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An In Vitro Assay for T Lymphocyte Progenitors (CFU-preT)

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Thy-1.2 negative progenitors give rise to Thy-1.2 positive colony cells when mouse bone marrow is cultured in vitro. The bone marrow cells are immobilized in a viscous medium containing methyl cellulose; discrete colonies are identifiable at 2 days and contain 30–60 cells by day 3 of culture. Colonies are tightly packed spheres (raspberries) and grow suspended in the gel. Growth of the raspberry colonies is absolutely dependent upon the presence of the appropriate serum (horse or human; not fetal calf) and conditioned medium from pokeweed mitogen-stimulated mouse spleen cells. As little as 0.1% of the conditioned medium is sufficient to promote raspberry colony growth. Under these conditions, nude mouse bone marrow yields as many colonies (1 per 1,000 nucleated cells plated) as normal marrow. Thymus, lymph node, and spleen (normal or nude) do not form colonies. Colony precursors are predominantly in S phase of the cell cycle, as determined by tritiated thymidine suicide of fresh bone marrow. Their numbers fall with age. Because the cells in colonies are Thy-1 positive, peanut agglutinin-positive, and active in a pre-T cell synergy assay, we conclude that their precursors are early committed T cell progenitors, and propose that they be called CFU-preT.

Key words: **T lymphocyte progenitors, colonies, CFU-preT, bone marrow**

Interest in the mechanisms that regulate differentiation and proliferation of hematopoietic cells has prompted the development of in vitro techniques for the culture of progenitors of monocytes and granulocytes, erythrocytes, megakaryocytes, and eosinophils [1], as well as mature T cells [2] and B cells [3]. These culture systems generally employ agar or methyl cellulose to immobilize the cells in a developing colony, and they require selected sera and growth factors. Using such culture systems, it has been possible to learn a great deal about the progenitor cells (colony-forming units; CFU) and the factors that control their growth and maturation. Until recently, it has not been possible to grow lymphoid precursors in this fashion.

Maturation of T lymphocytes is more complicated than that of other hematopoietic cells in that it takes place in 3 different anatomic sites. Differentiation begins in the bone marrow when a pluripotential stem cell (CFU-S or an earlier cell) gives rise, by as yet un-

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characterized means, to a committed T cell progenitor. Further differentiation takes place in the thymus, and finally in the periphery. The questions of when and where developing T cells acquire the ability to recognize "self" and respond to foreign antigens have not been resolved. Much evidence supports the idea that the thymus is the site for acquisition of both these characteristics, but recent reports [4–6] suggest that prethymic cells may recognize foreign antigens. We now describe an in vitro technique for culturing T cell progenitors that may allow us to study the early stages of T-lineage maturation in a carefully controlled manner.

MATERIALS AND METHODS

Mice

Male CBA/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Unless otherwise indicated they were 7–12 weeks old. Nude mice, the progeny of NIH nu/nu males and NIH nu/+ females, were a gift from Dr. Priscilla Campbell.

Cells

Bone marrow was flushed out of tibias and femurs with Hank's balanced salt solution and made into single cell suspensions by passage through a 25-gauge needle. Thymus, spleen, and lymph node cell suspensions were made by pressing the tissues gently through a 100-mesh stainless-steel screen. Cells were washed once in Hank's and resuspended in RPMI 1640 medium (GIBCO).

Conditioned Medium

CBA/J spleen cells were cultured at 3×10^6/ml in RPMI 1640 tissue culture medium (TCM) containing 5% fetal calf serum, 1% Gibco pokeweed mitogen, penicillin (100 U/ml), and streptromycin sulfate (100 μg/ml), for 7 days. The culture medium was harvested by centrifugation, and the supernatant was then passed through an 0.45 μm Millipore filter. It was stored at 4°C until used.

Tritiated Thymidine Suicide

Bone marrow at 3×10^6 cells/ml in TCM was incubated at 37°C for 1 h with 10 μCi of tritiated thymidine, specific activity 90 Ci/mmole (New England Nuclear). Cells were then washed twice in Hank's and made back to starting volume. Control cells were treated similarly, but thymidine was omitted.

