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Identification and Separation of Pre T-Cells from nu/nu Mice: Differentiation by Preculture with Thymic Reticuloepithelial Cells [1]

VICKI L. SATO, [2] SAMUEL D. WAKSAL, [3] AND LEONARD A. HERZENBERG

Department of Genetics, Stanford University School of Medicine, Stanford, California, 94305

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Spleens and lymph nodes of nu/nu, congenitally athymic, mice have 16–32% and 72–76%, respectively, of large, brain-associated-T (BAT) antigen positive cells. These BAT positive large cells were isolated from nu/nu spleens with a fluorescence-activated cell sorter (FACS-II) and then cultured on thymic reticuloepithelial cell (TRC) monolayers. Both concanavalin A and mixed lymphocyte reactivity was induced during this culture period. We concluded that these BAT positive cells are pre-T cells which can be induced to at least some T cell functions by an inductive factor produced by TRC.

INTRODUCTION

In normal mice, the thymus is responsible for the development of immunocompetent thymus-derived lymphocytes (T-lymphocytes) (1–3). Therefore it is not surprising that congenitally athymic (nu/nu) mice have greatly reduced T-cell functions. Despite the absence of mature, immunocompetent T-cells, precursors of T-lymphocytes do exist in these animals since bone marrow cells of nu/nu mice can repopulate the thymii of lethally irradiated normal recipients (4, 5). Similarly, a thymus implanted into a nu/nu is repopulated by cells of host (nu/nu) origin (6). It appears therefore, that these mice have no defect in either the lymphocyte stem cell population or in the development of pre-T cells (pro-thymocytes).

The thymus initially consists of a continuous epithelium of cells which later in fetal life becomes infiltrated by precursors from the liver and yolk sac and may be repopulated from the bone marrow during adult life (7–9). The epithelial cells of the thymus putatively induce the differentiation and maturation of thymocytes from these precursors (10, 11).

Our initial observations on Thy 1.2 bearing cells in nu/nu mice revealed small but significant numbers of splenic lymphocytes which stained with a wide range of

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[2] Fellow of the Giannini Foundation. Present address: The Biological Laboratories, Harvard University, Cambridge, Massachusetts, 02138.

[3] Present address: National Cancer Institute, Immunology Branch, National Institutes of Health, Bethesda, Maryland, 20014.

fluorescence intensities using this reagent. The staining control, AKR/J (Thy 1.1) showed no such stained cells (12).

There have been persistent indications since the initial reports of Raff (13) that Thy 1.2-positive cells exist in nu/nu mice in small to moderate numbers. Recent studies by Loor and Roelants (14, 15) have reported close to normal numbers of cells in the spleens of nu/nu mice which can be stained with a fluorescein-conjugated rabbit antibody raised against mouse brain and absorbed *in vivo* in nu/nu mice. They call this mouse brain associated θ antigen. They have suggested that such cells represent committed T-lymphocyte precursors. Several reports by Boyse and coworkers (16, 17) indicate that T-committed cells or prothymocytes can be found in spleen and bone marrow of nu/nu mice. Fractionation of these cell populations on albumin density gradients allows some enrichment for the prothymocyte population.

In this study we have used a rabbit antibody raised against mouse brain and rendered T-specific by absorption. To distinguish the T antigenic determinants detected by this from the alloantigenic determinants of Thy 1 (θ), we call the antigen it detects, brain associated T (BAT). We have used this antiserum to both identify and separate the BAT-positive cells from nu/nu spleen. This population was assayed for T-cell function both before and after cultivation on mouse thymic reticuloepithelial cells and fibroblasts. We have also demonstrated that the cells not bearing the BAT antigen are devoid of this T-cell precursor activity but do possess the B lymphocyte ability to respond to lipopolysaccharide.

MATERIALS AND METHODS

Animals. Congenitally athymic (nu/nu) mice were obtained from several sources. Dr. I. L. Weissman of Stanford University and Dr. Norman Reed of Montana State University kindly provided nu/nu mice on a BALB/c genetic background, and Dr. David Osoba, Ontario Cancer Institute, Toronto, Canada, provided them on a CBA genetic background. Sex and age-matched congenic mice, or normal (nu/+) littermates were used as controls. (BALB/c × C57Bl/6J)F_1, used for mixed lymphocyte culture, were also obtained from Dr. I. L. Weissman. All mice were maintained in filter-top cages and were apparently healthy when sacrificed.

