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Delayed Hypersensitivity to *Dirofilaria immitis* 1. Migration Inhibition Test

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Introduction

Uutil the present, immunological studies of filariasis have, in general, been limited to the investigation of reagin in association with the application of the immediate skin reaction in field surveys. However, the occurrence of delayed skin reaction caused by this parasite in humans has also been reported (Ishizaki *et al.*, 1964). As a further attempt to detect delayed hypersensitivity (DH) to *Dirofilaria immitis* (DI), our study was motivated by the fact that the delayed skin reaction can be observed in guinea pigs sensitized with DI antigen.

The migration inhibition test (MIT), first developed by George and Vaughan in 1962, is considered to be a phenomenon related to DH. This method is used to observe migration inhibition of macrophage from capillary tubes caused by a biologicaliy active substance called the migration ithibitory factor (MIF). This factor is released from sensitized small lymphocytes when they meet specific antigen.

This paper describes the confirmation of DH. of the tuberculin type in guinea pigs sensitized with DI antigen by means of MIT, the delayed skin reaction and its reaction by passive transfer with sensitized peritoneal exudate cells.

Materials and Methods

1. Animals

Female guinea pigs weighing around 300 g were used.

2. Antigen

Adult worms of *D. immitis* obtained from infected dogs were used. The purification of antigen was performed by a modification of the method described by Chaffee *et al.* (1954).

Lyophilised worms were homogenized and defatted with cold ether in a glass tissue-grinder. After centrifugation at 10^4 rpm for 30 minutes (0-5°C), the ether supernatant was discarded and the extract (lower phase) was dried overnight in a refrigerator. Then the extract was triturated again with a fixed volume (100 times the dry weight* for the MIT and intradermal test and 30 times the dry weight* for sensitization) of veronal bicarbonate buffered salt solution (VBS) and was extracted in a refrigerator overnight. The extract used for sensitization was stored at −20°C until required. The extract for MIT and intradermal test was subsequently centrifuged after dilution with the same

* This was arbitrarily calculated for 1 mg dry weight equivalent to 0.1 ml.

volume of VBS (0-5°C), and then dialyzed in running water. Finally the extract was passed through a Millipore filter and stored at -20°C.

3. Intradermal Test

Two hundredth ml of antigen diluted to 600 γ/ml with physiological saline was injected intradermally on a previously shaved abdomen of the animal. Tests in which the dimensions of the erythema were measured within 30 minutes were designated as immediate type, after 4-6 hours as Arthus and after 24-48 hours as delayed type. Those in which the mean size of the dimensions was 5 mm or greater were regarded as positive skin reactions.

4. Migration Inhibition Test

The improved method by Haga (1973) was employed. Peritoneal exudate cells were evoked by the intraperitoneal injection of 15 ml of liquid paraffin. Three to 4 days later, the exudate cells were collected in 100 ml of heparinized phosphate buffered saline (PBS), washed by centrifugation at least three times with PBS and the cells were resuspended in 2 ml of Minimum Essential Medium (MEM) with 1 % glutamine and 20 % calf serum.

The desired number of microcopillaries for MIT (Ikemoto Ltd.) were sunk into the cell suspension and centrifuged at 10^3 rpm for 2 minutes. After discarding the supernartant, cell packed capiliaries were placed with silicone grease in a culture chamber for MIT (Ikemoto Ltd.), followed by rapid pour of the maintenance medium, which contains 10 γ/ml of antigen protein in case of test groups. All chambers were incubated in a 5 % CO_2+95 % air atmosphere for 24 hours. The distance of cell migration from both edges of the capillary was measured. In each test group at least 4 capillaries were employed. The migration index (MI) was obtained for subsequent analysis.

$$MI=\frac{\text{average distance of migration with antigen}}{\text{average distance of migration without antigen}}$$

(MI values equal to or lower than 0.75 were regarded as positive cases).

5. Complement Fixation Test (CF)

The modified method of LBCF (Casey, 1965) by Matsuda *et al.* (1972) was employed.

6. Procedure for Sensitization

Five tenth ml of antigen (6 mg protein) in combination with the same volume of Freund's complete adjuvant was injected intradermally into the digital spaces of the footpads. In cases of successive sensitization, half volumes of antigen and Adjuvant were injected after the second sensitization. Both nontreated animals and animals injected with 0.5 ml of Adjuvant alone were used as control groups.

7. Passive Transfer

Peritoneal exudate cells were obtained from two animals 2 weeks after single sensitization. Four hundred million cells suspended in 5 ml of PBS was transferred intraperitoneally into a normal animal and 24 hours later, an intradermal test was done (Yoshida, 1972). Similarly, a total of 10 ml of sensitized serum was obtained from two animals 2 months after completion of 4 time sensitization and transferred into another normal animal.

