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Mutual Correlation of Human Thymocyte Receptors for Phytohemagglutinin, Sheep Red Blood Cell and Specific Antigens

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Abstract In vitro responses of human thymocytes to phytohemagglutinin (PHA), sheep red blood cells (SRBCs), anti-human thymus serum (AHTS) and their mutual correlation were evaluated. The PHA response of thymocytes was markedly depressed as compared with that of peripheral blood lymphocytes (PBL), while their SRBC rosette formation and AHTS cytotoxicity were more marked than those of PBL. The PHA response of PBL was not inhibited in the presence of AHTS, but it was considerably depressed by the combination of AHTS and guinea pig serum (complement). The SRBC rosette formation of thymocytes and PBL was inhibited by AHTS alone. Neither SRBC rosette formation nor AHTS cytotoxicity of thymocytes was disturbed by PHA. These results suggest that the AHTS binding antigen and the SRBC receptor are linked on the cell membrane of human thymocytes and PBL, but they are independent from the PHA receptor.

Key Words: Thymus; thymocyte, phytohemagglutinin. Antigen; thymus. Receptor; thymocyte, phytohemagglutinin

Introduction

Both phytohemagglutinin (PHA) response and sheep red blood cell (SRBC) rosette formation of human lymphocytes have been regarded as thymus derived (T)-lymphocyte markers. Anti-human thymus serum (AHTS) directed against thymus specific antigens can also be used for the identification of thymocytes or T-lymphocytes. Some reports suggest that the thymus specific antigen in some way effects on the responsiveness to PHA[1-3] and the SRBC rosette formation[4-8] of the human thymocyte and peripheral blood lymphocyte (PBL). These data, however, are discordant, and more systematic approaches to the problem are required.

In the present study, we have attempted to clarify the differences of the *in vitro* responses of human thymocytes and PBL by comparing their responsiveness to PHA, their SRBC rosette formation and their cytotoxicity

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to AHTS. We have also studied the mutual relationships of the receptor sites on the cell surface by competitive binding studies with PHA, SRBC and AHTS.

Materials and Methods

Lymphocyte suspension

Biopsy specimens of human thymus were obtained during open heart surgery from thirty-four patients with cardiac diseases, ranging in age from 3 months to 50 years. Cell suspension was prepared by mincing the tissue on a 60 gauge stainless-steel mesh in chilled Hanks' balanced salt solution, and then passing it through a tetron column (Termo Co, Ltd). The cells, thus obtained, were washed, counted and resuspended at the desired concentration.

Peripheral blood lymphocytes (PBL) were prepared from heparinized venous blood of the patients from whom thymus specimens were obtained and of healthy donors, using the Ficoll-Isopaque (Lymphoprep Nyeggard & Co. As., Oslo) density gradient method of Böyum[9] modified by Kato[10]. The preparations contained more than 90% lymphocytes while the other 10% or less consisting mainly of monocytes and granulocytes.

Anti-human thymus serum

The anti-human thymus serum (AHTS) was obtained from rabbits, as previously described[11,12]. Briefly, rabbits, 2 months of age, were given subcutaneous injections of 5×10^7 thymocytes emulsified in Freund's complete adjuvant (FCA) three times at 1 week intervals. One month after the third injection, intravenous booster injection of 5×10^7 thymocytes without FCA were done once a week for two weeks. The rabbits were bled to death one week after confirming a high antibody titer in the rabbit serum by the gel diffusion method of Ouchterlony[13]. The antisera were repeatedly absorbed with an extract of human liver homogenates and then with human red blood cells, each for 1 h at 4°C. The absorbents were then removed following centrifugation at 3,000 rpm for 20 min. The sera were stored at −20°C until they were used.

Lymphocyte culture

The lymphocytes (10^6/ml) from the thymus or peripheral blood were cultured in TC-199 medium containing 20% calf serum, and PHA-P (Difco, 10μg/ml), at 37°C for 72h in a humidified atmosphere of 5% CO_2−95% air. The percentages of blastoid cells from cultured cells were evaluated as a index of blastogenesis according to the method previously described[10].

A microculture method was also performed at 37°C for 72 h on plastic test plates (Falcon Plastics) containing 2×10^5 cells in 0.2ml TC-199 with 20% calf serum. For detection of DNA synthesis of cultured cells, 0.02 ml of 5 Ci/m mole ^{3}H-TdR (The Radiochemical Centre, Amersham) was added to each well in 0.2 ml of the culture medium 16h prior to culture termination. The cells were harvested with a multiple cell harvester (Minimash AM78, Dynatech Co.), and the radioactivity was counted with a Packard liquid scintillation counter.

