IN VITRO MATURATION OF IMMATURE THYMOCYTES INTO IMMUNOCOMPETENT T CELLS IN THE ABSENCE OF DIRECT THYMIC INFLUENCE*

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The thymus contains at least two populations of lymphoid cells which differ not only by their localization, respectively, the thymus cortex, or medulla, but also by their sensitivity to cortisone, the antigenic properties of their cell membrane, and their immunocompetence (1). The ontogenetic relationship of the cortical and medullary thymocytes (Thc)[1] are diversely interpreted (1, 2). Recently, it was observed (3, 4) that peanut lectin (PNL), which binds to galactose terminal residues, also binds to a majority of Thc, which appear to be cortical Thc. In the present work, use was made of this property, combined with cell affinity chromatography, to prepare almost pure populations of PNL$^+$ and $^-$ Thc which strikingly differ in their response in vitro to mitogens, cell membrane antigenicity, and immunocompetence. When PNL$^+$ Thc, which appear to be immunologically immature, were cultured in the presence of concanavalin A (Con A) in conditions allowing ther proliferation, this resulted in cells with new cell membrane properties, displaying immunocompetence towards allo or foreign antigens. This observation sheds some light on the ontogenetic pathway relating Thc and peripheral T lymphocytes.

Materials and Methods

Animals. Mice of C3H, C57/B16, BALB/c, AKR, CBA/ca, and CBA/H-T6T6 strains were purchased from The Jackson Laboratory (Bar Harbor, Maine) and bred in our laboratory.

Cell Preparation. Cell suspensions from thymus, peripheral lymph nodes, (LN) and spleen cells depleted in T cells with anti-Thy 1.1, and rabbit complement (C') treatment were obtained as described elsewhere (5). Fractions enriched in T cells were prepared by nylon wool filtration (6). Thc were fractionated by sedimentation on continuous sucrose gradients (7). Cortisone resistant Thc were prepared from mice treated with cortisone acetate (8). Whenever dead cells exceeded 10% of the cell preparations, they were removed (9).

Separation of Thc According to their PNL Binding. Thc, 10^7/ml, were incubated for 30 min at

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[1] *Abbreviations used in this paper:* C', agarose-absorbed rabbit serum; Con A, concanavalin A; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; GVHR, graft-versus-host reaction; [^{3}H]TdR, tritiated thymidine; HBSS, Hank's balanced salt solution; LN, lymh node; MICC, mitogen-induced cellular cytotoxicity; MLR, mixed lymphocyte reaction; PFC, plaque-forming cells; PHA, phytohemagglutinin; PNL$^+$ and $^-$, peanut-lectin binding (+) and non-binding (−) cells; SRBC, sheep erythrocytes; SUP, 24-h supernate of Con A-stimulated LN cells; Thc, thymocytes; Thy, thymus cell antigen.

4°C in Hank's solution (HBSS) with 10 μg/ml of purified peanut agglutinin (PNL) (4), then washed and applied to an anti-PNL Sepharose column. The column was prepared by coupling the IgG fraction of a rabbit anti-PNL antiserum (4) to cyanogen bromide-activated Sepharose 6 MB (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) and packing 6 ml of beads into a 10-ml syringe. After 30 min of incubation at 4°C, the column was washed with 50 ml of HBSS at a flow rate of 3 ml/min to remove unbound cells. PNL binding (PNL^+) Thc were recovered by a gentle resuspension of the beads in phosphate-buffered saline containing 0.1 M galactose. The cells collected from the effluent and eluate were examined by immunofluorescence to determine the frequency of PNL^+ Thc in each population (4). PNL^+ Thc contaminating the effluent were killed with anti-PNL antiserum and rabbit C′.

Cultures. Cells were cultured at 10^6/ml in RPMI-1640 (Microbiological Associates, Walkersville, Md.) supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Irvine, Scotland), 2-mercaptoethanol 5×10^{-5} M (Eastman Kodak Co., Rochester, N.Y.), streptomycin SO_4 (0.1 μg/ml), and penicillin G (10^{-2} IU/ml). Con A (Sigma Chemicals Co., St Louis, Mo.) and phytohemagglutinin (PHA) (a crude extract of *Phaseolus vulgaris*) were used at optimal mitogenic doses (4 μg/ml and 20 μl/ml, respectively). The following culture vials were obtained from Falcon Plastics (Oxnard, Ca.), tubes (no. 2003) for 1 ml, flask (no. 3013) for 15-ml cultures, microtest tissue culture plate (no. 3001), and lid (3041F) for 0.2-ml cultures.

Preparation of Culture Supernate (SUP). LN cells were cultured (10^7/ml) with Con A for 24h, the SUP harvested and filtered on sterile Milipore filters (0.45 μm). When indicated, Con A was removed from the SUP with Sephadex G-50 (Pharmacia) (10).

