Differentiation of Population of Peripheral Blood Lymphocytes Into Cells Bearing Sheep Erythrocyte Receptors In Vitro by Human Thymic Extract

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ABSTRACT A small population of human marrow cells has been shown to be differentiated in vitro by thymic extract into cells bearing T-lymphocyte (thymus-derived lymphocyte) characteristics. By a similar method, the differentiation of human peripheral blood lymphocytes has been studied. A discontinuous gradient of bovine serum albumin was used to isolate lymphocytes into four layers and cells from layers I and III demonstrated the greatest potential for differentiation by human thymic extract. Appearance of T-lymphocyte characteristics was recognized by the spontaneous E-rosette technique with sheep erythrocytes. Ability of human marrow cells to be differentiated under the influence of human thymic extract was abolished by specific inhibitors of nucleic acid and protein synthesis. The same inhibitors of nucleic acid synthesis, however, had no inhibitory effect on the maturation of peripheral blood lymphocytes during a 2 hr incubation with human thymic extract but puromycin, an inhibitor of protein synthesis, abolished this differentiative step in cells of layer I. It is suggested from these studies that many of the cells in peripheral blood that are differentiable by thymic extract are at a stage of maturation more advanced than those in human marrow that are also differentiable by thymic extract.

A certain population of lymphocytes from spleen and bone marrow in mouse have been shown to acquire specific surface markers, such as rosette forming capacity, TL and θ (θ-1) alloantigens, following a short-term incubation with thymic extracts (1, 2). In keeping with these findings, marrow cells in man could also be differentiated in vitro by thymic extracts into cells bearing T-lymphocyte (thymus-derived lymphocyte) characteristics (3-5). Appearance of specific T-cell markers on these cells was recognized by an anti-human T-cell serum (6) and by the E-rosette technique (5, 7) in the presence of sheep erythrocytes.

In the present paper, the possibility that a small number of lymphocytes in the peripheral blood could belong to a population of cells not yet completely differentiated into T-lymphocytes (8, 9) is evaluated by a technique similar to the one used for marrow studies. It was found that a small subpopulation of peripheral blood cells can, indeed, be induced to differentiate in vitro into cells bearing sheep erythrocyte receptors during a 2 hr incubation under the influence of a human thymic extract. T-lymphocyte characteristics were detected by the E-rosette formation technique with sheep erythrocytes (7).

Differentiation by thymic extracts was abolished in human marrow cells by specific inhibitors of nucleic acid and protein synthesis (4, 5). The effect of these inhibitors on induction of differentiation of peripheral blood cells was also determined and inhibition was not observed with inhibitors of nucleic acid synthesis. However, cells of layer I did not take on the marker of T-cells when treated with an inhibitor of protein synthesis. These findings suggest that some of the differentiable cells found in peripheral blood are more mature in terms of membrane receptor development than are the differentiable cells found in human marrow. Apparently, some marrow cells require RNA and protein synthesis to differentiate into T-lymphocytes under the influence of thymic extracts, whereas some cells in the peripheral blood may already have undergone a crucial step in differentiation and thus no longer require these same steps involving RNA synthesis to become T-lymphocytes. Further, it seems that other cells in the blood can take on some surface markers characteristic of T-lymphocytes without requiring protein synthesis.

MATERIALS AND METHODS

Thymic Extract. Human thymuses were obtained from infants undergoing cardiac surgery. Thymic extracts were prepared by the method of A. Goldstein (10) and their protein concentration determined by the method of Lowry using bovine serum albumin as a standard (11).

Inhibitors. Actinomycin D (Nutritional Biochemical Co., Cleveland, Ohio) inhibits RNA synthesis by reacting with the DNA template (12, 13). Alpha-amanitin (Boehringer, New York), the powerful toxin of the toad stool Amanita phalloides (14) is highly selective in its site of action. It interacts directly with two of the three DNA-dependent RNA polymerases, RNA polymerase B (15) or Form II of Roeder and Rutter (16), and specifically blocks RNA chain elongation. Puromycin (Sigma Chemicals, St. Louis, Mo.) inhibits protein synthesis by interrupting peptide chain elongation (17, 18).

