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Immunology

Expression of Thy-1 antigen is not limited to T cells in cultures of mouse hemopoietic cells

(bone marrow/colony-forming cell/T-cell hybridoma/T-cell growth factor/colony-stimulating factor)

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ABSTRACT Large numbers of Thy-1-positive cells were observed in cultures of bone marrow cells that had been depleted of T cells and grown for 3 to 4 days in the presence of medium conditioned by concanavalin A-activated spleen cells. Cells bearing levels of Thy-1 comparable with those on the bulk of thymocytes were isolated by using the fluorescence-activated cell sorter. Although many were large blasts, the Thy-1-positive cells failed to grow in response to T-cell growth factor and concanavalin A; about one-third, however, proliferated in the presence of factors stimulating hemopoietic progenitor cells. Furthermore, the Thy-1-positive population included cells capable of forming large colonies of macrophages and granulocytes in agar and cells forming splenic colonies in lethally irradiated mice. The appearance of the Thy-1-positive cells did not correlate with the presence of either T-cell growth factor or T-cell-derived granulocyte/macrophage colony-stimulating factor. These findings indicate that Thy-1 can occur on various murine hemopoietic stem and progenitor cells and myeloid cells; Thy-1 can no longer be regarded as an unambiguous marker of commitment to the T-cell lineage.

The Thy-1 antigen was first described in the mouse, where it was shown to exist in two allelic forms, Thy-1.1 and Thy-1.2, and to be present on neural and lymphoid tissues (1). Further studies showed that, in mice, Thy-1 served as a marker for T lymphocytes (2). In the past decade, immunologists studying the mouse have regarded the presence of the Thy-1 antigen as an unambiguous marker for distinguishing lymphoid cells of the T-cell lineage from B lymphocytes and other cells of the hemopoietic system.

Here, however, we show that Thy-1 antigen can be detected on a relatively large proportion of mouse bone marrow cells after a short period of tissue culture in the presence of medium conditioned by activated T cells or a myelomonocytic line, WEHI-3B. The Thy-1-positive cells included obvious myeloid cells together with hemopoietic stem and progenitor cells; these observations place important qualifications on the validity of Thy-1 as a marker for T cells in the mouse.

MATERIALS AND METHODS

Mice. CBA/H Wehi and AKR mice were bred under specific pathogen-free conditions at the Hall Institute.

Antibodies. Monoclonal anti-Thy 1.2, 30-H12, a rat antibody (3), and monoclonal anti-Thy 1.1, HO-22-1, a mouse antibody (4), were prepared from culture supernatants. The antibodies were directly conjugated with fluorescein isothiocyanate or were used in conjunction with fluorescein-conjugated rabbit anti-mouse immunoglobulin (Fl-anti-Ig).

Staining of Cells. Antibodies were centrifuged in a Beckman Airfuge prior to use to remove aggregates. Fresh thymocytes were stained in parallel as standards. Staining was carried out at 4°C, and cells were washed through an underlayer of fetal calf serum.

Tissue Culture—Generation of Thy-1 Positive Cells. One million viable nucleated bone marrow cells were cultured in 12-well Linbro plates in 1 ml of Dulbecco's modified Eagle's medium supplemented with 50 μM 2-mercaptoethanol, 2.8 mM glutamine, 0.1 mM asparagine, 10% fetal calf serum, and conditioned medium as indicated.

Assays of Cultured Cells. In attempts to recover T cells from cultured bone marrow cells, cells were cultured at various densities (10^2–10^6/ml) in the presence of concanavalin A (Con A; 2.5 μg/ml) and T-cell growth factor (TCGF) (5) that had been partially purified from medium conditioned by Con A-stimulated spleen cells by sequential ammonium sulfate precipitation and hydrophobic chromatography (6).

Colony-forming cells were assayed in cultures containing 0.3% agar (7) supplemented with medium conditioned by the myelomonocytic tumor WEHI-3B (8). The morphology of cells in colonies was analyzed by plucking off sequential colonies and staining with acetic acid/orcein and by pooling cells from multiple colonies, disrupting the agar by vigorous pipetting, making a cytocentrifuge smear, and staining with May-Grünwald-Giemsa.