Density Fractionation of Bone Marrow

The discontinuous density gradient method of Raidt et al [7] was used to fractionate bone marrow suspensions into 4 layers, labeled A, B, C, and D in order of increasing density.

Colony Assay

Cells were placed in a total volume of 0.1 ml in 96-well Costar Cluster dishes (flat bottom, 6.4 mm diameter wells). The incubation mixture consisted of 5 to 40×10^3 cells in RPMI 1640 with 0.8% w/v methylcellulose (4,000 centipoise, Fisher); 1–10% pokeweed mitogen-stimulated spleen conditioned medium; horse serum or human serum at 5% or 10%; penicillin (100 units/ml); streptomycin sulfate (100 μg/ml); and amphotericin B (2.5 μg/ml). Hydrocortisone sodium succinate and 2-mercaptoethanol were sometimes added (see Results).

Six to 12 replicates of each culture were set up. Plates were incubated at 37°C in humidified 5% CO_2 in air for 3–5 days. Colonies were counted with an inverted microscope using a 10 power objective; only tight spherical "raspberries" with more than 16 cells were scored.

Staining

Colonies were aspirated from the gel using a finely drawn Pasteur pipette, and several hundred colonies were pooled. They were washed with Hank's, and aliquoted. Some aliquots were cytocentrifuged and stained for peroxidase, non-specific esterase, or with Gurr's Giemsa. Others were incubated with fluorescein-labeled peanut agglutinin (Sigma, 0.125 mg/ml) or with monoclonal anti-Thy-1.2 (New England Nuclear, 10 μg/ml) followed by fluorescein-labeled goat anti-mouse μ chains (Cappel); controls were incubated only with fluorescein-labeled anti-μ.

Synergy Assay for Pre-T Cells

Although pre-T cells in bone marrow do not respond with significant mitogenesis to concanavalin A (Con A), they markedly enhance the response of mature T cells to that mitogen. This synergistic interaction has been used as the basis of a T cell progenitor assay [8]. It was applied to pooled colony cells by mixing them with small numbers of syngeneic lymph node cells and assaying the increase in tritiated thymidine incorporation following Con A stimulation, as described elsewhere [8].

RESULTS

Colony Growth

By 48 h of culture tight spherical colonies (raspberries) can be seen growing throughout the methylcellulose gel (Fig. 1). Under optimal conditions colonies contain 30–60 cells on day 3, indicating an average cycle time of 12 h or less. Individual colonies have had a mitotic index as high as 50%; there must be considerable synchrony. The colonies continue to grow until day 5 or 6, at which time other hematopoietic colony types overgrow them. Colony growth seems to stop at about 7 days, although many of the cells in a colony remain viable for up to 2 weeks by the fluorescein diacetate technique.

Colony growth is absolutely dependent upon a suitable serum, and conditioned medium; horse serum has been most effective, although certain human sera work nearly as well. No commercial horse serum works very well; our best results have been with blood obtained at a local abbatoir. Serum from this blood does not contain endotoxin detectable by the Limulus amebecyte lysate assay [9]. Five percent to 10% serum is optimal for colony growth (Fig. 2).

Conditioned medium (CM) from pokeweed mitogen-stimulated spleen cells is also required for colony growth. Typically, we use 10% horse serum and 10% CM, but as little as 0.09% CM will give a significant number of colonies (Fig. 2). Medium from phytohemagglutinin (PHA) or lipopolysaccharide (LPS) stimulated spleen cells does not stimulate colony growth; Con A-conditioned medium, which contains a high titer of T cell growth factor (TCGF [6]), stimulates the growth of only a very small number of colonies. Two nonessential additives will enhance colony growth: 2-mercaptoethanol at 5×10^{-6} M increases colony number by about 30%, and hydrocortisone at 10^{-6} M, by 100%. Under optimal conditions, without additives other than serum and CM, the yield of colonies from CBA/J marrow has been 1 per 1,000 cells plated in a very large number of experiments.