SRBC rosette test

The rosette test was performed by the method of Yata et al[14] modified by Kato et al[15]. Equal volumes (0.1 ml) of lymphocyte suspension (5×10^6/ml) and SRBC (2×10^8/ml) were mixed in short test tubes. Mixed cell suspensions were sedimented by centrifugation and left standing at 4°C for 16h. The cell pellets were gently resuspended and examined under a microscope at 400× magnification. The number of SRBC rosette forming cells (three or more attached SRBCs) was divided by the total number of lymphocytes present.

Cytotoxicity test

The cytotoxicity test was performed by the trypan blue dye exclusion method of Schlesinger[16]. Each 0.05 ml of lymphocyte suspension (5×10^6/ml) and AHTS were mixed and incubated at 37°C for 10 min. The mixture was further incubated with 0.01 ml of guinea pig serum, a source of complement (C), at 37°C for 30 min. 500 cells were counted in each experiment. The rate of killed (stained) cells was calculated using the following formula. Cytotoxic index (%)

$$=\frac{\%\text{ experimental cells killed}-\%\text{ control cells killed}}{100-\%\text{ control cells killed}}\times100$$

Results

PHA response

The percentages of PHA blastoid cells in thymocyte cultures were markedly lower than

Table 1 PHA Blastoid Transformation, SRBC Rosette Formation and AHTS Cytotoxicity of Human Lymphocytes from Thymus and Peripheral Blood

Source of lymphocytes	Expt. no.	PHA blastoid cells (%)	SRBC rosette forming cells (%)	Cytotoxic index (%)
Thymus (n=5)	Th 13	2.0	95.7	90.7
	15	3.1	97.0	71.1
	16	4.1	91.2	85.4
	18	7.7	86.1	94.4
	19	4.2	90.8	98.5
		4.2±1.0	92.2±1.9	88.0±4.7
Peripheral blood (n=5)	Bl 43	65.1	74.8	42.8
	45	54.5	65.1	36.5
	46	49.1	63.4	59.1
	47	55.6	60.1	49.5
	50	60.8	59.9	48.6
		57.1±2.7	64.7±2.7	47.3±3.8

Mean±SE

those in PBL cultures in all cases (Table 1). These findings were also confirmed on both thymocytes and PBL obtained from the same donor in 3 cases (thymocytes: 1.9%, 1.1%, 2.0% ; PBL: 58.4%, 51.6%, 61.6%). The degree of DNA synthesis of thymocytes (1,548 cpm, n=3) in PHA cultures, as estimated by ^{3}H-TdR incorporation, was also lower than that of PBL (31,915 cpm, n=4). Thus, the degrees of in vitro PHA responses of thymocytes and PBL measured by the morphological assessment correlated well with those of cells measured by ^{3}H-TdR incorporation.

SRBC rosette formation

Many SRBCs adhered to thymocytes and PBL under incubation at 4°C for 16h. The percentages of SRBC rosette forming cells in the thymus were considerably higher than those in PBL (Table 1).

The SRBC rosette test was performed on lymphocytes treated with PHA to determine whether or not the cell surface receptor for SRBC is affected by PHA treatment. Lymphocytes from thymus or peripheral blood were incubated with PHA (10μg/ml) for 30 min, washed three times with Hanks' balanced salt solution and then subjected to the SRBC rosette test. The degrees of SRBC rosette formation of PHA treated lymphocytes (thymus 90.9%, blood 67.5%) were similar to those of the PHA nontreated control (thymus 91.9%, blood 68.9%). This shows that the binding of PHA to the cell surface has no effect on SRBC rosette formation of lymphocytes.

Cytotoxicity of AHTS to lymphocytes

The AHTS alone showed no in vitro cytotoxic effect on thymocytes and PBL. However, the AHTS showed high cytotoxicity to thymocytes when it was added with guinea pig serum, as a source of complement. Aliquots of AHTS were estimated for cytotoxic index in both thymocytes and PBL from each of two donors (Fig. 1). The dilution of AHTS causing 50% lysis of thymocytes was over 3 times higher than that required to kill a comparable number of PBL. The AHTS of 5 or 10-fold dilution showed about 50% cytotoxicity to PBL. Such a higher cytotoxicity of AHTS (5-fold dilution) of thymocytes as compared to PBL was observed in all cases as shown in Table 1.

