FUNCTIONAL MATURATION OF THYMIC LYMPHOCYTE POPULATIONS IN VITRO*

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Lymphocytes which undergo maturation in the thymus are essential for cellular immunity and for the antibody response to some antigens. In the mouse it has been shown that thymus-derived (T)[1] lymphocytes are required for rejection of skin allografts (1, 2), initiation of graft-versus-host (GVH) reactions (2), development of killer cells specific for histoincompatible tumor cells (3), development of macrophage-mediated cellular immunity (4), initiation of the mixed lymphocyte interaction (MLI) (5), responsiveness to phytohemagglutinin (6), and provision of helper activity in the response to carrier-hapten conjugates and thymic-dependent antigens (7-10).

Two distinct subpopulations of thymic lymphocytes exist. The larger, functionally immature population resides primarily in the thymic cortex and is characterized by sensitivity to high dose steroid treatment (11, 12), relatively high cellular buoyant density (13), relatively high representation of the surface alloantigens θ, TL, Ly-A, Ly-B, and Ly-C, and relatively low representation of mouse H-2 histocompatibility antigens (14-18). The smaller functionally mature population resides primarily in the medulla and accounts for about 5% of the lymphocytes in the thymus (17-19). The mature cells are characterized by steroid resistance, lower cellular buoyant density, absence of the TL surface alloantigen, reduced representation of the θ and Ly antigens, and increased representation of H-2. The whole of graft-versus-host, mixed lymphocyte interaction, and phytohemagglutinin responsiveness found in the thymus seems to be a function of this smaller mature population (17-21). In addition,

[1] Abbreviations used in this paper: B, bone marrow derived; BUdR, 5-bromodeoxyuridine; Con A, concanavalin A; GVH, graft-versus-host; HBSS, Hanks' balanced salt solution; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MLI, mixed lymphocyte interaction; PAS, periodic acid-Schiff; PFC, plaque-forming cell; PHA-P, phytohemagglutinin-purified; PWM, pokeweed mitogen; T, thymus derived.

cells capable of cooperating with bone marrow-derived (B) lymphocytes are found in large numbers in the steroid-resistant thymus fraction (22). Maturation, or conversion of immunologically nonreactive to reactive thymic lymphocytes, thus appears to take place mainly within the thymus.

We have attempted to define the sequence of thymic lymphocyte maturation in more detail in hopes of learning more about cell function and heterogeneity. This proved difficult to accomplish in the intact animal (5), so we have devised an in vitro system which allows full functional maturation of thymic lymphocytes.

Materials and Methods

Mice.—8–12-wk old BALB/cJ or C57BL/6J mice from Jackson Laboratory, Bar Harbor, Maine, were maintained on laboratory chow and acidified-chlorinated water ad libitum. BALB/c cells were used in most experiments. Mice used as donors of steroid-resistant thymocytes were injected 48 hr before sacrifice with 2.5 mg hydrocortisone acetate (Merck Sharp and Dohme, West Point, Pa.) intraperitoneally.

Culture Techniques.—Spleens, thymuses, and lymph nodes were removed aseptically from mice and teased in cold, sterile Hanks' balanced salt solution (HBSS) lacking bicarbonate to obtain a single-cell suspension. Mediastinal lymph node contamination of thymus cells was avoided by injecting colloidal carbon intraperitoneally 2 hr before sacrifice and removing carbon-stained lymph nodes adherent to the thymus capsule and within the thymus. Cells were collected by centrifugation at 180 g for 10 min at 4°C and resuspended in complete culture medium. Nucleated cells were counted with a Model B Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) and adjusted to the appropriate cell density. The culture medium used in all experiments was RPMI 1640 (Associated Biomedic Systems, Inc., Buffalo, N. Y.) supplemented with 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, additional fresh L-glutamine (final concentration 4 mM; HEPES and glutamine obtained from Microbiological Associates, Inc., Bethesda, Md.), and 5% fetal bovine serum (Reheis Chemical Co., Kankakee, Ill., lot E 21806).

Mixed lymphocyte interactions and DNA synthetic responses to phytomitogens were incubated in 13 × 75 mm plastic tubes (Falcon No. 2003, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at 37°C in a humidified 5% CO_2-95% air atmosphere (23). Spleen cells to be assayed for antibody formation were cultured with modifications of the method of Mishell and Dutton (24, 25) using the supplemented RPMI 1640 medium described above.

Mixed Lymphocyte Interactions.—BALB/c cells derived from culture or from freshly sacrificed mice were adjusted to a density of 1 × 10⁶/ml, mixed with an equal number of allogeneic, C57BL/6 (*H-2ᵇ*), or syngeneic, BALB/c (*H-2ᵈ*), mitomycin-treated spleen cells and incubated in a final volume of 1.5 ml medium as described above. Target cells were exposed to 30 μg/ml mitomycin C (Calbiochem, San Diego, Calif.) for 30 min at 37°C and then thoroughly washed with medium at 4°C. After 48 hr, 0.5 μCi tritiated thymidine (Schwarz/Mann Div., Becton, Dickinson and Co., Orangeburg, N. Y.) was added to each tube; 24 hr later the cells were collected on glass fiber filters (No. 5270-D, Arthur H. Thomas Co., Philadelphia, Pa.), washed with cold phosphate-buffered saline containing 1 mM unlabeled thymidine (Schwarz/Mann), washed twice with cold 5% trichloroacetic acid containing 1 mM unlabeled thymidine, and finally with cold 95% ethanol. The dry filters were placed in Aquasol (New England Nuclear, Boston, Mass.) and counted in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Two or three replicate cultures were performed for each experimental group.

