DIFFERENTIATION FROM PRECURSORS IN ATHYMIC NUDE MOUSE BONE MARROW OF UNUSUAL SPONTANEOUSLY CYTOLYTIC CELLS SHOWING ANTI-SELF-H-2 SPECIFICITY AND BEARING T CELL MARKERS*

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Two quite different types of cytotoxic effector cells are presently being widely studied: cytotoxic T lymphocytes (CTL)[1] and natural killer (NK) cells. The first can recognize foreign modifications of syngeneic cells in association with self major histocompatibility complex products; the second can distinguish some tumor cells from normal cells. It is not known what recognition structures are used by either of these cell classes to distinguish cells that are attacked from cells that are not attacked, nor is the ontogeny of these recognition structures understood. CTL develop from specificity-restricted precursors (CTL precursor cells [CLP]) on exposure to the appropriate antigenic stimulus; NK are apparently spontaneously activated. Although the thymic environment has been implicated in the structuring of the CLP specificity repertoire (1, 2), it is also clear that CLP can develop in athymic nude mice (3, 4) and ultimately reach normal or near normal levels (5, 6). These CLP have been variously reported to show pronounced H-2 restriction (7, 8), no major histocompatibility complex (MHC) restriction (9), or MHC restriction that varies from mouse to mouse (6). NK cells also develop in nude mice and reach normal or above normal levels (10). The relationship, if any, between these two types of cells remains unclear at the present time.

We here present studies designed to provide information on the ontogeny of cytotoxic effector cells. The ability of precursor cells in nude bone marrow to give rise to cytotoxic effector cells has been measured. The studies were done in a microculture system in which small cell numbers (5–100) are cultured, in the absence of filler or feeder cells, in a total volume of only 20 μl of liquid culture

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[1] *Abbreviations used in this paper:* α-MEM, alpha-minimum essential medium; BSA, bovine serum albumin; CLP, cytotoxic T lymphocyte precursor; CM, conditioned medium; Con A, concanavalin A; CTL, cytotoxic T lymphocyte; FCS, fetal calf serum; IL-2, interleukin 2; MHC, major histocompatibility complex; NK, natural killer; PBL; peripheral blood leukocyte; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMA, phorbol myristyl acetate; SC, spleen cell.

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medium containing growth factor(s). This system appears to allow the growth of lymphomyeloid precursor cells. Cytolytic effector cells expressing both target cell specificity and T cell-specific membrane markers develop spontaneously in these cultures. The specificity of these effector cells is unusual. They preferentially lyse target cells of either tumor or normal origin that share H-2 determinants with the responder cells. Although the effector cells develop spontaneously, they do not appear to be NK cells. We suggest they represent an early stage in the ontogeny of the T cell repertoire.

Materials and Methods

Mice

(RNC × BALB/c)F_1 athymic (*nu/nu*) mice (H-$2^{k/d}$), their heterozygous (*nu*/+) normal littermates, and the heterozygous (*nu*/+) parental BALB/c (H-2^d) and RNC (H-2^k) strain mice were bred in the animal colony of the Ontario Cancer Institute. All mice used were ~12 wk old.

Culture Medium

The culture medium was alpha-minimum essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS), 10 mM Hepes buffer, and 5×10^{-5} M 2-mercaptoethanol.

Cell Suspension

Nude bone marrow cells were first freed of erythrocytes by a brief suspension in 0.84% ammonium chloride solution. Subsequently, they were passed through a nylon wool column (11); about 10% (range, 8–17% in >30 independent experiments) of the loaded cell number was eluted from the nylon wool column after a 1-h incubation at 37°C.

Conditioned Media

Phorbol myristyl acetate (PMA)-stimulated EL4 thymoma cell conditioned medium (PMA-EL4/CM). Murine EL4 thymoma cells (subclone EL4.1) suspended at 10^6 cells/ml in α-MEM plus 5% FCS were incubated for 48 h with 10 ng/ml PMA (12). The supernatant was collected, spun at 500 *g* for 10 min, filtered through a 0.22 μm filter, and stored at 4°C until use. This supernatant was used either crude or as a semipurified preparation, obtained by ammonium sulphate precipitation at 80% saturation, extensive dialysis against phosphate-buffered saline (PBS), and passage through a Sephadex G-100 column, pooling fractions from 10,000 to 70,000 D. In further processing steps (performed by Dr. V. Paetkau, Edmonton), this semipurified material was passed through DEAE-cellulose columns; the filtrate of these columns was devoid of interleukin 2 (IL-2) activity (as assayed in a quantitative microassay for IL-2-dependent proliferation of a cloned cytolytic T cell line [13]), while IL-2 activity could be eluted from this column and further purified by chromatofocussing (14). Material obtained at each of these processing steps was assayed for marrow cell growth-inducing activity in the microassay described below.

Concanavalin A-stimulated mouse spleen cell conditioned medium (Con A-SC/CM). Spleen cells (5×10^6/ml) from RNC mice were incubated for 48 h in culture medium containing 5 μg/ml Con A (15). The supernatant was harvested as described above.

Phytohemagglutinin (PHA)-stimulated human peripheral blood leukocyte conditioned medium (PHA-PBL/CM). Human PBL (10^6/ml) were incubated in tissue culture flasks for 7 d in the presence of 1% PHA in α-MEM plus 10% FCS at 37°C in a humidified atmosphere of 5% CO_2 in air (16). The supernatant was harvested as described above.

WEHI3 cell conditioned medium (WEHI3/CM). The murine cloned myeloid tumor cell line WEHI3 was grown in vitro in α-MEM plus 5% FCS. The supernatant was harvested as described above.

Microculture System

The system is based on that of Moreau and Miller (17), developed for limiting dilution analysis of T cells in human peripheral blood. Fractionated nude marrow cells, suspended

in α-MEM supplemented with 10% FCS and CM at a concentration that gave optimal growth-stimulating activity in preliminary tests, were transferred in 20-μl aliquots containing 10–100 cells into Terasaki microwells (30–60 wells per titration point) using a Hamilton syringe. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Growth within individual wells was scored with an inverted microscope at days 4–10 of in vitro incubation. A maximal number of wells containing growing cells was seen on days 6–7.

Fluoresceinated Antibody Labeling Procedures

Labeling of freshly prepared nude marrow cells. Nylon wool nonadherent nude marrow cells in PBS plus 10% FCS were suspended (2×10^5/1 ml) in ultracentrifuged fluorescein-conjugated, monoclonal rat anti-mouse Thy-1 and/or Lyt-1 antibody (18) (Becton, Dickenson & Co., Mountain View, CA). After a 30-min incubation on ice, cells were washed twice by layering over and spinning through 6% (wt/vol) bovine serum albumin (BSA) in PBS. The cells were then resuspended in PBS and placed on ice before flow cytometry analysis and/ or cell sorting.