Cell Isolation. Heparinized blood (50-150 ml) was obtained from 15 to 43-year-old normal volunteers and lymphocytes were isolated by ficoll-hypaque gradient centrifugation. Cells were washed twice with medium (RPMI-1640) containing antibiotics, penicillin 50 U/ml and streptomycin 50 μg/ml (Gibco, Grand Island, N.Y.) following centrifugation and the cell pellet resuspended in 3 ml of RPMI (3 to 4 x 10⁶ cells). An aliquot of the original cell suspension was saved for incubation with thymic extract and the rest layered on a bovine serum albumin discontinuous density gradient (5, 19) prepared in 16 x 125 mm plastic sterile tubes (Falcon Plastic, Oxnard,
Calif.). The gradient consisted of the following concentrations: 19, 21, 23, and 27% bovine serum albumin (Pentex, 35% solution, Miles Laboratories Inc., Kankakee, Ill.) diluted with RPMI and was centrifuged at 760 × g for 30 min at 5°. Four layers were obtained following centrifugation: layer I represents cells found on top of the gradient at interface between RPMI and 19% bovine serum albumin and layer II, cells between 19% and 21% bovine serum albumin, etc. Cells from each layer were washed once with RPMI by centrifugation and all concentrations were adjusted to 1.3 × 10^6 cells per ml prior to incubation with thymic extract.

Small volumes of bone marrow obtained from normal volunteers were aspirated into heparinized glass syringes from various points of the iliac crest and totaled 6–10 ml. Marrow cells were isolated and incubated with thymic extracts according to the procedure described earlier (5).

Incubation of Cells with Thymic Extracts. Peripheral blood lymphocytes or marrow cells from each layer were incubated for 2 hr with human thymic extract at 37° in a 5% CO2–95% air humidified incubator. Equal volumes of cell suspension and thymic extract were used and control tubes received RPMI instead of thymic extract. All cells were washed with RPMI by centrifugation prior to rosette formation with sheep erythrocytes. Inhibitors, in volumes of 5–10 μl, were added 5 min prior to addition of thymic extract at the beginning of the incubation period.

Formation of E-Rosettes. The ability of T-lymphocytes to form spontaneous rosettes with sheep erythrocytes was demonstrated according to the procedure of Bentwich et al. (7) with some modifications: 50 μl of a 0.5% solution of sheep erythrocytes and 15 μl of AB+ serum, heat inactivated and absorbed with equal volume of sheep erythrocytes, were added directly to the lymphocyte pellets. Tubes were gently mixed, incubated in a water bath at 37° for 5 min, and centrifuged (32 × g for 5 min) at room temperature. Tubes were kept 16–18 hr at 4° before counting the rosettes. Four hundred lymphocytes were counted per tube and large rosettes (R) are defined as lymphocytes to which four or more sheep erythrocytes were adherent.

RESULTS

Distribution of peripheral blood lymphocytes after bovine serum albumin gradient centrifugation

Four layers of cells were obtained after discontinuous density gradient centrifugation of the original suspension of peripheral blood lymphocytes. The number of cells remaining in layer I after washing to remove excess bovine serum albumin was very small and represented 0.36% (0.1–0.5%) of the total number of cells put on the gradient in nine separate experiments. The greatest number of cells was recovered in layer IV and represented 92% (87–96%) of the total in these experiments (Fig. 1). The greatest absolute number of cells differentiable by thymic extract into cells bearing multiple receptors for sheep erythrocytes was found in layer IV (Fig. 1a). However, the layer containing the greatest increase in the number of cells differentiable into T-cells was layer I (Fig. 1b).
TABLE 1. Differentiation of human peripheral blood lymphocytes by human thymic extract