Phytomitogen Stimulation.—The responses of cultured or fresh BALB/c lymphoid cells to

the phytomitogens phytohemagglutinin-P (PHA-P), concanavalin A (Con A), and pokeweed mitogen (PWM) were compared. 1 μg/ml final concentration of PHA-P (Wellcome Reagents Ltd., Beckenham, England), Con A (ICN Nutritional Biochemicals Div., International Chemical and Nuclear Corp., Cleveland, Ohio), or a 1:10 dilution of the stock solution of PWM (Grand Island Biological Co., Grand Island, N. Y.) were used for stimulation. Cells were cultured at a density of 1×10^6 cells/ml in 1 ml volumes. Control cultures received an equivalent volume of phytomitogen diluent (HBSS). DNA synthesis between 48 and 72 hr of culture was assayed as described above for MLI.

Antibody Formation In Vitro.—The ability of cultured or fresh lymphoid cells to cooperate in the in vitro plaque-forming cell (PFC) response was assessed in a series of experiments. Spleen cells from C57BL/6 mice were depleted of thymus-derived lymphocytes by treatment with anti-θ serum and complement. AKR anti-θ C3H serum was prepared by the method of Raff (26), collected, and frozen at $-20°$C until use. Freshly harvested spleen cells were washed twice in HBSS and resuspended in neat anti-θ serum at a density of 5×10^7 cells/ml. The cells were incubated at 4°C for 30 min, washed once with 50 ml HBSS, and resuspended in a 1:4 dilution of guinea pig complement (BBL, Division of BioQuest, Cockeysville, Md.), previously shown to be noncytotoxic for mouse cells, containing 10 μg/ml deoxyribonuclease (Worthington Biochemical Corp., Freehold, N. J.). The cells were incubated at 37°C for 30 min, washed twice with HBSS, and resuspended in medium. The cells were cultured with 5×10^6 sheep erythrocytes at a final density of 1×10^7 cells/ml with or without additional cell populations under conditions described in detail previously (25). Antibody formation was assayed after 5 days by enumerating both direct (IgM) and indirect (IgG) PFC using the slide modification of the Jerne hemolysis-in-gel technique previously described (25). Numbers of plaque-forming cells are expressed as the mean of replicate cultures $\pm$ standard error of the mean.

Cytotoxicity Assays.—The susceptibility of cultured or fresh lymphoid cells to killing by anti-θ serum and complement was determined in a series of experiments. Lymphoid cells were labeled with ^{51}Cr by incubation with 0.1 mCi $Na_2^{51}CrO_4$ (The Radiochemical Centre, Amersham, England)/50 $\times$ 10^6 cells in L-15 medium (Microbiological Associates, Inc.) supplemented with 10% bovine serum for 1 hr at 37°C (27). The cells were washed twice with 50 ml L-15, then mixed with appropriate dilutions of anti-θ serum and guinea pig complement and incubated at 37°C for 30 min. The amount of ^{51}Cr released into the supernate was assessed using a Packard gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The percentage ^{51}Cr released was calculated using a freeze-thaw control as 100% release and a complement-alone control as 0% (27).

5-Bromodeoxyuridine (BUdR) and Light Treatment.—Cells were exposed to 10^{-6} M 5-bromodeoxyuridine (Sigma Chemical Co., St. Louis, Mo.) for appropriate periods of time and lighted for 1 hr at a distance of 4 cm for a 15 w fluorescent lamp.

Supporting Cell Populations.—Three cell populations were tested for their ability to promote thymic lymphocyte maturation. The first, splenic adherent cells, were prepared by culturing 1×10^7 spleen cells in a 60 mm plastic culture dish (Falcon, No. 3002) for 1 hr at 37°C on a rocking platform. Nonadherent cells were removed by three successive vigorous washings with cold HBSS so that approximately 1% of the original cell number remained. The functional properties of adherent cells have been described previously (28, 29). 5 ml of a fresh thymocyte suspension containing 5×10^6 cells/ml was added to the adherent cells and incubated on a rocking platform (usually for 2 days) at 37°C in an atmosphere of 7% O_2, 10% CO_2, and 83% N_2. Cells were collected from culture by aspiration after gentle agitation of the dish.

