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DIFFERENTIATION OF HUMAN BONE MARROW CELLS INTO T LYMPHOCYTES BY *IN VITRO* INCUBATION WITH THYMIC EXTRACTS

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SUMMARY

Extracts of human or calf thymus influence differentiation of human bone marrow cells *in vitro*. Incubation of a putative stem cell fraction of human marrow with extracts of thymus for a period of 2 hr led to the appearance of lymphocytes with surface antigens recognized by a highly specific anti-T-cell antiserum. To a lesser degree, development of lymphocytes having the capacity to form rosettes with sheep erythrocytes was observed. Treatment of the so-called stem cell fraction with thymic extracts did not yield cells responsive to mitogenic influences of phytohaemagglutinin or allogeneic cells. This model should permit further analyses of immunodeficiency diseases and provide a useful technique for purification and analysis of thymus extracts active with human haematopoietic cells.

INTRODUCTION

The role of the thymus in immune development has been established by studies of rabbits, mice (Martinez *et al.*, 1962; Miller, 1961) and humans (DiGeorge, 1968; Good, Biggar & Park, 1971) deprived of thymus, especially in the neonatal period. How this lymphoepithelial organ exerts its influence has been a matter of some controversy even though it was recognized that humoral and cellular theories are not mutually exclusive. Over the past decade numerous studies, including diffusion chamber experiments, were strongly suggestive of the presence in the thymus of a diffusible humoral factor able to restore or expand several immune responses in thymectomized animals (Osoba & Miller, 1964; Stutman & Good, 1973). Although many data have accumulated in evaluating a variety of thymus 'extracts', 'factors' or 'hormones', the mode of action of these substances remains poorly understood and both purification and elucidation of the mechanism of action still remain before us because heretofore we have not had a simple, reproducible *in vitro* assay for this activity.

Recent investigations (Bach *et al.*, 1971; Komuro & Boyse, 1973) have shown that a fraction of mouse bone marrow or spleen cells can acquire T-cell characteristics after short-term

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incubation *in vitro* with certain thymic extracts. This important finding may shed light on the analysis of thymic factors as well as fundamental issues in cell differentiation. If such results could be extended to man, great benefit for the understanding of the lymphoid cell populations and treatment of many pathological states, including immunodeficiency diseases, might be forthcoming.

In the present study we have attempted to determine whether heteroantigens specific for T lymphocytes will appear at the surface of human bone marrow cells after *in vitro* incubation with thymic extracts. A heterologous anti-human T-cell serum has been prepared and its specificity verified (Touraine *et al.*, 1973a,c). In addition, the formation of spontaneous rosettes with sheep erythrocytes, which is characteristic of human thymocytes and putative T lymphocytes (Jondal, Holm & Wigzell, 1972; Wybran, Carr & Fudenberg, 1972), has been studied. Human bone marrow cells were fractionated on a density gradient in five layers, one of which is known to contain a higher concentration of stem cells and presumably of T-cell precursors (Dicke, Lina & Van Bekkum, 1970). In this layer, which initially contained a small percentage of T lymphocytes by either of the above criteria, a significant proportion of cells acquired the heteroantigens recognized by our antiserum, under the influence of extracts from both human and calf thymus. Further, the number of rosette-forming cells was also slightly increased after this treatment. On the other hand, the responsiveness to phytohaemagglutinin or to allogeneic cells, which is also characteristic of T cells but possibly related to more advanced stages of differentiation, has not yet been induced in our experiments.

MATERIALS AND METHODS

Thymus and spleen extracts

Human thymus and spleen were obtained from young children undergoing cardiac surgery and from autopsy (a few hours after accidental death). Calf thymus were purchased from a local slaughter house and obtained in the frozen state. Tissue extracts were prepared according to the method of Goldstein *et al.* (1972). Briefly, tissues were homogenized in 0·15 M NaCl and sonicated for 10 sec at 4°C. The homogenate was filtered and purified by several centrifugations. The supernatant solution obtained was heated at 80°C for 15 min, centrifuged again and filtered through a 0·45 μm millipore membrane (fraction 2). The acetone-insoluble precipitate was redissolved in 0·1 M phosphate buffer, pH 7·2 (fraction 3). Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Separation of bone marrow cells

