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IN VITRO DIFFERENTIATION OF HUMAN MARROW CELLS INTO T LYMPHOCYTES BY THYMIC EXTRACTS USING THE ROSETTE TECHNIQUE

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SUMMARY

The manner by which human and calf thymic extracts induce precursor cells of human marrow to differentiate *in vitro* into T lymphocytes has been studied using as a T-cell marker the spontaneous rosette formation technique of human T lymphocytes with sheep erythrocytes (E rosette). These findings confirm previous observations made in the study of the same process using a different T-cell marker, specific antigenicity recognizable by a heterologous anti-human T-cell serum in a microcytotoxicity test. The number of cells revealing evidence of differentiation demonstrated by the E rosette formation technique is smaller than that obtained with the anti-human T-cell serum, indicating perhaps that a different stage of maturation of T lymphocytes is recognized by the antiserum from the one detected by spontaneous rosette formation. Based on the effects of specific inhibitors of nucleic acids and protein synthesis, it can be concluded that these thymic extracts ultimately act by influences exerted in the cell nucleus and that RNA and protein synthesis are required for the differentiation of precursor cells into T lymphocytes induced by thymic extracts. In addition, continued protein synthesis appears to be required for maintenance of receptors for sheep erythrocytes on the cell surface.

INTRODUCTION

The manner by which the thymus induces precursor cells to differentiate into T lymphocytes is not well understood. Recent investigations in mouse (Komuro & Boyse, 1973) and in man (Touraine *et al.*, 1974b) have demonstrated that a certain population of precursor cells from spleen and bone marrow can acquire T-lymphocyte characteristics *in vitro* after a short incubation in the presence of thymic extracts. The extent of differentiation was assessed, using specific surface antigenicities as T-cell markers. In man, T lymphocytes were recog-

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nized by a heterologous anti-human T-cell serum (ATCS), the specificity of which had been carefully determined (Touraine *et al.*, 1974a).

In this study, the differentiative influence of thymic peptides *in vitro* was investigated further in man using another T-cell marker, the sheep red cell rosette (E rosette) (Bach *et al.*, 1969; Wybran & Fudenberg, 1971; Bentwich *et al.*, 1973). Using the ability of human T lymphocytes to form spontaneous rosettes with sheep red blood cells (SRBC), differentiation of marrow cells into T lymphocytes by thymic extracts could also be detected by the rosette technique (Incefy, Touraine & Good, 1974). In addition, the mode of action of thymic peptides on precursor cells was investigated at the molecular level using specific inhibitors of nucleic acid and protein synthesis. The results obtained indicate a requirement for RNA and protein synthesis in the mechanism of differentiation of human T lymphocytes by thymic extracts.

MATERIALS AND METHODS

Thymus and spleen extracts

Human thymus and spleen were obtained from young children undergoing cardiac surgery and at autopsy, a few hours after accidental death. Calf thymus was purchased frozen from a local slaughterhouse. Tissue extracts were prepared according to the method of Goldstein *et al.* (1972) and fractions 2 and 3 were used for these studies. Protein content of the extracts was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Precursor cell isolation

Bone marrow was obtained from normal human volunteers. Small volumes were aspirated into heparinized glass syringes from various points of the iliac crest, totalling up to 6–10 ml. Marrow cells were initially isolated from erythrocytes by Ficoll–Hypaque gradient centrifugation (400 *g* for 30 min at 23°C). Cells floating at the interface were collected and washed once with culture medium RPMI-1640 (Gibco, Grand Island, New York) containing 1% solution of antibiotics (penicillin, 50 u/ml and streptomycin, 50 μg/ml). The isolation of marrow precursor cells was performed by discontinuous density gradient centrifugation according to the procedures of Dicke, Lina & Bekkum (1970) or Tak Yan Yu *et al.* (1973) with slight modifications. Gradients were made up with bovine serum albumin (BSA) of the following concentrations: 17%, 19%, 21%, 23%, 25% and 27% prepared from 35% BSA (Pentex, Miles Laboratory, Incorporated, Kankakee, Illinois) with RPMI-1640 or Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) of the following concentrations: 13%, 15%, 17%, 20% and 25% prepared (w/w) with 0·1 M sodium phosphate buffer, pH 7·4. Marrow cells were layered on top of the BSA gradient resuspended in the 17% BSA and on top of the Ficoll gradient resuspended in RPMI-1640. Both gradients were centrifuged at 10°C for 30 min, the BSA gradient in a PRJ-International centrifuge at 840 *g* and the Ficoll in the SW40 rotor of the Model L5-65 Beckman Ultracentrifuge at 22,000 *g*.