The second population of supporting cells, thymic epithelial cells, was prepared by gently teasing apart a normal BALB/c thymus, removing the lymphocytes in suspension, and recovering the fragments of stroma and capsule. These were placed in a 75 cm^2 tissue culture flask (Falcon, No. 3024) and cultured in complete RPMI 1640 medium at 37°C in humidified

5% CO_2-95% air. After 3 wk, a monolayer consisting of epithelial cells and a few fibroblasts covered the bottom of the flask. Representative cultures were fixed and stained with the periodic acid-Schiff (PAS) reaction; epithelial cells did not stain but the few fibroblasts were PAS-positive. Several such primary cultures were maintained with weekly changes of medium, and subcultures prepared in 60-mm culture dishes after scraping cells from the primary culture with a rubber policeman. By 5 days after subculture of 1×10^6 cells/dish, a near-confluent monolayer had formed. At this standard time interval, 5 ml of fresh thymocytes at a concentration of 5×10^6 cells/ml was added and cultured for 2 days on a rocking platform in the humidified atmosphere of 7% O_2, 10% CO_2, and 83% N_2 at 37°C. Cells were recovered as above, resuspended in fresh medium, and their function assayed. Where C57BL/6 thymocytes were used, the epithelial cells were prepared in an identical manner using C57BL/6 mice.

The third population of supporting cells, kidney fibroblasts, was prepared by mincing and trypsinizing adult BALB/c kidneys and culturing them in large flasks as described above. Subcultures were prepared in 60-mm dishes and thymocytes added as above at a standard interval of time after subculture. Cultured thymocytes were recovered gently so that most of the fibroblasts remained attached to the dish.

RESULTS

Survival of Cultured Thymocytes.—Mouse thymocytes were cultured for different periods of time to determine the numbers of cells surviving and their functional activity. Normal thymocytes cultured without additional supporting cells at a density of 5×10^6 cells/ml survived poorly. Cell viability was determined by trypan blue exclusion. Only 10% of cells initially cultured survived 2 days in vitro (Table I). Few cells if any survived longer periods of culture.

If thymocytes were cultured in a dish containing adherent spleen cells, their survival was markedly improved (Table I). As few as 5×10^5 adherent cells increased the 2-day survival of 5×10^6 thymocytes from 10 to 35%. Conditioned medium from the same number of adherent cells had only a slight enhancing effect on thymocyte survival whether used undiluted or at a 1:10 or 1:100 dilution.

Thymocytes cultured on monolayers of thymic epithelial cells were similar to thymocytes cultured alone in survival (Table I). As will be demonstrated

TABLE I

Survival of Cultured Thymocytes

	Per cent viable cells* after			
	1 day	2 days	3 days	4 days
Normal thymus‡ (BALB/c)	32	10	4	1
Normal thymus + adherent cells	48	34	10	−§
Normal thymus + epithelial cells	35	12	4	—
Normal thymus + fibroblasts	30	9	2	—

* Cells excluding trypan blue. These results represent one typical experiment.

‡ Thymocytes cultured at 5×10^6 cells/ml in RPMI 1640, 5% fetal bovine serum, and 15 mM HEPES.

§ Not done.

below, the similarity in recovery of viable cells was in marked contrast to the difference in functional activity of the two cell populations.

Thymocytes cultured on kidney fibroblast monolayers were nearly identical in survival with thymocytes cultured alone or on thymic epithelium (Table I). As a control population, steroid-resistant thymocytes were cultured on either adherent or thymic epithelial cells. Fewer than 1% of the cells survived 2 days in culture; this population thus could not be used in further experiments.

Functional Activity of Cultured Thymocytes.—Thymocytes recovered from culture after 2 days were compared with freshly prepared steroid-resistant thymocytes, normal thymocytes, lymph node cells, and spleen cells for functional activity. Activity was assessed by the mixed lymphocyte interaction and the response to the phytomitogens PHA, Con A, and PWM.

The response of the various cultured and freshly prepared lymphoid populations to stimulation by mitomycin-treated allogeneic cells is shown in Table II. Normal thymocytes reacted poorly in the MLI, as did thymocytes cultured alone. Thymocytes cultured on either adherent or epithelial cells showed increased reactivity both to allogeneic and syngeneic cells; their activity closely resembled that of lymph node or spleen cells, while steroid-resistant thymocytes showed the greatest increase in thymidine incorporation in the MLI.

The same cell populations were compared for their response to PHA (Table III). Normal thymocytes responded poorly to PHA. Thymocytes cultured for 2 days were more responsive, and thymocytes cultured on adherent or epithelial cells were most responsive, being equal to lymph node and spleen cells

TABLE II

Response of Cultured Thymocyte Populations in the MLI

Cell populations 1 × 10⁶ BALB/c cells from:	cpm*	
	+ Allogeneic mitomycin-treated spleen cells	+ Syngeneic mitomycin-treated spleen cells
Normal thymus	2,083 ± 145	423 ± 118
Thymus cultured 48 hr alone	1,527 ± 267	298 ± 120
Thymus cultured 48 hr with adherent cells	15,922 ± 2,162	6,950 ± 2,427
Thymus cultured 48 hr with epithelial cells	7,962 ± 1,781	2,055 ± 122
Steroid-resistant thymus	4,579 ± 248	490 ± 74
Normal lymph node	15,561 ± 1,193	6,053 ± 1,560
Normal spleen	14,791 ± 1,127	7,096 ± 1,419

* To initiate the MLI, 1 × 10⁶ reacting (BALB/c) cells were mixed with 1 × 10⁶ mitomycin-treated allogeneic (C57BL/6) or syngeneic (BALB/c) spleen cells and incubated for 72 hr. Tritiated thymidine incorporation during the final 24 hr of culture was determined. Numbers are the mean ± standard error of the mean for five replicate experiments. Mitomycin-treated C57BL/6 spleen cells cultured alone had a mean thymidine incorporation of 452 ± 127 cpm. Mitomycin-treated BALB/c spleen cells cultured alone had a mean thymidine incorporation of 337 ± 144 cpm.

and greater than steroid-resistant thymocytes. Con A significantly stimulated normal thymocytes, but this responsiveness was further increased by culture with either adherent or thymic epithelial cells (Table IV).