Immunofluorescence and radioautography were performed as described previously (11). C3H or AKR Thc proliferating in culture were detected by anti-Thy 1.1 or anti-Thy 1.2 alloantisera, binding to cells, and revealed with the rhodamine-labeled IgG fraction of a rabbit anti-mouse Ig serum.

C′-Mediated Cytotoxicity. AKR anti-C3H Thc (anti-Thy 1.1) and BALB/c anti-EL 4 lymphoma (anti-$H_2{}^d$) were raised as described elsewhere (12). Cytotoxicity in the presence of agarose absorbed normal rabbit serum as a C′ source was tested by using the trypan blue exclusion method (13).

Cell proliferation was determined by [^{3}H]thymidine ([^{3}H]TdR) incorporation (5). Caryotypic analysis of the proliferating cells was performed according to Moorhead and Nowell (14).

Mitogen-Induced Polyclonal Cellular Cytotoxicity (MICC). A microtest modification of the Brunner et al. method (15) was employed, with P-815 mastocytoma cells (kindly provided by Dr. K. T. Brunner). Mastocytoma cells, grown in tissue culture, were labeled with ^{51}Cr, washed three times, and suspended at 5×10^5/ml in Dulbecco's modified Eagle medium (DMEM-5% FCS). Of this suspension 0.1 ml was added to 0.1-ml duplicates of lymphoid cell suspensions of various concentrations. For each cell population tested, serial dilution duplicates were pipetted into a Linbro microplate, (Flow Laboratories) and 20 μl PHA was added. The trays were incubated for 4 h at 37°C and centrifuged at 1,000 rpm. The SUP were harvested with a TITRITEC supernatant Harvester system (Flow Laboratories), and counted in a Packard gamma counter. The specific ^{51}Cr release was calculated as the

$$\text{cpm}\ \frac{\text{experimental} - \text{spontaneous}}{\text{maximal} - \text{spontaneous}} \times 100\%$$

where experimental is the release in the presence of lymphocytes, spontaneous the release in absence of lymphocytes, and maximal the release after freezing and thawing three times. The spontaneous release after the 4-h incubation never exceeded 10% of the maximal release.

In Vitro Response to Sheep Erythrocytes (SRBC). The generation of anti-SRBC direct plaque-forming cells (PFC) was assayed in the culture conditions of Mishell and Dutton (16) with the modification of Click et al. (17).

Popliteal Graft-Versus-Host Reaction (GVHR). 10^7 parental cells were injected in the footpad of an F_1 hybrid and the reaction measured by the increased cellularity of the popliteal LN 7 days later, as described elsewhere (12, 18).

Mixed lymphocyte reactions (MLR) were performed with responding cells (4×10^5) and irradiated (850 REM) stimulator spleen cells (6×10^5) in 0.2 ml as described by Peck and Bach (19). SUP used in these experiments was prepared with Click's medium supplemented with 0.5% mouse serum.

Results

1. *Fractionation of* PNL^+ *and* PNL^- *Thc.* Thc suspensions which usually contain 85% ± 5 PNL^+ Thc as judged by indirect immunofluorescence, were fractionated into PNL^+ and $^-$ Thc with an anti-PNL-Sepharose column as described in Materials and Methods. After washings and specific elution, from 65 to 80% of the Thc loaded were recovered as viable cells. Samples of these cells were then incubated with PNL and stained with the fluorescent anti-PNL antibody. Usually 96% or more of the adherent cells, eluted with galactose from the anti-PNL-Sepharose column were PNL^+. Between 15 and 20% of the cells did not adhere to the column (effluent) and about half of these cells were PNL^+. This fraction was further depleted in PNL^+ cells by treatment with anti-PNL antiserum and C′. Thereafter, populations referred to as PNL^+ or $^-$ Thc were at least 96% pure; populations not showing this degree lof purity were discarded.

2. *Characterization of* PNL^+ *and* $^-$ *Thc*

(*a*) SUSCEPTIBILITY TO ANTI-THY 1 AND ANTI-H_2 ALLOANTISERA. The anti-Thy 1.1 and anti-$H_2{}^d$ antiserum titers killing, in the presence of rabbit C′, 50% of C57 whole Thc, PNL^+ and $^-$ Thc, and LN T lymphocytes are shown in Table I. As shown in Table I, PNL^+ Thc are highly susceptible to anti-Thy 1 and relatively resistant to anti-H_2 + C′ lysis. The reverse is found for PNL^- Thc, which behaved like LN T cells.

(*b*) HYDROCORTISONE SENSITIVITY. The thymuses of 6–8 wk old C57 mice contain $120 \pm 20 \times 10^6$ cells, of which 85 ± 5% are PNL^+. 2 days after injection of 2 mg of cortisone, the thymus contained $12 \pm 4 \times 10^6$ cells, 25 ± 5% being PNL^+, and after 8 mg cortisone, $4 \pm 1.5 \times 10^6$ cells, 8 ± 3% being PNL^+.