Labeling of cultured nude marrow cells. At 4–8 d after the initiation of the microcultures, wells containing growing cells were marked, 15 μl medium was carefully removed and replaced with 15 μl PBS plus 6% BSA containing 1 μl of ultracentrifuged fluorescein-conjugated monoclonal rat anti-mouse Thy-1, Lyt-1, or Lyt-2 antibody (Becton, Dickenson & Co.). After a 60-min incubation on ice, cultures were washed twice by replacing 15 μl of the culture volume with 15 μl of fresh ice-cold PBS containing 6% BSA. Just before visual assessment of the cultures for stained cells with a fluorescence microscope, 15 μl of the culture supernatant was again removed.

Flow Cytometry and Cell Sorting

The flow cytometer/cell sorter used in these experiments was designed and built at the Ontario Cancer Institute and is basically similar to the instrumentation in widespread use elsewhere. Procedures were as described elsewhere (19).

Antiserum and Complement Treatment

Monoclonal anti-Thy-1.2 IgM antibody (donated by R. A. Phillips, Ontario Cancer Institute), monoclonal anti-Lyt-1.1 IgG2a antibody (clone 7-20, 6/3; Cedarlane Laboratories, Hornby, Ontario), and monoclonal anti-Lyt-2.2 IgM antibody (clone AD4; Cedarlane Laboratories [15]) were used at a final concentration of 1:10. Antibody preparations (diluted 1:5), nontoxic rabbit complement (Cedarlane Laboratories) (diluted 1:5), and 50 μg/ml propidium iodide (Calbiochem-Behring, San Diego, CA) in PBS-6% BSA were prepared for a one-step antibody-mediated cytotoxicity assay in 20-μl microwells. For the test, 10 μl of the culture volume from a microwell containing growing nude marrow cells were removed and replaced by 10 μl of the antibody-complement-propidium iodide–containing medium. Wells were incubated for 2 h at 37°C. Just before reading these cultures with a fluorescence microscope, 15 μl of the culture volume was carefully removed. Dead cells displayed a bright red fluorescence under these conditions. Before day 7 of culture, the background of dead cells in cultures treated with complement alone was always <5% of the total cell number.

Cytotoxicity Assay

Microversion. After a 6–14-d incubation, individual microwells were tested for the presence of cytolytic effector cells in a modified microversion of the standard 4-h ^{51}Cr-release cytotoxicity assay. From a 20-μl microwell that contained growing nude marrow cells, 10 μl was removed and replaced with 10 μl tissue culture medium in which 500 ^{51}Cr-labeled target cells were suspended. The target cell panel included P815 (H-2^d) mastocytoma cells, RBL5 (H-2^b) T lymphoma cells, YAC (H-2^a) leukemia cells, and 2–3-d splenic Con A blasts from allogeneic C57BL/6 (H-2^b), parental RNC (H-2^k), parental BALB/c (H-2^d), and syngeneic (RNC × BALB/c)F_1 mice. After a 4-h incubation at 37°C, 5 μl supernatant was collected from each microwell and counted in a standard γ-counter.

The fractional specific ^{51}Cr-release was defined as (observed release − spontaneous release)/(total release − spontaneous release). The spontaneous release was obtained in 20-μl microwells containing 500 labeled target cells in tissue culture medium; 5 μl was removed and counted after a 4-h incubation at 37°C. The total release was obtained by incubating 500 target cells in 20-μl wells in acetic acid for 4 h at 37°C, and removing 5 μl for γ-counting.

Macroversion. With short-term cell lines, derived from in vitro propagated proliferating nude marrow cells (that had originally been seeded in 20-μl microwells at 5–10 cells per culture), cytotoxicity assays against the target cell panel were performed at various time points of in vitro culture. Cells were cultured in 200-μl V-bottomed wells with 10^3 ^{51}Cr-labeled targets at an effector/target cell ratio of 3:1 to 10:1. After the 4-h incubation, 100 μl supernatant was removed for γ-counting.

Short-term Cell Lines

Nude marrow cells grown for 10 d in 20-μl microwells from an original inoculum of 5–10 cells per well were transferred to 200-μl wells containing fresh medium supplemented with conditioned supernatants and 10^5/ml irradiated (1,500 rad) syngeneic F_1 or parental RNC spleen 'filler' cells. After another 5 d, cells were transferred into 1-ml wells. Cultures were fed with fresh medium, conditioned supernatants, and irradiated filler cells every 4–5 d. They were routinely continued for no more than 4 wk.

Limiting Dilution Analysis

All cultures at each cell dilution were scored as growing or not growing. The precursor frequency was then calculated using the procedure of Porter and Berry (20), which yields not only a frequency with 95% confidence limits but a χ^2 value, so that one can assess whether the data are consistent with one-hit limiting dilution theory. All frequencies shown here satisfied this criterion.

Results

In Vitro Growth of Low Numbers of Murine Marrow Cells in CM. We first describe conditions that enabled the in vitro growth of small numbers of cells derived from the marrow of athymic nude mice. Nude marrow cells were freed of nylon wool adherent cells by passage through a nylon wool column; they were then seeded into microcultures at cell numbers varying from 20 to 140 per microculture and 60 replicate microcultures per cell concentration. Each microculture contained 20 μl of culture medium supplemented with PMA-EL4/CM (see Materials and Methods). After 7 d of culture each microwell was scored for growth. Wells showing growth contained a minimum of 100 and usually >500 cells; wells not showing growth contained no cells. In microwells showing growth, cells tended to adhere together to form a tight ball and are often here referred to as a colony.

In Fig. 1, the logarithm of the fraction, f, of cultures not showing growth at each cell concentration is plotted as a function of cell concentration. The points fit a straight line that intercepts the y-axis at f = 1. According to limiting dilution theory (20–22), this is consistent with a single precursor cell being able to give rise to a colony. An analysis using limiting dilution theory gives a frequency for this cell of 1/156 (95% confidence limits of 1/121–1/201; χ^2 = 0.421). In subsequent experiments presented here, titrations were done and the results expressed as a precursor cell frequency calculated using limiting dilution analysis as in Fig. 1.

Growth was observed only in the presence of an appropriate CM. Several

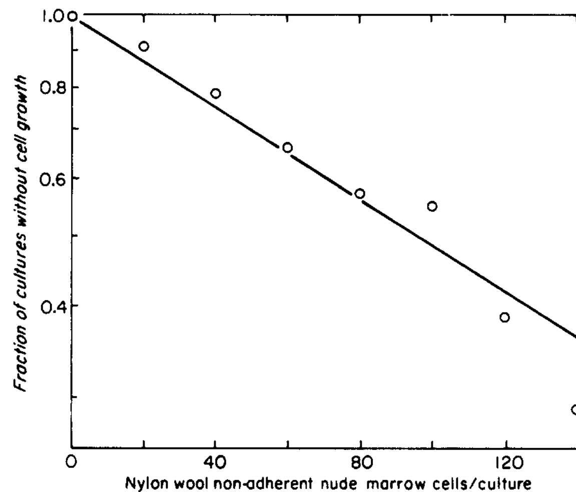

FIGURE 1. Limiting dilution analysis of growth-inducible nude marrow cells. Replicate cultures (60 per group) containing from 20 to 140 nylon wool nonadherent marrow cells from (RNC × BALB/c)F_1 nude mice were cultured in the presence of 20% PMA-EL4/CM (see Materials and Methods). Individual microwells were scored for the presence of growth after 7 d of culture. The graph shows the fraction of nongrowing cultures versus the number of cells per culture. The solid line is the limiting dilution analysis fit to the data obtained using the method of Porter and Berry (20). It gives a precursor frequency of 1/156 with 95% confidence limits of 1/121–1/201 and $\chi^2 = 0.421$.