Antisera. Antiserum against brain associated T-lymphocyte antigen (BAT) was prepared after the method of Golub. Rabbits were injected along multiple sites with homogenized BALB/c brain in complete Freund's adjuvant, boosted weekly for 2 weeks and bled 1 week following the final boost. Following precipitation with 50% saturated ammonium sulfate, the globulin fraction was passed over a mouse immunoglobulin (Ig) column, and absorbed extensively with BALB/c red blood cells and bone marrow. Treatment of hapten-carrier primed cells with this antiserum and complement showed no loss of antibody forming cell precursor activity, although there was quantitative loss of cooperator function. $(Fab')_2$ fragments were prepared by pepsin digestion followed by gel filtration (18) and then conjugated with fluorescein (19).

Rabbit anti-mouse Ig (RAMIG) was purified by repeated injections of rabbits with 50% saturated ammonium sulfate precipitated mouse Ig in complete Freund's adjuvant. Goat anti-rabbit Ig (GARIG) was prepared by repeated injections of

goats with DEAE-cellulose purified rabbit Ig in complete Freund's adjuvant and a fluorescein conjugate of the ammonium sulfate purified Ig was prepared (19).

All serum preparations were deaggregated by centrifugation at 80,000*g* for 1 hr, sterilized by passage through a 0.22 micron Millipore filter, and stored at 4°C.

Cell suspensions. Mice were sacrificed by cervical dislocation. The spleens and peripheral lymph nodes were excised and cell suspensions prepared in Dulbecco's phosphate buffered saline (D-PBS) supplemented to 5% v/v with fetal calf serum.

Fetal calf serum. Fetal calf serum (FCS), obtained from Grand Island Biological Co., was heat inactivated at 56°C for 2 hr, passed through a 0.45 micron Millipore filter and tested for mitogenic activity against splenic lymphocytes. Lots giving low background stimulation were selected for use in all experiments involving ^{3}H-thymidine uptake.

Fluorescence staining of cells. Pelleted cells (1×10^7) were resuspended in 0.1 ml of the labeling reagent and left at room temperature for 20 min. The cell suspension was pelleted through FCS and subsequently washed in 2 ml of medium. In the case of the RAMIG, the cell pellets were resuspended in 0.1 ml of fluoresceinated GARIG at 0.4 mg/ml and left at room temperature for 20 min. The cells were washed as above, resuspended to 1×10^7 cells/ml and held on ice for analysis or separation on the FACS.

Mitogen stimulation. Cell suspensions were maintained sterilely in RPMI 1640 medium (H-18), supplemented with 2 m*M* L-glutamine, 10 m*M* HEPES buffer (#1330440), 100 U/ml penicillin and streptomycin, and 5% v/v FCS (all reagents obtained from Grand Island Biological Co.). Aliquots of 4×10^5 cells in 100 μl were dispensed into Falcon Microtest II plates either with RPMI 1640 (control), 2.5 μg/ml phytohemagglutinin (PHA, Burroughs Wellcome, Beckenham, England), 1.5 μg/ml concanavalin A (Con A, A grade, Calbiochem, San Diego, California), or 100 μg/ml lipopolysaccharide W, *E. coli* 011:B4 (LPS, Difco Laboratories, Detroit, Michigan). The final culture volume was 200 μl/microwell. Cultures were incubated for 40 hr at 37°C in a humidified CO_2 incubator, pulsed at 40 hr with 1 μCi of ^{3}H-thymidine, and harvested 8 hr later on a multiple automated sample harvester (MASH). All samples were performed in triplicate and reported as the mean counts per minute ± one standard deviation.

Separations. Extensive descriptions of the fluorescence activated cell sorter (FACS-1) can be found in several publications (20, 21). Using as measuring parameters either intensity of fluorescence and/or intensity of low-angle light scattering, this instrument is able to rapidly detect and separate single cells on the basis of their fluorescence and their relative cell volume.