PWM at high concentrations caused an increase in thymidine incorporation

TABLE III

Response of Cultured Thymocyte Populations to PHA

Cell populations 1 × 10⁶ BALB/c cells from:	cpm*	
	+ PHA	Unstimulated
Normal thymus	1,191 ± 121	467 ± 130
Thymus cultured 48 hr alone	4,064 ± 1,780	652 ± 134
Thymus cultured 48 hr with adherent cells	44,771 ± 16,406	9,885 ± 574
Thymus cultured 48 hr with epithelial cells	43,752 ± 12,764	2,679 ± 585
Steroid-resistant thymus	11,350 ± 1,486	543 ± 110
Normal lymph node	47,643 ± 11,640	2,080 ± 167
Normal spleen	68,706 ± 15,668	9,141 ± 1,706

* Incorporation of tritiated thymidine from 48 to 72 hr of culture of equal numbers of reacting cells was measured. PHA was added at time 0 at a concentration of 1 μg/ml. Numbers are the mean ± standard error of the mean for five replicate experiments.

TABLE IV

Response of Cultured Thymocytes to Con A

Cell populations 1 × 10⁶ BALB/c cells from:	cpm*	
	+ Con A	Unstimulated
Normal thymus	12,459 ± 1,545	494 ± 132
Thymus cultured 48 hr alone	14,223 ± 1,659	728 ± 143
Thymus cultured 48 hr with adherent cells	87,498 ± 15,885	11,722 ± 1,625
Thymus cultured 48 hr with epithelial cells	137,720 ± 12,217	2,679 ± 158
Steroid-resistant thymus	22,059 ± 1,671	466 ± 126
Normal lymph node	72,277 ± 12,189	2,328 ± 139
Normal spleen	116,680 ± 12,589	8,091 ± 1,538

* Numbers are mean ± standard error of the mean for five replicate experiments. Con A was present at 1 μg/ml in stimulated cultures. Tritiated thymidine incorporation between 48 and 72 hr of culture was determined.

in normal thymocytes. This stimulation decreased if normal thymocytes were cultured alone for 2 days, but was significantly increased if the thymocytes were cultured with adherent or epithelial cells (Table V).

Cultured thymocytes thus behave similarly by each of these four in vitro criteria. Thymocytes cultured alone are variable in their responsiveness, but not greatly different from normal thymocytes. Thymocytes cultured with a

TABLE V

Response of Cultured Thymocyte Populations to PWM

Cell populations 1 × 10⁶ BALB/c cells from:	cpm*	
	+ PWM	Unstimulated
Normal thymus	4,834 ± 1,074	975 ± 174
Thymus cultured 48 hr alone	1,203 ± 312	617 ± 117
Thymus cultured 48 hr with adherent cells	13,034 ± 2,446	5,612 ± 1,212
Thymus cultured 48 hr with epithelial cells	12,672 ± 1,823	1,069 ± 215
Steroid-resistant thymus	18,302 ± 5,029	867 ± 189
Normal lymph node	28,379 ± 6,250	3,901 ± 851
Normal spleen	38,159 ± 8,762	8,102 ± 2,094

* PWM was added to stimulated cultures as 50 μl of 1:10 stock solution. Numbers represent means ± standard errors of the mean of three replicate experiments. Tritiated thymidine incorporation between 48 and 72 hr of culture was measured.

supporting cell population displayed increased responsiveness, often exceeding that of steroid-resistant thymocytes and equaling that of lymph node or spleen cells. Since the response to PHA was a good index of increased reactivity, it was used in further experiments to be described below.

Although both supporting cell populations, adherent cells and thymic epithelial cells, produced enhanced activity of surviving thymocytes, the level of thymidine incorporation without specific stimulation was considerably higher when adherent cells were used. Accordingly, the actual increment in thymidine incorporation was greater for thymocytes cultured on epithelial cells. As mentioned, thymocytes cultured with adherent cells incorporated tritiated thymidine at a much greater rate than thymocytes cultured alone in the absence of specific mitogen (e.g., Tables II–IV). Since adherent cells cultured alone for 2 days gave rise to some detached cells, the possibility that detached adherent cells contributed to the mitotic activity of cultured thymocytes was examined. Adherent cells were prepared as usual, but 5 × 10⁶ thymocytes irradiated with 2000 rads (G.E. Maximar 250 Type III, General Electric Co., Schenectady, N. Y., 250 kvp, 15 ma, 1 mm Al, 0.5 mm Cu filters) in vitro instead of normal thymocytes were added. After 2 days, cells were harvested and stimulated with PHA. No incorporation of thymidine was obtained, so detached adherent cells could not have been contributing directly to the increased mitotic activity of thymocytes cultured with adherent cells.