alternative sources of supernatants were active and gave comparable results. All supernatants were titrated and subsequently used at a concentration that gave a plateau response. Experiment 1, Table I gives titration data for PMA-EL4/CM, the supernatant used in Fig. 1. Experiments 2 and 3 show that similar numbers of marrow precursor cells were stimulated to proliferate by PMA-EL4/CM, Con A-SC/CM, or PHA-PBL/CM. Mixing these three supernatants in pairs did not increase the number of growth-inducible nude marrow precursor cells. Two of the pairs tested are shown in experiments 2 and 3. Thus, PMA-EL4/CM, Con A-SC/CM, and PHA-PBL/CM all appear to support growth of the same precursor cells. In contrast, WEHI3/CM stimulated proliferation of many more marrow precursor cells than the three lymphoid cell-CM described (experiment 4). The plating efficiency of marrow cells stimulated by WEHI3/CM plus PMA-EL4/CM (experiment 4) or Con A-SC/CM (data not shown) was indistinguishable from that obtained by culturing marrow cells in WEHI3/CM alone, hence, the populations of WEHI3/CM-responsive and PMA-EL4/CM-responsive marrow precursor cells were, although not identical, largely overlapping.

Semipurified (see Materials and Methods) preparations of PMA-EL4/CM (at an optimal stimulating concentration) stimulated marrow cell growth as efficiently as the crude supernatant (experiment 5). Further biochemical purification of semipurified PMA-EL4/CM uniformly resulted in dramatic loss of the marrow cell growth-stimulating activity. Thus, material extensively purified for IL-2 activity by adherence to DEAE-cellulose with subsequent elution and chromatofocussing (14) (kindly provided by Dr. V. Paetkau, University of Alberta, Edmonton) did not stimulate or support nude marrow cell growth in vitro, whereas

TABLE I

Conditioned Media from Different Sources Stimulate Nude Marrow Precursor Cell Growth

Experiment	Conditioned medium*	Frequency of precursors giving rise to marrow cell growth‡		
		Mean frequency	95% confidence limits	χ^2
1	PMA-EL4/CM 20%	1/171	1/131–1/232	1.165
	10%	1/184	1/141–1/240	5.360
	5%	1/471	1/322–1/690	6.935
2	Con A-SC/CM	1/156	1/121–1/201	0.481
	PHA-PBL/CM	1/221	1/166–1/215	1.160
	PHA-PBL/CM + Con A-SC/CM	1/214	1/161–1/284	3.061
3	PMA-EL4/CM	1/153	1/120–1/194	1.365
	PHA-PBL/CM	1/152	1/119–1/194	2.543
	PMA-EL4/CM + PHA-PBL/CM	1/170	1/134–1/217	1.333
4	PMA-EL4/CM	1/119	1/90–1/156	4.446
	WEHI3/CM	1/60	1/47–1/78	5.652
	PMA-EL4/CM + WEHI3/CM	1/53	1/40–1/68	3.084
5	Crude PMA-EL4/CM	1/254	1/187–1/344	1.130
	Semipurified PMA-EL4/CM§	1/205	1/155–1/269	1.505
	Highly purified IL-2 preparation‖	<1/8,000¶	—	—
	Preparation devoid of IL-2**	1/699	1/434–1/886	2.182
6	PMA-EL4/CM‡‡ 0 filler cells	1/91	1/73–1/111	2.660
	100 filler cells	1/104	1/83–1/130	4.525
	300 filler cells	1/124	1/98–1/157	6.236
	1,000 filler cells	1/146	1/114–1/187	9.638

* An optimal stimulating concentration of crude supernatant was used (if not indicated otherwise).

‡ Growth of marrow cells was scored at day 7 of in vitro incubation.

§ Supernatant processed by $(NH_4)_2$ SO_4 precipitation at 80% saturation and Sephadex G-100 filtration; fractions of 10,000–70,000 mol wt pooled.

‖ Semipurified PMA-EL4/CM, passed through DEAE-cellulose columns. The eluted preparation further purified for IL-2 by chromatofocussing.

¶ Upper limit—no growth seen.

** Semipurified PMA-EL4/CM, filtered through DEAE-cellulose columns. This preparation does not contain IL-2 activity.

‡‡ Cells cultured in 20% vol/vol crude PMA-EL4/CM, with or without irradiated (1,600 rad) syngeneic filler cells (100, 300, or 1,000 cells/well).

the DEAE-cellulose column filtrate, which is devoid of IL-2 activity, did produce some growth (experiment 5). Two other supernatant preparations known to contain high levels of IL-2 also failed to produce growth: CM from human PBL stimulated 48 h with PHA (kindly provided by J.-F. Moreau, Ontario Cancer Institute) and a murine 'allogeneic effect factor' preparation (kindly provided by T. Delovitch, Best Institute, Toronto).

Pooled data from a number of independent experiments (Table II) show relatively small variations in plating efficiency from one experiment to another, certainly no larger than those seen in other functional cell titrations (22, 23). At this stage, the factor(s) requirement(s) for triggering/supporting marrow cell

TABLE II

Conditioned Media from Different Sources Stimulate Nude Marrow Precursor Cell Growth

Culture medium*	Number of independent experiments	Calculated nude marrow precursor cell frequency‡	
		Mean	Range
PMA-EL4/CM	14	1/198	1/85–1/315
Con A-SC/CM	7	1/155	1/116–1/229
PHA-PBL/CM	4	1/154	1/99–1/221
WEHI3/CM	3	1/60	1/45–1/83

* Nylon wool nonadherent nude (RNC × BALB/c)F_1 marrow cells were cultured at 10–160 cells per 20-μl microwell with an optimal stimulating concentration of CM as in Table I and Fig. 1.

‡ Cell growth was scored at day 7–10 of in vitro incubation.

proliferation in vitro at low initial cell densities remain relatively undefined except that, if IL-2 is required, it is certainly not the only factor.

Attempts to further increase the plating efficiency were unsuccessful. Mitogenic (PHA-P, 0.5–5% vol/vol; ConA, 1–10 μg/ml) or nonmitogenic (peanut agglutinin, 25–250 μg/ml; wheat germ agglutinin, 10–100 μg/ml) lectins, lipopolysaccharide (0.5–500 μg/ml), or PMA (1–100 ng/ml) did not increase plating efficiency of marrow precursor cells inducible to growth by various CM (data not shown). Nor did they by themselves stimulate growth. Furthermore, the addition of 100–1,000 irradiated syngeneic or autologous marrow, spleen, or lymph node filler cells to the microcultures decreased the plating efficiency. Experiment 6 in Table I shows one such test.