Fractionation of human bone marrow cells was performed by the technique of Dicke *et al.* (1970). Bone marrow was aspirated from a 16-year-old cadaver a few hours after accidental death (donor A) and from an adult volunteer in good health (donor B). After centrifugation at 200 *g* for 5 min, the buffy coat was removed and resuspended in a 17% solution of bovine serum albumin (BSA Pentex, 35% solution, Miles Laboratories Incorporated, Kankakee, Illinois) prepared in tissue culture medium RPMI 1640 (Grand Island Biological Company, Grand Island, New York). This cell suspension was carefully layered upon a discontinuous albumin density gradient made of the following dilutions of BSA in RPMI 1640: 19%, 21%, 23%, 25% and 27%. Following centrifugation (750 *g*, 30 min, 10°C), the cells appearing at the interface between the albumin layers were removed and are called layers I (at the 17–19%

interface), II (19–21%), III (21–23%), IV (23–25%) and V (25–27%). The cell yield of layer III was between 1 and 8% of the total number of nucleated cells in the starting suspension. Cells from each layer were washed in Hanks' balanced salt solution (BSS) and the cell suspensions were adjusted to a concentration of 2×10^6 cells/ml. Viability was checked by Trypan Blue exclusion method and found to be above 95%.

Incubation of bone marrow cells with tissue extracts

Equal volumes of cell suspension from each layer and of tissue extract solution were mixed together and incubated for 2 hr (for cytotoxicity and rosette assay) or for 3 hr (for lymphocyte cultures) at 37°C, in a humidified atmosphere of 5% CO_2 and 95% air. Cells were washed in Hanks' BSS. Viability was then greater than 90% in every sample.

Anti-human T-cell serum (ATCS)

The preparation and the specificity for T lymphocytes versus B lymphocytes and monocytes, as well as some applications of this antiserum have been previously reported (Touraine *et al.*, 1973a, b and c). ATCS was raised in rabbits by injecting peripheral blood lymphocytes (PBL) from a patient with X-linked agammaglobulinaemia. The serum was rendered specific for T cells by several absorptions especially with B lymphoblasts from a cultured cell line, chronic lymphatic leukaemia cells and adherent cells. The surface antigens recognized by this antiserum have been called human-specific T-lymphocyte antigens (HTLA).

Two-stage microlymphocytotoxicity test (Amos *et al.*, 1969)

A suspension of 2×10^6 cells per ml was incubated with ATCS in the well of microtest tissue culture plate (Falcon Plastics 3034, Los Angeles, California), then washed, and rabbit complement (C) was added. Every test was done in duplicate. The Trypan Blue exclusion method was used to evaluate the percentage of live and dead cells. Three hundred cells were counted in each well. The cytotoxic index (or percentage of HTLA-positive cells) was calculated as follows (Klein & Klein, 1964):

$$\text{cytotoxic index} = \frac{\text{percentage alive with normal rabbit serum and C} - \text{percentage alive with ATCS and C}}{\text{percentage alive with normal rabbit serum and C}}.$$

'Non-immune' rosette formation

Human rosette-forming cells (RFC) were quantified with a technique previously described (Kiszkiss *et al.*, 1973) using untreated sheep red blood cells (SRBC) in the presence of foetal calf serum.

Lymphocyte cultures

To each tube a suspension of $0{\cdot}4 \times 10^6$ cells in 1·0 ml of Eagle's minimum essential medium supplemented with L-glutamine (2 mM/l), heat-inactivated AB serum (10%), penicillin G (50 u/ml), and streptomycin (50 μg/ml) was added. Cultures were done in triplicate. Phytohaemagglutinin (PHA MR68/69 Wellcome Laboratories, Research Triangle Park, North Carolina) was added at the optimal concentration of 1·5 μg per tube. For mixed leucocyte cultures (MLC), mitomycin-treated PBL from an unrelated donor were added at the concentration of $0{\cdot}4 \times 10^6$ cells per tube. Cultures with PHA were carried out for 3 days and MLC for 5 days, in a humidified atmosphere of 5% CO_2 and 95% air. Twenty-four hours

before the harvest, 1 μCi of [^{3}H]methyl thymidine (6·7 Ci/ mmole New England Nuclear, Boston, Massachusetts) was introduced in each tube. At the end of the culture, cold thymidine and saline were added; the cells were subsequently treated with 10% trichloracetic acid, 80% alcohol and solubilized in NCS (tissue solubilizer, Amersham/Searle). Radioactivity was determined in a Packard liquid scintillation spectrometer in the usual way.