(*c*) MITOGEN RESPONSIVENESS. PNL^+ Thc were weakly responsive to Con A as judged by [^{3}H]TdR incorporation after 3 days, and unresponsive to PHA (Table II). In the presence of Con A, the cell recovery was very poor (10–20% of input) and only 10% of the recovered cells were blasts. PNL^- cells, in contrast, responded strongly to both mitogens by [^{3}H]TdR incorporation, with a cell recovery of 50–90% of the input, and 80–90% of the recovered cells were blasts (Table III).

(*d*) IMMUNOLOGICAL COMPETENCE. PNL^+ and $^-$ Thc were tested for their ability to help B-cell differentiation into anti-SRBC PFC in culture (Table IV), and for their responsiveness in a popliteal GVHR (Table V) and in MLR (Table VI). In all assays, PNL^- Thc were as effective as LN T cells, and PNL^+ cells were ineffective.

3. *Induction of Proliferation of* PNL^+ *Thc in Culture.* SUP of 24-h cultures of Con A or PHA stimulated LN cells or spleen cells are known to stimulate the proliferation of Thc (20–26). When PNL^+ Thc were cultured with Con A in a medium supplemented with 30% of such a SUP, or in presence of lethally irradiated LN cells, a strong proliferative response was observed (Table II). After 3 days of culture with Con A, cell recovery was high, in some instances higher than the input into the culture, and 80–100% of the recovered cells were blasts. SUP alone was also efficient, but after depletion of most of its Con A content (see Materials and Methods), it was found to be only weakly mitogenic (Table II), whether dialyzed or not (27). Addition of Con A to cultures containing

TABLE I
Cytotoxic Effect of Anti-Thy 1.1 and Anti-$H_2{}^d$ Antisera + C' on Thc, LN Cells, and PNL$^+$ and $^-$ Thc Subpopulations, Freshly Prepared or after Proliferation in Culture

Cells	Antiserum titers killing 50% of cells*	
	Anti-Thy 1.1 + C'	Anti-$H_2{}^d$ + C'
LN T Lymphocytes	1:250 (180–350)	1:500 (420–600)
Thc	1:1,200 (1,050–1,320)	1:100 (50–130)
Fresh PNL$^+$ Thc	1:1,400 (1,120–1,650)	1:50 (30–80)
Fresh PNL$^-$ Thc	1:300 (220–410)	1:450 (400–490)
"Cultured"‡ PNL$^+$ Thc	1:100 (60–150)	1:550 (520–580)
"Cultured"‡ PNL$^-$ Thc	1:100 (50–140)	1:600 (420–750)

Results are the means of three experiments performed with cells of C57 mice. Extreme values in brackets.
* Titers of antisera dilutions killing 50% of the cells, as determined on cytotoxicity curves.
‡ For definition of cultured cells, see Results, section 4.

Con A-depleted SUP led again to strong proliferation; addition of PHA in these conditions was also mitogenic (Table II). However, when PNL$^+$ Thc were incubated for 24 h in mitogen-depleted SUP, and then the recovered viable cells cultured in fresh medium containing Con A and no SUP, no proliferation was observed (not shown). Thus, the simultaneous presence of both mitogen and SUP is necessary to induce the proliferation of PNL$^+$ Thc. It may also be noted that cultures of LN cells (data not shown) and PNL$^-$ Thc always showed a stronger proliferative response to Con A when SUP was present (Table II). The following questions were then asked concerning the proliferating cells in cultures of PNL$^+$ Thc stimulated by Con A in the presence of SUP.

Are the proliferating cells really of PNL$^+$ origin? The PNL$^+$ Thc population contained at most 4% of contaminant PNL$^-$ Thc. To rule out the possibility of selective proliferation of these contaminants, the following experiments were performed: (*a*) cultures were arrested after 15 and 35 h, after a terminal 30-min pulse of [^{3}H]TdR, labeling DNA synthesizing cells. A combined radioautographic and immunofluorescence study, shown in Table III, indicated that most of the proliferating cells are PNL$^+$, and suggested also that the proliferating cells are progressively losing their PNL$^+$ character, since their staining was of uneven intensity (see below). (*b*) Since T blasts from AKR or C3H origin can be identified by their Thy alloantigens, PNL$^+$ C3H Thc (bearing Thy 1.1) were contaminated with 3% of PNL$^-$ AKR Thc (bearing Thy 1.2) before culture. After 3 days, only 2% of the cells bore the Thy 1.2 antigen as detected by indirect immunofluorescence. (*c*) Since CBA/ca and T6T6 cells can be caryotypically identified during mitosis, T6T6 PNL$^-$ Thc were added to CBA/ca PNL$^+$ Thc. In a culture supplemented at the beginning with 3% of contaminant T6T6 Thc, and arrested after 35 h of culture, 6 out of 89 mitoses (7%) were T6T6, and in a culture containing 6% of contaminant T6T6 Thc, and arrested after 3 days, 17 out of 144 mitoses (12%) were of T6T6 origin. Altogether, these experiments showed that PNL$^+$ Thc are indeed proliferating and that the small percentage of contaminant PNL$^-$ Thc have no selective advantage.

