's edema and 23 cases of tuberculosis were tested for their responses on F P T antigen. Moreover, 8 cases of ascariasis, 9 cases of ancylostomiasis, 10 cases of trichocephaliasis, 2 cases of heterophyiasis, 4 cases of

paragonimiasis, 1 case of taeniasis, 15 cases of gnathostomiasis and 7 cases of schistosomiasis were also examined about their cross-reactivities. These results were summarized in Table 4.

No cases except one gnathostomiasis and one schistosomiasis showed positive reaction. However, those who showed false positive reactions had lived in tropical region in the past for several years and consequently the filarial infections of them could not be neglected.

6) Epidemiological observations with skin reaction on the inhabitants in endemic regions

Skin reactions among the inhabitants of endemic regions of bancroftiasis, Hoino and Iojima,

Table 4. Nonspecific- and cross-reaction among helminthic and several diseases examined with F P T antigen

Diseases examined	Number of cases tested	Positives in skin reaction (%)
Asthma	6	0(0.0)
Urticaria	9	0(0.0)
Quincke's edema	5	0(0.0)
Tuberculosis	23	0(0.0)
Ascariasis	8	0(0.0)
Ancylostomiasis	9	0(0.0)
Trichocephaliasis	10	0(0.0)
Heterophyiasis	2	0(0.0)
Paragonimiasis	4	0(0.0)
Taeniasis	1	0(0.0)
Gnathostomiasis	15	1(6.7)
Schistosomiasis	7	1(14.3)

were shown in Fig. 4. The positive rate of microfilaria carriers in Hoino and Iojima were 12.5 % and 3.3 %, respectively. Frequency distribution curve in Hoino was divided into two groups with boundary of 5.0 mm - 6.5 mm as shown in Fig. 4. The first and the second group were analytically assumed to be composed chiefly of the uninfected and infected inhabitants with filariasis, respectively. In Iojima, however, the frequency distribution curve had one peak. According to the criterion mentioned previously, 64.4 % and 62.4 % of inhabitants in Hoino and Iojima were positive, respectively.

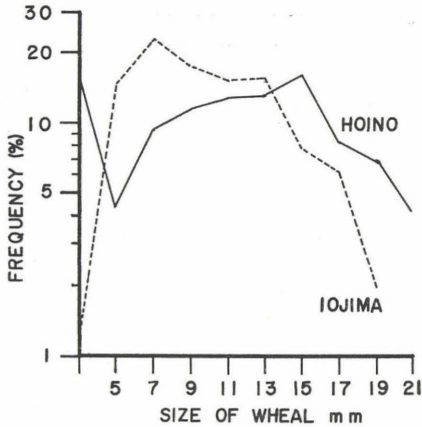


Fig. 4. Frequency distribution curves among the inhabitants of two villages, Hoino and Iojima, in skin reactions.

Soldiers of the Japan Air Self Defense Force in two bases of two different districts known as endemic areas of filariasis, Nagasaki and Kagoshima, were examined. In these cases, as shown in Fig. 5, persons examined were clearly divided into two groups; the one below 8.5 mm and the other over 9.0 mm in size of wheal. Patterns of frequency distribution curves obtained among them were quite similar to those of normals and microfilaria carriers shown in Fig. 2. Positive rate of the carriers was below 1.0 % in each air base, and both of the two microfilaria carriers found in them showed positive in the skin reaction.

Summarizing these observations, the pattern of frequency distribution curves might be influ-

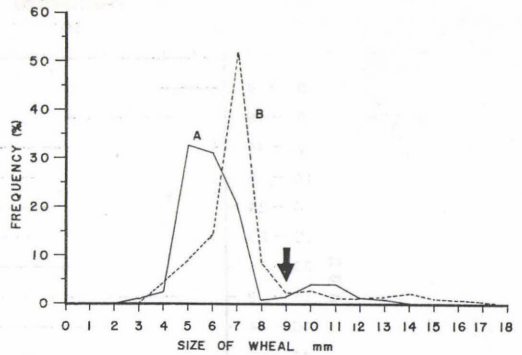


Fig. 5. Frequency distribution curves obtained from the soldiers in two different air bases, Shimokoshiki in Kagoshima Pref. (B) and Goto in Nagasaki Pref. (A).

enced by the rate of population of the infected and the uninfected inhabitants with filariasis.