<table>
<thead>
<tr>
<th>Percentage of E-rosettes</th>
<th>Original cell suspension</th>
<th>Gradient layers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C* T*</td>
<td>C  T  C  T  C  T  C  T  C  T</td>
</tr>
<tr>
<td>R</td>
<td>8.2  2.0</td>
<td>3.0  4.0  6.2  7.1  8.1  2.7  8.3  5.9</td>
</tr>
<tr>
<td>R₂</td>
<td>4.2  1.7</td>
<td>4.3  1.5  3.5  2.7  6.4  1.5  5.3  3.5</td>
</tr>
<tr>
<td>R₃</td>
<td>2.7  2.0</td>
<td>2.8  1.0  3.7  1.2  3.7  1.7  4.3  2.2</td>
</tr>
<tr>
<td>R₄</td>
<td>36.2  56.9</td>
<td>16.3  35.2  24.7  26.3  30.1  51.8  40.5  52.9</td>
</tr>
<tr>
<td>Total R</td>
<td>51.3  62.6</td>
<td>26.4  41.7  38.1  37.3  48.3  57.7  58.4  64.5</td>
</tr>
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R₁ + R₂ + R₃ (C-T)† | 9.4  3.6  2.4  12.3  6.3 |
| R (T-C)              | 20.7  18.9  1.6  21.7  12.4 |
| Total R (T-C)        | 11.3  15.3  -0.8  9.4  6.1 |

Percent increase in number of E-rosettes

<table>
<thead>
<tr>
<th></th>
<th>R  57  116  6  72  31</th>
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<tr>
<td>Total R</td>
<td>22  58  -2  19  9</td>
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Human peripheral blood lymphocytes from the original cell suspension and from each gradient layers, obtained after fractionation on a discontinuous bovine serum albumin gradient, were incubated for 2 hr with fraction 3 of a human thymic extract (10), containing 0.82 mg of protein per ml. Each E-rosette value represents the mean of four separate counts per 100 nucleated cells. R₁, R₂, R₃, and R indicates 1, 2, 3, 4, or more sheep erythrocytes adhering to a lymphocyte.

† (C-T) is percentage of E-rosettes formed by control cells (C) treated with medium alone minus the percentage formed by human thymic extract treated cells (T) during a 2 hr incubation; (T-C) is treated minus control.

Differentiation of peripheral blood lymphocytes by human thymic extract

An increase in the number of E-rosette forming cells was observed when the original lymphocyte suspension, prior to being subjected to density gradient centrifugation, was treated with human thymic extract during a 2 hr incubation (Table 1). This expansion in the number of rosette-forming cells under the influence of thymic extract was also noted when cells were subjected to a bovine serum albumin gradient centrifugation and each isolated cell layer was treated with the same thymic extract for 2 hr (Table 1). The greatest proportional response to this stimulation (116%) was noted with a cell population found in layer I where 15.3% of the cells which had the ability to be induced to form large rosettes (R) under the influence of thymic extract were derived from the non-E-rosetting population of peripheral blood lymphocytes. Cells from the other layers and from the original cell suspension, which were stimulated by thymic extract to form large R, appeared to be derived from the peripheral blood lymphocytes forming small rosettes with sheep erythrocytes (R₁, R₂, or R₃).

Since the enhancement of E-rosette formation induced by thymic extract differed from person to person, an effort was made to correlate the differentiative response observed in the various original lymphocyte suspensions with the proportion of E-rosettes originally present in the peripheral blood. The results shown in Fig. 2 indicate that the response to thymic extract is, in general, inversely proportional to the number of E-rosettes present in the blood.