Results

1) The presence of DH shown by MIT (Table. 1)

Migration inhibition was observed in 6 of 8 sensitized animals, thus confirming the presence of DH by this method. On this occasion, these migration inhibition cases were associated with positive delayed skin reaction and developed more or less elevated humoral antibody.

2) Relation between cellular antibody and humoral antibody (Table 2)

The relation between cellular and humoral antibodies was investigated over a period of 17 weeks by means of MIT, intradermal test and CF in animals sensitized 4 times. Neither antibody appeared on the third day after the first sensitization. On the 4th day, migration inhibition was observed with positive delayed skin reaction. Humoral antibody was first deteced on the 7th day by CF (titer=1 : 20) in spite of negative immediate and Arthus skin reactions. Afte-

Table 1 Migration inhibition of guinea pig peritoneal exudate cells incubated in the presence of *Dirofilaria* antigen

Animal	MIT	CF	Intradermal test		
			Immediate	Arthus	Delayed
No2~1	−	1 : 160	+	+	+
No2~3	+	1 : 160	+	+	+
No2~4	+	1 : 160	+	+	+
No2~5	+	1 : 40	+	+	+
No3~1	+	1 : 160	+	+	+
No3~2	−	1 : 160	+	+	+
No3~5	+	1 : 160	+	+	+
No3~6	+	1 : 20	−	−	+

Table 2 Relation between cellular antibody and humoral antibody

	sen															
week	↓ 1	3					4					7				
day	1															
type of test		MIT	CF	Imm*	I.T* Art*	Del*	MIT	CF	Imm	I.T Art	Del	MIT	CF	Imm	I.T Art	Del
No 2~3		−	0	−	−	−						+	20	−	−	+
No 3~1							+	0	−	−	+					

	sen							sen					sen			
week	↓ 3	4	5					↓ 6					↓ 10	12	13	17
type of test		MIT	CF	CF	Imm	I.T Art	Del	MIT	CF	Imm	I.T Art	Del		MIT	MIT	CF
No 2~3		+	80	160	+	+	+	+	0	−	−	+			+	80
No 3~1		+	160	160	+	+	+	+		+	+	+		+		160

*I.T : Intradermal test, Imm : Immediate type, Art : Arthus type, Del : Delayed type, sen : Sensitization

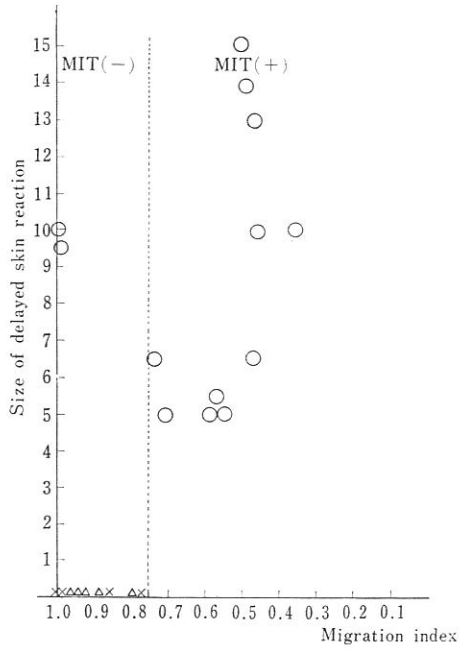
rwards sensitization was repeated 3 times at 3-4 week intervals, but no correlation was found between humoral and cellular antibodies.

3) Relation between the size of delayed skin reaction and migration index (Fig. 1)

In all migration inhibition cases, the size of the delayed skin reaction was 5 mm or greater. However, 2 animals exhibiting 9.5 mm, 10 mm reactions respectively, showed no migration inhibition. As shown in Fig. 1, no correlation was found between migration index and the size of delayed skin reaction ($r=0.182$).

4) Establishment of DH by passive transfer with pertoneal exudate cells (Table 3).

The purpose of this experiment was to determine whether this cellular antibdy is of



○ sensitized with *Dirofilaria* and adjuvant
△ sensitized with adjuvant
× non sensitzed

Fig. 1 Relation between migration index and the size of delayed skin reaction

Table 3 Reactions to *Dirofilaria* antigen in normal guinea pigs transferred with sensitized peritoneal exudate cells or sensitized serum

Type of transfer	Intradermal test (mm)		
	Immediate	Arthus	Delayed
Sensitized cells	−	−	6×5
Sensitized serum	−	6×7	−

the tuberculin type or not (Chase, 1945; Metaxas & Metaxas, 1948; Stavitsky, 1948). Passive transfer of DH by peritoneal exudate cells from 2 sensitized animals was achieved as shown by delayed skin reaction in normal animals. An additional attempt to show passive transfer by serum from 2 sensitized animals revealed positive skin reaction of the Arthus type only.