Cells were also cultured at limiting dilution (2.5–1,000 per well) in Terasaki plates in 10 μl of medium supplemented as indicated with conditioned medium and Con A. Plates were examined by using an inverted microscope. In limiting dilution analyses, one Terasaki plate (60 wells) was set up for each dilution of cells.

Multipotential hemopoietic stem cells were assayed by using the splenic colony-forming unit (cfu-s) assay of Till and McCulloch (9) and scoring colonies on day 9.

Use of the Fluorescence-Activated Cell Sorter (FACS). Analysis and sorting was performed on a modified FACS II (Becton-Dickinson) using a single-laser two-color system and computerized data handling. For analysis, 25,000–50,000 cells were run; dead cells were excluded on the basis of light scattering and uptake of propidium iodide (1 μg/ml in the final suspension). Autofluorescence (seen in red and green channels) was assessed in control preparations by cross-correlating red and green fluorescence and was allowed for when necessary.

Conditioned Medium. Medium conditioned by Con A-stimulated spleen cells (CAS) was prepared as described (6). Medium conditioned by the Con A-stimulated T-cell hybridomas

Abbreviations: Con A, concanavalin A; CAS, Con A-stimulated spleen cell supernatant; FACS, fluorescence-activated cell sorter; Fl-anti-Ig, fluorescein-conjugated anti-Ig; Fl-anti-Thy-1.2, fluorescein-conjugated monoclonal anti-Thy-1.2 antibody; TCGF, T-cell growth factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; P cell, persisting cell; PSF, P-cell stimulating factor; cfu-s, splenic colony-forming unit.

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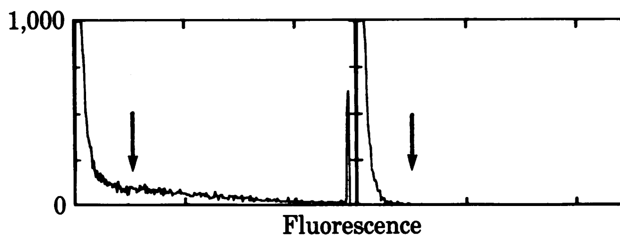

FIG. 1. FACS analysis of cultured CBA (Thy-1.2) (*Left*) or AKR (Thy-1.1) (*Right*) bone marrow using fluorescein-conjugated monoclonal anti-Thy-1.2 antibody (Fl-anti-Thy-1.2). Bone marrow cells were cultured for 3 days in the presence of medium conditioned by WEHI-3B (3%). ↓, Level of fluorescence above which was found the major (cortical) subpopulation of a preparation of thymocytes stained in parallel. For the CBA bone marrow, 18% of the population had levels of fluorescence exceeding this mark.

123 (7, 10) and T6 and T19.1 (11) were prepared as described (7, 11). Medium conditioned by the myelomonocytic tumor WEHI-3B (8) was prepared in the presence of 10% fetal calf serum and concentrated to 1/10th vol.

Treatment of Bone Marrow with anti-Thy-1 Antibodies and Complement. Freshly prepared bone marrow suspensions were incubated at 10^7/ml for 45 min at 37°C in the presence of previously optimized concentrations of monoclonal anti-Thy-1 antibodies and, as a source of complement, selected rabbit serum previously absorbed with mouse spleen cells. The levels of viable Thy-1-positive cells in bone marrow cells so treated were $<1/10^5$ cells.

RESULTS

Rapid Appearance of Thy-1-Positive Cells in Cultured Bone Marrow. Bone marrow cells from CBA mice were depleted of viable Thy-1 positive cells using monoclonal anti-Thy-1 antibody and complement and cultured with or without addition of CAS (3%). After 3 to 4 days, when the numbers of cells in the CAS-supplemented cultures were $1–1.5 \times 10^6$ per culture, the cells were harvested and stained with a directly fluoresceinated monoclonal anti-Thy-1.2 antibody. A significant population of positively stained cells was present; the cells, round and the size of large lymphoblasts, occurred in clusters that could be disrupted only by vigorous pipetting. In cultures not supplemented by CAS, cell numbers had decreased and Thy-1-positive blast cells were not found. Comparable levels of Thy-1-positive cells were found at day 8 in cultures split and fed with conditioned medium on day 4. The appearance of the Thy-1-positive blast cells was not dependent on the removal of Thy-1-positive cells from the starting population of bone marrow cells; however, the experiments reported here have been done with bone marrow depleted of Thy-1-positive cells to simplify interpretation of the results.