Electronic thresholds for separation into BAT-positive and BAT-negative fractions were set on the basis of fluorescence distributions such as that shown in Fig. 1-b. This compares the fluorescence profiles of viable BALB and nu/nu spleen cells stained with fluorescein conjugated rabbit-anti-BAT (RABAT). The BAT-positive fraction in the normal control was defined as those cells with a fluorescence intensity greater than 30 (arbitrary) fluorescence units. This represented 43% of total live cells. BAT-positive cells from nu/nu spleens were then defined as all cells with a fluorescence intensity greater than 30 units. For the experiment illustrated 32% of total live cells were BAT-positive. To decrease the contamination of one fraction with the other, 10–15% of cells with fluorescence intensities between

those defined for positive and negative fractions were discarded. Thus, for distributions given in Fig. 1-b, cells with fluorescence intensities between 0 and 25 were called BAT-negative, while cells with intensities between 35 and 250 were called BAT-positive.

Thymus reticuloepithelial cell and fibroblasts cultures. Monolayers of thymus reticuloepithelial cells (TRC) were prepared by aseptically removing thymii from 4-week-old CBA or BALB/c mice and mincing the thymii until all solid pieces were removed. The minced thymus tissue was put into a trypsinizing flask. The tissue was washed in Hank's balanced salt solution (HBSS) for 10 min using a magnetic stirrer. The HBSS was then poured off and replaced with 0.25% trypsin-EDTA solution (Grand Island Biological Co.) and trypsinized for 10 min. The tissue was put into a flask containing collagenase solution (CLSP, Worthington Biochemical) 150 units/ml in MEM to incubate at 37°C for 3 hr. The fragments, which were swollen and partially dispersed, were further dispersed by vigorous pipetting and poured through double pieces of sterile gauze to remove remaining solid tissue pieces. The cells were then centrifuged at 800*g* and washed in cold HBSS. The cells were then added to Falcon plastic 60 mm tissue culture dishes with Waymouth's medium (Grand Island Biological Co.) without serum. Two mouse thymii per 60 mm plate were used for culturing the cells. The Waymouth's medium does not allow for lymphocyte survival but does allow for growth of the adherent cell population. After 2 days of culture, the Falcon dishes were washed with cold HBSS, Eagle's minimal essential medium (MEM, Grand Island Biological Co.) plus 15% FCS was added and the cells maintained. Secondary cell cultures were prepared by trypsinizing the primary cell cultures with 0.1%

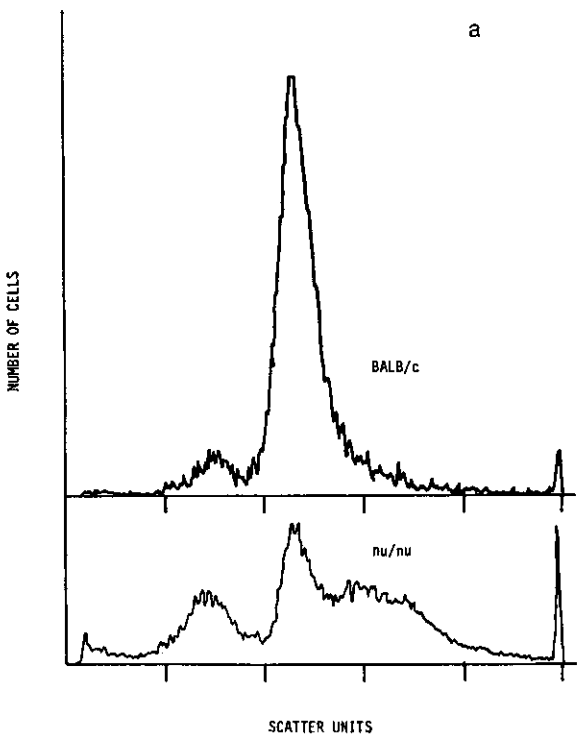

FIGURE 1a

trypsin. Only secondary cell monolayers were used in the experiments to rule out the possibility of contaminating thymocytes.

Control fibroblast monolayers were prepared by mincing 14–16 day old mouse embryos and trypsinizing them for 10 or 20 min in 0.25% trypsin-EDTA solution. The cells were maintained on MEM plus 10% FCS.

