CYTOTOXICITY ASSAY AGAINST CULTURED HEART CELL IN RHEUMATIC HEART DISEASE

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Non-specific cytotoxicity of peripheral lymphocytes from healthy subjects was observed by stimulation of PHA and PWM.

Peripheral lymphocytes from patients with rheumatic fever and rheumatic heart disease was cytotoxic against ^{51}Cr labelled cultured heart cells in vitro.

SEVERAL investigators have detected auto-antibodies to the heart tissue in the serum of rheumatic fever patients.¹ They showed a correlation between the presence of circulating anti-heart antibodies and clinical course of the disease. Although circulating anti-heart antibodies may play some role in human disease, especially rheumatic fever, the pathogenesis of human heart disease has not been established yet. It is not clarified whether these auto-antibodies are produced as a sequela of cardiac damage or prior to the onset of the inflammatory state as a cause of rheumatic carditis. Further studies on the immunological process occurring in rheumatic fever are needed. Holm first reported the use of the isotope release method as a cytotoxicity assay and it has been used widely in immunological studies by many workers.² As a result, it is well recognized that a cytotoxicity is one of the most important activity in immunological reactions. In this paper, in order to investigate a possible role of cellular immunity and humoral immunity in rheumatic fever, cytotoxic activity against ^{51}Cr -labelled heart cell was studied using peripheral lymphocytes and sera.

Key Words:

RF, RHD
Cytotoxicity

METHOD AND MATERIAL

Tissue culture

Heart cells were obtained through the courtesy of the American Type Culture Collection and were cultured in Eagle's minimal essential medium (MEM) containing 10% heart-inactivated fetal calf serum (FCS) for further use. Cells were gently trypsinized for 4 minutes at 37°C in atmosphere of 5.0% CO₂, then resuspended in MEM containing 10% FCS to a final concentration of 2×10^6 cells/ml. The cell suspension was incubated with 100 $\mu\text{Ci/ml}$ ^{51}Cr for 45 minutes at 37°C in atmosphere of 5.0% CO₂. Finally, the ^{51}Cr -labelled heart cells were washed three times in MEM containing 10% FCS and resuspended to a concentration of 1×10^5 cells/ml.

Lymphocytes

Twenty milliliters of venous blood was obtained from the subjects. Samples were collected in heparinized syringes, and were layered onto an equal volume of Ficoll-Conray solution and centrifuged at 1,550 rpm for 30 minutes. Ficoll-Conray solution was prepared by mixing 10 parts of 33.4% Conray with 24 parts of 9.0% Ficoll and this solution was adjusted to a specific gravity of 1.078. Lymphocytes were centrifuged at 1,550 rpm for 30 minutes and appeared as small band. The lymphocyte-rich fraction was

(Received on February 7, 1977; Accepted on March 6, 1978)

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removed carefully and washed three times in MEM containing 10% FCS. Finally, the concentration of the lymphocytes was adjusted to 2×10^6 cells/ml. The fraction was stored at 37°C in atmosphere of 5.0% CO_2 until use.

Mitogens

An optimal concentration for each mitogen was determined by adding varying amount of mitogen to normal lymphocytes in a standard culture. PHA-P (Difco Laboratory, Detroit, Michigan) was used at an optimal concentration of $20 \mu\text{g/ml}$. Poke-weed mitogen (PWM) (Grand Island Biological Company, Grand Island, New York) was used at a concentration of $100 \mu\text{g/ml}$.

Patients

Rheumatic fever and rheumatic heart disease patients who visited the Juntendo University Hospital were investigated. The diagnosis was made on the basis of Jones' revised criteria (Guidelines in the Diagnosis of Rheumatic Fever, 1965).

Cytotoxicity Assay

1) 2×10^6 peripheral lymphocytes and 1×10^5 radiolabelled heart cells in a volume of 1.5 ml of tissue medium were incubated in an atmosphere of 5.0% CO_2 at 37°C for 12, 24 and 48 hours. After centrifugation at 800 rpm, isotope release from heart cells into the supernatant was counted by a Well-Type gamma counter. The percent release was estimated as follows:

Cytotoxic index =

$$\frac{\text{Radioactivity of the supernatant}}{\text{Total radioactivity}} \times 100$$

2) 0.1 ml of test sera, 1×10^5 ^{51}Cr -labelled heart cells and Guinea pig complement were incubated in a atmosphere of 5.0% CO_2 at 37°C for 12 hours. Similarly, after centrifugation at 800 rpm, isotope release was counted.

