[Jap. J. Parasit., Vol. 23, No. 4, 226-231, 1974]

Delayed Hypersensitivity to *Dirofilaria immitis* II Blast Transformation Test

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(Received for publication May, 22, 1974)

Introduction

In the previous paper (Kobayakawa *et al.*, 1973), the authors confirmed the presence of delayed hypersensitivity (DH) to *Dirofilaria immitis* (DI) antigen in guinea pigs by means of the delayed skin reaction and migration inhibition test (MIT) using sensitized peritoneal exudate cells. Since this cellular antibody was transferrable to a normal animal, it was determined to be a tuberculin type of antibody.

The lymphocytic blast transformation test (BTT) (Bender & Prescott, 1962) is also known as a method for the detection of cellular antibody *in vitro*. Since lymphocytes from individuals with DH are transformed into blastoid cells *in vitro* when specific antigen is added, this test can be used to observe morphological effects on cells and to determine the [^{3}H]-thymidine incorporated into the cells.

In the field of parasitic immunology, the antigenic character of cell-mediated immunity makes it possible to apply this kind of test to protozoan infections in which the organisms act as intracellular proliferative antigens; for example, leishmaniasis by Bryceson *et al.* (1970) and Blewett *et al.* (1971), toxoplasmosis by Tremonti & Walton (1970) and trypanosomiasis by Hare and Soulsby (1969). However, there are few studies on helminthic infection, the only one known to the authors being Colly's in 1972 on schistosomiasis.

This paper confirms the occurrence of lymphocytic blast transformation in peripheral blood and spleen from guinea pigs sensitized with DI antigen by determining the incorporation of [^{3}H]-thymidine into cells.

Materials and Methods

1. Animals

Female guinea pigs of Hartley strain 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), the details being described in our previous paper (Kobayakawa *et al.*, 1973).

3. Procedure for Sensitization

0.5 ml of antigen (6 mg protein) in combination with the same volume of Freund's complete adjuvant was injected into the digital spaces of the footpads.

4. Blast Transformation Test

(1) Harvest of peripheral lymphocytes

Peripheral blood was collected by heart puncture from sensitized and nontreated animals. A mixture of 9% Ficoll solution and 32% Conray (12 : 5 v/v) was made (F-C solution). 7 ml of blood was gently layered on 13 ml of F-C solution and centri-

fuged at 1100 rpm for 40 minutes (4°C). Lymphocytes obtained as a white layer were centrifuged at 2000 rpm for 20 minutes and the sediment was washed 3 times with phosphate buffered saline at 1,200 rpm for 10 minutes. This sediment contains lymphocytes with a purity of 95~98%.

(2) Harvest of splenic cells

Sensitized and nontreated animals were killed, their spleens removed, cut into small pieces and passed through 4 layers of gauge. The cell suspensions were prepared in RPMI 1640 medium and washed at least 3 times with the medium.

(3) Culture conditions

$0.5\sim1.0\times10^6$/ml cells were cultured in 2 ml of RPMI 1640 medium with 20% fetal calf serum and 100 units/ml of penicillin and 100 γ/ml of streptomycin for suspended culture in Falcon 3033 TC tubes. All cultures were prepared in duplicate. 10 γ/ml of antigen was added in 0.1 ml medium for test groups at the beginning of the culture. Groups for nonspecific stimulation contained Concanavalin A (Con A) in the range of 1 γ, 3 γ, 7 γ and 70 γ and Phytohemagglutinin (PHA), 1 γ, 2 γ and 10 γ per ml, respectively. The mixtures were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air, at the end of which period 0.1 μ Ci of [^{3}H]-thymidine was introduced into each culture tube.