Inducing cell cultures. Spleens were removed aseptically from nu/nu mice and teased in cold, sterile HBSS to obtain a single cell suspension. The population collected from FACS separation was washed and centrifuged at 400*g* for 10 min at 4°C and resuspended in MEM plus 10% FCS at a concentration of 1×10^7 cells/ml. Three milliliters of each cell population were added to TRC monolayers or control fibroblast monolayers and incubated for 24 hr. The cells were removed from the monolayers by gentle pipetting and examined for mitogen and mixed lymphocyte reactivity (MLR) responsiveness.

RESULTS

Analyses of size and fluorescence of spleen cells from nu/nu mice using the fluorescence activated cell sorter (FACS) are compared with BALB controls in

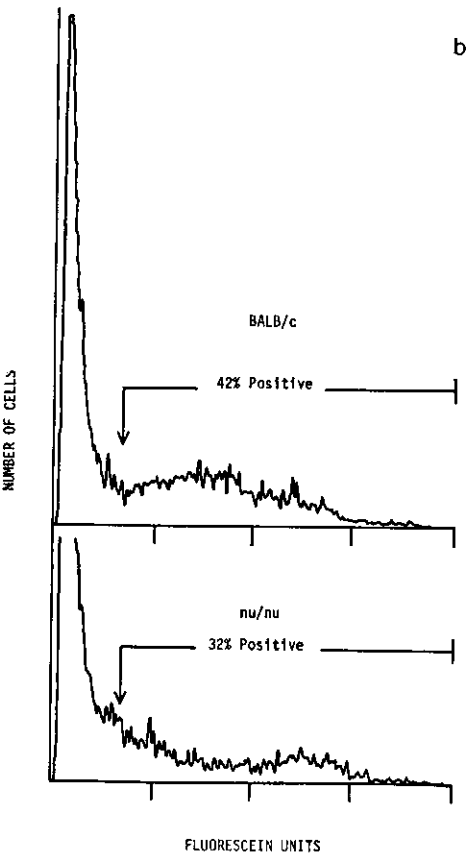

FIG. 1. Comparison of FACS scatter and fluorescence distribution of BALB/c and nu/nu spleen cells. a) FACS scatter profile. b) FACS fluorescence profiles obtained after staining spleen cells with fluorescein-conjugated RABAT.

TABLE 1

Relative Percentages* of BAT and Ig Positive Cells in Nu/Nu and BALB/c Mice

	BAT	Ig
BALB Spleen	35–45	40–45
Lymph Node	72–76	25–28
nu/nu Spleen	16–32	44–55
Lymph Node	26–35	60–72

* As estimated by the FACS; see *Methods*.

Fig. 1. Figure 1-a compares the scatter profiles of nu/nu and BALB spleen cells. The small peak between Channels 50 and 100 contains dead cells, and the major peak centered at Channel 115 represents small lymphocytes. Nu/nu spleen cells show a prominent shoulder of large cells (Channels 140–250) which fluctuates between 10–40% in different pools of spleens, as well as among different individuals. Fluorescence-gated scatter (scatter of fluorescent cells only) analysis of BAT-positive cells reveals that this population is enriched for these large cells. Wright-Giemsa stained smears of separated BAT-positive cells showed a group of large, highly basophilic cell which morphologically resemble lymphoblasts.

In Fig. 1-b the fluorescence profiles of BALB and nu/nu spleens, stained with the fluorescein-conjugated RABAT are compared. BAT-positive cells were defined as those cells possessing a fluorescence intensity greater than 30 fluorescence units. In BALB spleen preparations these represented 35–45% of the total live population, and were highly enriched after nylon column purification of T-lymphocytes. This is consistent with the adoptive transfer data which indicates that RABAT recognized the population of T cells contained in spleen. The numbers of cells staining with either the fluorescein-conjugated RABAT or RAMIG plus GARIG in nu/nu spleen and lymph node are given in Table 1. These figures are obtained by integration of the fluorescence profiles seen on the FACS. It is apparent that substantial numbers of BAT-positive cells can be found in both spleen and lymph node of nu/nu mice. We also observe Ig-bearing cells in numbers reported by others, but we have not done double labeling studies to determine the existence of Ig^+BAT^+ cells.