Cells from individual microcultures were examined both by phase-contrast microscopy and after staining with polychromatic May-Gruenwald-Giemsa stain. Morphologically different cells were found both within a colony and from colony to colony. Lymphoid, fibroblastoid, and myeloid cells (i.e., mature monocytes and granulocytes as well as their respective immature precursor cells) could be distinguished. At this point, we concentrated our attention on cultures showing spontaneous cytotoxic activity and on cultures containing cells with T cell markers.

Cytolytic Effector Cells are Spontaneously Generated In Vitro in Nude Marrow Cell 'Colonies'. Cell populations growing in individual wells were assayed for spontaneous cytolytic activity against a panel of different targets in a microversion of the ^{51}Cr-release cytotoxicity assay (see Materials and Methods). Data of some representative experiments are shown in Fig. 2, in which the cytolytic activity of groups of 80–160 individual microcultures was tested against a panel of syngeneic, semisyngeneic, and allogeneic target cells. Clearly, many of the colonies contained cytolytic activity. The colonies tested were derived from nylon wool nonadherent marrow cell suspensions of (RNC × BALB/c)F_1 (H-$2^{k/d}$) nude mice cultured as in Fig. 1 and most of the data are derived from cultures initially containing 60–80 cells/well. The target cell panel included Con A blasts (syngeneic F_1 [H-$2^{k/d}$], semisyngeneic parental RNC [H-2^k], semisyngeneic parental BALB/c [H-2^d], allogeneic C57BL/6 [H-2^b]) and tumor cells (P815 [H-2^d],

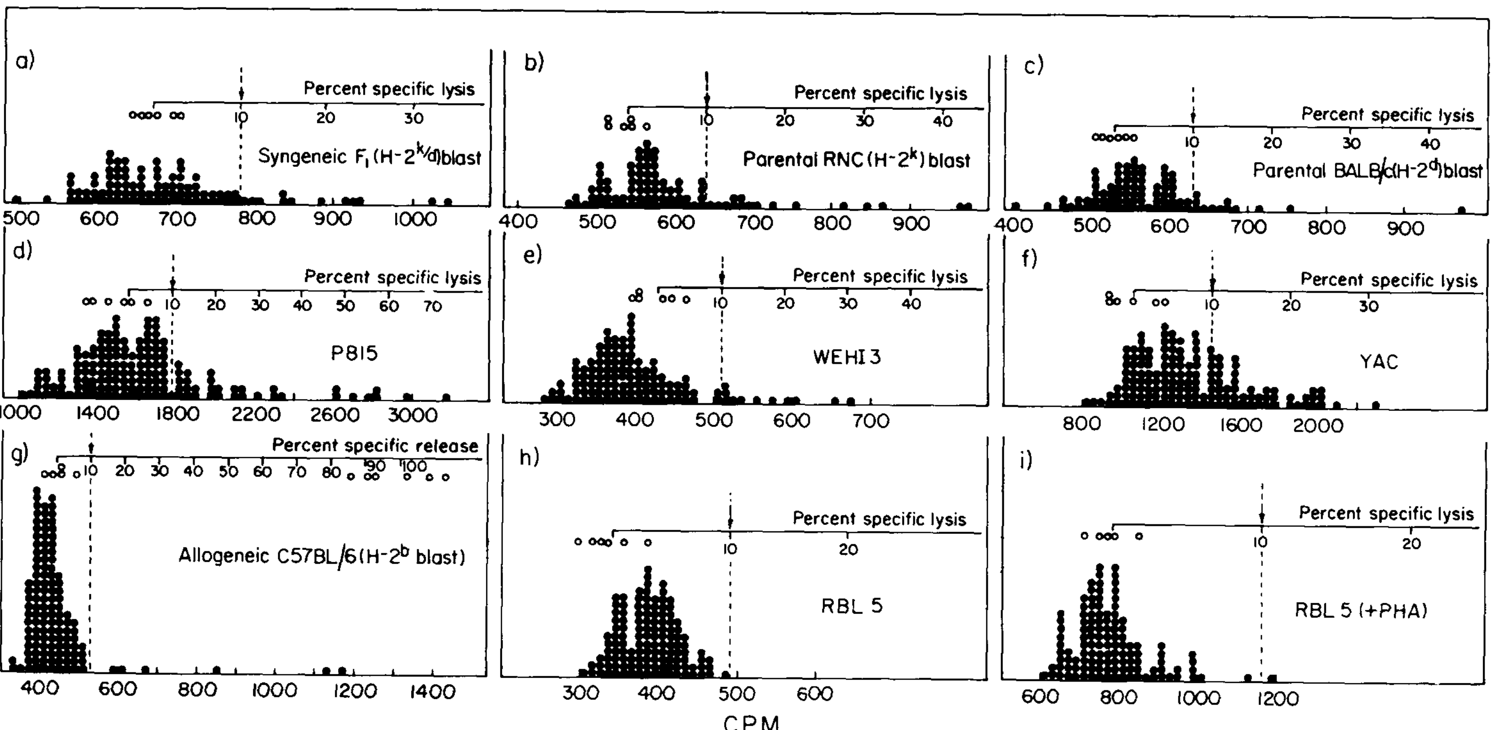

FIGURE 2. Spontaneous cytolytic activity of microcultures against various targets. Groups of 80–160 microcultures containing proliferating (RNC × BALB/c)F_1 nude marrow cells stimulated with PMA-EL4/CM were assayed at day 8–11 of incubation against different syngeneic, semisyngeneic, and allogeneic targets in a microversion of the standard 4-h ^{51}Cr-release cytotoxicity assay. Cultures showing >10% specific lysis were scored positive. (*a*) Syngeneic F_1 (H-$2^{k/d}$) Con A blasts, 12/92 (13%), (*b*) semisyngeneic RNC (H-2^k) Con A blasts, 16/86 (19%), (*c*) semisyngeneic BALB/c (H-2^d) Con A blasts, 12/83 (14%), (*d*) P815, 33/155 (21%), (*e*) WEHI3, 11/117 (9%), (*f*) YAC, 43/146 (29%), (*g*) allogeneic B6 Con A blasts, 6/123 (5%), (*h*) allogeneic RBL5, (H-2^b), 0/124, (*i*) RBL5 in the presence of PHA, 1/118 (1%). Data shown in *a*, *b*, *c*, and *i*; *d*, *e*, and *h*; *f*; and *g* are independent experimental series. Each closed circle is the ^{51}Cr release from a single culture. The open circles are spontaneous release values and, in *g* only, 100% release values.

WEHI3 [H-2^d], YAC [H-2^a, a k/d recombinant haplotype], RBL-5 [H-2^b]). Cultures were also assayed in a lectin-facilitated test against RBL5.