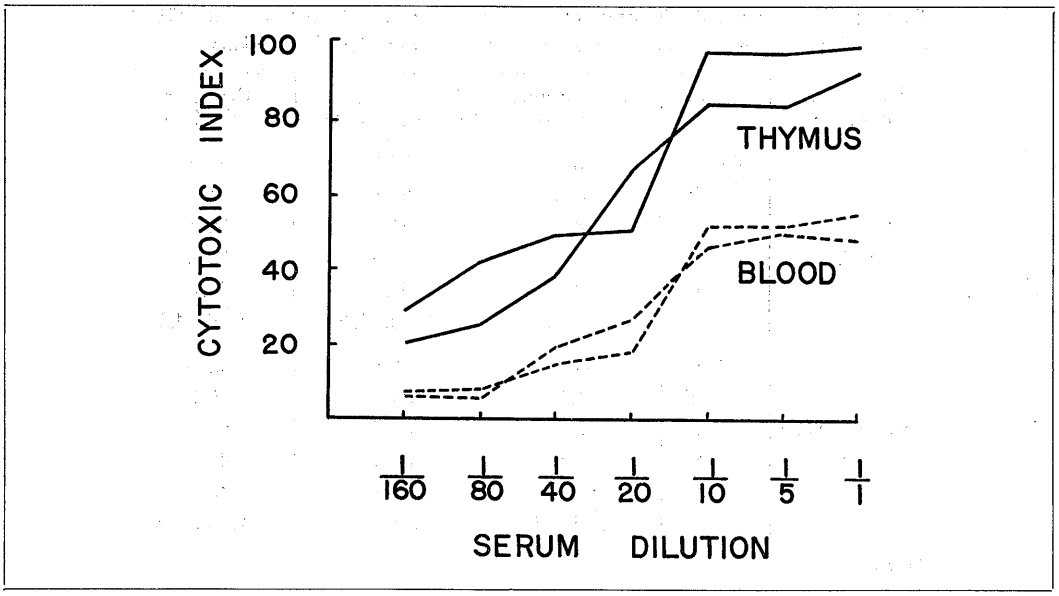

Fig. 1 Cytotoxic index of anti-human thymus serum (AHTS) in both thymocytes and peripheral blood lymphocytes (PBL) from each of two donors.

Inhibition of PHA response and SRBC rosette formation by AHTS

The AHTS was added to lymphocyte suspension for PHA culture or SRBC rosette test to investigate the in vitro effect of AHTS on PHA response or SRBC rosette formation. AHTS and guinea pig serum (complement) or normal rabbit serum were simultaneously added to PBL suspension with PHA and then the lymphocytes were cultured for 72h. The PHA response of PBL was greatly suppressed by treatment with AHTS and complement, whereas no significant reductions in PHA responses of PBL were noted after the addition of AHTS, guinea pig serum or normal rabbit serum alone (Table 2).

On the other hand, SRBC rosette formation of thymocytes and PBL were almost completely inhibited by treatment with AHTS alone (Table 3-Expt. 1). It was found that degree of the inhibition depended on the concentration of AHTS and the time at which AHTS was added to the mixture of SRBCs and lymphocytes.

Table 2 Effects of AHTS on Human PBL Responses to PHA

Groups	Blastoid cells (%)	Incorporation of ^{3}H-TdR (cpm)/ 2×10^5 cells
PHA+AHTS+C	6.5	2950
PHA+AHTS	69.9	20855
PHA+C	62.5	18740
PHA+NRS	60.1	17650
PHA	65.4	19864

Peripheral blood lymphocytes (PBL) were cultured with PHA (10μg/ml) for 72h. AHTS: Anti-human thymus serum, C: Guinea pig serum (Complement), NRS: Normal rabbit serum. PHA was simultaneously added with AHTS and C, AHTS, C and NRS alone.

Cytotoxicity of AHTS to PHA treated cells and SRBC rosette forming cells

The cytotoxicity test was performed on thymocytes and PBL previously treated with PHA (10μg/ml) or SRBCs to investigate whether or not the cytotoxicity of AHTS to lymphocytes changes after such treatments of

Table 3 Effects of AHTS and/or Guinea Pig Serum on SRBC Rosette Formation of Human Thymocytes and PBL

Experiments groups		SRBC rosette forming cells (%)	
		Thymus	Peripheral blood
Expt. 1.	SRBC+AHTS	1.2	2.4
	SRBC−30min+AHTS	41.5	39.6
	SRBC	94.6	66.8
Expt. 2.	AHTS+C−30min+SRBC	2.5	3.9
	SRBC	98.5	65.7