Following separation of BAT-positive and BAT-negative cells, the mitogen responsiveness and mixed lymphocyte reactivity were compared to those of normal controls and unseparated nu/nu spleen cells.

Compared to the responses of normal splenic lymphocytes, unseparated nu/nu spleen cells responded only slightly to the T-cell mitogens PHA and Con A, with stimulation indices of 2.5 and 3.0, respectively (Table 2). In contrast, nu/nu lymphocytes demonstrated levels of LPS responsiveness which were equivalent to those elicited from BALB spleen. These data are consistent with previous observations and have been used to support the idea that few, if any, functional T-lymphocytes exist in the nu/nu strain, although a normal complement of active B lymphocytes can be found. Similarly, mixed lymphocyte reactivity by unseparated nu/nu spleen cells was negligible when compared to normal controls.

Cultivation of unfractionated nu/nu splenic lymphocytes on TRC monolayers resulted in a significant increase in responsiveness of this mixed cell population

TABLE 2

Mitogen Responsiveness of BALB/c and nu/nu Spleen Lymphocytes

Spleen cells	Expt.	Control	PHA			Con A			LPS		
		cpm*	cpm	SI**	Δ***	cpm	SI	Δ	cpm	SI	Δ
Unseparated BALB/c	1	0.2 ± 0.03	34.2 ± 7.0	163	34.0	15.5 ± 0.6	74	15.3	6.2	29	6.0
Unseparated nu/nu	1	0.4 ± 0.02	9.5 ± 0.02	2.5	9.1	1.1 ± 0.01	2.9	0.7	3.8	9.5	3.4
	2	0.2 ± 0.04	0.5 ± 0.04	2.0	0.3	0.6 ± 0.04	3.0	0.4	6.5 ± 0.8	27.6	6.3
	3	18.3 ± 0.4	8.2 ± 0.1	0.4	−10.1	1.2 ± 0.1	0.1	−17.2	162.7 ± 27	8.8	154.4
	4	0.6 ± 0.05	0.3 ± 0.01	0.5	− 0.3	0.7 ± 0.08	1.2	0.1	6.5 ± 0.6	10.8	5.9
BAT+	1	1.2 ± 0.4	0.8 ± 0.2	0.6	− 0.4	3.4 ± 0.9	3.2	2.2	16.8 ± 2.7	16.0	15.6
	2	1.8 ± 0.2	1.5 ± 0.2	0.8	− 0.3	2.3 ± 0.3	1.3	0.5	126 ± 6	71	174
	3	1.6 ± 0.4	0.4 ± 0.1	0.3	− 1.2	0.6 ± 0.3	0.4	− 1.0	5.8 ± 1	3.7	4.2
	4	0.7 ± 0.09	0.7 ± 0.1	1.0	1.0	3.7 ± 0.8	5.2	3.0	10.5 ± 1.2	15.0	9.6
BAT−	1	0.5 ± 0.1	0.2 ± 0.03	0.4	− 0.3	0.4 ± 0.05	0.8	− 0.1	46.4 ± 12.1	95	45.9
	2	0.3 ± 0.01	0.3 ± 0.01	1.0	0.0	0.5 ± 0.3	1.6	0.2	35.2 ± 13	120	34.9
	3	9.0 ± 0.5	3.2 ± 1.9	0.4	− 5.8	4.5 ± 1.4	0.5	− 4.5	415 ± 13	46	406
	4	0.15 ± 0.02	0.2 ± 0.05	1.3	0.05	0.35 ± 0.1	2.3	2.15	38.0 ± 10.	253	37.8

* ^{3}H-thymidine uptake in cpm × 10^{-3} ±standard deviation.