The killer cells detected displayed a definite preference for syngeneic or semisyngeneic targets. This is particularly apparent in comparing the bottom three panels of Fig. 2 (allogeneic H-2^b targets) with the top six panels (targets carrying self-H-2^k and/or H-2^d antigens). Note that even the presence of the lectin, PHA, did not facilitate the lysis of allogeneic RBL5 targets. A summary of all data derived from these and similar cytolytic assays is given in Table III.

No spontaneous cytolytic activity against any of the targets tested was found in freshly prepared nylon wool nonadherent nude marrow cell populations. The spontaneous cytolytic activity of in vitro cultured nude marrow cell colonies first appeared at day 8 of incubation, peaked at day 10, and markedly declined around day 12 if the cells were not fed or transferred into larger culture volumes. A fraction of repeatedly fed and subcultured short-term lines established from microcultures retained cytolytic activity for at least 4 wk.

Patterns of Lysis of Individual Nude Marrow Cell Colonies. It would be interesting to see if a microculture positive for one of the targets in the panel of Fig. 2 could also lyse one or more of the other targets. However, these cultures were assayed only 8–12 d after set up and not enough cells were available to split the cultures into several different aliquots. We therefore established short-term lines from these cultures. Starting with an original cell inoculum of five nylon wool nonadherent nude marrow cells per microwell, cultures showing growth on day 10 were picked and grown up in medium supplemented with PMA-EL4/CM over a further 2-wk period. At this time, ~5% of the expanded cultures expressed cytolytic activity when tested against a target cell panel consisting of Con A blasts derived from F_1 (syngeneic) and the two parental (semisyngeneic) strains. Table IV shows the results for 15 such lines. Cultures 1–5 contained self-reactive cytolytic effector cells that lysed F_1 targets as well as one or both parental targets. Cultures 6–15 showed unusual patterns of lysis that contradict the classic rules of transplantation: these cell lines lysed either both parental targets without lysing the F_1 targets or only lysed one of the parental or self-F_1 targets. Hence

TABLE III

Fraction of Microcultures Showing Spontaneous Cytolytic Activity Against Various Targets

Target	H-2	No. of colonies producing >10% lysis (%)
F_1 Con A blast	k/d	110/709 (15.5)
RNC Con A blast	k	156/887 (17.6)
BALB/c Con A blast	d	26/166 (15.7)
P815	d	123/761 (16.2)
WEHI3	d	50/306 (16.3)
YAC	a	75/372 (20.2)
RBL5	b	6/224 (2.7)
B6 Con A blast	b	6/114 (5.3)
RBL5 + PHA	(b)	12/542 (2.2)

Results from 11 independent experiments performed as in Fig. 2. The threshold for significant lysis was taken as 10% specific ^{51}Cr release.

TABLE IV
Lytic Patterns of Spontaneously Cytolytic Effector Cells Derived from Individual Nude Marrow Microcultures

Culture No.	Target*		
	F_1	k	d
1	32.2	21.3	15.3
2	26.4	18.2	22.8
3	19.1	22.7	—
4	21.5	11.4	—
5	24.4	—	45.6
6	18.5	—	—
7	—	10.9	—
8	—	23.6	—
9	—	35.9	—
10	—	36.8	—
11	—	18.8	—
12	—	—	16.8
13	—	—	17.4
14	—	—	46.9
15	—	—	14.7

Nylon wool nonadherent (RNC × BALB/c)F_1 nude marrow cells (five cells per well) were stimulated with PMA-EL4/CM in 20-μl microwells for 10 d. Proliferating colonies with a predominantly small lymphoid morphology were selected and expanded in 1-ml cultures (in the presence of PMA-EL4/CM and irradiated (1,600 rad) semisyngeneic RNC spleen filler cells) for a further 14 d.

* Each expanded culture was tested for its ability to lyse ^{51}Cr-labeled Con A blasts derived from F_1 and both parental strains. Entries show specific ^{51}Cr release from 15 of the more active cultures (of ~200 tested). (—) indicates that the ^{51}Cr release was <2 SD above the mean spontaneous release. This set a threshold for a positive response of 3–5% specific ^{51}Cr release.

the observed pattern of lysis can be classified as anomalous.

Expression of T Cell Markers by Cells in Nude Marrow Colonies. The serologically defined T cell differentiation markers Thy-1, Lyt-1, and Lyt-2 were looked for on cells from microcultures seeded with nylon wool nonadherent bone marrow using either fluorochrome-labeled monoclonal antibodies or a complement-dependent antibody-mediated cytotoxicity assay. A microversion of these serological techniques (see Materials and Methods) allowed typing of proliferating cells within microwells in such a way that there was minimal disturbance of the topographical arrangement of the cells in the microwell. Tests were done 8–10 d after initial culture set up. Both serological assays gave concordant results: ~30% of colonies grown from low numbers of PMA-EL4/CM-stimulated nude marrow cells contained up to 40% cells expressing the Thy-1 and/ or Lyt-1 antigen; only occasional cells were stained by anti-Lyt-2 antibody (data not shown).

We found that 15–20% of cells in nude nylon wool nonadherent marrow could be labeled with fluorescent anti-Thy-1 monoclonal antibodies before culture. Similar values have been recently reported for normal marrow (24). Thus, we

next asked whether colonies containing cells with T cell markers could be grown from nylon wool nonadherent nude marrow depleted of cells carrying this T cell marker as well as the Lyt-1 marker. Nonadherent marrow cells were labeled simultaneously with fluorescein-conjugated anti-Thy-1 and anti-Lyt-1 antibodies, and sorted into labeled and unlabeled fractions using a flow cytometer/cell sorter as depicted in Fig. 3. Forward angle, light-scattering intensity is known to be roughly proportional to cell size (25). Fig. 3*a* shows the forward angle, light-scattering spectrum for all cells (top line) and fluorescent cells (bottom line). A window was set as indicated by the dotted line in the figure and all cells above this window were discarded. The fluorescence spectrum of the remaining cells is shown in Fig. 3*b*. There is a well-defined fluorescence peak, representing ~10% of the starting population. A window was set as indicated by the dotted line in the figure. Cells above and below this window were sorted into two pools, a Thy-1^+/Lyt-1^+ and a Thy-1^-/Lyt-1^- subset.