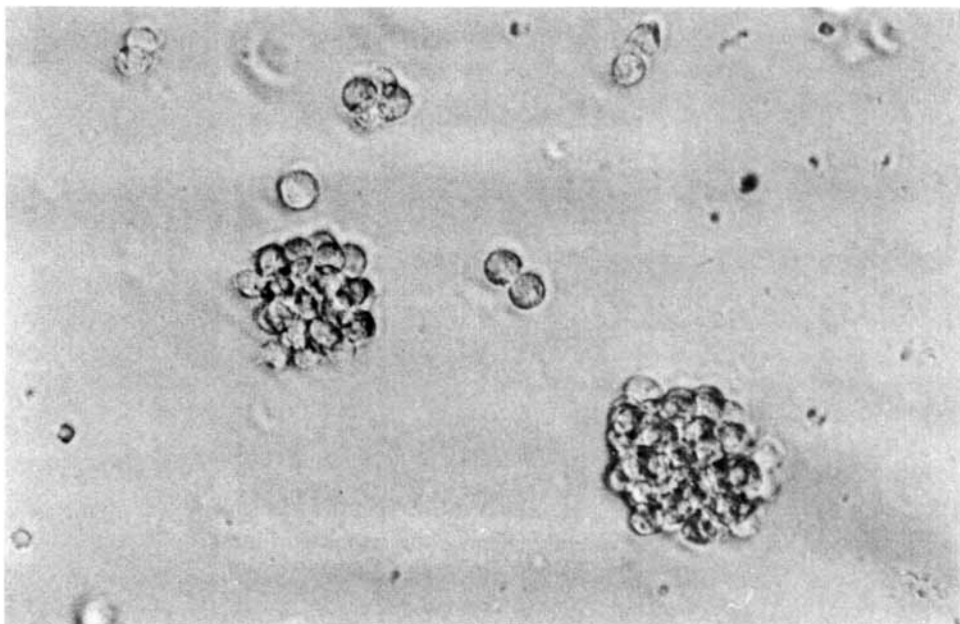

Fig. 1. Phase-contrast photograph of two "raspberry" colonies on day 3 of culture. Enlarged from a 100 × photograph.

Characteristics of the Colony-Forming Unit

Colony precursors are found in bone marrow of normal and athymic (nude) mice in approximately equal numbers. Colonies do not grow from thymus, lymph node, or spleen (nude or normal). There is a characteristic decline in the number of colony precursors in the marrow of aging mice, with 30-week-old mice having about half as many as young (6–10-week-old) mice (Fig. 3).

When bone marrow was separated on the basis of density on a discontinuous albumin gradient, a considerable enrichment of colony-forming cells was obtained in the lightest layers, A and B (Fig. 4). These layers are enriched in actively cycling blast cells, as opposed to resting cells, and it is thus not surprising that between 75% and 95% of colony-forming units could be eliminated by in vitro tritiated thymidine suicide of young bone marrow. This percentage fell in older mice (30–70 wks) by about 50% (Fig. 3), so that while actively cycling cells fall with age, the slowly cycling or non-cycling population seems quite constant, at least from 6 to 70 weeks of age.

Colony precursors do not have detectable amounts of the Thy-1 differentiation antigen on their surface: bone marrow treated with hybridoma-derived anti-Thy-1.2 (New England Nuclear) at 1:200 (500 times the titer) and complement gave as many colonies as untreated bone marrow.

Characteristics of the Cells in Colonies

Colonies were individually harvested to minimize contamination by non-colony cells, and then pooled to obtain sufficient cells for histochemistry and immunofluorescence. The cells appeared to be early lymphoid cells on Giemsa staining, with large nuclei,