RESULT

Cytotoxic lymphocytes

First, viability of the heart cells in culture was measured by the method of ^{51}Cr release assay. The death rate of the cell was 19.48% at 12 hours, 29.97% at 24 hours and 40.97% at 48 hours after beginning of the culture. No significant difference in death rate was found when cells were stimulated by PHA-P and PWM. The result indicated that mitogen such as PHA-P or PWM did not damage the target cells in the medium in the absence of lymphocytes (Table I). The cytotoxic activity of normal lymphocytes, taken from healthy subjects, was determined at various concentrations after stimulation by PHA-P and PWM (Figure 1, 2).

Cytotoxic activity induced by PHA-P and PWM was well correlated with a concentration of the lymphocytes. Lymphocytes stimulated by PHA-P and PWM showed cytotoxicity at a concentration of 3.0×10^6 cells/ml. They showed less cytotoxicity at a concentration of 6×10^6 cells/ml (Figure 1, 2). The effect of incubation time on cytotoxic activity of lymphocytes stimulated by PHA-P and PWM was studied. The result showed that the longer incubation time produced the more cytotoxicity (Figure 3, 4).

In order to obtain an optimum concentration of mitogen, cytotoxic activity of normal lymphocytes was studied after stimulation by mitogen at various concentrations. A maximum cytotoxicity was obtained when PHA-P was present in a final concentration of $10 \mu\text{g/ml}$ and less cytotoxic activity was observed at a concentration of PHA-P $10 \mu\text{g/ml}$ (Figure 4). Maximum cytotoxicity was also obtained with PWM in a final concentration of $100 \mu\text{g/ml}$ (Figure 3). Cytotoxic activity of peripheral lymphocytes from patients with rheumatic fever and rheumatic heart disease was shown in Table II. The mean of cytotoxic index of lymphocytes from patients with rheumatic fever and rheumatic heart disease was 30.12 ± 10.9 , which was much

TABLE I VIABILITY OF HEART CELL IN THE CULTURE

	Incubation time (h)	Medium	PHA	PWM
Heart cell	12	19.48 ± 2.8	18.20 ± 2.6	17.73 ± 1.5
	24	27.97 ± 2.0	24.71 ± 0.7	26.31 ± 2.9
	48	40.97 ± 2.0	35.31 ± 6.0	40.03 ± 9.6

Fig.1.

Fig.2.

Fig.3.

Fig.4.

TABLE II CYTOTOXICITY OF LYMPHOCYTE FROM PATIENT WITH RHEUMATIC FEVER FOR CULTURED HEART CELL

Patient		Incubation time (h)	Cytotoxic Index with		PWM
			Medium	PHA-P	
M.Y.		24	33.33	46.19	39.64
N.S.		24	34.34	37.59	36.10
N.K.		24	31.56	29.59	39.62
H.K.		24	31.56	35.10	40.11
T.T.		24	27.33	37.72	36.62
H.Y.		24	29.26	29.01	29.15
Control (n=11, mean±1 SD)		24	30.12 ± 10.9	35.86 ± 33.3	36.78 ± 13.8
			22.83 ± 6.8	33.41 ± 17.5	31.06 ± 4.9
*Med/RHD—Med/Cont	P < 0.01	Med/RHD—PHA/RHD	P < 0.1	Med/Cont—PHA/Cont	P < 0.001
PHA/RHD—PHA/Cont	NS	Med/RHD—PWM/RHD	P < 0.05	Med/Cont—PWM/Cont	P < 0.001
PWM/RHD—PWM/Cont	P < 0.05	PHA/RHD—PWM/RHD	NS	PHA/Cont—PWM/Cont	NS

TABLE III CYTOTOXICITY OF SERA FROM PATIENT WITH RHEUMATIC FEVER FOR CULTURED HEART CELL AND FIBROBLAST CELL

Patient	Heart cell	Fibroblast cell (L)
M.O.	33.45	28.84
Y.S.	36.42	26.82
S.S.	29.94	30.18
A.Y.	29.34	29.72
O.Y.	37.99	25.67
J.Y.	27.60	75.82
S.H.	41.83	37.05
S.T.	27.92	83.92
H.K.	25.15	73.09
A.O.	27.14	63.37
	31.75 ± 6.7	47.44 ± 10.2
R = (1, 2, 3, 4)	75.50 ± 14.8	78.25 ± 9.4
Controls =	28.08 ± 8.6	32.62 ± 6.4

R = Rabbit anti-Human Heart Sera

more than that of lymphocytes from normal subjects without mitogenic stimulation. ($p < 0.01$)

After PHA-P stimulation, cytotoxic index of lymphocytes from patients with these disease was 35.86 ± 33.3 and after PWM stimulation, it was 36.78 ± 13.8 .