AHTS: Anti-human serum, SRBC: Sheep red blood cell, C: Guinea pig serum (Complement), PBL: Peripheral blood lymphocytes,
Expt. 1 SRBC+AHTS: AHTS was added with SRBC simultaneously. SRBC-30 min+AHTS: AHTS was added 30 min after SRBC rosette test.
Expt. 2 AHTS+C-30 min+SRBC: AHTS and C were added 30 min. before SRBC rosette test.

the cells. Lymphocytes from thymus or peripheral blood of a single donor were allowed to incubate with or without PHA for 30 min and then the cytotoxicity test was made using AHTS. The cytotoxic index of PHA treated cells (thymus 94.1%, blood 51.8%) was almost the same as that of PHA non-treated cells (thymus 93.8%, blood 52.9%). This shows that the binding of PHA to the cell surface also has no effect on the cytotoxicity of AHTS to the lymphocytes.

High viability of thymocytes treated with SRBCs at 4°C for 16 h was also observed. The cytotoxicity test was then performed on thymocytes previously subjected to SRBC rosette test. Almost all SRBC rosette forming cells among the thymocytes were stained with trypan blue and their SRBC rosettes disintegrated gradually.

The SRBC rosette test was also performed on thymocytes and PBL subjected to the cytotoxicity test to discover the relationships between populations of SRBC rosette forming cells and the cells which have AHTS binding antigen. Lymphocytes were incubated with AHTS and complement at 37°C for 30 min, washed three times with Hanks' balanced salt solution and then subjected to the SRBC rosette test. The number of SRBC rosette forming cells among these lymphocytes markedly decreased after the cytotoxicitytest (Table 3-Expt. 2).

Discussion

It was found that the responsiveness of thymocytes to PHA was markedly lower than that of PBL (Table 1). In the previous study[15] we found that there was no significant difference between human thymocytes and PBL in their ability to bind PHA on their cell surface. This suggests that the factors other than the binding of this lectin on the cell surface may be required for the induction of blastogenesis.

Claman[17] and Winkelstein and Craddock[18] suggest from their data that thymus contains two populations of lymphocytes: a major component which shows autonomous and unsustained proliferative activity and does not respond to PHA, and probably a minor cellular component which transforms and proliferates in response to PHA. The second cell group appears to respond identically with some of the PBL. This suggestion

seems to offer an explanation for the difference in response of thymocytes and PBL to PHA.

The ratio of SRBC rosette forming cells to all the thymocytes counted was more than 90%, while the ratio of these cells to PBL about 65%. These findings are in sharp contrast to those seen in the PHA response.

The SRBC rosette formation of thymocytes and PBL was not disturbed by their preincubation with PHA for 30 min. Thus, the receptor sites for SRBC and PHA seems to be mutually independent on their cell surface.

AHTS used in the present study killed the majority of human thymocytes and some of the PBL in the presence of complement. The number of the killed thymocytes almost corresponded to that of SRBC rosette forming cells estimated by rosette test. A similar tendency was seen in PBL, although there was some discordance between the numbers estimated by cytotoxicity and rosette test (Table 1). These findings show that our AHTS is specific for human thymocytes and some of the PBL. Our AHTS, therefore, will be useful as a marker of thymocytes and their descendant T-cells in further experiments.

As summarized in Table 2, the PHA response of PBL was not suppressed by AHTS alone; however, the response was markedly inhibited by the combination of AHTS and complement. This almost agrees with the findings of some investigators[1-3]. On the other hand, there was no difference in the cytotoxic activity of AHTS to the thymocytes and the PBL regardless of whether or not these cells were preincubated with PHA for 30 min. This suggests that the AHTS binding antigen (thymus specific antigen) and the PHA receptor may constitute distinct entities on cell surfaces.

The SRBC rosette formation of thymocytes and PBL was inhibited by AHTS alone. Therefore, this inhibition may not be caused by lysis or death of cells but by direct action of AHTS on the SRBC receptor on the cell surface. Our data, in combination with the observation of other investigators [4-6], suggest that the AHTS binding antigen (thymus specific antigen) and SRBC share the common receptor site on the cell membrane of human thymocytes and PBL.

SRBC rosette forming cells of thymocytes and PBL were considerably decreased after the cytotoxicity test with AHTS. These suggest that the population of SRBC rosette forming cells may be identical with that of cells killed by the combination of AHTS and complement.

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