TABLE II
Proliferation of PNL⁺ and PNL⁻ Thc in Response to Mitogens in Various Culture Conditions

Culture conditions*		Culture recovery ($\times 10^{-6}$ cells/culture)	[^{3}H]TdR Incorporation
Cells (10^6 cells/culture)	Addition to cultures		
			cpm $\times 10^{-3}$
Thc	0	0.15 (0.05–0.25)	0.6 (0.3–0.9)
Thc	Con A	0.27 (0.15–0.35)	26.0 (14–33)
Thc	PHA	0.12 (0.10–0.15)	0.6 (0.3–0.9)
PNL$^+$ Thc	0	0.15 (0.1–0.25)	0.5 (0.3–1.5)
PNL$^+$ Thc	Con A	0.2 (0.15–0.30)	5.0 (2.5–7.5)
PNL$^+$ Thc	PHA	0.1 (0.05–0.2)	0.6 (0.4–1.4)
PNL$^+$ Thc	LN cells$_{rx}$‡ + Con A	1.1 (0.8–1.3)	70.0 (45–95)
PNL$^+$ Thc	SUP§	0.6 (0.4–0.7)	45.0 (25–65)
PNL$^+$ Thc	SUP§ + Con A	0.75 (0.4–1.2)	80.0 (29–180)
PNL$^+$ Thc	SUP (depl.)‖	0.35 (0.2–0.5)	8.5 (4.0–12)
PNL$^+$ Thc	SUP (depl.)‖ + Con A	0.85 (0.6–1.2)	55.0 (27–85)
PNL$^+$ Thc	SUP (depl.)‖ + PHA	0.20 (0.1–0.3)	17.0 (10–30)
PNL$^-$ Thc	0	0.25 (0.2–0.35)	1.2 (0.8–1.4)
PNL$^-$ Thc	Con A	0.9 (0.7–1.1)	80.0 (50–95)
PNL$^-$ Thc	PHA	0.6 (0.4–0.7)	25.0 (13–35)
PNL$^-$ Thc	SUP§ + Con A	1.2 (1.0–1.6)	100.0 (80–119)
PNL$^-$ Thc	SUP (depl.)‖	0.45 (0.2–0.6)	10.0 (5–15)
PNL$^-$ Thc	SUP (depl.)‖ + Con A	1.2 (0.9–1.4)	125.0 (80–180)

Cells of (C3H × C57)F_1 mice were tested. Results are the means of three or more experiments, with extreme values in brackets, 3rd day of culture.

* 1-ml cultures.

‡ LN cells: lethally (850 rads) irradiated LN cells, 0.5 × 10^6/culture.

§ Culture medium containing 30% supernate (see Materials and Methods).

‖ SUP (depl.): culture medium containing 30% supernate depleted in Con A (see Materials and Methods).

Are the proliferating cells derived only or mainly from the large PNL$^+$ Thc already undergoing cell division in the thymus, or can small, nondividing PNL$^+$ Thc (probably corresponding to small cortical Thc) also be induced to proliferate? PNL$^+$ Thc were fractionated by sedimentation according to their size (see Materials and Methods). The efficiency of separation was determined by cytologic criteria, and by [^{3}H]TdR incorporation: the slowly sedimenting, small cells synthesized almost no DNA (20–40 times less than the more rapidly sedimenting cells). When cultivated in SUP in the presence of Con A, nonfractionated PNL$^+$ Thc and such small PNL$^+$ Thc responded equally well (data not shown).

4. *Differentiation of PNL$^+$ Thc in Culture.* Time curves of cell recovery and [^{3}H]TdR incorporation in cultures of PNL$^+$ Thc stimulated by Con A in presence of SUP showed that proliferation peaked around day 3 and 4, at which time 80–100% of the cells were blasts. Cultures were then left untouched until the 8th

TABLE III

Immunofluorescence and Radioautographic Study of the Response of PNL⁺ Thc to Con A in a SUP: Binding of PNL to the Responding Cells as a Function of Time*

Time of culture arrest	Cells		Blasts‡	
	Of initial	PNL binding	Among recovered cells	PNL binding
	%		%	
Onset	100	97 (96–97)	3 (2–4)	98 (96–100)
15 h	60 (50–70)	95 (90–100)	15 (12–18)	86 (80–92)
35 h	70 (60–80)	80 (70–90)	50 (35–65)	70 (62–78)

Results are the means of two to three experiments. (C3H × C57)F_1 thc were used. After culture, cells were incubated for 30 min in HBSS with 0.05 M α-methyl mannoside and washed twice in HBSS before processing for PNL staining. Extreme values in brackets.

* For definition of SUP see Materials and Methods.