Table 1. Generation of Thy-1 positive cells in 3-day cultures of T-cell-depleted bone marrow

Strain	CAS, %		WEHI-3B-CM, %	
	Thy-1.2⁺	Thy-1.1⁺	Thy-1.2⁺	Thy-1.1⁺
CBA (Thy-1.2)	36	<0.5	36	<0.5
AKR (Thy-1.1)	<0.5	60	<0.5	22

Bone marrow cells from CBA or AKR mice were depleted of Thy-1 positive cells and cultured with CAS or medium conditioned by WEHI-3B (WEHI-3B-CM). After 3 days, cells were harvested and aliquots were stained for fluorescence microscopy using either monoclonal anti-Thy-1.1 as a first step and Fl-anti-Ig as a second step or Fl-anti-Thy-1.2. Levels of fluorescent cells in control preparations were <0.5%.

FACS analysis showed that 20–40% of the cells had an intensity of staining comparable with that of the bulk of thymocytes (Fig. 1). The forward light-scatter characteristics of the cultured bone marrow cells confirmed that the positive cells were larger than the bulk of thymocytes.

Specificity of Staining. It was crucial to establish that the immunofluorescence technique was not detecting artefacts—for example, due to binding of the anti-Thy-1 reagent by Fc receptors. Against this possibility was the observation that the monoclonal anti-Thy-1.2 antibody failed to bind to AKR (Thy-1.1) bone marrow cells that had been cultured in parallel (Fig. 1). In other experiments, aliquots from parallel cultures of bone marrow from CBA mice (bearing the Thy-1.2 allele) and AKR mice (bearing the Thy-1.1 allele) were stained with either anti-Thy-1.1 or anti-Thy-1.2 monoclonal antibody. The results of one such criss-cross experiment, showing that each of the mono-

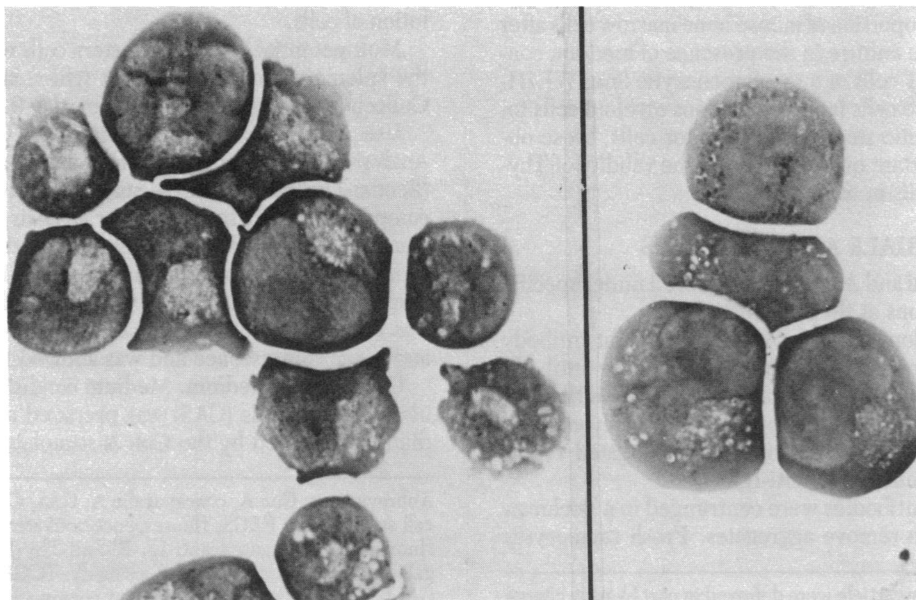

FIG. 2. Cytocentrifuge smear of Thy-1-positive cultured bone marrow cells (May–Grünwald–Giemsa stain).

