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POPULATIONS OF LYMPHOCYTES SEPARATED FROM HUMAN THYMOCYTES*

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SUMMARY

Thymocytes from twenty-two human foetal and post-natal thymuses were separated according to their buoyant density. Thymocytes from eight were separated into multiple fractions by means of continuous gradients of bovine serum albumin (BSA), pH 5·1 and iso-osmolar with human cells, and thymocytes from fourteen were separated into two fractions of density less than and greater than 1·068 g/cm^3. Fractions were tested for antigen-binding lymphocytes (ABL) to ^{125}I-labelled human thyroglobulin, for response to phytohaemagglutinin (PHA) and for rosette-forming cells (RFC) using sheep red blood cells. For subjects of all ages there was a pronounced enrichment of both ABL and thymocytes responsive to PHA among low or 1·064–1·065 g/cm^3 density thymocytes. In older subjects there was a second smaller enrichment of ABL among high or 1·072–1·073 g/cm^3 density thymocytes. RFC were distributed over a wider range of densities, and although they did not form a discrete subpopulation they predominated among high density thymocytes.

INTRODUCTION

Human thymic lymphocytes specifically bind radioiodine-labelled antigens, including flagellin and haemocyanin, presumably by means of an antigen-binding receptor of immunoglobulin character (Dwyer & Mackay, 1970; Dwyer, Warner & Mackay, 1972). Antigen binding by thymic lymphocytes has been shown also by an enzymatic reaction, using beta-galactosidase as antigen (Sercarz *et al.*, 1971). Counts of antigen binding thymocytes per 1000 cells are highest in early foetal life, and fall progressively thereafter, according to several studies (Dwyer & Mackay, 1970; Hayward & Soothill, 1972; Roberts, Whittingham & Mackay, 1973). However, difficulty is encountered in demonstrating antigen binding

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by peripheral T cells in mice (reviewed by Warner, 1974), and antigen-binding cells were very scarce among blood lymphocytes (presumably T cells) in agammaglobulinaemic children (Dwyer & Hosking, 1972). Possibly the intrathymic lymphocyte and the peripheral thymus-derived lymphocyte function differently in regard to antigen attachment to their surface; moreover there could exist, even within the thymus itself, functionally different types of thymic lymphocytes. These considerations, and the availability of techniques for fractionating populations of human thymic lymphocytes according to buoyant density, led to the studies reported here.

MATERIALS AND METHODS

Thymic tissue

Cell suspensions were prepared from thymic tissue (Roberts, Whittingham & Mackay, 1973) from three human foetuses obtained from therapeutic termination of pregnancy between 16 and 24 weeks of gestation, and from nineteen biopsy specimens from children aged from 1 to 11 years, the latter being obtained during cardiac surgery in the course of access to the operative site. A portion of each thymus was prepared for histological examination.

Thymocytes were separated into populations of different buoyant density by two alternative procedures (Shortman, 1968; Shortman, Williams & Adams, 1972). Eight thymuses, two foetal and six post-natal, were processed by the first fractionation procedure. Suspensions containing 10^8–10^9 thymocytes were centrifuged at 4000 *g* for 30 min at 4°C in continuous density gradients of bovine serum albumin (BSA) in salt solution, pH 5·1, and isoosmolar with human cells. Cells of uniform buoyant density were collected as eleven to twelve fractions and equal volume over a density range of 1·057–1·089 g/cm^3, and for each fraction the precise density was determined. Cells in each fraction were washed free of albumin with Dulbecco's balanced salt solution, and fractions containing less than 10^7 cells were pooled with the smaller of the two adjacent fractions. Of the eight thymuses thus processed, one was tested for ABL and cells responsive to PHA, three were tested for ABL only, two for cells responsive to PHA, and two for rosette-forming cells (RFC). For further substantiation of results from fractionations, fourteen thymuses were processed by the simpler procedure of neutral density separation, in which thymocytes were separated into two populations, of low density ($<1{\cdot}068$) and high density ($>1{\cdot}068$). Thymocytes were suspended in 2 ml of BSA (density 1·068 g/cm^3), layered over 2 ml BSA of the same density, and centrifuged at 2600 *g* for 10 min, and cells in the supernate and pellet were separated and washed free of albumin; four thymuses thus processed were tested for ABL, five for cells responsive to PHA, and five for RFC.