The possibility that the enhancing effect of thymic epithelial cultures might be due to the minor population of fibroblasts was examined. Thymocytes were cultured on adherent cells, thymic epithelium, or cultures of kidney fibroblasts and their responses to PHA compared (Table VI). Fibroblasts were much less efficient than thymic epithelial cells in promoting the increase in thymocyte

TABLE VI

Relative Efficiency of Different Supporting Cells in Promoting Thymocyte PHA Responsiveness

Supporting cells	cpm*		Δcpm‡	Relative efficiency§
	+ 1 μg PHA	Unstimulated		
Spleen adherent (approximately 10^5)	17,825 ± 3,126	6,187 ± 1,043	11,638	24.6
Thymic epithelium (monolayer)	14,752 ± 1,216	1,202 ± 89	13,550	28.6
Kidney fibroblasts (monolayer)	6,148 ± 842	1,638 ± 128	4,510	9.5
None	1,328 ± 129	855 ± 42	473	1

* Data from one representative experiment. Mean cpm ± SE for three replicate cultures.
‡ Δcpm = stimulated − unstimulated cpm.
§ Arbitrary ratio with activity of thymocytes cultured alone equal to 1.

responsiveness. The probability that the few fibroblasts contaminating the epithelial cell cultures contribute significantly to their function thus is lessened.

The cultured thymocyte populations finally were assayed for "helper" cell function in the in vitro antibody response to sheep erythrocytes. This response requires the participation of three cell types, one of which is thymus derived, and can be abrogated by elimination of θ-positive cells. Accordingly, spleen cells were treated with anti-θ serum and complement and cultured thymus cells or other sources of thymus-derived lymphocytes were compared for their ability to restore the PFC response in vitro. Table VII shows the results of three such experiments. Normal thymocytes were totally unable to restore helper cell function. Cultured thymocytes partially restored the response. Thymocytes cultured alone were least effective, thymocytes cultured on adherent cells were more effective, and thymocytes cultured on thymic epithelium completely restored helper cell activity. Of the control cell populations, both steroid-resistant thymocytes and normal lymph node cells restored the antibody response.

These experiments have shown that normal thymocytes cultured for 2 days with suitable supporting cells develop into an immunologically competent population identical in activity with peripheral mature thymus-derived lymphocytes. The key question to be answered is whether this increase in functional activity represents maturation of immature thymocytes to reactive mature lymphocytes (equivalent to thymus-derived lymphocytes) or selective survival of mature cells by death of irrelevant immature cells in a way analogous to the presumed action of in vivo steroid treatment.

Susceptibility of Cultured Thymocytes to Anti-θ Serum and Complement.—The average number of θ determinants per cell can be estimated by the titer of anti-θ serum required to kill 50% of a given cell population. Since one measure of the maturity of a thymic lymphocyte is the decreased number of θ deter-

TABLE VII

Restoration of Anti-θ-treated Spleen Cell PFC Response by Cultured Thymocytes

C57BL/6 Spleen cells (10 × 10⁶)	C57BL/6 T cell supplement (2 × 10⁶)	Day 5 PFC/culture* ± SE	
		IgM	IgG‡
Anti-θ spleen§	—	518 ± 180	253 ± 85
Anti-θ spleen	Normal thymus	430 ± 21	467 ± 82
Anti-θ spleen	48 hr thymus	959 ± 60	700 ± 40
Anti-θ spleen	48 hr thymus + adherent cells	1968 ± 343	1213 ± 168
Anti-θ spleen	48 hr thymus + epithelial cells	5100 ± 348	1453 ± 217
Anti-θ spleen	Steroid-resistant thymus	5750 ± 137	1600 ± 140
Anti-θ spleen	Normal lymph node	4814 ± 334	2520 ± 230
Brain-absorbed‖ anti-θ spleen	—	3471 ± 329	1587 ± 105
Brain-absorbed anti-θ spleen (background)	No antigen	140 ± 15	33 ± 7

* Antigen = sheep erythrocytes; numbers are the mean ± standard error of the mean of three replicate experiments.

‡ IgG PFC were developed according to the method of Pierce et al. (25).

§ Spleen cells bearing θ determinants were depleted by treatment with neat anti-θ serum and complement (see text).

‖ Anti-θ activity was absorbed by reacting brain cells from two C3H mice with 4 ml anti-θ C3H serum at 4°C for 1 hr.

