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Delayed Hypersensitivity to *Dirofilaria immitis* III. The *in vitro* Cytotoxic Activity of sensitized Lymphocytes and their Effect upon the Mortality of Microfilariae in Diffusion Chambers implanted intraperitoneally into Guinea Pigs

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Introduction

In our previous paper (Kobayakawa *et al.*, 1973), the authors reported the occurrence of delayed hypersensitivity (DH) to *Dirofilaria immitis* (DI) antigen in guinea pigs as proved by means of delayed skin reaction and migration inhibition test using sensitized peritoneal exudate cells. Since this cellular antibody was transferrable to normal animal, it was concluded to be a tuberculin type of antibody. Subsequently, DNA synthesis in cultured peripheral and splenic lymphocytes with DI antigen confirmed by incorporation of [^{3}H]-thymidine into blast cells was reported (Kobayakawa & Ishiyama, 1974).

The present study was undertaken to elucidate the role of this cellular antibody in the immune mechanism against DI infection with the *in vitro* cytotoxicity test and the *in vivo* diffusion chamber method using microfilariae (Mf) of this species as target cells.

Materials and Methods

1. Animals

Female guinea pigs of the Hartley strain weighing around 300 g were used.

2. Antigen

Adult worms of DI obtained from infected dogs were used. The purification of antigen was performed by a modification of the method described by Chaffee *et al.*, (1954) and detailed procedure has been described in our previous paper (Kobayakawa *et al.*, 1973).

3. Procedure for sensitization

A mixture of 0.5 ml of antigen (6 mg protein) and an equal volume of Freund's complete adjuvant was injected into the digital spaces of the footpads of the animals.

4. Harvest of peritoneal exudate cells

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 with PBS by centrifugation at least three times and the cells were finally suspended in physiological saline (PS).

5. Harvest of splenic cells

The spleens were removed aseptically and dispersed by pressure through 4 layers of gauze. Cells were washed with PBS at least 3 times and finally resuspended in PS.

6. Separation of microfilariae (Mf)

The blood collected in a heparinized syringe from infected dog was centrifuged at 800 rpm for 5 minutes. After discarding the supernatant, the sediment was suspended in an appropriate volume of distilled water

and left for 2 minutes allowing hemolysis to occur. After the addition of an equal volume of PS, the suspension was centrifuged at 800 rpm for 5 minutes. The final sediment containing Mf was suspended in an appropriate volume of PS.

7. *In vitro* cytoxicity test

Two ml of a mixture of Mf and cells in the various combinations as described in Table 1 was cultured in a Falcon 3001 tissue culture petri dish (35×10 mm). The number of cells was adjusted to 5×10^6/ml and the antiserum to DI antigen (titer in complement fixation test=1 : 160) and complement were diluted 20 folds with PS. Two dishes were prepared for each group and the experiment was duplicated.

At 2, 4 and 6 hours after incubation, one drop from each culture dish was placed on a slide with a cover slip and promptly examined microscopically for surviving Mf. The criteria for the death of Mf included lack of motion and morphological changes in the worm surface membrane. In most of the dead worms, the surface membrane was wrikled, thus making the determination comparatively easy.

8. *In vivo* diffusion chamber method

Diffusion chambers were made using Millipore Lucite-rings (diameter : 10 mm thickness : 2 mm) and filter membranes (pore size : 0.45 μ). They were closed after the injection of 0.05 ml of cell suspension adjusted to the number of 5×10^5/ml and 0.05 ml of Mf suspension (in groups without cells, 0.1 ml of Mf suspension) and 5–7 chambers were implanted intraperitoneally into a guinea pig. Two animals were used for each experimental group and 3 animals for the control group. The combinations of cell types in the diffusion chambers and implanted animals are presented in Table 2.

The diffusion chambers were removed 2 and 3 days after the implantation. The content of the chambers was then washed with 0.1 ml of PS, and Mf was examined for death or survival by the procedure described above.

Results

1) *In vitro* cytotoxicity test (Table 1)

The mortality rate of Mf at 6 hours of incubation in the groups cultured with sensitized peritoneal exudate cells, regardless the presence or absence of complement, was about 3 times higher than that in group with Mf only and 2 times higher than that in groups with normal peritoneal exudate cells. There was no apparent difference in rate between the groups with antiserum and the control group.