(4) Measurement of DNA synthesis

24 hours later, each test tube was centrifuged and the sediment suspended in 0.2 ml of physiological saline was trapped on 2 filter discs (2.5 cm in diameter). The dried filter discs were washed with cold 10 and 5% trichloracetic acid for 30 minutes each, then once with cold ethanol 10 minutes followed by 1~2 washes with ethanol at room temperature 10 minutes each, and a final wash with ethyl ether 10 minutes. They were transferred to counting vials and prepared for scintillation counting by adding 5 ml of toluene phospher scintillator (containing 4 g/l PPO and 0.4 g/l dimethyl POPOP). The incorporation of [^{3}H]-thymidine expressed counts per minutes for each sample was measured with a LS-500 liquid scintillation counter (Horiba Ltd).

Results

1. Effect on DNA synthesis by nonspecific stimulants. (Figs. 1, 2)

DNA synthetic responses in both splenic and peripheral lymphocytes from nontreated animals by varying doses of Con A and PHA at the 3rd day of incubation were 2~4 times that of the control, thus confirming that the cells were viable and capable of being stimulated by nonspecific mitogens.

2. Effect on DNA synthesis in peripheral lymphocytes by DI antigen. (Fig. 1)

Presensitized groups stimulated with DI antigen indicated that the first peak of responsiveness occurred at the 11th day and suddenly decreased to control level by the 13th day. A second peak occurred at the 18th day of incubation.

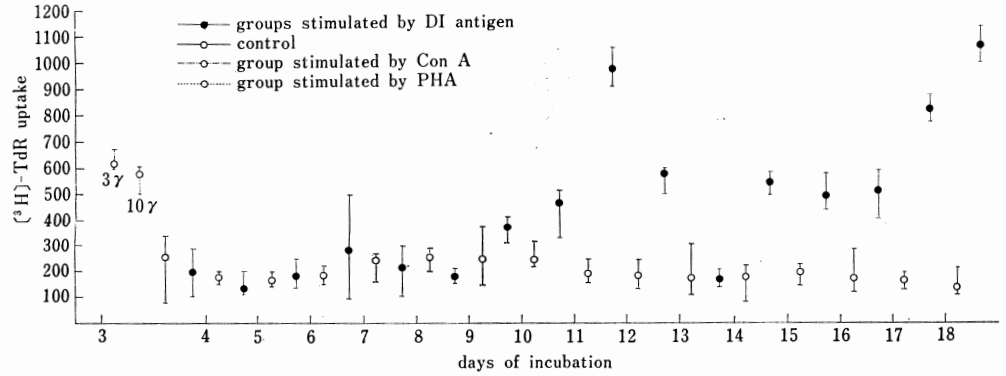

Fig. 1 The rate (mean values) of DNA synthesis in peripheral lymphocytes as measured by pulse labeling with [^{3}H]-TdR

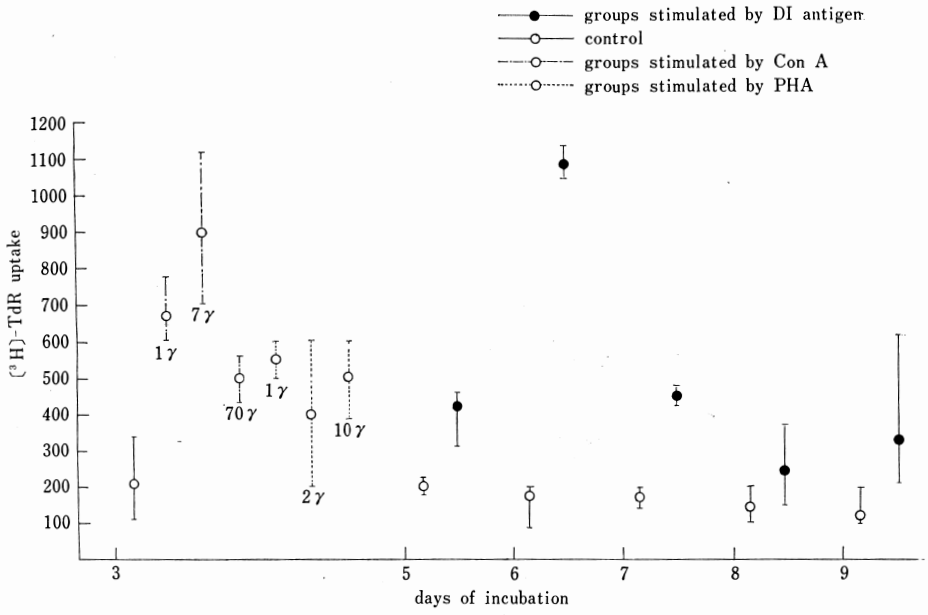

Fig. 2 The rate (mean values) of DNA synthesis in splenic lympocytes as measured by pulse labeling with [^{3}H]-TdR