minants, "mature" cells should be more difficult to kill with anti-θ serum and complement. Normal thymocytes, lymph node cells, steroid-resistant thymocytes, and various cultured thymocytes were labeled with ^{51}Cr and treated with serial dilutions of anti-θ serum and complement. The proportion of cells killed by this treatment as measured by the percentage ^{51}Cr release is shown in Fig. 1. Normal thymocytes are highly susceptible to lysis by anti-θ serum, suggesting that they have a high average θ content per cell. Thymocytes cultured alone for 2 days were similar in their killing curve, even though their functional activity had increased by some parameters of thymic lymphocyte function (Tables II–V). There thus had been no significant selection for the mature θ-poor thymocyte fraction. In contrast, thymocytes cultured on adherent or epithelial cells resembled mature steroid-resistant or peripheral cells in their susceptibility of killing; i.e., their average θ content per cell was lower. Since it was in the thymocytes cultured with supporting cells that the greatest increase in functional activity was seen, this finding means either that all immature θ-rich cells matured into θ-poor cells in vitro, or that all θ-rich immature cells died. The latter possibility seemed remote since the number of highly reactive thymocytes surviving 2 days culture on adherent cells was much higher (35%) than the estimated 10% of mature cells in the normal thymus. The possibility that selective cell death of the immature thymocyte population in culture gave rise to a surviving mature population was evaluated in the following experiments.
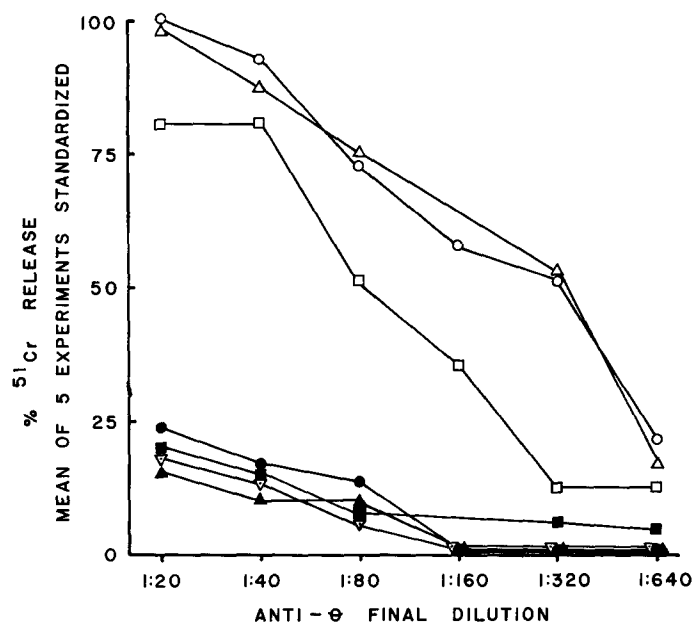
Fig. 1. Susceptibility of various BALB/c lymphoid cell populations to killing by anti-θ C3H serum and guinea pig complement. Data points represent the mean percentage of ^{51}Cr release in five replicate experiments. The data were standardized by setting the percentage kill of normal thymocytes at 1:20 dilution of anti-θ serum to 100% in each experiment and expressing the killing of other lymphoid populations as a proportion of thymocyte killing. The actual percentage of ^{51}Cr release for normal thymocytes ranged between 78 and 95% in the five experiments. O ——O, normal thymocytes; △——△, thymocytes cultured 48 hr alone; □——□, normal lymph node cells; ●——●, normal spleen cells; ■——■, steroid-resistant thymocytes; ▽——▽, thymocytes cultured 48 hr with epithelial cells; ▲——▲, thymocytes cultured 48 hr with adherent cells.

Requirement for Division for In Vitro Thymocyte Maturation.—The requirement for cell division for increase of thymocyte reactivity during culture was assessed. Most rapidly dividing cells in the thymus belong to the immature pool in the cortex, whereas the mature medullary population seems to be in a relatively quiescent state. It was hypothesized, therefore, that if only mature thymocytes survived in culture, cell division might not be required for the functional increase in activity. Thymocytes were cultured in the presence of BUdR so that dividing cells could subsequently be eliminated by light treatment. After lighting, the ability of the surviving cells to react to PHA (Table VIII) or to act as helper cells (Table IX) was determined. BUdR and light treatment abolished the responsiveness of the cultured thymocytes by both criteria of function. Cell division thus was required for the increase of cell reactivity in vitro. If, however, mature thymocytes were required to divide to sustain themselves in vitro, this same result would have been obtained. The following

TABLE VIII

Requirement for Division for In Vitro Thymocyte Maturation: PHA Response

Reacting cells (BALB/c)	BUdR*	Light	cpm‡	
			+ 1 µg PHA	Unstimulated
Thymocytes cultured alone	--	—	1,438 ± 128	976 ± 36
	10^{-6} M	—	1,207 ± 104	1,021 ± 143
	10^{-6} M	+	957 ± 42	1,000 ± 62
Thymocytes + adherent cells	—	—	16,756 ± 804	6,761 ± 129
	10^{-6} M	—	21,532 ± 1,106	7,331 ± 277
	10^{-6} M	+	8,203 ± 219	6,456 ± 854
Thymocytes + epithelial cells	—	--	15,300 ± 1,244	1,140 ± 62
	10^{-6} M	—	16,033 ± 822	1,335 ± 93
	10^{-6} M	+	1,186 ± 120	1,111 ± 86

* BUdR was present throughout initial 48 hr of culture. Cells were exposed to light for 1 hr, washed, then cultured for 72 hr with or without PHA.

‡ Cells were exposed to 0.5 µCi thymidine-^{3}H between 48 and 72 hr of the second culture interval. Numbers are the mean ± SE of three replicate cultures in one representative experiment.