The presence of inhibitors of RNA synthesis, actinomycin D or a-amanitin, during the incubation period with thymic extract did not reduce significantly the ability of the lymphocytes from gradient Layers I and III to develop into cells with T-cell markers under the influence of thymic extract (Fig. 3, PBL I and III). This seemed to be the case even when both low (0.5–1.2 μg/ml) and higher (2.5 μg/ml) doses of actinomycin D were used; at 1.2–2.5 μg/ml mRNA synthesis should be inhibited. These findings are in contrast with those obtained previously (5) with bone marrow cells of gradient layer III as shown in Fig. 3 (B.M. III). Here both inhibitors of mRNA synthesis, acting by different mechanisms (12–14), abolished the increase produced by thymic extract; in addition, actinomycin D seemed to stimulate the control cells somewhat and we have no explanation for this effect at the present. Puromycin (17), however, inhibited the differentiative influence of thymic extract in cells of layer I (Fig. 3, PBL I) but did not inhibit the change in cells found in the other layers or failed to inhibit a sufficient number of cells to alter the differentiative response observed (PBL III). This finding would suggest that induced changes in protein synthesis are required for expansion of the T-lymphocyte population in at least one very small subpopulation of cells in the periphery. For bone marrow cells, both control and human thymic extract-treated cells were inhibited by puromycin (Fig. 3, B.M. III) and in the presence of this inhibitor the inducive influence of the thymic factor was considerably reduced even at the low concentration used in these studies. Viability of cells treated with each of the inhibitors even at the higher doses, as determined by trypan blue exclusion, was found to be greater than 80%.

DISCUSSION

These findings indicate that a small population of peripheral blood cells are induced by thymic extracts to develop into a population of cells that possess receptors for sheep erythrocytes. This observation is compatible with prior investigations which have defined a population of post-thymic cells that, although clearly influenced by the thymus and probably already committed to the T-cell line, are not completely differentiated (8, 9). These cells exist in the periphery as perhaps two definable lymphoid populations. Both still require thymic influence to become fully differentiated immunocompetent.
quires transcription of receptors for sheep erythrocytes (SRBC) in peripheral blood lymphocytes (PBL) and human marrow cells after a 2 hr incubation with thymic thymic extract. Each group represents cells from a control (C) and cells induced by a human thymic extract (H.T.) untreated with inhibitors; cells from a control (C) and cells induced by a human thymic extract (H.T.) treated with actinomycin D, α-amanitin, or puromycin respectively. Cells were isolated on a discontinuous bovine serum albumin gradient and were from layers I and III for PBL I and PBL III, respectively, and from layer III for bone marrow cells, B.M. III. Each column represents the mean of three experiments for PBL I, or more than three experiments for the others, with one standard deviation indicated by vertical lines on the top of each column.

T-lymphocytes (9). Our findings would be compatible with the prediction of Stutman and Good (9) that the influence of thymus is exerted in several distinct steps. It would seem that among the cells in bone marrow influenced by thymosin is a population that undergoes a differentiative process that requires transcription and translation. In the peripheral blood are cells, especially those in layer I, that do not seem to require RNA synthesis for differentiation to cells with T-cell markers but that do require protein synthesis. In addition, a separate population is found in other cell layers (Fig. 3, PBL III) that does not require either RNA or protein synthesis for this change. Thus, the influence on the latter may depend on relatively superficial changes in the surface properties of such cells. It could well be that a post-thymic T-cell population is the major population that responds to thymic extract in the experiments with peripheral blood lymphocytes presented herein. That this population differs from a bone marrow stem cell population that can also be induced to T-cell differentiation by thymic extracts is suggested by the differences in the influence of inhibitors of nucleic acid synthesis presented here.

It would not be surprising that RNA synthesis is required for basic stem cell differentiation but might not be required for final stages of maturation of cells already programmed to become T-lymphocytes. However, protein synthesis might be required even for some changes in cell surface to occur and so would be required to some degree for differentiation at a second step but perhaps not at a final step involved in the differentiative process. Since many substances which seem to have no appreciable influence on T-lymphocyte development on athymic mice in vivo do induce T-cell markers in mouse lymphocytes in vitro (20), it would seem that they are involved in a step or steps relatively late in differentiation of cells already committed to the T-cell line by thymic or other influences. Further experimental study will be needed to sort out these relationships and to characterize fully the action of both thymic and non-thymic factors which can signal lymphoid cells to take on the markers of the fully differentiated T-lymphocytes.

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