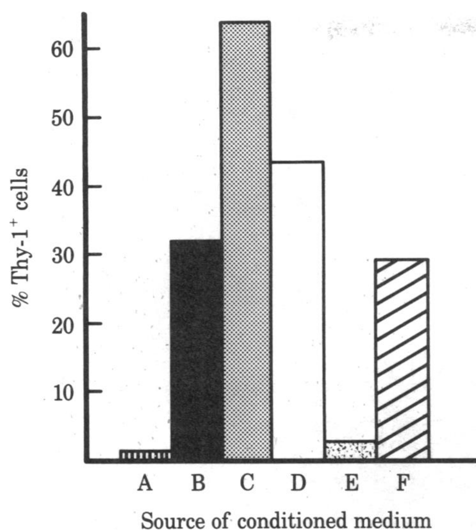

FIG. 3. Two T-cell hybridomas support the appearance of Thy-1-positive cells in bone marrow cultures. CBA bone marrow cells were depleted of Thy-1-positive cells and cultured (10^6) in duplicate in medium alone (bar A) or in the presence of CAS (bar B) or medium conditioned by Con A-stimulated T-cell hybridomas 123, T6, and T19.1 (bars C–E) or by WEHI-3B (bar F). At day 4, cells were harvested, counted, and stained with Fl-anti-Thy-1.2, and the percentage of Thy-1-positive cells was determined with a microscope. Yields of cells ($\times 10^{-6}$) per culture were A, 0.03; B, 1.0; C, 0.5; D, 1.0; E, 0.5; and F, 1.8.

clonal anti-Thy-1 antibodies stained only cultured bone marrow cells of the appropriate genotype, are given in Table 1. Similar experiments using the FACS and directly fluorescein-conjugated monoclonal anti-Thy-1.1 and Thy-1.2 antibodies gave the same result. The appearance of Thy-1.1 (but not Thy-1.2) on the AKR cells (Table 1) also excluded the possibility that the Thy-1 detected in these experiments had merely been absorbed from the conditioned medium.

Morphology of Thy-1-Positive Cells. Using the FACS, we isolated the population of Thy-1-positive cells with levels of fluorescence corresponding to those exhibited by the bulk of thymocytes—i.e., those with high levels of Thy-1. In cytocentrifuge smears, the most prevalent cell was a large blast cell having a somewhat irregular nucleus and basophilic cytoplasm with a lighter Golgi area (Fig. 2). Vacuoles and granules were often seen in the cytoplasm of these cells. Less frequent were obvious granulocytic cells with horseshoe or annular nuclei and vacuolated monocytic cells. Smaller cells resembling lymphocytes were absent, being found only in the low-Thy-1 or Thy-1-negative fractions, which also contained some more heavily granulated cells.

Nature of the Factor Involved in the Appearance of Thy-1-Positive Cells. The presence of the activity stimulating the appearance of Thy-1-positive cells in CAS suggested that activated T cells were the source. However, as shown in Table 1, not only CAS but also medium conditioned by the myelomonocytic tumor WEHI-3B was active.

To further examine the question of the T-cell origin of the factor, we tested medium conditioned by three Con A-stimulated T-cell hybridomas (7, 11). Two of the T-cell hybridomas, 123 and T6, produced the factor supporting the appearance of Thy-1-positive cells in cultures of bone marrow cells while a third, T19.1, did not (Fig. 3). Elsewhere we have documented that hybridomas 123 and T6 produce a variety of biological activities, including TCGF (6, 7, 10) and at least two classes of molecules affecting hemopoietic progenitor cells (*i*) granulocyte/macrophage colony-stimulating factor GM-CSF (6, 7, 11),

Table 2. Response of Thy-1-positive cells from cultured bone marrow to TCGF or WEHI-3B-conditioned medium

		Frequency of response	
Exp.	Subpopulation	Con A/TCGF	WEHI-3B-CM
1	Thy-1$^+$	None detected	0.2 ± 0.08
	Remainder	None detected	0.3 ± 0.1
2	Thy-1$^+$	None detected	0.36 ± 0.12
	Remainder	None detected	0.04 ± 0.014

CBA bone marrow cells depleted of Thy-1-positive cells were cultured with WEHI-3B-conditioned medium (WEHI-3B-CM) for 3 days, giving 39% of cells Thy-1$^+$ (Exp. 1), or for 8 days with splitting and feeding on day 4, giving 37% of cells Thy-1$^+$ (Exp. 2). Cells having levels of Thy-1 corresponding to those of cortical thymocytes (Thy-1$^+$) were isolated using the FACS. Thy-1$^+$ cells were cultured, in parallel with the remaining population depleted of these cells (remainder), at limiting dilution with either Con A (2.5 μg/ml)/TCGF (1%) or WEHI-3B-conditioned medium (3%). No cells except the occasional adherent macrophage survived in medium alone or with TCGF/Con A. Wells were considered positive if they contained clusters of four or more viable nonadherent cells at day 4. Results represent frequency of cultures showing growth of WEHI-3B responsive cells ± 95% confidence limits (19).