TABLE IX

Requirement for Division for In Vitro Thymocyte Maturation: Helper Cell Function

C57BL/6 Spleen cells (10 × 10^6)	C57BL/6 T cell supplement (2 × 10^6)	BUdR + light‡	Day 5§ PFC/culture
Anti-θ spleen*	—	--	188 ± 58
Anti-θ spleen	Normal thymus	—	205 ± 60
Anti-θ spleen	48 hr thymus	—	275 ± 76
Anti-θ spleen	48 hr thymus	+	180 ± 50
Anti-θ spleen	48 hr thymus + adherent cells	----	643 ± 136
Anti-θ spleen	48 hr thymus + adherent cells	+	135 ± 5
Anti-θ spleen	48 hr thymus + epithelial cells	---	868 ± 218
Anti-θ spleen	48 hr thymus + epithelial cells	+	190 ± 20
Anti-θ spleen	Normal lymph node	--	1360 ± 158

* Spleen cells treated with anti-θ serum and complement as described in the text.

‡ BUdR and light used as described in Table VIII.

§ IgM PFC. Numbers are the means ± standard error of the mean for two replicate experiments. Antigen = sheep erythrocytes.

rather complicated experiment was designed to more definitely answer whether active cultured thymocytes arose from immature or mature precursors.

Original State of Maturity of Thymocytes Surviving In Vitro.—Immature thymocytes are characterized by a relatively high average number of θ determinants and a low number of *H-2* determinants per cell. Mature thymocytes have a relatively sparse representation of θ, but a high number of *H-2* determinants per cell. Anti-θ and anti-*H-2^b* sera were titrated to determine the dilu-

tion that would kill 50% of normal C57BL/6 thymocytes. Treating thymocytes with this dilution of anti-θ serum would cause a relative enrichment of θ-poor mature cells, and treating with anti-H-2 serum would cause a relative enrichment of H-2-poor immature cells. The two thymocyte populations surviving after such treatment were then cultured for 2 days either alone, with adherent cells, or with thymic epithelial cells. BUdR and light treatment was employed as before to determine if cell division were required for cells surviving antiserum and complement treatment to maintain or increase their functional activity. The activity of the cultured C57BL/6 cells was determined both by PHA responsiveness and ability to restore the PFC response of anti-θ-treated spleen. Depletion of θ-rich immature cells had no effect on the activity of cells surviving in culture. Depletion of H-2-rich mature cells, in contrast, prevented the increase in activity of cultured thymocytes (Table X). BUdR and light impaired the responsiveness of active cultured thymocyte populations (not shown). Culture of thymocytes, particularly on adherent or epithelial cells, thus seems to promote the proliferation and final maturation of cells in the minor, mature thymocyte fraction characterized by reduced θ antigen and increased H-2 antigen content.

TABLE X

Selective Survival of H-2-rich, θ-poor Thymocytes In Vitro

Initial treatment of thymocytes*	Cultured 48 hr on	PHA response‡ cpm		Day 5 PFC/culture of anti-θ-treated spleen + 2 × 10⁶ cultured cells§
		+ PHA	Unstimulated	
1:40 Anti-θ + C	—	4,665 ± 456‖	1,317 ± 225	343 ± 63
	Adherent	24,372 ± 665	3,673 ± 260	643 ± 92
	Epithelial	18,398 ± 342	3,311 ± 75	728 ± 82
1:40 anti-H-2^b¶ + C	—	665 ± 92	361 ± 38	100 ± 31
	Adherent	5,629 ± 731	2,483 ± 138	55 ± 5
	Epithelial	2,707 ± 777	2,517 ± 277	65 ± 8
None	—	3,894 ± 617	1,557 ± 102	275 ± 30
	Adherent	22,077 ± 4,568	3,590 ± 899	680 ± 22
	Epithelial	13,195 ± 22	3,138 ± 146	950 ± 85
No thymocytes added	—	—	—	188 ± 58

* Thymocytes were exposed to either 1:40 anti-θ serum or 1:40 anti-H-2^b serum, then complement, then washed before initial 48 hr culture.

‡ Cells cultured with PHA after recovery and washing after initial 48 hr culture. Tritiated thymidine incorporation between 48 and 72 hr of second culture interval was determined.

§ 10 × 10⁶ anti-θ-treated spleen cells were reconstituted with 2 × 10⁶ cultured thymocytes and the IgM PFC response was measured after 5 days' incubation with sheep erythrocytes.

‖ Numbers are the mean ± standard error of the mean for three replicate cultures in one representative experiment.

¶ Anti-H-2^b serum was obtained from DBA/2 mice 14 days after the last of six biweekly immunizations with 25 × 10⁶ C57BL/6 spleen cells.

DISCUSSION

We have developed techniques for the production of immunologically reactive thymic lymphocytes in vitro. These relatively simple techniques provide an almost pure population of lymphocytes with reactivity equal to or greater than peripheral T lymphocytes by several parameters. The active population of thymic lymphocytes recovered after incubation on adherent or thymic epithelial cells was derived from the small subpopulation of relatively mature cells characterized by reduced sensitivity to anti-θ serum and complement and increased sensitivity to anti-H-2 serum and complement.