Following centrifugation, five cell layers were obtained with both gradients, layer I representing the first cell layer at the top, for example the interface between 17% and 19% BSA for the BSA gradient and the interface between RPMI-1640 and 13% Ficoll for the Ficoll gradient. Cells from each layer were washed twice with RPMI-1640 prior to incubation with thymic or splenic extracts.

Density measurements of the various solutions used in the preparation of gradients were performed in a Gay–Lussac pycnometer (Thomas, Philadelphia, Pennsylvania). Refractive indices were determined in an Abbe refractometer (Bausch & Lomb, Rochester, New York) using 1-bromonaphthalene as a standard.

Incubation of cells with extracts

Cells from each layer were resuspended to a concentration of 1×10^7 cells/ml or slightly less in RPMI-1640 containing 10% AB^+ serum and antibiotics and incubated with equal volumes of thymic and splenic extracts for 2–14 hr in a humidified 5% CO_2–95% air incubator. After incubation, cells were washed twice in RPMI-1640 prior to formation of rosettes with sheep erythrocytes. Viability, assessed by Trypan Blue exclusion, was greater than 90% in each sample.

'Non-immune' rosette formation

Human rosette-forming cells were detected by a procedure similar to the one described by Bentwich *et al.* (1973). In brief, 50 μl of cells (2×10^6 cells/ml or smaller) were incubated for 5 min at 37°C in a water bath with 50 μl of a 0·5% solution of SRBC and 15 μl of AB^+ serum, decomplemented and absorbed with equal volume of packed SRBC. Immediately after incubation, tubes were centrifuged at room temperature for 5 min at 50 ***g*** and kept for several hours at 4°C before counting.

Inhibitors

Actinomycin D was purchased from Nutritional Biochemical Corporation, Cleveland; α-amanitin was from Boehringer, New York; and puromycin from Sigma Chemicals, St Louis, Missouri. Inhibitors were diluted with RPMI-1640 to the desired concentrations and added in 5–10-μl volumes to control and extract-treated cells at the beginning of the incubation period.

RESULTS

Fractionation of bone marrow cells

Bone marrow aspirated from ten normal healthy volunteers, aged 20–35 years, contained an average $1{\cdot}46 \times 10^8$ nucleated cells ranging from 0·3 to $2{\cdot}4 \times 10^8$ cells. Recovery of cells after discontinuous gradient centrifugation and two washings with RPMI-1640 gave a mean of 48% for the BSA gradient and 54% for the Ficoll; however, excellent recovery can be obtained, as high as 86%, if extreme care is taken and if no small clots are present in the marrow aspirates. Some of the characteristics of the gradients used are shown in Fig. 1. The distribution of cells separated by these gradients ranged in densities between 1·045 and 1·085. Precursor cells able to be differentiated *in vitro* by human thymic extracts are found predominantly in layer III of the BSA gradient, whereas they are found mostly in layer I and II of the Ficoll gradient. These precursor cells are therefore found in the density range of 1·055–1·065. The relative distribution of the total number of marrow cells isolated by these two types of gradient is shown in Fig. 2. The apparent difference observed between the two graphs is due to the different densities of the lower part of the gradient, layer V of the BSA gradient corresponding in density to layer III of the Ficoll, but the higher parts of the gradient are similar.

Density (ρ)	Refractive index (n)	Centrifugation	Volume (ml)		Cell layers
				BSA (%)	
1·0459	1·3670		2·50	17	
1·0495	1·3705		1·85	19	I
1·0552	1·3742	International PR-J centrifuge 840 *g* 30 min, 10°C	1·85	21	II
1·0618	1·3782		1·85	23	III
1·0663	1·3820		1·85	25	IV
1·0729	1·3856		3·50	27	V
				Ficoll (%)	
1·0059	1·3369		2·5	0	
1·0599	1·3572		2·0	13	I
1·0655	1·3601	Beckman L5-65 ultracentrifuge 22,000 *g* 30 min, 10°C	2·0	15	II
1·0736	1·3635		2·0	17	III
1·0851	1·3686		2·0	20	IV
1·1049	1·3771		1·0	25	V

FIG. 1. Characteristics of the BSA and Ficoll discontinuous density gradients used for the isolation of precursor T cells from human normal bone marrow.