Cells from the two sorted pools and from an unsorted, unlabeled control population were cultured in microcultures in a limiting dilution assay precisely as in Fig. 1. Growth was observed from all three samples and all three were well-fitted by limiting dilution analysis (Fig. 4). The frequency of the colony-forming unit in the Thy-1^-/Lyt-1^- subset was nearly the same as that in the unsorted

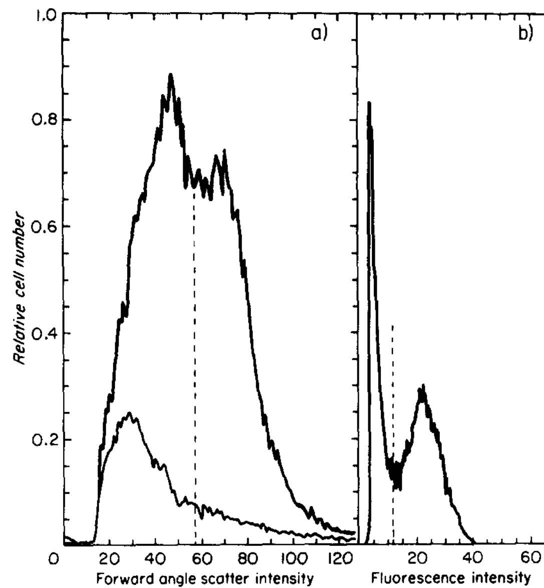

FIGURE 3. Cell sorting of Thy-1/Lyt-1-labeled nude nylon wool nonadherent bone marrow cells. Nylon wool nonadherent nude marrow cells were stained with fluorescein-labeled anti-Thy-1 and anti-Lyt-1 monoclonal antibodies. (*a*) shows the scatter profile of the total cell population (upper line) and of the Thy-1^+/Lyt-1^+ subset (lower line), 16% of the cell population. The large size cells (above the dotted line), comprising ~43% of the total cell population, were discarded. (*b*) shows the fluorescence histogram of the remaining cells. These were sorted into labeled and unlabeled cells as defined by the dotted line in the figure.

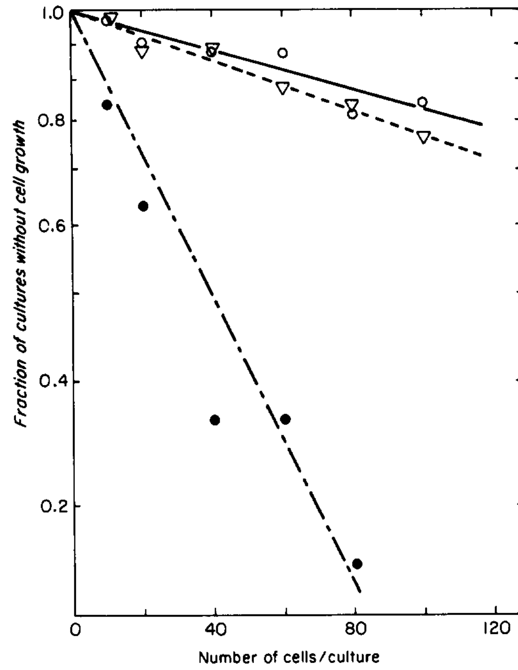

FIGURE 4. Nylon wool nonadherent nude marrow cells were fractionated as shown in Fig. 3*a* and *b* and then cultured for 7 d in PMA-EL4/CM (as in Fig. 1) before limiting dilution analysis. (○) Nylon wool nonadherent, unsorted, unlabeled control cells (f = 1/278; 1/192–1/388 95% confidence limits; $\chi^2 = 0.584$; (▽) sorted (small size) Lyt-1⁻/Thy-1⁻ cells (f = 1/224; 1/161–1/311 95% confidence limits; $\chi^2 = 3.950$); (●) sorted (small size) Lyt-1⁺/Thy-1⁺ cells (f = 1/42; 1/33–1/53 95% confidence limits; $\chi^2 = 1.254$).

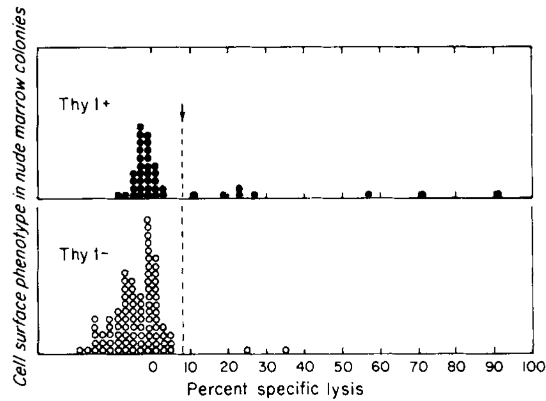

FIGURE 5. Simultaneous characterization of 130 colonies grown from sorted Thy-1⁻/Lyt-1⁻ cells for presence of Thy-1⁺ cells and for spontaneous cytolytic activity. (●) Thy-1⁺ cells in colony; (○) Thy-1⁺ cells absent. Lysis values to right of dotted line were scored as positive. See Results.

control (1/224 vs. 1/278) but much lower than that of the Thy-1$^+$/Lyt-1$^+$ subset (1/42).

Spontaneous Cytoxic Activity in Nude Marrow Colonies Grown from Thy-1$^-$/Lyt-1$^-$ Precursors. We next asked whether the colonies grown from Thy-1$^-$/Lyt-1$^-$ precursors exhibited spontaneous cytotoxic activity and, if so, whether this correlated with the expression of T cell markers. Cells from 130 individual colonies were split into two aliquots. One aliquot was tested for expression of Thy-1 using fluorescein-conjugated anti-Thy-1 monoclonal antibody and the other was tested for its ability to lyse ^{51}Cr-labeled semisyngeneic RNC Con A blast target cells. The results are shown in Fig. 5. 40 of the colonies were Thy-1$^+$ and, of these, 8 produced significant lysis. Of the 90 Thy-1$^-$ colonies, only 2 produced significant lysis. There is a significant correlation ($P < 0.01$) between the expression of Thy-1 and the ability to produce lysis.

Using flow cytometer analysis and fluorescein-labeled monoclonal antibodies, cells from pooled, expanded colonies containing spontaneous cytolytic activity were tested for expression of Thy-1, Lyt-1, and Lyt-2 (Fig. 6). A significant fraction of the cells (at least 25%) labeled with each of the three monoclonal antibodies. These tests were done about 4 wk after initial culture set up. In the time interval between 8–10 d and 4 wk after culture set-up, the fraction of Thy-1$^+$ and Lyt-1$^+$ cells has changed little whereas the fraction of Lyt-2$^+$ cells has markedly increased.

As a final question, we asked whether the lytic effector cells themselves carry Thy-1 or Lyt-2. Cells from short-term lines already established as being cytolytic were pooled and treated with anti-Thy-1 or anti-Lyt-2 plus complement before

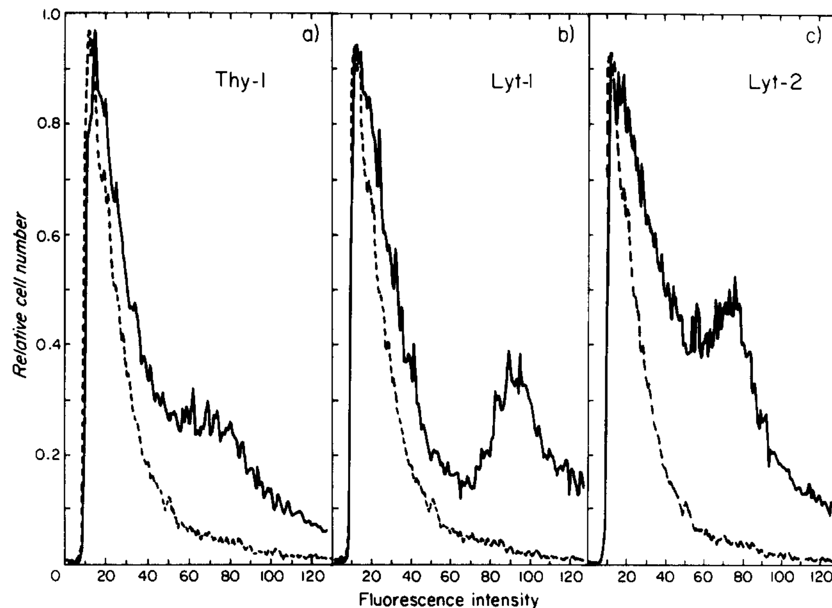

FIGURE 6. Surface phenotype of pooled nude marrow cells from colonies with spontaneous cytolytic activity. The upper line is from labeled cells; the lower line is autofluorescence from unlabeled control cells. (*a*) Thy-1, (*b*) Lyt-1, (*c*) Lyt-2.