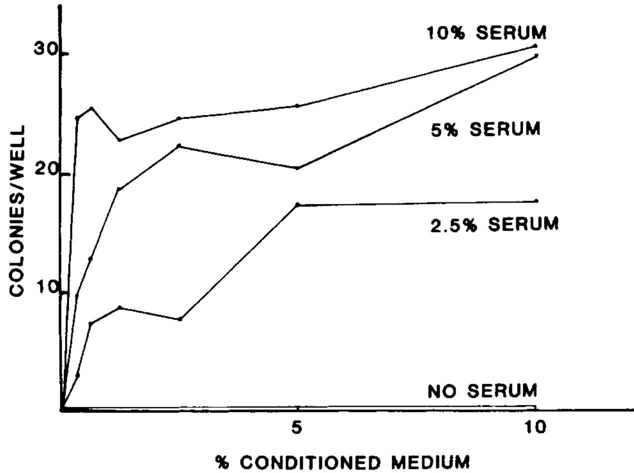

Fig. 2. Dose-response curves for various concentrations of conditioned medium in the presence of 0, 2.5%, 5%, or 10% horse serum. Serum and conditioned medium are both essential for colony growth. In all cases, 40×10^3 bone marrow cells were plated.

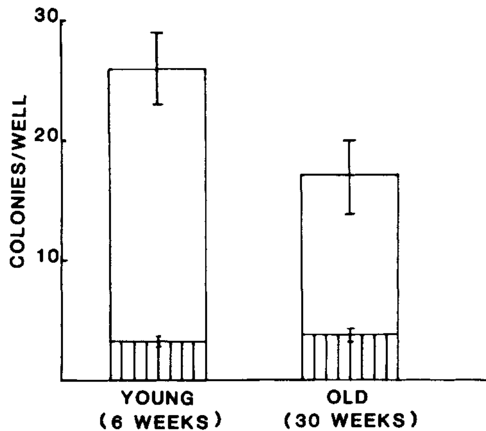

Fig. 3. Colony formation by 6- and 30-week-old CBA/J bone marrow cells. On the average, mice older than 30 weeks have about 50% as many CFU-preT as young mice. The hatched portions of the bars represent colonies obtained from bone marrow exposed to tritiated thymidine suicide. All fractions were plated at 20×10^3 cells/well.

prominent nucleoi, and undifferentiated cytoplasm. They are negative for nonspecific esterase and peroxidase and do not phagocytose latex beads. They are negative for surface and cytoplasmic IgM by immunofluorescence.

Bone marrow cells from CBA/J (Thy-1.2) and AKR/J (Thy-1.1) mice were cultured, and colonies were collected with a micropipette after 75 h. Only colonies of the very tight "raspberry" morphology were chosen; these were easily distinguished from granu-

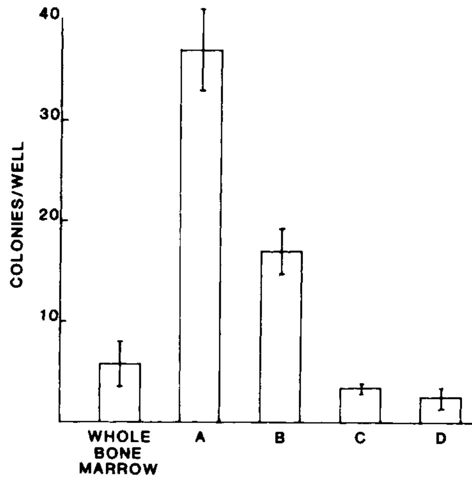

Fig. 4. Colony formation by whole bone marrow, and by the 4 fractions obtained on a bovine serum albumin density gradient. Cells were plated at 5×10^3 / well, using 10% horse serum and 10% conditioned medium.

locyte-macrophage colonies, which are small but evident at 3 days, and have the typical scattered cell distribution characteristic of highly motile cells. Four hundred colonies were pooled and the cells stained with anti-Thy-1.2 followed by fluorescein-labeled goat anti-mouse μ chains, or with the latter reagent only. In both cases about 10% of the cells stained with goat anti-mouse μ chains, but these were quite clearly phagocytic cells, having ruffled borders and containing many refractile granules. Of lymphoid cells, 88% were positive for Thy-1.2 in the CBA/J cultures, and 3% in the AKR/J cultures. Thus the anti-Thy-1 staining is specific for the Thy-1.2 allele. There was an inverse relationship between size and intensity of staining with anti-Thy-1.2. Over 90% of cells stain with fluoresceinated peanut agglutinin (under conditions which stain 94% of thymus, 8% of spleen, and 14% of bone marrow cells); in this case, there was a direct relationship between size and fluorescence intensity, with many small cells being negative.