‡ Labeled cells on radioautography, after incubation with [^{3}H]TdR (20 μl/ml) for the last 30 min of culture.

TABLE IV

In Vitro Response to SRBC of T-Depleted Spleen Cells after Restoration with Fresh or Cultured PNL^+ and $^-$ Thc

Spleen cells (10^7 cells/culture)	T Cells (4×10^6 cells/culture)	PFC/Culture SRBC/culture:	
		0	2×10^6
Normal mouse serum + C′	0	40	750
Anti-Thy 1.1 + C′	0	80	70
Anti-Thy 1.1 + C′	LN cells	120	740
Anti-Thy 1.1 + C′	Fresh PNL^+ Thc	40	40
Anti-Thy 1.1 + C′	Fresh PNL^- Thc	30	820
Anti-Thy 1.1 + C′	"Cultured"* PNL^+ Thc	60	620
Anti-Thy 1.1 + C′	"Cultured"* PNL^- Thc	40	640

Results from the 5th-day of culture of cells from C57/B16 mice (means of triplicates).

* For definition of cultured cells, see Results, section 4.

day, placed for 2 days in fresh medium, and the cells recovered on day 10 (20 ± 5% of the input) analyzed. The cells had the appearance of medium-sized lymphocytes, and only 15 ± 10% of these were now PNL^+. Since the proliferation of PNL^+ Thc results in the appearance of cells which are mostly PNL^-, the properties of these were explored by studying (*a*) changes in the antigenicity of the cell membrane; (*b*) response to PHA; (*c*) possible acquisition of immunological competence and functions characteristic of mature T lymphocytes. With the exception of MICC, these studies were performed with medium-sized cells from 10-day cultures, as described above, and these will be referred to as cultured PNL^+ Thc, although the majority of them were PNL^- at that stage.

(*a*) SUSCEPTIBILITY TO ANTI-THY 1.1 AND ANTI-H_2 ALLOANTISERA. As shown in Table I, the susceptibility of the cultured cells to anti-Thy 1.1 and anti-H_2 alloantisera + C′ was completely modified. The cultured PNL^+ Thc cells now resembled PNL^- Thc or peripheral T lymphocytes.

TABLE V
Popliteal GVHR Elicited by Fresh and Cultured PNL$^+$ and PNL$^-$ Thc

Donor	Cells injected*	Recipient	Cells per popliteal LN $\times 10^{-6}$
C3H	Thc	(C3H × C57)F_1	3.3 (2.6–4.8)
C3H	LN Cells	(C3H × C57)F_1	18.5 (16–22)
C3H	Fresh PNL$^+$ Thc	(C3H × C57)F_1	3.0 (2.2–4)
C3H	Fresh PNL$^-$ Thc	(C3H × C57)F_1	19.0 (17–22)
C3H	"Cultured"‡ PNL$^+$ Thc	(C3H × C57)F_1	18.0 (16–23)
C3H	"Cultured"‡ PNL$^-$ Thc	(C3H × C57)F_1	16.0 (15–19)
C3H	LN Cells	C3H	3.0 (2.5–5)
C3H	"Cultured"‡ PNL$^+$ Thc	C3H	4.0 (3–5.5)

Results are the means of four experiments, with range in brackets.
* 10^7 viable cells from C3H were injected into the footpad of either C3H or (C3H × C57)F_1 recipients.
‡ For definition of cultured cells, see Results, section 4.

TABLE VI
Mixed Lymphocyte Culture Response of Fresh and Cultured C3H PNL$^+$ Thc and LN Cells in the Presence or Absence of Supernate

Exp.	Responder cells (C3H mice)	Culture medium	Stimulator spleen cells (irradiated)	[^{3}H]TdR Incorporation*
				cpm
A	LN Cells	Fresh	C3H	700 (650–800)
			(C3H × C57)F_1	7,000 (5,900–8,400)
	Fresh PNL$^+$ Thc	Fresh	C3H	180 (150–220)
			(C3H × C57)F_1	350 (250–400)
	"Cultured"‡ PNL$^+$ Thc	Fresh	C3H	700 (580–850)
			(C3H × C57)F_1	2,800 (2,100–3,000)
	Fresh PNL$^-$ Thc	Fresh	C3H	550 (400–650)
			(C3H × C57)F_1	2,450 (2,150–2,800)
B	Fresh PNL$^+$ Thc	Fresh	C3H	250 (180–350)
			(C3H × C57)F_1	600 (450–700)
	Fresh PNL$^+$ Thc	SUP§	C3H	350 (300–450)
			(C3H × C57)F_1	3,200 (2,500–4,400)
	Fresh PNL$^-$ Thc	Fresh	C3H	350 (250–390)
			(C3H × C57)F_1	4,600 (3,500–6,100)
	Fresh PNL$^-$ Thc	SUP§	C3H	400 (250–500)
			(C3H × C57)F_1	6,000 (5,000–6,800)

* Results of two representative experiments arrested at day 5; mean values of triplicate culture, with the range in brackets.
‡ For definition of cultured cells, see Results, section 4.
§ Culture medium prepared as described in Materials and Methods.