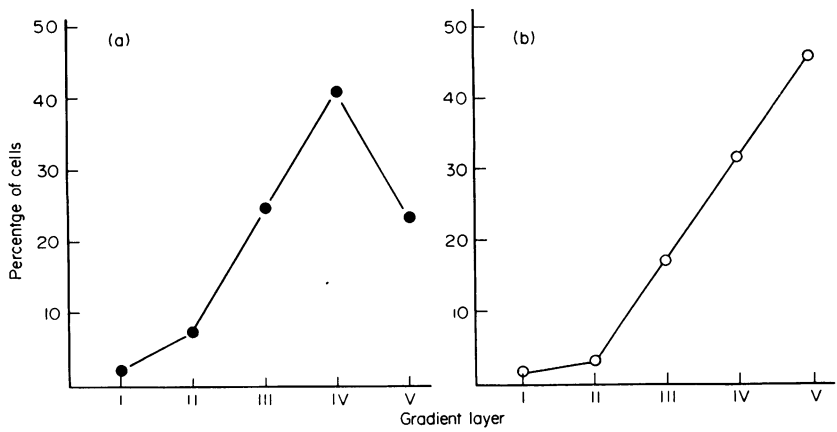

FIG. 2. Relative distribution of human marrow cells isolated by (a) Ficoll or (b) BSA discontinuous density gradient centrifugation.

Induction of receptors for SRBC

Marrow cells from each gradient layer were tested for their ability to synthesize receptors for SRBC during incubation in the presence of human or calf thymic extracts. As shown in representative experiments in Table 1, an increased proportion of cells bearing receptors for

TABLE 1. Differentiation of human marrow cells into cells bearing T-lymphocyte characteristics after *in vitro* treatment with thymic extracts

Extracts	Incubation (hr)	E rosettes (% large R) in layer: I	II	III	IV	V
Control	2	3·5	1·5	3·8	0·8	—
Human thymic extract F_3	2	1·5	3·2	8·3	0·8	—
Calf thymic extract F_3	2	0·0	2·8	4·8	0·5	—
Human splenic extract F_3	2	4·0	5·2	2·5	0·1	1
Control	14	—	—	8·0	4·1	0·7
Human thymic extract F_3	14	—	—	26·0	6·5	1·5

Cells ($1{\cdot}8 \times 10^6$) from each layer of a BSA gradient were incubated with extracts having the following protein concentrations: human thymic extract = 0·82 mg protein/ml; calf thymic extract = 1·15 mg/ml; human splenic extract = 0·58 mg/ml. T lymphocytes were recognized by the spontaneous rosette technique with SRBC as described in the Materials and Methods section.

SRBC are detected in layer III obtained from a BSA gradient after a 2-hr incubation with human thymic extract. After stimulation, the number of rosette-forming cells was more than twice that of the control value and the difference is statistically significant at a P level less than 0·01, as assessed by Student's t-test. A smaller stimulation is noticeable in layer II but no appreciable changes are visible in layers I, IV and V, or when cells of these layers were treated with human splenic extract as additional control. When the incubation was pro-

TABLE 2. Differentiation of human marrow cells into cells bearing T-lymphocyte characteristics after *in vitro* treatment with thymic extracts

Extracts	Incubation (hr)	E rosettes (% large R) in layer: I	II	III	IV	V
Control	2	2·8	8·0	7·5	1·5	3·7
Human thymic extract F_3	2	9·5	4·3	11	1·7	—
Calf thymic extract F_3	2	—	10	7·5	7·0	5·6
Human splenic extract F_3	2	—	18	6·0	5·5	5·3

Cells ($1{\cdot}3 \times 10^6$) from each layer of a Ficoll gradient were incubated with thymic extracts having the following protein concentrations: human thymic extract = 0·82 mg protein/ml; calf thymic extract = 1·15 mg/ml; human splenic extract = 0·58 mg/ml. T lymphocytes were recognized by the spontaneous rosette technique with SRBC as described in the Materials and Methods section.