Pooled colony cells do not respond to Con A, PHA, LPS, or pokeweed mitogen. They do, however, synergistically support the proliferation of a small number of mature T cells stimulated by Con A, as do pre-T cells [8]. One hundred thousand lymph node cells cultured alone gave a Con A response of 4,736 cpm; in the presence of 50,000 bone marrow cells this was 11,795 cpm, and in the presence of 50,000 colony cells, 16,892 cpm.

DISCUSSION

This report describes a technique for growing colonies from what appear to be T lymphocyte progenitors from mouse bone marrow. The colony-forming unit is Thy-1 negative, as shown by its resistance to killing by very high titer anti-Thy-1 and complement. The cells themselves, however, are largely Thy-1 positive; of mouse hematopoietic cells only T lymphocytes bear this antigen. The cells also bind peanut agglutinin, which

has been described as a characteristic of immature cells of the T lineage [10]. Thus cells in the colonies we have described, being both Thy-1 and peanut agglutinin positive, have the phenotype of cortical (immunoincompetent) thymocytes. We are further characterizing these cells in terms of their content of TL and Lyt antigens, and the enzyme terminal deoxynucleotidyl transferase. The colony cells also synergize with mature T cells in response to concanavalin A, another characteristic of T cell precursors [8]. Because of this evidence, we propose that the cells that give rise to these colonies be referred to as CFU-preT (colony-forming units, pre-thymocytic).

Jacobs and Miller [11] have also described a colony-forming technique that appears to permit the growth of T cell progenitors, in that nude mouse spleen gave rise to Thy-1 positive colonies. There are several differences between their procedure and ours. Most important is that their technique also allows the growth of mature T cells, whereas ours is selective for progenitors. The conditioned medium used by Jacobs and Miller is from the PHA-stimulated lymphocytes of a patient with hemochromatosis, and was used at 40%; clearly, the PHA carried over into the colony assay may be responsible for the response of mature T cells. This is not a problem in our method, where 0.1–1.0% conditioned medium gives a significant colony response. Since the requirements for hemochromatosis-patient conditioned medium in the Jacobs and Miller technique are apparently quite stringent, we have not been able to compare directly their colonies and ours. Their group refers [12] to the colony precursors in their system as TL-CFU (T lymphocyte), which seems appropriate since both mature and immature cells are detected.

We were surprised that nude mouse spleen did not give rise to colonies in our assay, since this organ contains 10–20% cells that can be induced to express Thy-1 [13]. This may mean that the pre-T cells in nude spleen are different from those found in bone marrow; perhaps they are more mature and as such are no longer responsive to the growth factors in our medium.

It is interesting to note that the vast majority of CFU-preT are in active cell cycle. We have suggested elsewhere [14] that the generation of T cell diversity does not occur in the thymus, as is commonly thought, but in the bone marrow. The finding of a rapidly cycling pre-T cell population in the marrow is a prerequisite for such a model. The fall in cycling CFU-preT with age is similar to what we have shown using the synergy assay [15]. It will be interesting to see the effects of soluble thymic factors on this age-related decline; in our previous study the fall in cycling pre-T cells with age was completely reversed by in vivo administration of a thymic extract.

In preliminary experiments (unpublished) we have found that the growth of CFU-preT can be enhanced by the addition of a crude thymic extract to the culture medium. We are working to make this a reliable assay for the thymic factor, which will then allow us to purify and characterize it.

We are hopeful that the ability to grow individual clones of T lymphocyte progenitors in culture will allow us to study the physiology of these cells more precisely than has heretofore been possible. In particular, we want to observe the acquisition of antigen receptors, subpopulation restriction, and differentiation markers, and to learn the ways in which this is regulated.

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