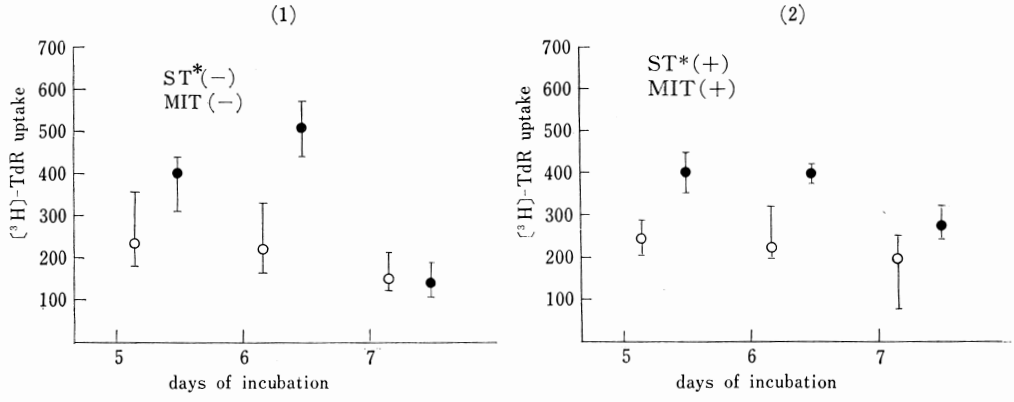

Fig. 3 The rate (mean values) of DNA synthesis in splenic lymphocytes from animals sensitized 3 (1) and 4 (2) days prior as measured by pulse labeling with [^{3}H]-TdR

——●—— groups stimulated by DI antigen

——○—— control

S.T*: skin test (delayed skin reaction)

These data make us consider that blast cells might be produced around the time of the first peak, that they mature around the 13th day and then resume DNA synthesis due to continuous stimulation by antigen.

3. Effect on DNA synthesis in splenic lymphocytes by DI antigen (Fig. 2)

Presensitized groups stimulated by DI antigen showed peak responsiveness at the 6th day of incubation.

4. Comparison of BTT results with those obtained in MIT and delayed skin reac-

tion. (Fig. 3)

In the previous study, one animal at 4 days postsensitization showed both the delayed skin reaction and migration inhibition (MI) of macrophages, while another sensitized 3 days prior showed neither reaction.

2 animals sensitized 3 and 4 days prior were used on BTT for comparison. Both groups seemed to start DNA synthesis though uptake was not conspicuous.

Discussion

There have been many arguments as to whether lymphocytic blast transformation *in vitro* correlates directly with the cellular immune response or not. However, it became increasingly apparent that this manifestation specifically requires the T-cell.

For instance, Stobo *et al.* (1972) reported that treatment of bone marrow cells of mice with complement and iso-antisera directed against the thymus dependent differenciation antigenθ abolishes the Con A and PHA responsiveness of these cells. Ray *et al.* (1973) found that lymph node cells from C3/HeJ mice incubated with Con A or PHA interfered with the cytotoxic activity of C57 anti-C3H antibody and complement and explained that, by some unknown mechanism, these mitogens interfere with the binding and/or expression of the cytotoxic activity of murine-antisera. Additionally in this study, cell treatment with pokeweed mitogen (PWM), a nonspecific B-cell stimulant, had no effect on the lysis of these cells by antibody and complement. The former study showed that lymphocytic DNA synthesis occures specifically in the T-cell. The latter revealed that the cytotoxic effect by allo-antisera is inhibited by the mitogen agglutinated T-cells bacause a large proportion of lymph node cells are T-cells.