Age distribution of positives, divided into 10 groups, in Hoino and Iojima was calculated and shown in Fig. 6. Positives increased in accordance with the increase of age from 1 to 19 or 24 years old; i. e. positive rate was 0.0 % in 0 to 4 years old, 33.3 % in 5 to 9 years old, 72.4 % in 10 to 14 years old and 92.3 % in 15 to 19 years old in Hoino, while the rate was almost 100 % over 20 or 24 years old. Age distribution pattern in Iojima showed a similar tendency.

Discussion

Up to the present, many kinds of antigens have been used for diagnosis of human filariasis. However, most of them have been used under chemically undetermined value. However, some workers such as, Franks (1947) used 3.2 mg N/100 ml of antigen, Zeligs (1947), 0.0005 mg protein/ml and Wharton (1947) used 0.26 mg N/ml male worm antigen for tests. In F P T antigen, it was suggested that this was composed of low-molecular protein, probably of peptides, and the nitrogen content was determined as 10.8 %. Use of 1 μ g/0.05 ml of antigen was enough for the diagnosis of human filariasis.

In this investigation, corresponding to the time of lapsed after medication, positives in the skin reaction decreased year by year. A similar

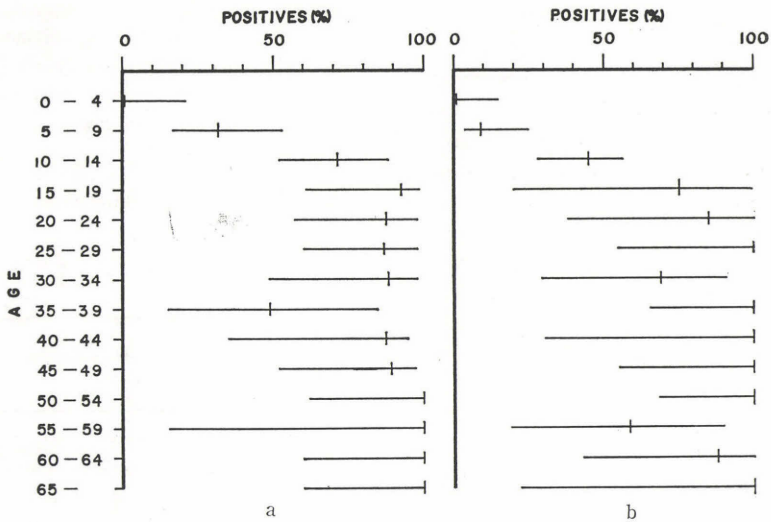


Fig. 6. Age distribution of the inhabitants of Hoino (Fig. 6-a) and Iojima (Fig. 6-b) in skin reactions.

result was recognized by Stefanopoulo (1940).

The reactivity of medicated persons might be influenced in considerably short time by the treatment of parasites. At the same time, as to the false negative reactions observed in some cases of microfilaria carriers, desensitization mechanism or the early stage of infection might have played some roles.

Skin reactions, carried out among the inhabitants living in endemic regions, revealed higher rate of positives than that expected from the positive rate of microfilaria carriers. This fact indicated that positive reaction in individuals did not run necessarily parallel with the absence or presence of microfilariae in the peripheral blood, but probably with the sensitization with filarial infections. This consideration was somewhat supported with the fact that the strong skin reactivity used to remain even in the stage which could not be found microfilariae in the peripheral blood. Moreover, microfilarial counts had no relations with the strength of the skin reaction. For this reason, false positives among the inhabitants without microfilariae were found frequently in endemic areas. Wharton *et al.* (1946) and Wright *et al.* (1944) also reported this kind of false positiveness, which seemed inevitable in the skin reaction in this disease.