TABLE V
Nude Marrow Cell Lines with Spontaneous Cytolytic Activity Are Thy-1⁺

No.	Treatment*	Viable cells recovered	Specific lysis‡
		%	%
1	None	100	34.6
2	C′	86	44.7
3	Anti-Thy-1 + C′	38	16.0
4	Anti-Lyt-2 + C′	53	56.8
		Threshold:	2.1

* Cells from various different short-term lines were pooled. Aliquots were either not treated (No. 1) or treated with either (1:10 diluted) rabbit complement, C′ (No. 2), monoclonal anti-Thy-1.2 antibody (diluted 1:10) plus C′ (No. 3), or monoclonal anti-Lyt-2.2 antibody (diluted 1:10) plus C′ (No. 4). The number of viable cells recovered after these treatments was assessed by eosin uptake.

‡ Viable (treated or nontreated) effector cells were incubated with 10^3 ^{51}Cr-labeled YAC tumor target cells at an effector/target ratio of 10:1 in a 4-h assay. The specific lysis values were calculated as described in Materials and Methods.

being tested for cytolytic activity. A substantial fraction of the effector cells appeared to carry Thy-1 but not Lyt-2 (Table V).

Discussion

We have described here experiments that appear to demonstrate the in vitro differentiation of functional T cells from less mature precursor cells found in the bone marrow of athymic nude mice. We first discuss these results in the context of previous studies of myeloid differentiation from precursor cells in bone marrow. The most widely used procedure has been to observe the development of colonies from marrow cells cultured in a relatively large volume of semi-solid medium (typically ~1 ml) in the presence of growth factor(s) from CM (reviewed in 26, 27). Colony growth is seldom observed in these cultures when the initial cell inoculum is $<10^5$ cells/ml unless the cell density is raised by addition of irradiated feeder cells. As well as providing nutritive and/or essential conditions required for colony growth, these added cells might also prevent the growth of some potential precursor cells and have a profound influence on differentiation events taking place in the colonies developing from those that do grow. It may also be that use of a semi-solid medium selects for a subset of all lymphomyeloid precursors. Thus, for many cell lines, anchorage-independent growth in semi-solid medium correlates with the ability to form tumors in vivo (28).

Our objective here was to obtain differentiation of T cells from immature precursors in marrow without having to use high initial cell numbers or added feeder cells; this we appear to have achieved by culturing the cells in very small volumes in liquid medium containing growth factor(s). Our use of nylon wool nonadherent nude marrow cells as the standard starting cell suspension was a

consequence of earlier experiments. We initially observed that 500–5,000 unfractionated cells from murine marrow, spleen, thymus, or lymph nodes, cultured in 20-μl microwells in the presence of CM, produced cell growth in a fraction of seeded wells. Many more microwells containing proliferating cells were found in cultured cell suspensions with many mature T cells than in cell suspensions obtained from central or T cell-deficient lymphoid organs. Minimum estimates for precursor cell frequencies were 1/200 for lymph node cells, 1/350 for euthymic spleen cells, 1/5,000 for marrow cells, 1/20,000 for thymocytes, 1/2,000 for nude marrow cells, and 1/15,000 for nude spleen cells. This suggested that mature T cells proliferated under our microculture conditions. Furthermore, limiting dilution analysis of these data indicated that more than one precursor cell population could give rise to the proliferating cells within an individual microwell. As our interest was focussed on the early extrathymic in vitro differentiation of T cell precursors and not on the expansion of mature T cell clones, we chose nude marrow cells as the object for further studies. The introduction of a simple fractionation step, i.e., the passage of marrow cells through nylon wool columns, strikingly improved two aspects of the in vitro microsystem. The plating efficiency increased >10-fold; transfer of 10–100 cells into a 20-μl microwell resulted in cell growth in a substantial fraction of seeded wells (Fig. 1). In addition, limiting dilution analysis of the data was consistent with all proliferating cells within individual microwells being the progeny of a single precursor cell.

The growth of precursor cells in these cultures was induced and/or supported by CM containing FCS. The crude and semipurified supernatants used contain a very heterogenous mixture of biological activities, e.g., granulocyte/macrophage-colony stimulator factor, IL-2 and IL-3, and interferons (29–31). Hence, it was to be expected that different lymphomyeloid precursor cells might be triggered by these supernatants. However, we were surprised at how many precursor cells were apparently inducible to in vitro clonal growth and differentiation, with a comparably high plating efficiency, in a microsystem with an initial input of only 5–100 cells per well. Thus, the presence of CM was apparently sufficient to initiate and/or support the in vitro proliferation and differentiation response of lymphomyeloid precursor cells, and there was no evidence of a requirement for 'inductive', 'microenvironmental', 'instructive', or 'accessory' cell interactions in the early phase of the response. The addition of filler cells actually depressed growth (Table I).

The factor(s) requirement(s) for in vitro T cell differentiation in the microsystem have not been fully defined. The activity is present in crude and semipurified supernatants of stimulated murine or human lymphoid cell populations (i.e., PMA-EL4/CM, Con A-SC/CM, PHA-PBL/CM). Further purification steps of ammonium sulphate-precipitated material (with a molecular weight of 10,000–70,000) from stimulated EL4-thymoma cell supernatant resulted in a loss of activity. Clonal nude marrow cell growth was stimulated by WEHI3/CM, but we did not find functional cytolytic cells in any of these colonies (data not shown). Interestingly, WEHI3/CM is devoid of IL-2 activity (32). Evidence against an exclusive role for IL-2 was obtained by comparing fractions of PMA-EL4/CM highly purified for IL-2 (no growth obtained) and devoid of IL-2 (some growth

obtained) (see Table I, experiment 5). Possibly, different factors are required to act simultaneously or sequentially in the course of consecutive developmental events, which would make a factor analysis very complex.