Antigen-binding lymphocytes

Human thyroglobulin of high purity was prepared from thyroid glands obtained at autopsy (Roberts *et al.*, 1973), and iodinated with ^{125}I (Radiochemical Centre, Amersham) using a modified chloramine-T oxidation procedure (Ada *et al.*, 1964). The specific activity of ^{125}I-labelled human thyroglobulin varied between 6 and 11 μCi/μg. Ten million thymocytes were brought into contact with 20 μg of ^{125}I-labelled human thyroglobulin in a final volume of 0·25 ml for 30 min at 4°C and processed by the autoradiographic technique described by Roberts *et al.* (1973). Four Giemsa-stained smears were independently coded

and 1000 undamaged thymocytes, which morphologically appeared to be lymphocytes with intact nuclei and which were not in contact with other cells or debris, were counted. 'Lightly labelled' lymphocytes were defined as those having more than five grains of silver above background distributed over their surface and within one cell diameter of their edge, and 'heavily labelled' cells those with more than twenty-five grains so distributed.

PHA-responsive lymphocytes

One-millilitre aliquots containing 1×10^6 thymocytes in Eagle's minimum essential medium and 10% foetal calf serum were transferred to sterile Falcon tubes. The dose of 20 μg of PHA (Burroughs Wellcome) optimal for transformation was determined from a dose–response curve established from preliminary tests using an unfractionated thymocyte suspension and doses of 5, 10, 20, 50 and 100 μg of PHA (Fig. 1). Thymocytes were cultured

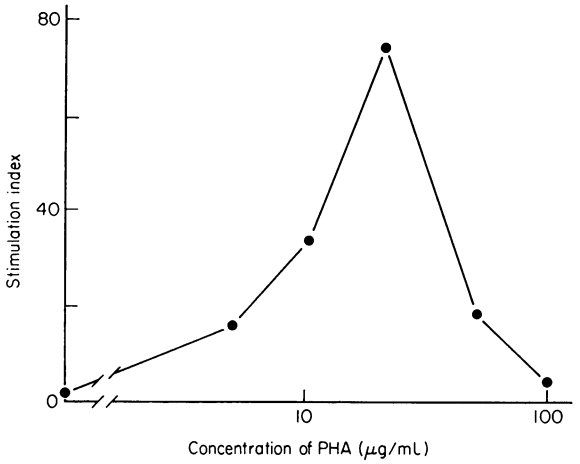

FIG. 1. Response by human thymocytes to 0, 5, 10, 20, 50 and 100 μg phytohaemagglutinin (PHA). Ct/min of tritiated thymidine incorporated into DNA are expressed as a stimulation index, i.e the ratio of mean ct/min incorporated into PHA-stimulated cultures to mean ct/min incorporated into non-PHA-stimulated cultures.

in triplicate at 37°C in 5% CO_2 and air, with and without the addition of PHA. At 66 hr 1 μCi of tritiated thymidine was added and at 72 hr the cultures were processed according to the method described by Byrd, von Boehmer & Rouse (1973) and modified by Toh *et al.*, (1973). The results, as ct/min of tritiated thymidine incorporated into DNA obtained for the test on unfractionated thymocyte suspensions, were expressed as the absolute response above background, i.e. the difference between the mean ct/min per 10^6 cells obtained for stimulated and non-stimulated (background) cultures.

Rosette-forming cells

Thymus cell suspensions were tested for counts of RFC, i.e. cells which had five or more adherent sheep red blood cells (SRBC). For all of the fractionation experiments and most of the neutral density separations, RFC were tested for under conditions as described by Whittingham & Mackay (1973); 10^7 washed thymocytes suspended in 1 ml of Eisen's

balanced salt solution were held at 37°C for 15 min with 0·04 ml of 10% SRBC, centrifuged at 200 *g* for 3 min, resuspended and counted in a Neubauer counting chamber. For one study using neutral density separation, RFC were tested for by the method of Stjernswald *et al.* (1972) in which 10^6 thymocytes in 0·5 ml of medium are held with 0·5% SRBC at 37°C for 15 min and 4°C overnight.