We interpret the results of the experiments presented in Tables VIII–X to indicate that the generation of immunologically active thymus cell populations in vitro results from both differentiation and selection processes. The requirement for cell division for the increased activity of cultured thymocytes, particularly those selected for low θ content by previous treatment with anti-θ serum and complement, strongly suggests that differentiation accompanies the final cycles of division leading to the generation of fully active thymus-derived lymphocytes in vitro. Mature thymus-derived lymphocytes generated in vitro were more active on a cell-per-cell basis than steroid-resistant thymocytes.

Both steroid resistance and reduced θ content therefore seem to define an intermediate cell type with some functional activity, but which requires further differentiation to express its full potential. On the other hand, the demonstration that the cells with already increased H-2 determinants are the cells which participate in this differentiation process indicates that a clear selection process operates to the detriment of less mature cells richer in θ and poorer in H-2 determinants.

The activity of cultured thymocytes was greatly enhanced by the presence of splenic adherent cells or thymic epithelium. These cells may have functioned as "feeder layers" providing some limiting culture nutrient to the thymocytes. No evidence of a soluble factor produced by either cell type could be found. Such negative evidence does not, of course, exclude products which are labile or act at short range, and stimulatory products have been described which potentiate thymocyte reactivity (30). Adherent cells and thymic epithelial cells were somewhat different in their effects on cultured thymocytes. Adherent cells promoted better cell survival, greater thymidine incorporation both in stimulated and unstimulated cultures, but only an intermediate level of helper cell activity. Thymic epithelial cells did not enhance thymocyte survival, but were more active than adherent cells in thymocyte activation. The background level of thymidine incorporation was less for epithelial cells than for adherent cells, and helper cell activity was greater when thymic epithelial cells were used. Adherent and thymic epithelial cells thus seem to differ in their supporting roles in thymocyte maturation. The rationale for using thymic epithelium cultures was their demonstrated role in inducing thymic lymphopoesis in ontogeny (31, 32). Perhaps epithelial cells continue to produce an "inducing

factor" during adult life which aids the in vivo maturation of thymic lympho-cytes. Such a factor, which stimulates lymphoid differentiation, has been isolated recently from a clonal tumor cell line (33).

The requirement for division for increased activity of cultured thymocytes is somewhat surprising since the most rapidly dividing population in the thymus seems to be the immature cortical cells (34). Perhaps the fetal calf serum com-ponent of the medium or the products of the many dying thymocytes suffice to stimulate cell division. Another possibility is that division is normally required for the final steps of thymic lymphocyte maturation, but the number of cells in that particular stage of differentiation in vivo is quite small and therefore difficult to detect.

These experiments incidentally confirm a number of previous observations made by several authors (18, 19, 21). Two minor exceptions were noted. First, steroid-resistant thymocytes as well as cultured thymocytes responded to pokeweed mitogen in appropriate doses, in contradiction to the report of Janossy and Greaves (35) that PWM is only a B cell stimulant. Secondly, no separation between cellular immunity (as reflected by MLI or phytomitogen responses) and helper cell activity was observed, in contrast to the report of Segal et al. (36). We envision maturation of thymic lymphocytes to proceed through a number of stages, each involving the acquisition of more immuno-logical reactivity, and involving two or more mature stages with cells having the surface markers characteristic of the mature thymic lymphocyte but vary-ing in functional activity.

Thymus-derived lymphocytes have been shown to be heterogeneous with regard to their migration patterns; some are lymph node-seeking and others spleen-seeking (37). It is possible that these migration properties reflect the stage of maturation at which the lymphocytes emigrate from the thymus, i.e., that some T lymphocytes leave the thymus at earlier stages than others. This interpretation would suggest that the synergy between different T lymphocyte populations in producing GVH reactions (38) might represent cooperation between two cells of the same cell line but different stages of maturation. Whether further maturation of thymic lymphocytes can occur in vivo after the cells have left the thymus may also depend upon the stage of maturation at the time of emigration. This experimental system provides a relatively simple, reproducible model for obtaining pure populations of thymus-derived lymphocytes in vitro, and may lead to further understanding of the cellular events leading to the production of the thymus-derived cell so ubiquitous in immune phenomena.

SUMMARY

Mouse thymocytes were cultured for short periods of time either alone or with one of two supporting cell populations, splenic adherent cells or thymic epithelial cells. The thymus-derived (T) cell activity of thymocytes cultured on

supporting cell populations increased dramatically during 2 days of culture, as assayed in the mixed lymphocyte interaction (MLI), response to phytomitogens, and helper cell activity in the in vitro antibody response. The level of activity attained was equal to that of spleen and lymph node lymphocytes and greater than that of steroid-resistant thymocytes. The cultured thymocytes had surface antigens characteristic of mature T lymphocytes with regard to θ and H-2. The appearance of functionally active lymphocytes in vitro depended upon cell division. Most of the active cultured cells arose from cells already undergoing maturation, i.e., from cells with reduced θ determinants and increased H-2 determinants. We therefore have generated a population of thymocytes indistinguishable from peripheral T lymphocytes using simple in vitro techniques. The extent to which the production of these active lymphocytes depends upon in vitro differentiation is discussed.

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