RESULTS

'Induction' of HTLA-positive phenotype

In bone marrow layers I, II, IV and V from both donors, the percentage of ATCS-sensitive cells was low and was not significantly modified by a 2-hr incubation with thymic extracts. By contrast, in layer III, an increased proportion of cells bearing surface HTLA was noted after such an incubation (Table 1). No major difference in the capacity to induce HTLA was

TABLE 1. Percentage of T lymphocytes (HTLA-positive cells) after *in vitro* incubation of human bone marrow cells with thymic or splenic extracts

Bone marrow cells	Cytotoxic index after incubation with extracts						
	Human thymus		Calf thymus		Human spleen		Medium
	F2*	F3*	F2	F3	F2	F3	Control†
Donor A							
Layer I	4		4		2		4
Layer II	9		7		6		5
Layer III	36		44		15		10
Layer IV	2		5		5		4
Layer V	1		2		2		1
Donor B							
Layer I		10		7		5	6
Layer II		9		8		5	4
Layer III	29	30	19	27	10	7	4
Layer IV		8		7		8	6
Layer V		4		3		3	4

* F2 and F3 = fractions 2 and 3.

† Control = medium RPMI 1640.

Bone marrow cells from each layer were incubated for 2 hr at 37°C with tissue extracts. The presence of surface HTLA was then assayed in cytotoxicity with ATCS and rabbit complement.

Human thymus extracts F2 and F3 were prepared from surgical specimens of 33 and 1-year-old children respectively (protein content of F2 = 1·8 mg/ml and F3 = 0·8 mg/ml). Calf thymus were obtained frozen from a slaughter house (protein content of F2 = 1·0 mg/ml and F3 = 1·9 mg/ml). Human spleens were obtained from autopsy and cell viability was 84–94% (protein content of F2 = 1·7 mg/ml and F3 = 0·6 mg/ml).

seen when human and calf thymus extracts were compared. Nor was a difference between fraction 2 and the more purified fraction 3 noted. Using a formula (Klein & Klein, 1964; Komuro & Boyse, 1973) which gives the percentage of cells converted from HTLA$^-$ to HTLA$^+$ phenotype, it was calculated that the four preparations of thymic extracts tested

induced between 16 and 38% of cells from layer III to become $HTLA^+$ (assuming no proliferation of a cell population during the short-term incubation).

Although much less effective than thymus extracts, the preparation of spleen extract increased slightly the proportion of $HTLA^+$ cells in bone marrow layer III from both donors (Table 1).

The absence of HTLA in the thymic extracts was verified by absorption experiments. ATCS was incubated with the various preparations of extracts and its anti-T-lymphocyte activity was then assayed on normal PBL. The cytotoxic index was not at all decreased by this procedure, which eliminates the possibility of a significant amount of free HTLA in the preparations tested.

'Induction' of receptors for SRBC

Much evidence has linked human RFC to the T-lymphocyte population (Jondal *et al.*, 1972; Wybran *et al.*, 1972; Kiszkiss *et al.*, 1973) and, although the precise nature of the lymphocyte receptor for SRBC is still unknown, the technique of 'spontaneous' rosette formation is now widely used as a method for quantitation of human T cells (Wybran *et al.*, 1972; Kiszkiss *et al.*, 1973). We investigated the appearance of this T-cell characteristic after a 2-hr incubation with tissue extracts. The proportion of RFC was slightly increased (from 1–2% to 4–7%) after incubation of layer III cells from both donors with human and calf thymic extracts. However, this percentage of RFC remained much lower than that representing $HTLA^+$ cells.

Responsiveness to PHA and allogeneic cells

The ability of bone marrow cells from donor B to be stimulated by PHA or allogeneic PBL was studied. In both systems a virtual absence of response was observed in cells from each layer and in no case did we find an increased incorporation of [^{3}H]thymidine in cells which had been initially subjected to a 3-hr incubation with thymic or splenic extracts. These extracts tended to be more inhibitory than stimulatory on cells cultured alone as well as cells cultured in presence of PHA or in one-way MLC.