(*b*) PHA RESPONSIVENESS. In contrast to LN T lymphocytes (data not shown) and PNL$^-$ Thc, fresh PNL$^+$ Thc are not stimulated by PHA (Table II). However, cultured PNL$^+$ Thc, washed and further cultured in the presence of PHA responded strongly, but the peak values of [^{3}H]TdR incorporation ($35 \pm 0.4 \times 10^3$ cpm, mean of three experiments) were already reached within 24 h of culture, in contrast to what is observed with primary cultures of LN T lymphocytes or PNL$^-$ Thc.

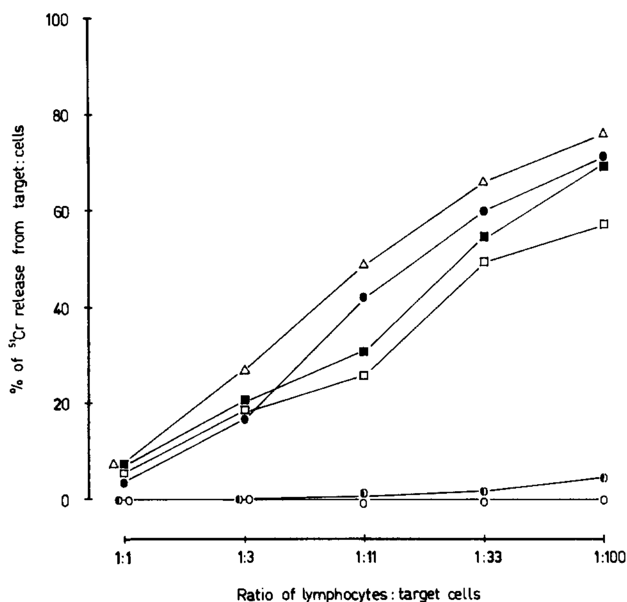

FIG. 1. MICC of PNL$^+$ and $^-$ Thc. Cells of (C3H × C57)F_1 mice were cultured 5 days with Con A in normal medium or in supernate. PHA revealed cell-mediated cytotoxicity. △——△ LN cells, cultured in normal medium; ○——○ PNL$^+$ Thc, cultured in normal medium; □——□ PNL$^-$ Thc, cultured in normal medium; ◐——◐ whole Thc, cultured in normal medium; ●——● PNL$^+$ Thc, cultured in SUP; ■——■ PNL$^-$ Thc, cultured in SUP.

(*c*) MICC. Con A induces in lymphoid cell cultures the appearance of polyclonal cytotoxic T cells whose killer activity on a given type of target cells is revealed in the presence of Con A or PHA (28–30). Cells recovered from 4-day Con A-stimulated cultures of LN cells, whole Thc, PNL$^+$, and $^-$ Thc in the presence of SUP developed comparable cytotoxicity, while total Thc and PNL$^+$ Thc cultured in the absence of SUP were not cytotoxic (Fig. 1).

(*d*) HELPER EFFECT IN A PRIMARY IN VITRO ANTIBODY RESPONSE TO SRBC. While fresh PNL$^+$ Thc were unable to help PFC differentiation in T-depleted primary spleen cell culture, cultured PNL$^+$ Thc were as effective in this respect as LN cells or PNL$^-$ Thc, fresh or cultured (Table IV).

(*e*) INDUCTION OF POPLITEAL GVHR. When injected in the foot pads of (C3H × C57)F_1 mice, C3H LN cells, PNL$^-$ Thc, and cultured PNL$^+$ Thc gave strong cellular increase in the draining popliteal LN, while injection of whole Thc or PNL$^+$ fresh Thc gave no response. Culture of PNL$^+$ Thc for 24 h in SUP alone did not confer these cells the capacity to elicit a GVHR (not shown).

(*f*) MLR. Fresh and cultured PNL$^+$ Thc, PNL$^-$ Thc, and LN cells of C3H mice were cultured for 5 days in the presence of irradiated spleen cells, either syngeneic or of (C3H × C57)F_1 origin. Fresh PNL$^+$ Thc did not react in these conditions, but an MLR, less intense than with LN cells, was consistently observed with cultured PNL$^+$ Thc and PNL$^-$ Thc (Table VI Expt. A). However, if mitogen-depleted SUP was added to the culture medium (Table VI, Exp. B), fresh PNL$^+$ Thc were also responsive.