** Stimulation index given as (cpm with mitogen)/(cpm without mitogen).

*** Delta, or difference; value given as (cpm with mitogen) − (cpm without mitogen).

TABLE 3

Mitogen Responsiveness after Inductive Preculture

Cell population	Expt. no.	Preculture§ on:	Control*	Con A*	SI°	Δ†	PHA*	SI°	Δ†	LPS*	SI°	Δ†
nu/nu unstained	2	Fibroblasts	0.47 ± 0.08	0.44 ± 0.13	0.9	−0.03	0.73 ± 0.08	1.5	0.25	7.2 ± 1.6	15.3	6.8
		TRC	0.88 ± 0.05	0.88 ± 0.08	1.0	0.01				8.2 ± 0.9	9.3	7.3
nu/nu stained with anti BAT	1	Fibroblasts	0.61 ± 0.16	0.70 ± 0.17	1.1	0.08	0.41 ± 0.16	0.67	−0.19	6.5 ± 1.5	10.5	5.9
		TRC	0.96 ± 0.12	1.4 ± 0.16	1.4	0.43	1.0 ± 0.10	1.1	0.1	6.6 ± 4.7	6.9	5.6
	2	Fibroblasts	0.35 ± 0.06	0.51 ± 0.09	1.4	0.16	0.42 ± 0.08	1.2	0.07	5.4 ± 0.9	15.2	5.0
		TRC	0.54 ± 0.08	1.2 ± 0.12	2.3	0.68	0.83 ± 0.12	1.5	0.29	7.6 ± 1.7	14.0	7.0
BAT positive	1	Fibroblasts	0.72 ± 0.18	3.7 ± 0.4	5.1	3.0	0.67 ± 0.26	0.9	−0.04	10.5 ± 1.2	14.6	9.8
		TRC	1.2 ± 0.54	12.8 ± 2.6	10.4	11.5	2.7 ± 0.43	2.2	1.5	14.1 ± 2.3	11.5	12.9
	2	Fibroblasts	0.52 ± 0.4	2.7 ± 0.26	5.1	2.2	0.96 ± 0.06	1.8	0.43	8.2 ± 1.5	15.7	7.7
		TRC	0.95 ± 0.05	13.6 ± 0.09	14.2	12.6	1.3 ± 0.10	1.4	0.40	4.5 ± 0.5	4.6	3.5
BAT negative	1	Fibroblasts	0.23 ± 0.10	0.35 ± 0.18	1.5	0.13	0.35 ± 0.13	1.5	0.13	37.9 ± 4.8	167	37
		TRC	0.43 ± 0.10	0.72 ± 0.11	1.7	0.29	0.64 ± 0.07	1.5	0.21	31.3 ± 6.0	73	31
	2	Fibroblasts	0.49 ± 0.07	0.46 ± 0.17	0.9	−0.03	0.72 ± 0.15	1.4	0.22	21.9 ± 4.4	44	21.4
		TRC	0.64 ± 0.14	0.92 ± 0.14	1.4	0.28	0.60 ± 0.17	0.93	−0.04	18.2 ± 3.6	28	17.6

§ See *Methods*.

* ^{3}H-Thymidine uptake expressed as cpm × 10^{-3} ±standard deviation.

° SI = stimulation index = (cpm with mitogen)/(cpm without mitogen).

† Δ = difference = (cpm with mitogen) − (cpm without mitogen).

TABLE 4

Mixed Lymphocyte Reactivity

Responding population	Preculture* on:	Stimulators for MLR: Syngeneic cpm†	Allogeneic (Irradiated F_1) cpm	Allogeneic / Syngeneic
BALB/c	—	855 ± 165	6104 ± 877	6.9
nu/nu	—	271 ± 27	442 ± 84	1.6
	Fibroblasts	1250 ± 115	1886 ± 95	1.5
	TRC	1356 ± 135	4077 ± 475	3.0
BAT^+	—	473 ± 84	725 ± 196	1.5
	Fibroblasts	793 ± 92	1469 ± 165	1.4
	TRC	1103 ± 162	4055 ± 370	3.7
BAT^-	—	8815 ± 242	8155 ± 940	0.9
	Fibroblasts	698 ± 113	598 ± 70	0.9
	TRC	1090 ± 183	877 ± 64	0.8

* See *Methods*.

† ^{3}H-thymidine uptake in cpm ± standard deviation.

to both Con A and semiallogeneic cells (Tables 3 and 4). In contrasts, LPS responsiveness was unaffected by culture on either fibroblast or TRC monolayers.

It seemed apparent from these data that cells in nu/nu spleen can be induced to respond to Con A and allogeneic lymphocytes by thymic epithelial cell cultures. The likely candidates for these cells are the ones already bearing the antigen BAT. These were separated from the BAT-negative cells, and both populations were tested for responsiveness before and after inductive culture.