Cytotoxicity of PHA and PWM activated lymphocytes from patients with rheumatic fever and rheumatic heart disease was not significantly different as compared with lymphocytes from normal controls.

Cytotoxic sera

Cytotoxic activity of sera from patient with rheumatic fever and rheumatic heart disease against cultured heart cells was 31.75 ± 6.7 . It was of no significant difference as compared with

normal subjects. However, against fibroblast cells (L cells), it was 47.44 ± 10.2 , which was much more than that of sera from normal subjects. In short, cytotoxic activity of sera from rheumatic fever and rheumatic heart disease cases was observed against fibroblast cells in the presence of complement (Table III).

DISCUSSION

There is some evidence on the presence of an autoimmune mechanism in rheumatic fever. Circulating anti-heart antibodies has been detected by a number of workers on rheumatic fever. The antibodies were also found in post-myocardial infarction and post-cardiotomy syndrome or collagen disease such as systemic

lupus erythematosus. Zabriski stressed the correlation between the presence of circulating anti-heart antibodies and clinical course of rheumatic fever.³ As well, we could show the presence of anti-heart antibodies by using tanned red blood cell hemagglutination test and immunofluorescent technique in rheumatic fever and rheumatic heart disease and the decline and disappearance of high titer antibodies after inactivation of rheumatic inflammation. On the other hand, Kuch could find a correlation between the presence of cardiac lesions and presence of anti-heart antibodies.⁴ The role of circulating anti-heart antibodies in the pathogenesis on the cardiac damage is not clear yet.

Recently, in addition to humoral immunity, the role of a cellular immunity has been studied on the pathogenesis of organ lesions in various immunological diseases.⁵⁻¹⁰ Cytotoxicity is a phenomenon that target cells are destroyed by immune lymphocytes sensitized against specific antigens. Cytotoxic factor could be released from immune lymphocytes stimulated by specific antigen and from non-immune lymphocytes stimulated by mitogens. In 1972, Podelski reported the cytotoxic effect of lymphocytes from patients with Hashimoto thyroiditis for mouse mastocytoma cells coated with either thyroglobulin or the microsomal fraction of the human thyrotoxic gland.¹¹ In addition, several workers have shown that patients with Hashimoto thyroiditis possessed lymphocytes which are specially sensitized to a compartment of the thyroid gland and are capable of cell damage activity.¹² Friedmann (1970) demonstrated that cytotoxicity has been obtained with lymphoid cells sensitized to the rabbit heart extract and group A streptococci.¹³ In addition, the author showed that passive transfer of lymphocytes from Lewis rat sensitized to the heart tissue could bring about a myocarditis. Our result showed that lymphocytes from normal healthy subjects get more cytotoxic activity to the cultured heart cells in the presence of PHA-P or PWM. Peripheral lymphocytes from patients with rheumatic fever and rheumatic heart disease were much more cytotoxic than those from normal subjects to the human heart cells in vitro.

In this paper, cytotoxic activity of lymphocytes from patients with rheumatic fever was determined using heart cell antigen with the ⁵¹Cr release technique. It appeared that patients with rheumatic fever and rheumatic heart disease are carrying circulating lymphocytes which are

specially sensitized to heart cells.

On the other hand, cytotoxic activity of sera from patients with these diseases was much more than that of normal subjects against fibroblast cells (L cells) in the presence of complement. Further investigation will be needed to determine whether these lymphocytes and sera could destroy the heart tissue in vivo and whether this cytotoxic mechanism be of a primary importance in the production of cardiac lesions in rheumatic fever. But it is probable that the cytotoxicity could contribute to the formation of cardiac lesions in rheumatic fever and rheumatic heart disease. In conclusion, the presence of not only anti-heart antibodies in the sera but also cytotoxicity of lymphocytes due to cellular immunity was established and these factors could participate in the pathogenesis of cardiac lesions in rheumatic fever and rheumatic heart disease.

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