and (*ii*) a group among which are P-cell stimulating factor (PSF), stimulating the persisting (P) cell, a mast cell progenitor (12–14) and cfu-s stimulating activity, stimulating a multipotential hemopoietic stem cell (11). In contrast, the only activity that we have found in medium conditioned by T19.1 (which did not support the appearance of Thy-1-positive cells in cultured bone marrow) is GM-CSF (11, 15). WEHI-3B produces PSF (16), and also cfu-s stimulating activity and colony-stimulating factors but does not produce TCGF (17), a fact we have confirmed in this laboratory. These results indicate that the activity responsible for the appearance of Thy-1-positive cells did not correlate with the presence of either TCGF or GM-CSF.

Thy-1-Positive Cells Include Hemopoietic Progenitor Cells. Naturally, a key question was whether the Thy-1-positive cells were T cells. We have been unable to detect any cells that survived or proliferated in response to TCGF or to a combination of TCGF and Con A. In this respect, the large Thy-1-positive cells found in these cultures differ from mature T-cell blasts, which in general have receptors for TCGF and continue to grow in the presence of TCGF (18). However, these negative experiments do not exclude the presence within the Thy-1-posi-

Table 3. Thy-1-positive cultured bone marrow cells include *in vitro* colony-forming cells

Exp.	Subpopulation	Agar colonies, no. per 10^3 cells
1	Thy-1$^+$	24.0
	Remainder	8.0
2A	Thy-1$^+$	10.0 ± 1.0
	Remainder	2.0 ± 0.7
2B	Thy-1$^+$	6.0 ± 1.0
	Thy-1$^-$	0.8 ± 0.4
3	Thy.1$^+$	36.0 ± 2.2
	Thy.1$^-$	0.5 ± 0.3

CBA bone marrow cells depleted of Thy-1-positive cells were cultured with WEHI-3B-conditioned medium for 8 days with feeding at day 4 (Exp. 1), for 4 days (Exp. 2), or for 3 days (Exp. 3). Cells were labeled with Fl-anti-Thy-1.2 and were separated with the FACS into Thy-1$^+$ fractions (corresponding to fluorescence levels of cortical thymocytes) and either remaining cells (remainder) or a fraction having background levels of fluorescence (Thy-1$^-$). Cells were plated in agar with 5% WEHI-3B-conditioned medium at 5×10^3 per plate. Colonies were scored at day 7.