Discussion

Some of the present observations confirm and extend the findings of Reisner et al. (3), showing that mouse Thc can be divided into two classes of cells, distinguished by the presence or absence of surface membrane receptors for PNL. This property allowed us to devise methods of Thc preparation, based mainly on cell affinity chromatography, yielding populations of PNL^+ and $^-$ Thc almost 100% pure, as judged by immunofluorescence. PNL^+ cells have a high Thy 1, low H_2 cell membrane content, are unresponsive to PHA and poorly responsive to Con A, do not express MICC, and are immunologically incompetent, as judged by an assay of in vitro helper function as well as by responsiveness in GVHR and MLR. PNL^- cells have opposite characteristics and behave in all these respects very similarly to peripheral T lymphocytes. These features, as well as the high percentage of PNL^+ cells and the observation that most cortisone-resistant cells are PNL^-, suggest that PNL^+ and $^-$ cells can be considered as cortical and medullary Thc, respectively (1–3, 31). It must be emphasized, however, that there is presently no way to ensure that the coincidence between these cell characteristics and localization in the thymus is total: for instance high doses of cortisone probably kill some PNL^- Thc, since cell recovery after cortisone administration is markedly lower than 15%, the average percentage of PNL^- Thc, and since a few PNL^+ Thc resist this treatment.

The salient feature of this work is the finding that PNL^+ immunoincompetent Thc can be induced to proliferate and differentiate in vitro, in the absence of any direct influence of the thymus, into lymphocytes which are immunocompetent both in vitro and in vivo, and which possess new cell membrane characteristics, comparable to those of peripheral T lymphocytes. The proliferating and differentiating cells very probably arise mainly from small cortical Thc, since these cells represent the bulk of the PNL^+ Thc, and since removal of large cells from the PNL^+ population did not reduce the ability of the small cells to proliferate in response to Con A. The implications of these observations can be discussed in relation to (*a*) the requirements for the differentiation of cortical Thc (*b*) the ontogenetic pathway of thymic-derived lymphocytes, and finally (*c*) the traffic of Thc within and from the thymus.

Proliferation and differentiation of cortical Thc require the presence in the culture medium of Con A and, in addition, of factor(s) produced by activated peripheral lymphoid cells (SUP). Either Con A or mitogen-depleted SUP alone stimulate PNL^+ Thc only marginally. However, in the presence of mitogen-depleted SUP the weak response to Con A is strongly enhanced and the PNL^+ Thc become able to respond to PHA and to alloantigenic stimulation (MLR). The observation that PNL^+ Thc can respond in an MLR in the presence of mitogen-depleted SUP indicated that these cells, although immunoincompetent, have already acquired specific surface receptors. On the other hand, SUP by itself is not a sufficient signal to induce maturation, since prior incubation of PNL^+ Thc in a mitogen-depleted SUP does not enhance a subsequent response to Con A stimulation, nor does it confer on them the capacity to induce a GVHR. Thus, SUP appears to represent for PNL^+ Thc a nonspecific second signal, which acts only in addition to a first signal, immunologically specific

(alloantigens) or not (mitogenic lectins) and which is necessary for the proliferation of these immature cells in response to the first signal. This role of SUP is consistent with observations showing that cultures of whole Thc are unable to proliferate and generate MICC in the presence of Con A or specific cytotoxic cells in MLR, except when irradiated spleen cells are added to the culture (26, 32, 33). In our experiments too, addition of irradiated lymphoid cells to the culture of PNL$^+$ Thc played the same role as addition of SUP, and presence of SUP was necessary to observe cells displaying MICC (Fig. 1). This SUP factor(s) is probably identical to the lymphocyte activating factor(s) first described by Gery, then by a number of other authors, and whose production might involve the participation of mature T lymphocytes or macrophages, or both (20–27, 34).

Thus, we can distinguish two steps in the differentiation of immature precursors into mature, immunologically reactive T lymphocytes: (*a*) acquisition of specific surface receptors, including alloantigen receptors, a process which might perhaps take place during the extensive cell division giving rise, in the cortex, to small cortical Thc. Small cortical Thc should not be considered immunologically incompetent, since they probably bear immunologically specific receptors, but immunologically unreactive; (*b*) development of the capacity to respond to receptor triggering by cell division without the help of factor(s) released by activated lymphoid cells. This second step of differentiation occurs in Thc cultures stimulated by Con A in the presence of SUP after marked cell proliferation, and may therefore result from cell division itself, if cell division of immature cells produces cells progressively more reactive to receptor triggering. The diversity of antigenic recognition of the differentiated cells may be attributed to the fact that Con A, a polyclonal mitogen, has stimulated a variety of cells with receptors for different antigenic specificities. Mature T cells do not need extracellular factor(s) to proliferate in response to mitogens or alloantigens, although there is evidence that helper factor(s) released by the activated cells themselves (35) or by the alloantigenic stimulating cells (36) may play a role in the response of mature T cells.