Discussion

In the field of parasitological immunology, there have been many studies related to humoral antibody, while little attention has been paid to cellular antibody because of the difficulties involved in quantitative determinations and clinical application. Above all there was no satisfactory technique available for the investigation of cellular antibody in vitro. Even MIT has only recently been acknowledged to have qualitative value.

In general, there are three classes of methods now applied to the investigation of cellular antibody in vitro.

1) In vitro cytotoxicity test by sensitized lymphosytes.

This test is used to observe the phenomenon of direct injury to parasites or other kinds of organisms as antigen in vitro by added sensitized lymphocytes or macrophages but not by immune serum. This is now considered to be the most direct method of detecting the activity of cellular antibody.

2) Blastformation of lymphocytes in vitro.

Lymphocytes from the individuals sensitized with DH are transformed into blastoid cells in vitro when specific antigen is added (Bender & Prescott, 1962; Polar & Prescott, 1970). This method is used to observe morphological changes or to count the radioactivity incorporated into the cells. However, the significance is inclined to be suspected because blastformation also occurs on addition of non-specific antigen such as PHA, an extract of the kidney bean, *Phaseolus vulgaris*. The recent developments in studies related to T-cell and B-cell of lymphocytes also give cause for caution in interpreting this phenomenon.

3) Migration Inhibition Test (described in Introduction).

In the field of Parasitology, Bray and Bryceson (1968) reported that sensitized lymphocytes from convalescent animals destroyed all cultured macrophages infected with *Leishmania enterietti* and Toyokawa (1969) reported that the adhesion of lymphocytes sensitized with Anisakis antigen from guinea pig to Anisakis larvae and the impediment of larval mobility was observed by Immuno-Leuco-Adhesion.

Successively, Tremonti and Walton (1970) applied MIT and blastformation test to the cases with *Toxoplasma gondii* and *Leishmania braziliencis* and Wolfson *et al.* (1972) demonstrated migration inhibition on the peripheral leucocytes from human schistosomiasis. However, these kinds of technique have not been applied yet to *D. immitis* to date. With respect to the first purpose of our study, i.e. to detect DH to this parasite, it must be emphasized that DI antigen strongly gives rise to DH. Namely, all sensitized animals revealed delayed skin reaction and, except for two cases, migration inhibition. In the two cases showing positive skin reaction without migration inhibition, a phenomenon resembling "enhancement" in vivo was suspected because of their high titer of humoral antibody in CF.

It is impossible to qualitatively determine cellular and humoral antibodies by the same technique, and moreover, it seems unreasonable to compare results obtained by different techniques. However, according to our results with MIT and CF, the cellular antibody appeared earlier than the humoral antibody. In our experiments, delayed skin reaction was observed at the same time as migration inhibition. The absence of correlation between the appearance of the cellular and the humoral antibodies through the whole course of observation makes us consider a miscellaneous immune response in the host.

Finally, evidence that this cellular antibody is of the tuberculin type was obtained by the achievement of passive transfer of DH using sensitized peritoneal exudate cells. Further study is required to elucidate the role of this cellular antibody in the onset of filariasis and in the protective mechanism activated by reinfection.

Summary

1. The presence of delayed hypersensitivity to *D. immitis* in guinea pigs was confirmed by means of delayed skin reaction and MIT using sensitized peritoneal exudate cells.

2. All migration inhibition cases were necessarily associated with positive delayed skin reaction.

3. The cellular antibody confirmed by MIT first appeared with positive delayed skin reaction on the 4th day after single sensitization.

4. The cellular antibody seemed to appear earlier than the humoral antibody.

5. No correlation was found between migration index and the size of delayed skin reaction.

6. The cellular antibody could be transferred passively to a normal guinea pig using sensitized peritoneal exudate cells.

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犬糸状虫免疫にみられる遅延型過敏症
1. マクロファージ遊走阻止試験

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1) 犬糸状虫抗原で，モルモットを感作し，その腹腔細胞の遊走阻止現象と同時に，遅延型皮内反応の出現を確認した．

2) 細胞性抗体は，感作後4日目に，遅延型皮内反応と共に出現した．

3) 細胞性抗体は，液性抗体に比し，早期に出現した．

4) 腹腔細胞の遊走阻止率と遅延型皮内反応の大きさとの間には，相関関係はみられなかった．

5) この細胞性抗体は，感作腹腔細胞による受動感作が可能であった．