longed to 14 hr as shown below, a more pronounced differentiation could be demonstrated with cells of layer III in the presence of human thymic extract. Table 2 shows results obtained when bone marrow cells were fractionated on a Ficoll density gradient. A significant increase in cells bearing receptors for SRBC is demonstrated in cells of layer I after 2-hr incubation with human thymic extract and in layer IV with calf thymic extract; no appreciable differentiation being observed in the other layers with calf or splenic extracts, except in layers II and IV where small increases are observed after treatment with splenic extract. In addition, cell proliferation, as reflected by the greater number of cells present, occurred in layers II, III and IV when marrow cells were treated with the same human thymic extract for 14 hr (Fig. 3).

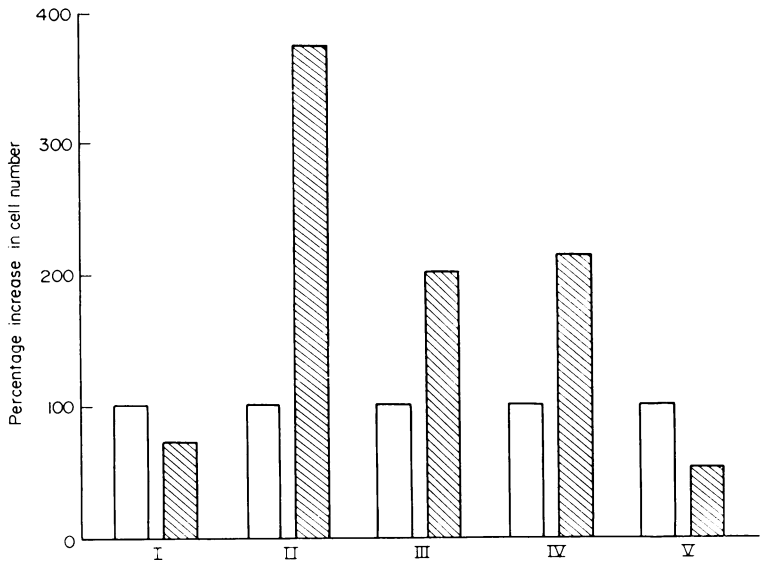

FIG. 3. Cell proliferation observed in cells of layers II, III and IV isolated by BSA gradient and treated for 14 hr with human thymic extract. Open columns, control. Hatched columns, human thymic extract-treated cells.

Effect of inhibitors

To determine the mode of action of thymic extracts on precursor cells *in vitro*, the effect of specific inhibitors of nucleic acids and protein synthesis were determined and compared to control cells that had been treated with inhibitors. Fig. 4 indicates that actinomycin D and α-amanitin completely abolished the differentiation produced in cells of layer III by human thymic extract during a 2-hr incubation. Both these inhibitors block RNA synthesis, but by different mechanisms (Reich & Goldberg, 1964; Lindell *et al.*, 1970). Actinomycin D inhibits transcription of DNA into RNA by binding to the double-stranded DNA and α-amanitin inhibits RNA chain elongation by its effect on the DNA-dependent RNA polymerase II. The control cells also treated with these inhibitors were not affected in their ability to form E rosettes with SRBC. When cells were treated with puromycin, a potent inhibitor of protein synthesis (Yarmolinsky & Haba, 1959), receptors for SRBC were