With regard to the ontogenetic pathway of thymic-derived cells, the present results provide conclusive evidence against the possibility, suggested by Shortman et al., Shortman and Jackson, and Hopper and Shortman (2, 37, 38) that Thc rich in Thy 1 antigen (high θ-lineage) represent a sterile differentiation pathway, independent of that of peripheral T lymphocytes, and perhaps destined to eliminate self-reactive cells. It is apparent, on the contrary, that the PNL$^+$ Thc population, probably corresponding mainly to high θ small cortical Thc, contains precursors for T lymphocytes with helper or cytotoxic function, and reactivity against allo and foreign antigens. However, this does not imply that the correct ontogenetic pathway is from small cortical Thc to nondividing medullary Thc to peripheral T cells, as often accepted. In fact, this is not compatible with the data of Shortman et al. and Shortman and Jackson (2, 37) which led to the formulation of the sterile differentiation pathway and which show that, under continuous [^{3}H]TdR availability, the increase of labeled small Thc occurs in parallel in both high θ (cortical) and low θ (medullary) Thc, while a direct pathway from small cortical to medullary Thc would imply a sequential appearance of the label. These data are, however, entirely consistent with the

observations of the present work strongly suggesting that further cell division is required for maturation of immunoincompetent high θ-low H_2 cortical Thc into low θ-high H_2 immunocompetent lymphocytes. The generation of nondividing medullary Thc would result from further division in the medulla of high θ cortical Thc, a process during which θ concentration decreases, accounting for the sizable proportion of dividing cells observed in the low θ Thc (37, 39, 40).

The traffic of cells within the thymus is still frequently considered to go from the cortex to the medulla and only then to the periphery (40, 41). Although selective labeling with [^{3}H]TdR of the dividing outer cortical Thc has shown that a fraction of medullary Thc are certainly derived from these cells (41), the number of labeled cells ultimately found in the medulla is, however, considerably lower than the number of cortical cells labeled. Furthermore, there is strong evidence that an important fraction of competent medullary Thc behaves as a resident population which does not leave the thymus, at least for a long time (1, 42, 43). This is compatible with the view that a large number of cortical Thc may in fact leave the thymus, mainly for the spleen (1, 43, 44), possibly being shortlived if they do not mature quickly; the presence of some PNL^+ T lymphocytes in peripheral lymphoid organs is in keeping with this idea (4). The observation that properly stimulated PNL^+ Thc can mature outside the thymus, in the absence of direct thymic influence, provides an interesting alternative, or addition, to the possibility that immature thymic cells may mature in peripheral lymphoid organs under the humoral influence of the thymus (43). A mechanism of differentiation similar to that observed in culture would indeed represent an efficient way of regulating the immune response by the recruitment, among immature T cells constantly emigrating from the thymus, of new immunocompetent T lymphocytes specific for an antigen already stimulating the peripheral lymphoid tissue: the cell division required for maturation would be initiated by the combined effects of antigen and, as in culture, of a second signal resulting from the stimulation of immunocompetent cells by this antigen.

Summary

Peanut lectin (PNL) binds to a majority of mouse thymocytes (Thc) in suspension. By using cell affinity chromatography on a column of anti-PNL antibody, Thc populations at least 96% pure in PNL^+ or $^-$ cells, as judged by immunofluorescence, were obtained. PNL^+ cells are rich in Thy 1 and poor in H_2 antigens, cortisone sensitive, unresponsive to phytohemagglutinin (PHA), and immunologically incompetent, as judged by mixed lymphocyte reaction, popliteal lymph node graft-versus-host assay, and by testing helper activity in a primary in vitro antibody response to sheep erythrocytes; the converse is true of PNL^- cells. Thus, PNL^+ and $^-$ cells appear to correspond to cortical and medullary Thc, respectively, as previously suggested. In culture, PNL^+ Thc show poor viability and a weak proliferative response to concanavalin A (Con A), except when supernate (SUP) of 24 h Con A stimulated lymph node lymphocyte cultures, or irradiated lymph node cells, are added, in which cases a strong proliferative response to the mitogen is observed. A variety of control experiments showed that the proliferating cells did not result from preferential stimulation of a few contaminating PNL^- Thc present in the PNL^+ Thc cultures.

The blasts resulting from PNL$^+$ Thc proliferation display mitogen-induced cytotoxicity, and give rise to a population of medium-sized lymphocytes, mostly PNL$^-$, poor in Thy 1 and rich in H_2 antigens, PHA responsive, and immunologically competent in the above-mentioned assays.

Fresh PNL$^+$ Thc responded in mixed lymphocyte reaction in the presence of SUP (lectin depleted) and since incubation in SUP alone did not confer reactivity on PNL$^+$ Thc, it appears therefore that (*a*) immature Thc possess alloantigen and mitogen-specific surface receptors but lack the capacity to respond by proliferation to receptor triggering without the help of extracellular factor(s) released by mature lymphoid cells stimulated by mitogens (*b*) cell division is associated with the acquisition of immunological responsiveness, characteristic of mature T lymphocytes. The implications of these findings for the ontogenesis of thymus-derived lymphocytes, and for the possible traffic of Thc within and from the thymus, are discussed.

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