2) *In vivo* diffusion chamber method (Table 2)

The mortality rates of Mf in the groups implanted with sensitized splenic or peritoneal exudate cells and the group of sensitized animals implanted with normal peritoneal exudate cells were significantly increased

Table 1 *In vitro* cytotoxicity test on Mf

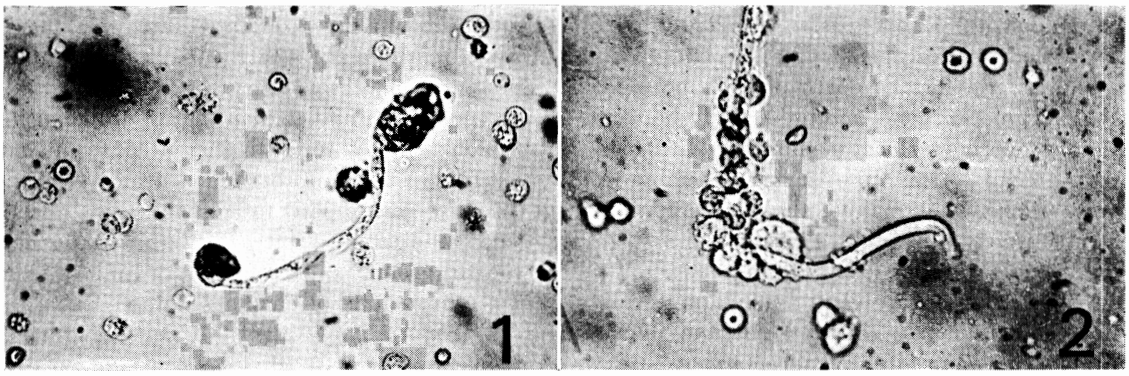
Hours of incubation	2 hours			4 hours			6 hours		
	No. of Mf.		Mortality rate(%)	No. of Mf.		Mortality rate(%)	No. of Mf.		Mortality rate(%)
	dead	alive		dead	alive		dead	alive	
Sensitized PE cells*	11	51	17.7	37	48	43.5	37	39	48.6
Sensitized PE cells + complement	1	32	3.0	12	53	18.4	27	31	46.5
Normal PE cells	12	58	17.1	23	83	21.6	45	115	28.1
Normal PE cells + antiserum	2	92	2.1	10	74	11.9	14	42	25.0
Antiserum	2	40	4.7	3	83	3.4	13	71	15.4
Mf only	1	48	2.0	1	67	1.4	16	103	13.4

PE cells* : peritoneal exudate cells

Table 2 The effect of sensitized lymphocytes on the mortality of Mf in diffusion chambers

Animal	Type of cells	Days after DC* implantation					
		2 days			3 days		
		No. of Mf.		Mortality rate (%)	No. of Mf.		Mortality rate (%)
		dead	alive		dead	alive	
Sensitized	Sensitized splenic cells	6	54	10.0	70	247	22.0
Sensitized	Sensitized PE cells	36	121	22.9	20	83	19.4
Sensitized	Normal PE cells	7	35	16.6	33	128	20.4
Sensitized	Mf only	1	51	1.9	4	78	4.8
Normal	Sensitized splenic cells	1	44	2.2	31	176	14.9
Normal	Sensitized PE cells	8	137	5.5	14	64	17.9
Normal	Normal PE cells	13	198	6.1	10	128	7.2
Normal	Mf only (control)	1	50	1.9	10	597	1.6

DC*: diffusion chambers



Explanation of Figures

Fig. 1 Dead or dying Mf apparently excreting exudate-like substances.
Fig. 2 Effect of cell adhesion on Mf. Upper left shows adhesion of sensitized cells on immobilized Mf. Lower center of Mf without cell adhesion is still motile and smooth.

over that of controls.

No increase of the mortality rate in the sensitized group implanted with Mf only (in which the worms would be affected exclusively by humoral factors from the host) was observed.

Sensitized cell adhesion to dead or dying Mf and the exudate-like substances probably excreted from dying Mf were observed (Figs. 1, 2).

Discussion

To date, the lymphocyte cytotoxicity test *in vitro*, one of the experimental models of cell-mediated immunity, has been applied chiefly to tumor target cells (Goldstein *et al.*, 1972), but in some studies this test has been applied to allotransplantation antigens (Osoba & Miller, 1969) and tubercle bacilli (Fong *et al.*, 1956). In the field of parasitic immunology, this kind of study has been strictly confined to protozoan infection, in which the organismus act as intracellular proliferative antigens (Bray & Bryceson, 1968) and few studies, if any, have been performed on helminthic infection.

In this study, peritoneal exudate cells were used as the source of effector cells. There were little differences among the results of studies using peritoneal exudate cells (Fong *et al.*, 1956), splenic cells (Cerottini *et al.*, 1970b) and lymph node cells (Rosenau & Moon, 1961). Due to recent striking advances in the study on behavior of small lymphocyte subpopulations, the T-cell (thymus derived cell) has come to be regarded as an effector cell which directly injures the target cells (Cerottini *et al.*, 1970a).

The viability of Mf in PS was reported by Kume *et al.* (1942) who also observed that the first death of Mf occurred at around 5 hours and all Mf were dead within 10 hours. In the experiments reported here, the data show that only the groups with sensitized peritoneal exudate cells showed a striking cytotoxic activity against Mf. This fact may possibly be due to *in vitro* cell-mediated immunity, since the presence of sensitized serum or complement had no appreciable effect on cytotoxic activity.