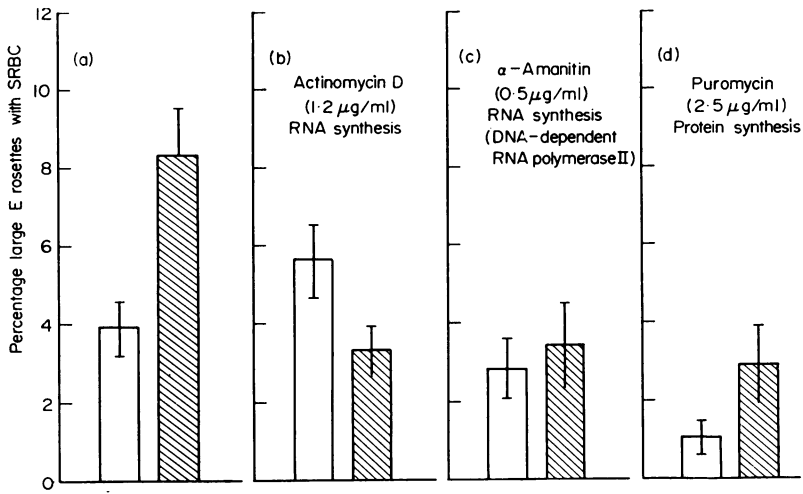

FIG. 4. Effects of inhibitors of nucleic acids and protein synthesis on the synthesis of receptors for sheep erythrocytes in cells of layer III during a 2-hr incubation in the presence of human thymic extracts. The standard deviation is indicated by the vertical line on top of each column. Open columns, control. Hatched columns, human thymic extract-treated cells. (a) Control, untreated with inhibitors. (b) After treatment with 1·2 μg/ml of actinomycin D. (c) After treatment with 0·5 μg/ml of α-amanitin. (d) After treatment with 2·5 μg/ml of puromycin.

diminished in control, and the observed stimulation usually produced in human thymic extract-treated cells was completely abolished. Cellular viability, determined by Trypan Blue exclusion, was found to be 97–100% in all cellular fractions treated with inhibitors, thus eliminating the possibility that cell death could produce the inhibition observed.

DISCUSSION

These studies confirm and extend previous observations from our laboratories indicating that both human and calf thymic extracts can induce evidence of differentiation of human marrow cells *in vitro*. In our prior studies we showed that antigens specific for thymus derived lymphocytes can be induced by thymic extracts to appear on the surface of human marrow cells lacking these antigens (Touraine *et al.*, 1974b). This change in cell surface occurred after periods of incubation of only a few hours. These findings seemed consonant with the previous observations of Komuro & Boyse (1973) using immunological markers on mouse lymphocytes. In the present study, it was found that a completely different marker of T lymphocytes, namely the capacity for spontaneous rosette formation with sheep red blood cells, could also be induced in bone marrow cells after only brief incubation with extracts of human or calf thymus. By contrast, with our earlier observations, fewer bone marrow cells could be induced to express the SRBC marker after incubation with thymic extract for only 2 hr. However, with longer incubation periods of 14 hr the number of marrow cells expressing this T-cell marker approached the number defined by HTLA. The differences in these results could be due to different precursor cells being activated

to develop the separate markers, differences in the processes involved in the activation, or simply and most likely differences in the processes required for full expression of the two separate markers at the cell surface.

These experiments also showed that the means used to fractionate the marrow cells could influence to some degree the results obtained. Ficoll gradients as well as BSA gradients were used and both yielded cell fractionations in which the apparent induction of differentiation could be demonstrated. It seemed, however, that the Ficoll gradient had some advantages in that the yield of cells that were susceptible to differentiation was generally higher and in most experiments a larger proportion of cells with the SRBC marker could be obtained after exposure to the thymic extracts.

That the process being studied does not simply involve rearrangement at the cell surface is indicated by the studies reported here using specific metabolic inhibitors. Actinomycin D and α-amanitin, which inhibit RNA synthesis in different ways, inhibited induction of SRBC rosettes. This finding indicates that RNA synthesis is essential to induction of SRBC marker by thymic extracts. Further, puromycin, an inhibitor of protein synthesis, also prevented induction of the capacity to make rosettes with SRBC.

In the experiments using longer periods of incubation, evidence of stimulation of cellular proliferation by thymic extract was also observed. This is of real interest since proliferation is a prominent feature of the lymphoid cells in the thymus. This observation raises several important questions since maximal stimulation to proliferate did not occur in the same cell layer as did maximal stimulation for the differentiation of T-cell markers. The proliferative response could be due to a different factor in the crude extracts used rather than the factor involved in the differentiative influence. Alternately, cells in different stages of differentiation might be responding differently to the same factor. Careful dissection of the extracts influencing these two processes and also fractionating the cells of the marrow by different techniques can resolve these questions. Finally, the influence of puromycin to reduce the number of rosette-forming cells in unstimulated cell preparations can be interpreted to indicate that ongoing protein synthesis must be present to maintain the apparatus for rosette formation.

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