RESULTS

Unfractionated thymocyte suspensions

All thymuses tested contained ABL for thyroglobulin, cells responsive to PHA and RFC. All ABL were lightly labelled cells; their number was highest in the early foetal thymus and according to thymic age varied between 3·5 and 12·8 per 1000 thymocytes. The absolute response to PHA varied between 13,500 and 112,000 ct/min. The number of RFC per 1000 thymocytes, demonstrable by the method of Whittingham & Mackay (1973) ranged between 50 and 135, and in one study by the method of Stjernswald *et al.* (1972) was 836.

Fractionation of thymocyte suspensions

Cell suspensions from eight thymuses were separated into various fractions of different cell density and, for each fraction, we calculated the number of nucleated cells, ABL or

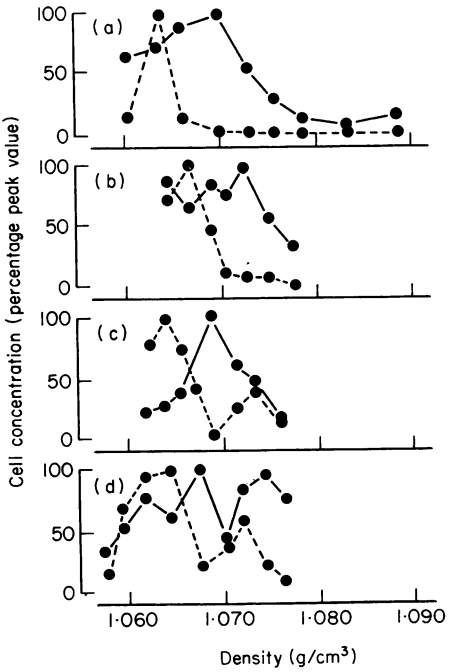

FIG. 2. The curves represent the density distribution of nucleated thymocytes or of antigen-binding lymphocytes (ABL) to ^{125}I-labelled thyroglobulin. In all thymuses ABL appeared as subpopulations distinct from the main peak of nucleated cells. The major subpopulation was present in the low density region. A minor subpopulation appeared among high density thymocytes from older subjects. (——) Total nucleated cells. (– – –) ABL. (a) Twenty-four-week-old foetus. (b) Fourteen-month-old child. (c) Seven-year-old child. (d) Eight-year-old child.

RFC; PHA responsiveness of lymphocytes for each fraction was expressed as absolute ct/min above background. Because all fractions did not cover an equally sized density range, cell counts were corrected by expressing them as the number of cells per density increment, rather than the number of cells per fraction. The final density distribution profiles for nucleated cells, ABL, PHA-responsive lymphocytes or RFC were obtained by expressing the corrected counts as a percentage of the number of such cells in the fraction containing the maximum number of such cells.

The main proportion of thymocytes in the eight thymuses tested was in a density range between 1·067 and 1·075 g/cm^3. For ABL there was, in all subjects, a major peak of low density, 1·064–1·065 g/cm^3. In older subjects there was also a minor peak of high density, 1·072–1·073 g/cm^3 (Fig. 2). For PHA-responsive thymocytes there was one peak and this was in the low density region (Fig. 3). By contrast, the RFC were distributed as a broad peak but were detected maximally among thymocytes of high density 1·070–1·085 g/cm^3 (Fig. 4).