Prior to cultivation, neither BAT-positive nor BAT-negative cells demonstrated significant responses to PHA or Con A, although all of the Con A responsiveness seen in whole nu/nu spleen can be seen to reside in the BAT-positive population (Table 2). After exposure to TRC monolayers, however, the BAT-positive population of cells can be seen to respond well to Con A. This increase in responsiveness does not occur with the T-cell mitogen PHA but is echoed in the small but significant increase in the mixed lymphocyte response to irradiated semiallogeneic cells (Table 4). It should be noted, however, that the most striking increase occurs in the response to Con A.

In distinct contrast to the responsiveness of the BAT-positive cells to the stimulating influence of TRC monolayers, the BAT-negative population exhibited no responsiveness to PHA, Con A or semiallogeneic cells either before or after cultivation on fibroblasts or TRC monolayers (Tables 2, 3, 4). This population of cells, containing less than 1% of BAT-positive cells after FACS separation, seems to be decidedly depleted for the cells capable of responding to the inductive signals of the TRC. However, it should be noted that removal or BAT-positive cells has resulted in a dramatic rise in the LPS responsiveness—an enhancement far greater (10-fold) than can be accounted for by the 30% enrichment for BAT-negative cells resulting from separation. This strongly suggests that the BAT-negative cells,

although unable to respond to T-cell maturation signals, may be under a regulatory influence exerted by the pre-T, BAT-positive cells.

It should be noted that in Tables 2, 3, and 4 we have chosen to present the actual cpm of the different cell populations, the ratio of response cpm to control cpm (stimulation index, S.I.), and the difference between response cpm and control cpm (Δ). We feel that the variability in background (unstimulated) cpm seen in the various culture conditions makes it necessary to consider the data in both these ways.

Although methodological factors such as excessive FCS may affect the background in random ways, biological differences may also be reflected in these figures. For example, the BAT-positive fraction demonstrates a consistently higher endogenous cpm than the BAT-negative, which fits with the morphological evidence that there are many lymphoblasts in the BAT-positive fraction. At this stage we cannot tell if the background represents DNA synthesis in the same cells or different cells than those that are specifically stimulated.

DISCUSSION

In the studies reported above, we have used the analytical and sorting capabilities of the FACS to detect and separate a population of cells bearing a T-cell associated antigen, BAT, from nu/nu spleen. The BAT-positive cells are shown to possess a low reactivity to the T-cell mitogen Con A which was markedly enhanced after 24 hr cultivation on thymic epithelial cell monolayers. TRC monolayers also induced a small but significant MLR responsiveness in the BAT-positive cells. In contrast, the 70% of cells which were BAT-negative demonstrated no Con A or MLR reactivity either before or after exposure to TRC.

Earlier studies using a fluorescein-conjugated anti Thy 1.2 and the highly sensitive FACS demonstrated 5–10% of nu/nu spleen cells to be positive, although only 1–2% of these cells were as bright as the T-cells staining in spleens of normal controls (12). The heterologous antiserum RABAT used in this report stained approximately 30% of nu/nu spleen lymphocytes, often with a range of fluorescence intensities very similar to that of normal spleen cells. Our results indicating that greater numbers of cells are detectable with RABAT than with anti Thy 1.2 are consistent with the observations of Roelants *et al.* (16), who find only 1–2% positive cells with the alloantiserum against Thy 1.2 but over 30% of cells positive with their rabbit antiserum directed against mouse-brain-associated theta antigen. We feel that our heterologous antiserum defines a larger proportion of cells staining than the alloantiserum because the former reagent detects more epitopes on the same antigenic molecules. This is supported by our ability to block anti Thy 1.2 staining by pre-treatment with RABAT. Recent biochemical analysis of the antigens detected by T-cell-specific allo and heteroantisera (22), as well as extensive immunofluorescent studies on the staining and cytotoxic properties of these antisera [4] indicate that the same antigenic molecules are seen by these reagents.