Effect of tissue extracts on PBL from a patient with severe combined immunodeficiency

The same studies as above were carried out on PBL from an infant with severe combined immunodeficiency. This patient had a relatively moderate lymphopenia (1000 lymphocytes/mm^3) and, among his PBL, 30% possessed surface characteristics of T cells (30% $HTLA^+$, 29% RFC). No capacity to respond with proliferation to PHA was present and little response occurred with allogeneic cells. After *in vitro* incubation with thymic extracts the percentage of cells with surface HTLA and with receptors for SRBC was not increased, suggesting that there was not an abnormally high percentage of T-cell precursors in peripheral blood. Yet in these experiments we did not fractionate PBL, so that a percentage of precursors equal to that found in unfractionated normal bone marrow would not have been analysed. Furthermore, no appearance or increase in the response to PHA and allogeneic cells was observed. Thus the thymic extracts were unable, in this circumstance, to induce the transformation of lymphocytes into cells with those characteristics by which the more mature immunocompetent T lymphocytes are recognized.

DISCUSSION

The changes which are induced in human bone marrow after *in vitro* incubation with thymic extracts can be interpreted in several ways. The proliferation of the small, but existent, mature T-lymphocyte population present in the bone marrow (and perhaps increased by a minimal contamination by peripheral blood) is very unlikely in view of the short period of incubation and of the absence of certain characteristics of mature T cells (response to PHA and allogeneic cells). A direct alteration of the cell surface is not excluded but would not be a mere fixation of antigens because thymic extracts were shown to lack HTLA. A more probable explanation is that we have achieved induction of some stages of differentiation through a derepression or activation of the T-cell precursor. This hypothesis is reinforced by the results of Komuro & Boyse (1973) showing that mouse spleen cells incubated with thymic extracts express only the Thy-1 or TL phenotype for which they are genetically determined. Furthermore, some antigens characteristic of T-cell differentiation can similarly appear on T-cell precursors when incubated with a variety of agents such as endotoxin, poly A:U, and cyclic AMP (Sheid *et al.*, 1973).

The observed difference between the percentage of cells converted to $HTLA^+$ phenotype and of those which have acquired the capacity to form rosettes may have several explanations: the receptors for SRBC might appear more slowly or in an insufficient concentration on the cell surface, these receptors might also be related to a slightly more advanced stage of differentiation than is associated with appearance of heteroantigens recognizable by our anti-T-cell serum. More mature T cells are probably needed for the response to allogeneic cells and even more to phytohaemagglutinin. This may be the reason why we did not observe the acquisition of the cell responses to stimulation after incubation with thymic extracts. It is still unclear whether our thymic extracts lack the ability to induce this advanced T-cell differentiation. Other factors, possibly found in peripheral lymphoid tissues, could be required for this later step in differentiation. Further it could be that technical factors, for instance a too short period of incubation, were responsible for this perhaps abortive differentiation. A recent report (Woody *et al.*, 1973) suggests that the responsiveness of mouse bone marrow cells to PHA-P can be increased by prior incubation (for more than 2 hr) with calf thymus extracts. In another study (Wilson & Bhaumick, 1973) it was shown that some thymic extracts slightly stimulate [^{3}H]thymidine incorporation into DNA of mouse spleen cells when injected *in vivo*.

If the *in vitro* induction of antigens specific for T cells is related to the *in vivo* mode of action of thymic factors, the method and findings presently described could be of value in comparing various preparations, e.g. preparations from different species. Our results show that calf thymus extracts are effective in inducing antigenic changes on human T-cell precursors. The method would also be of great value in purification of active thymic factors and possibly in development of accurate radioimmunoassays.

The present study also provides some information on the method of bone marrow cell fractionation. The technique used certainly increased the concentration of T-cell precursors in one layer and also the ratio of precursor cells to mature cells. Yet this layer contained a few cells with surface characteristics of T lymphocytes even though these cells seemed less responsive to PHA than did unfractionated bone marrow cells (Dicke *et al.*, 1970). Perhaps these cells are the equivalent of the post-thymic cells or T_1 cells of the mouse. The presence in layer III of a few T cells, at least in early stages of differentiation, was ob-

served even in cases when the cell yield of this fraction was low (1–2% of the initial number of nucleated cells) and the contamination by peripheral blood quite minimal.

This new approach to the study of human immunodeficiencies may help to discriminate between genuine deficiency of stem cells or precursor cells and deficiency in inducers of early cell differentiation. In this regard, it will be of interest to analyse the inductive capacity of thymic extracts such as those used in this study on bone marrow cells from patients with the different forms of immunodeficiencies, especially those in which numbers and functions of T cells seem diminished.

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