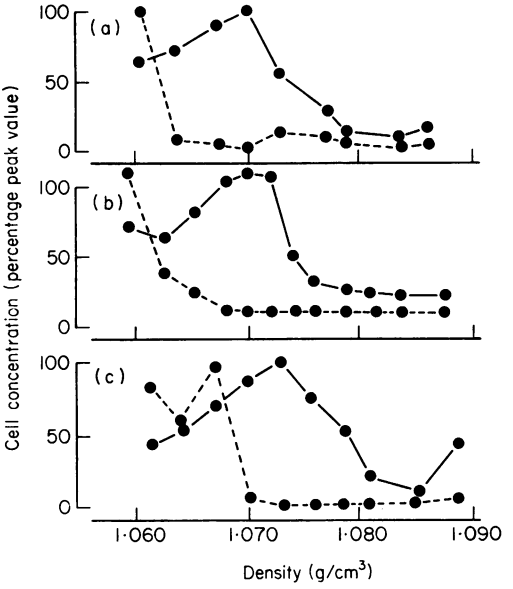

FIG. 3. The curves represent the density distribution of nucleated thymocytes or phytohaemagglutinin (PHA)-responsive lymphocytes. In all thymuses PHA-responsive cells appeared as a subpopulation among low density thymocytes. (——) Total nucleated cells. (– – –) PHA-responsive cells. (a) Twenty-four-week-old foetus. (b) Two-year-old child. (c) Five-year-old child.

Neutral density separation of thymocyte suspensions

Cell suspensions from fourteen thymuses were 'cut' at a density of 1·068, and the total number of ABL, RFC or PHA-responsive thymocytes in the low density fraction was expressed as a percentage of the sum obtained for both fractions; this result showed the percentage distribution of such cells in the two fractions (Table 1). The findings were consistent with results obtained in the cell fractionation experiments, in that most ABL and most PHA-responsive lymphocytes were in the low density fraction; with increasing age there

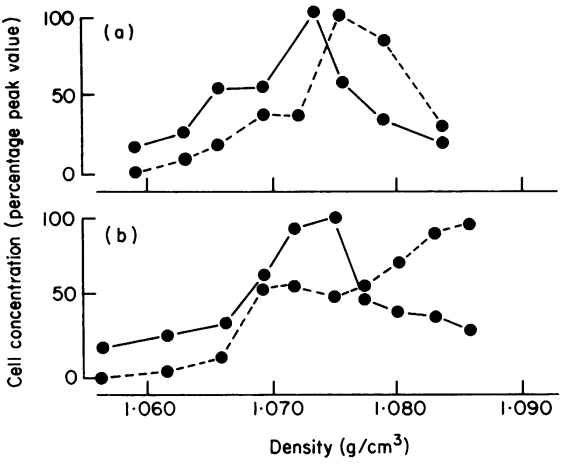

FIG. 4. The curves represent the density distribution of nucleated thymocytes or of high 'avidity' rosette-forming cells (RFC). RFC were distributed broadly among thymocytes but maximally among thymocytes of high density. (——) Total nucleated cells. (– – –) RFC. (a) Five-year-old child. (b) Eleven-year-old child.

TABLE 1. Percentage distribution of antigen-binding lymphocytes (ABL), phytohaemagglutinin (PHA) responsiveness, and rosette-forming cells (RFC) among thymocytes of density $< 1{\cdot}068$ and density $> 1{\cdot}068$ g/cm³

Population*	Percentage distribution among thymocytes of density (g/cm³)†	
	$< 1{\cdot}068$‡	$> 1{\cdot}068$‡
ABL (foetus) (2)	94 (1·9)	6 (0·1)
	90 (1·5)	10 (0·6)
ABL (post-natal) (2)	78 (1·2)	22 (0·4)
	75 (1·3)	25 (0·3)
PHA responsiveness (5)	92–99	1–8
RFC a§ (4)	17–33	67–83
RFC b¶ (1)	15	85

* The numbers in brackets refer to the number of thymuses tested.

† The numbers in brackets are the percentages of ABL in that fraction.

‡ The numbers of nucleated cells in the two fractions were approximately equal.

§ 'a' Refers to data using the method of Whittingham & Mackay (1973).

¶ 'b' Refers to data using method of Stjernswald *et al.* (1972).

was an increase in numbers of ABL of high density. By contrast, most RFC were present in the high density fraction; results were similar for RFC demonstrable by the two methods described, as indicated in Table 1.

Histological examinations of thymus

All thymus specimens were read as being histologically normal; the only features to be remarked upon were slight degrees of age involution, with fat replacement, in specimens from some of the older children.