The point should be stressed that the microculture system used here has unique features: the initial cell inoculum is exceedingly small; we never used feeder or filler cells, and we assayed for function or marker expression as early as possible. This is in contrast to in vitro techniques favored by most current research work on lymphomyeloid differentiation (e.g., 33–36) in which fairly large initial cell inocula are grown up in the presence of feeder cells in bulk cultures. Subsequently, cell lines are derived from individual colonies or single cells cloned out of bulk cultures and propagated in vitro, again in the presence of feeder cells. Assays for function and marker or specificity expression are often done many months after the initiation of culture, at which time the cells may be locked into a frozen state of maturation arrest. In contrast, we feel we are asking questions about the phenotypic stability (or irreversible commitment) of differentiating cells to certain functions, marker expressions, or specificities at different stages along a developmental pathway.

Though not formally established, we think we are observing early T cell differentiation in our microculture system. We have demonstrated the de novo generation from marrow cells of cells that express T cell markers phenotypically. More importantly, we have detected the generation of functional cytolytic effector cells that show specificity. Although some NK cells are known to express some T cell markers (37, 38), there is no precedent in the literature for NK cells that discriminate between a Con A blast target of the syngeneic $(A \times B)F_1$ versus the semisyngeneic A or B mouse strains, although cells generated spontaneously in short-term cultures and having this kind of specificity have been previously observed (39, 40). The fact that we observe lysis of YAC target cells (Table III), an excellent NK target, does not necessarily mean we are observing NK cells. YAC is $H\text{-}2^a$, an $H\text{-}2^k$-$H\text{-}2^d$ recombinant, and therefore shares H-2 antigens with the syngeneic and semisyngeneic Con A blast targets that are lysed. RBL5 ($H\text{-}2^b$) is also a good NK target (41) and is not lysed (Table III). We also consider it unlikely that we are observing conventional H-2-restricted CTL responses against modified self-determinants induced, for example, by the FCS in the CM. Not only do our effectors lack the Lyt-2 markers typically found on conventional CTL (Table V), but effectors of the H-2-restricted type should lyse both F_1 and appropriate parental targets. This was not seen (Table IV). In addition, target cells maintained by in vivo passage (e.g., P815) were as effectively lysed as those produced in vitro, whereas other target cells maintained by in vitro passage (e.g., RBL5) were not lysed.

We consider our data as a contribution to the problem of the ontogenetic development of the T cell repertoire. Three striking features of the early repertoire were unexpected: (*a*) the definite preference for syngeneic and semisyngeneic targets by early cytolytic effector cells; (*b*) the anomalous patterns of lysis of syngeneic and semisyngeneic targets, which did not conform with the rules of classic transplantation genetics; and (*c*) the absence of alloreactivity in early cytolytic effector cells.

The pronounced bias of early cytolytic cells for (semi-) syngeneic targets may

support a hypothesis that suggests self-reactivity of early T cell precursors from which the final repertoire is proposed to be generated by positive selection through somatic mutation (42). The anomalous patterns of lysis have to be considered with reference to long-standing observations of $F_1(A \times B)$ anti-parent (A or B) reactivity (43–50). In the in vitro version of this system, $F_1(A \times B)$ CTL are activated by parental (A or B) stimulator cells in a conventional mixed leukocyte culture. The F_1-derived CTL recognize (MHC-encoded) A or B only on target cells with a homozygous set of the appropriate A or B alleles, but not on self-F_1 target cells heterozygous for A and B (although the gene products of A and B are codominantly expressed on F_1 target cells). The present study extends these observations, as some in vitro differentiated effector cells lysed exclusively self-$F_1(A \times B)$ targets (but not A or B targets). We have obtained similar results in F_1 anti-parent mixed leukocyte cultures performed at limiting dilution (51). The further analysis of these early appearing unusual patterns of lysis is being pursued in experimental studies.

Differentiation of myeloid cells from stem cells has been observed in liquid culture systems, but only when a feeder layer of unknown function had also been established in the culture vessel (52). The same cultures also appear to contain cells committed to the T cell lineage (53), but in general there has been only limited success in obtaining lymphoid differentiation in vitro (54, 55). Although B cell colonies can be grown in semisolid medium, their precursor appears to be a nearly mature B cell (56).

There is an extensive literature on T cell colonies grown in semisolid medium. Most such colonies appear to arise from mature T cells that have been activated and undergone clonal expansion although, given appropriate growth factors, colonies that appear to arise from much less mature precursors can arise (57). In particular, colonies containing CLP can be grown from normal mouse spleen cells in a methylcellulose system with an initial cell input of $>10^5$ cells. CLP of several different specificities can develop within a single colony from a single less mature precursor (58, 59). The relative frequency of different characteristic CLP specificities is essentially the same as that found in the intact animal (59). The colony-forming unit for the development of CLP had two components in this system, one Thy-1^- and the other Thy-1^+, the latter being the precursor of mature CLP. These two different components were identified through differential organ-specific distribution patterns in vivo and the expression versus nonexpression of T cell-specific markers (57–59). Cells found in nude marrow could function only as the Thy-1^- component in this system and did not have the potential to develop into cytotoxic cells (57). In contrast, we show in this paper that the introduction of a modified microversion of the in vitro technique allowed maturation of functional cytolytic cells from precursors in nude marrow. The specificity repertoire of these cytolytic cells was very different from that found in either the T cell colonies or the peripheral lymphoid tissues of an intact animal. We assume that the present system dilutes out negative cell-mediated regulatory influences that prevent the expression of cytolytic effectors in marrow cell populations in the methylcellulose system. The data presented here suggest a Thy-1^-/Lyt-1^- surface phenotype for some precursors of mature cytolytic effector cells activated in the liquid microsystem in vitro, which is in contrast to

the Thy-1$^+$/Lyt-1$^-$ surface phenotype of progenitors of mature CTL detected in the methylcellulose system (59). This raises the possibility that the cytolytic cells generated in the microsystem represented the progeny of a very early precursor cell, possibly close to the actual initiation point of the lineage committed to T cell differentiation. We hope to elucidate this question in further studies.

Summary

We describe an in vitro limiting dilution culture system that supports growth and differentiation of nylon wool nonadherent bone marrow cells from athymic nude mice. Cells were seeded at low cell numbers (5–120 cells per 20-μl microculture well) in the absence of added filler or feeder cells but in the presence of conditioned medium. Microwells positive for growth appeared to contain a single clone of cells that adhered together to form a tight cluster referred to here as a colony. A fraction of colonies contained cells that expressed an unusual spontaneous cytolytic activity. They lysed syngeneic or semisyngeneic Con A blast or tumor cell targets but seldom lysed H-2-incompatible Con A blast or tumor target cells, even in a lectin-facilitated assay. A large fraction of colonies contained lymphoid cells that expressed the T cell markers Thy-1 and Lyt-1. Colonies expressing spontaneous cytolytic activity and also containing cells with Thy-1$^+$ and/or Lyt-1$^+$ markers could be grown from nylon wool nonadherent nude marrow cells depleted rigorously by cell sorting of cells expressing either of these markers. Expression of Thy-1 and spontaneous cytolytic activity in a particular colony was significantly correlated. Short-term lines established from cytolytic colonies with T cell markers maintained both characteristics. The cytolytic effector cells observed in these cultures may represent an early stage in the development of the T cell repertoire.

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