Kent (1963) reported in his review on the immunodiagnostic tests employing soluble antigens that the early soluble preparations were simple extracts of larvae or adult worms which contained much inactive material as well as cross-reactive and specific antigens. Consequently the undesirable non-specific reactions were often observed. Some purified antigens would solve this problem. As to the cross-reaction, Woodruff (1958) with strongyloides cases and Lippelt *et al.* (1938) with hook worm and strongyloides diseases reported that the filarial antigens crossly reacted with them. Furthermore, Stemplen (1944) and Wright *et al.* (1944) also proved nonspecific reactions with intestinal parasites in the filarial antigens. On the other hand, Thompson *et al.* (1945), Hunter *et al.* (1945), Saunders *et al.* (1946), Franks *et al.* (1947) and Benetazzo (1949) found no cross-reactions with intestinal parasites in them. In the authors' result, not only the intestinal parasites but also some other helminths did not influence on the specificity of the skin reaction. Asthma, urticaria, Quincke's edema and tuberculosis cases also gave no false positive reactions. From these results, F P T antigen was assumed highly specific against the sensitization with filarial infections.

Summary

A purified antigen, F P T, was prepared from the adult worms of *Dirofilaria immitis* and considered to be mainly composed of polypeptides. An absorption maximum in ultraviolet region was observed at 275 millimicrons and the nitrogen content was determined as 10.8%. Intradermal injection of 1 μ g of antigen in 0.05 ml of phenolized physiological saline was considered as adequate for the diagnostic purpose of human filariasis. It was concluded that the positive reaction was 9.0 mm or more in size of wheal and negative was 8.5 mm or less in 15 minutes after injection. One hundred and twelve cases (91.0%) out of 123 microfilaria carriers were shown as positive according to this criterion. However, only 3 cases (3.0%) out of 100 normals reacted positive. The number of microfilaria carriers in the inhabitants of an endemic region of filariasis increased corresponding to the increase in size of wheal among them. An increasing tendency in the positive rate among the individuals from 1 to 19 or 24 years old was also shown. Treatment of patients with 1-diethylcarbamil-4-methyl-piperazine citrate (Supatonin) seemed to show the decrease of the positive rate in skin reaction year by year. There seemed no correlations between the number of microfilariae and the size of wheal in microfilaria carriers. Nonspecific reactions were not found with asthma, urticaria, Quincke's edema and tuberculosis. Furthermore, no cases of ascariasis, ancylostomiasis, trichocephaliasis, paragonimiasis, heterophyiasis and taeniasis reacted with this antigen. Besides, 1 case out of 15 gnathostomiasis and 1 out of 7 schistosomiasis showed positive.

Applications of this method on the epidemiological survey of bancroftiasis revealed interesting results. A highly specific activity of F P T antigen for the diagnostic purpose of human filariasis was reported in this paper.

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犬糸状虫精製抗原によるフィラリア症の皮内反応に関する研究

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フィラリア症の皮内反応に関する研究は従来主として粗抗原により追求されて来た。著者らは精製抗原による皮内反応の診断的特異性を研究する目的で犬糸状虫体酸性抽出物を等電点沈澱・ピクリン酸沈澱・塩酸エタノール抽出などを順次用いて精製した。得られた物質は蛋白性で N 量 10.8% で 275 m μ に吸収を示し、抽出条件から考えペプチド画分と考えられるので FPT と名づけ活性を追求した。本抗原は 1 μ g/0.05 ml で充分な活性を示し陽性判定基準は 15 分後の丘疹径 9.0 mm にあると考えられた。仔虫保有者はその 91% が陽性反応を示すのに対し健康人ではわずか 3% の陽性反応が認められたにすぎない。腸管寄生虫を含む 8 種の寄生虫保有者合計 66 名について見ると 2 名が陽性であるにすぎず交叉反応は無いと考えられた。また結核症・数種アレルギー疾患においても非特異反応は見られず特異性が高いと考えられた。皮内反応の強さは血中の仔虫密度と無関係だがジエチルカルバマジン治療例では仔虫消失後の期間が長いほど反応陽性者は減弱し、治療 4 年後の人では 22.2% が陽性であるにすぎない。フィラリア症流行地住民の皮内反応発現状況から地域の同症浸淫が疫学的に推定される点に興味を持たれた。