On the other hand, in the sensitized animals the cytotoxic activity affecting the mortality of Mf in chambers with normal peritoneal exudate cells increased to almost the same level as those in chambers with sensitzed splenic or peritoneal exudate cells. This phenomenon is reproducible and some possible explanations for it are (1) passage of sensitized cells into the chamber from the host through the "Millipore filter", (2) sensitization of peritoneal exudate cells by continuous antigenic stimulation from the host, (3) non-specific phagocytosis by macrophages and neutrophiles, and (4) elaboration of humoral factors. However, the possibilities of (1), (2) and (3) are considered negligible for the following reasons : the passage of cells through the filters with the pore size of 0.45 μ diameters might be impossible and the period of 2 or 3 days might be too short for the sensitization of normal peritoneal exudate cells to constitute cell-mediated immunity. Moreover, the fact that the cytotoxic activity with normal peritoneal exudate cells implanted into normal animals was low may exclude the passiblity of non-specific phagocytosis. Thus the possibility (4) is the most likely one.

Humoral factors related to the expression of cell-mediated immunity have been termed "Lymphokines" by Dumonde *et al.* (1969) and they considered that, among the cytotoxic factors acting on the target cells, there is a soluble mediater as well as migration inhibitory factor and the blastogenic factor. Osoba & Miller (1969) suggested that this humoral factor is released from T-cells or thymocytes. The report by Valentine & Lawrence (1969) that supernatants, prepared from sensitive human lymphocytes cultured with specific antigen, activate lymphocytes from non-sensitive individuals *in vitro* is in line with Goldstein & White's observation (1967) that a soluble product termed thymosin from thymic tissue can restore an immunological deficit in thymectomized mice. Another study by Fishman & Adler (1973) described the formation of complexes between antigen ingested and solubilized by peritoneal macrophages and ribonucleic acid (RNA) of such cells which induced *in vitro* antibody response. On the other hand, Decker & Pilch (1972) succeeded in the mediation of an immune response to tumor-specific antigens by syngeneic immune RNA and suggest that cell-mediated immunity is transferrable by RNA. These results as well as those of our previous studies may support the view of the former mechanism. However, there are no conclusive evidences to deny the latter.

Comparing the results between *in vivo* and *in vitro*, it is clear the Mf could be no more maintained *in vitro* at a viable condition after 6 hours. The probable effect of humoral factors upon normal peritoneal exudate cells therefore could not be determined.

The phenomenon of sensitized cell adhesion to a parasite and the exudate-like substances which was probably excreted from dying worm have already been reported with *Anisakis* larvae by Toyokawa *et al.* (1969). In Soulsby's study (1967) using *Ascaris* larvae implanted into rabbits, the adhering cells

were found to consist almost entirely of eosinophiles and this adhesion was confined to the third stage larvae. The specificity of this cell adhesion was suspected by some workers; Jeska stated (1969) that cell adhesion occurred in both sensitized and normal mice in which *Ascaris suum* larvae were implanted intraperitoneally.

In any case, the mode of action by which sensitized cells react to parasitic worms has not necessarily been completely stronly clarified yet. However, the present study strongly suggests that the cellular antibody would play the most important role in the immune mechanism against Mf of DI species.

Summary

1) The parasiticidal effect of sensitized lymphocyte from guinea pigs on the Mf of DI was investigated by means of the *in vitro* cytotoxicity test and *in vivo* diffusion chamber method.

2) The cytotoxicity test revealed that in the groups cultured with sensitized peritoneal exudate cells, Mf mortality rate was 3 times higher than that of group with Mf only and 2 times higher than that of group with normal peritoneal exudate cells. The addition of sensitized serum and complement had no effect on Mf mortality.

3) In the diffusion chamber studies, sensitized splenic and peritoneal exudate cells in both sensitized and normal animals, and normal peritoneal exudate cells in sensitized animals, had a significantly high parasiticidal activity. Sensitized cell adhesion to Mf and exudate-like substances of dead or dying Mf were observed. The nature of the cytotoxic activity of cellular antibody was discusssd.

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犬糸状虫免疫にみられる遅延型過敏症
III. In vitro 細胞毒性試験，および拡散小室法における細胞性抗体の，ミクロフィラリアに対する障害性

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犬糸状虫免疫モルモットより得られた，感作細胞の，同種ミクロフィラリアに対する障害性を，In vitro 細胞毒性試験，続いてモルモット腹腔内に，埋入された，拡散小室を用いて検索した．

1) In vitro 細胞毒性試験の結果では，培養後6時間目で，感作腹腔細胞を用いた群では，ミクロフィラリア単独培養群に比し3倍，正常腹胞細胞を用いた群に比し，2倍のミクロフィラリア死亡率がみられた．なお，感作血清，補体の添加は，何ら効果がみられなかつた．

2) 拡散小室法では，感作腹腔細胞，感作脾細胞，また感作動物に入れた正常腹腔細胞が，高い障害性をみせた．

3) 拡散小室法において，死滅虫体，および死滅寸前と思われる虫体に，感作細胞，また虫体からの滲出物と思われる物質の付着が観察された．