DISCUSSION

Human thymocytes, after the 10–12th week of gestation, bind radioactively labelled antigens (Dwyer & Mackay, 1970), respond to mitogenic stimulation by PHA (Papiernik, 1972), and form rosettes with sheep erythrocytes (Whittingham & Mackay, 1973). Antigen binding is specific, and is mediated by an immunoglobulin-like receptor (Dwyer & Mackay, 1971) which does not appear to be passively acquired cytophilic antibody (Dwyer *et al.*, 1972; Roberts *et al.*, 1973). A high proportion, some 2%, of antigen-binding cells has been demonstrated in both the human and mouse thymus by autoradiography using various radioiodine-labelled antigens including flagellin, haemocyanin and thyroglobulin (Dwyer & Mackay, 1970; Dwyer *et al.*, 1972; Roberts *et al.*, 1973) and by enzymatic reactions using beta-galactosidase (Sercarz *et al.*, 1971). By contrast the formation by thymocytes of rosettes with sheep erythrocytes is not immunologically specific, and is claimed to be a general property of human T cells, in thymus and blood (Jondal, Holm & Wigzell, 1972).

In the mouse, Shortman and co-workers (Shortman, Brunner & Cerottini, 1972; Shortman *et al.*, 1973) defined, by buoyant density separations and various functional assays, a minor light fraction of thymocytes which were relatively cortisone-resistant, had a high level of H-2 antigen and low level of theta antigen, responded to mitogens and allogeneic lymphocytes, and provided progenitors of cytotoxic lymphocytes. Using the criterion of buoyant density, we found for the human thymus that there was a discrete low density subpopulation which contained most cells capable of binding antigen (thyroglobulin) and responding to PHA; after birth, there was demonstrated a minor high density subpopulation of ABL. Also, there was a less discrete but predominantly high density subpopulation which contained RFC. We suggest that our light density antigen-binding and PHA-responsive subpopulation of thymocytes represents the high H-2 low theta cortisone-resistant subpopulation of mouse thymocytes. However this claim cannot be pressed too far, as we lack for human thymocyte strain-specific antigenic markers and antisera, and it would not be feasible to demonstrate cortisone-resistant thymocytes, at least by prior *in vivo* injection, as is done in the mouse.

Our findings allow certain conclusions to be drawn as well as raising some interesting questions. First, the low density antigen-binding subpopulation contains fully viable cells with the 'T-cell like' property of a mitogenic response to PHA; however, whether the same cells in this subpopulation bind antigen, respond to PHA and form rosettes with sheep red blood cells has not been ascertained. Secondly, the number of antigen-binding cells in the foetal thymus is high, for some antigens comprising up to 2% of all thymocytes; we now show that most of these are present in a limited light density range of thymocytes, among which there would be an estimated tenfold enrichment of ABL. This favours the suggestion

(Dwyer & Mackay, 1970) that these low density antigen-binding thymocytes in the human foetal thymus are 'polyvalent' in terms of antigen recognition.

The questions raised include the vexed issue of the nature of the T-cell receptor, and differentiation pathways of thymocytes. The receptor for antigen-binding by thymocytes was judged to be immunoglobulin, from blocking studies with anti-immunoglobulin sera, although steric hindrance effects could explain this (Dwyer *et al.*, 1972). The postulated immunoglobulin receptor could be passively acquired (cytophilic) antibody, but it seems unlikely that antibody to thyroglobulin would be acquired by early foetal thymocytes. We note that for thymocytes of certain defined densities, and for human peripheral T cells (Dwyer & Hosking, 1972), antigen recognition appears to be undemonstrable by our autoradiographic technique.

It cannot be stated whether the different properties of thymocytes separated by buoyant density reflect the existence of different classes of thymocytes, or whether thymocytes exist in 'activated' (immature) and 'non-activated' (mature) forms, with only the former having the capacity for antigen-binding and PHA responsiveness. The question that then arises, and is discussed by Schlesinger (1972), is the nature of the intrathymic influences which may drive thymocytes in either of these two directions.

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