We do not yet know whether all RABAT-staining cells are indeed early T precursors, but the BAT-positive fraction of nu/nu spleen lymphocytes does include all the cells capable of being induced to Con A or allogeneic cell responsiveness. The fact that the cells in this population not only bear T-cell associated antigen but also

[4] Sato, V. L., and I. L. Weissman, unpublished observations.

possess a low endogenous responsivity to Con A strongly supports previous suggestions (16, 17, 23) that the nu/nu mouse has a complement of lymphocytes already committed to be T-cells but which are blocked in further maturation.

It is worth noting that of the parameters studied here the increase in Con A responsiveness of the BAT-positive cells is the most striking effect of inductive culture. The response to semi allogeneic cells, though consistent, is small, and no consistent response to PHA was found after 24 hr culture. Previous studies have indicated that Con A responsiveness is acquired early in ontogeny, at a time when PHA responsiveness and MLR reactivity are still very low (24, 25). Since the separated BAT-positive lymphocytes are cultured on fibroblast and TRC monolayers for only 24 hr prior to assay, it is possible that longer periods or more effective inducing conditions might lead to stronger PHA and MLR responsiveness.

Our studies in nu/nu mice indicate that committed pre-T cells, or prothymocytes, exist in substantial numbers, bear the T-cell characteristic surface marker BAT, and are responsive to the inductive signals of the TRC. These pre-T cells also appear to have the migratory properties of T lymphocytes. Examination of frozen spleen and lymph node sections from nu/nu mice reveals that cells staining with a T-cell specific reagent, known to stain the same cells as RABAT, are localized to T-dependent areas.[5] Thus, even in the absence of a thymus, pre-T cells are programmed to migrate to appropriate anatomical spots.

The BAT-negative population of cells in the nu/nu, on the other hand, is quantitatively depleted for T precursor cells. This fraction does contain, however, the LPS-responsive cells, indicating the presence of B cells and/or their precursors. Recent work by Scheid, *et al.* (26) provides evidence that prothymocytes and immature B cells are two separable populations occurring in nu/nu spleen. T-cell precursors are recovered in the less dense fractions of albumin density gradients than are the B-cell precursors, and are also responsive to the inductive effects of thymopoietin. The immature B cells are impervious to thymopoietin but can be induced by ubiquitous immunopoietic polypeptide (UBIP). Whether the FACS-separated BAT-negative cells include the UBIP-responsive population is certainly an issue for further study.

It has been suggested that the immunological defect in the nu/nu mouse is the lack of a functional thymic epithelium (27). Thymus grafts to nu/nu mice from normal mice become infiltrated by cells of host origin and can reconstitute T-cell function in these recipients (27). The culture of nu/nu spleen cell populations on TRC monolayers devoid of lymphocytic elements may provide an *in vitro* analog of *in vivo* thymic grafts. In the studies reported here, the TRC monolayers seem able to induce further T-cell maturation, although to a much lesser extent than normally occurs *in vivo*. The mechanism by which thymic epithelial cells exert their inductive effects is unclear, but it most likely involves control of cyclic AMP levels, or regulation of thymic factors which in turn affect intracellular CAMP concentrations.

The work reported here has provided evidence that a brain-associated T-cell marker is one of a number of antigenic determinants which define not only mature T-lymphocytes but precursor populations of this line as well. In addition, we have shown by separation studies that BAT-antigen bearing cells include the population of precursor lymphocytes responsive to T-cell induction signals. Cell populations

[5] Weissman, I. L., unpublished observations.

depleted of this BAT-bearing lymphocyte are correspondingly devoid of any ability to be induced by TRC monolayers.

The BAT antigen itself may be a protein related to those reported by Trowbridge *et al.* (22) and Morris *et al.* (28). Such a protein could be available for glycosylation by enzymes activated *in vivo* by the thymus, or *in vitro* by preculture on TRC or with thymic hormones, resulting in a glycosylated protein antigen which could then react maximally with determinants detected with anti Thy 1 antibodies. This would be consistent with the suggestion of Morris *et al.* (28) that the rat brain associated xenoantigen and the Thy 1.1 antigen in mature T cells are on the same molecule.

These data strongly support the hypothesis that lymphocytes already committed to the T-cell lineage are formed and maintained in the absence of a thymus. These precursors can be characterized by surface markers, migration properties, and responsiveness to inducers of T-cell maturation. The ability to define and separate this population of cells in nu/nu mice will allow further studies on the mechanism of T-cell maturation.

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