Comparing our results obtained by the three methods for the detection of cellular antibody, i.e. MIT, BTT and delayed skin reaction, a difference in the time of appearence of the positive reaction was noted. Namely, the animal sensitized 3 days prior showed lymphocytic stimulation without MI and delayed skin reaction. These results are in line with the studies of Spitler *et al.* (1970) and Kirkpatrick *et al.* (1972). Guinea pigs sensitized with tobacco mosaic virus protein showed delayed type skin reaction and MI but no blastogenic response to the antigenic peptide of the protein. In patients with mucocutaneous candidiasis, there can be MIF (migration inhibitory factor) production to candida antigen in the absence of lymphocytic DNA synthesis, and conversely, there can be patients whose cells show the reverse pattern.

In considering the causes of these results, first place should be given to the fact that splenic lymphocytes or peripheral lymphocytes were used to measure the blastogenic response, whereas peritoneal exudate cells were used in MI studies. This gives rise to the possibility that the underlying cause might be due to varying subpopulations or proportions of responding cells from two different sites. Another possibility suggested by Spitler *et al.* (1970) is that there may be two populations of lymphocytes responsible for these functions, one which undergoes division in response to antigen and another which produces MIF.

Dumonde & Maini (1971) envisage that one mediator system may exist and function in the absence of another type of mediator system, but in all the lymphokines there are substances which "amplify and regulate" the responses of lymphoid cell populations to specific antigens. On the contrary, from the antigenic point of view, the possibility that different antigen preparations of the same materials contain different determinants, some of which are more discriminating for one or other of the *in vivo* or *in vitro* tests, has to be noted.

With respect to our results that DNA synthesis in splenic lymphocytes started earlier than that in peripheral lymphocytes, the authors considered two reasons: (1) Splenic cells contain more macrophages in the cell population and (2) the splenic cells are less mature than the peripheral cell population. The studies of Vischer & Stastny (1967)

suggest that the transforming activity is mainly localized in spleen.

In our previous and present studies, it was demonstrated that MIF and blastogenic factor was produced in guinea pigs sensitized with DI antigen. However, whether the DI worm provides the target cells for those lymphokines and sensitized lymphocytes or not is left to further studies.

Summary

1. DNA synthesis in cultured peripheral and splenic lymphocytes from guinea pigs sensitized with DI antigen in combination with Freund's complete adjuvant was confirmed by incorporation of [^{3}H]-thymidine into blast cells when DI antigen was added.

2. Splenic lymphocytes showed peak responsiveness at 6th day of incubation, however, that of peripheral lymphocytes was 11th and 18th day of incubation.

3. In animal sensitized 3 days prior, DNA synthesis already started in the absence of MI and delayed skin reaction.

4. The significance of blast transformation on cell-mediated immunity and its correlation between MIT and delayed skin reaction was discussed.

Acknowledgemeht

The authors wish to thank Dr. T. Ishizaki and the staff of the Department of Parasitology, National Institute of Health, for their helpful advice and assistance.

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犬糸状虫免疫にみられる遅延型過敏症
II. リンパ球幼若化試験

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1) 犬糸状虫抗原で，モルモットを感作し，その末梢リンパ球，及び脾細胞を抗原の添加のもとに培養しリンパ球の幼若化現象を，トリチウムチミジンの取り込みにより確認した.

2) 脾臓からのリンパ球は，培養後6日目にDNAの合成を最も盛んに行つたが，末梢リンパ球のそれは11日目，18日目であつた.

3) 感作後3日目の動物では，遅延型皮内反応，マクロファージ遊走阻止は未だみられぬが，脾臓からのリンパ球は，既にDNA合成を行つた.

4) 本試験の細胞性免疫における意義，及び遅延型皮内反応，マクロファージ遊走阻止試験との相関を討議した.