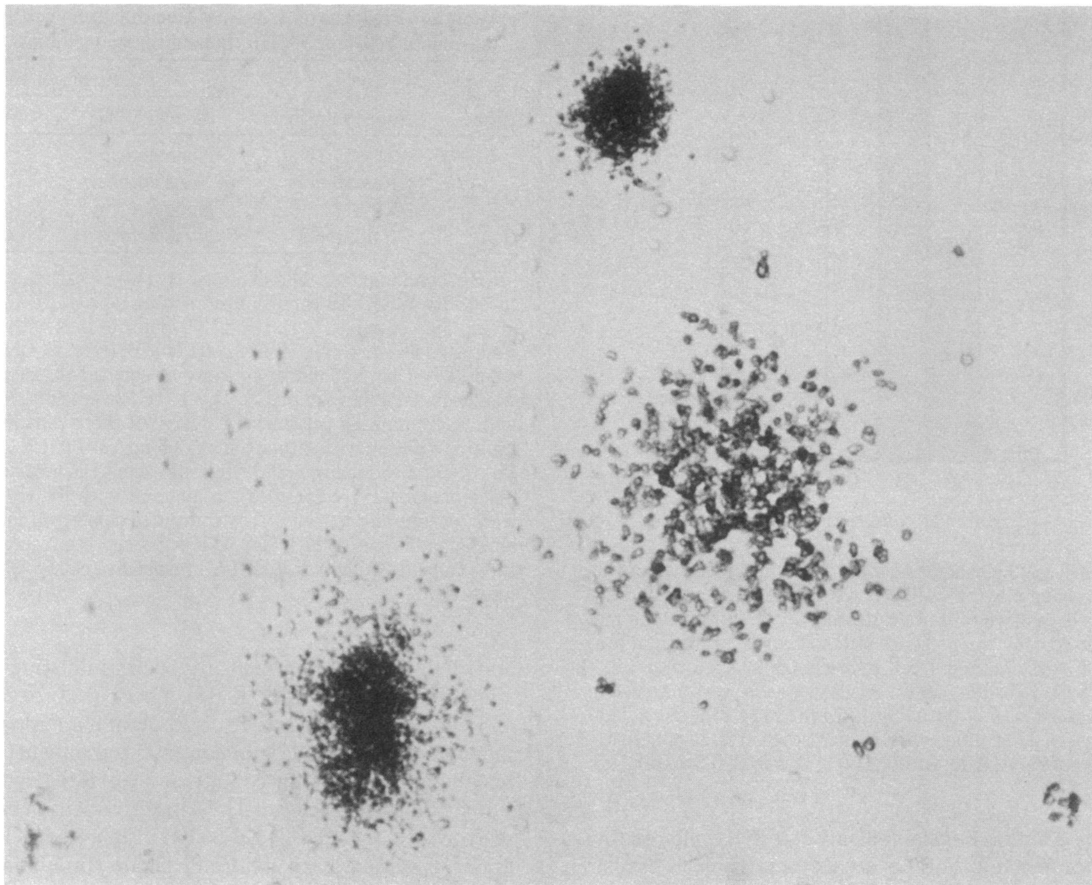

FIG. 4. Colonies grown in agar from Thy-1-positive cultured bone marrow cells. Shown are 10-day-old colonies grown in the presence of WEHI-3B-conditioned medium (5%) from the Thy-1^+ cells used in experiment 3 of Table 3.

tive population of precursors of T cells or thymocytes.

Several lines of evidence indicated that, whether or not T cells or their precursors were present, a proportion of the Thy-1-positive cells were clearly *not* T cells and were in fact progenitors of a variety of hemopoietic cells. About one-third of the cells in the Thy-1-positive fraction of cells proliferated to some degree in response to growth factors present in medium conditioned by WEHI-3B but not in response to TCGF/Con A (Table 2). The clone size varied, some clones grew to 16 cells and stopped while others (about one-third) were still growing vigorously at 3 wk. These continuously growing clones consisted of cells having the morphological, staining, and growth characteristics of P cells (12, 13). No cells, apart from the occasional adherent macrophage, survived in cultures containing medium alone or Con A (2.5 μg/ml)/TCGF that had been separated from GM-CSF and PSF (6, 14). Differences between the experiments shown in Table 2 in the fraction of responsive cells that were highly Thy-1-positive may reflect differences in culture conditions.

Smears of the cells that grew in the presence of WEHI-3B-conditioned medium showed macrophages, polymorphonuclear cells, blast cells (many of which contained intracytoplasmic granules), and cells having the coarse intracytoplasmic metachromatic granules characteristic of mast cells and P cells (12, 13).

The Thy-1-positive population included cells capable of forming large colonies in agar in the presence of medium conditioned by WEHI-3B (Table 3 and Fig. 4). Relative to the remaining cells or Thy-1-negative cells, the Thy-1-positive fraction was reproducibly enriched for colony-forming cells, eliminating the possibility that the colony-forming cells in the Thy-1-positive population were Thy-1-negative contaminants. The majority of these colonies contained readily identifiable macrophages and in 16–50% of cases polymorphonuclear granulocytes. Of pooled cells from colonies taken at day 7, ≈30% stained with monoclonal Fl-anti-Thy-1.2. These agar cultures also contained smaller clusters of proliferating cells.

The Thy-1-positive population also contained cells capable of forming macroscopic colonies in the spleens of lethally irradiated mice (cfu-s) (9), indicating the presence of pluripotential hemopoietic stem cells (Table 4). Histological examination of these colonies showed erythroid and granulocytic cells.

DISCUSSION

These experiments, showing that Thy-1 can occur on hemopoietic stem and progenitor cells, call for reappraisal of experiments with hemopoietic tissues in which the presence of Thy-

Table 4. Presence of cfu-s among Thy-1-positive cells in cultured bone marrow

Fraction injected	cfu-s, no. per 10^5 cells
None (control)	0*
Thy-1^{+}†	16 ± 1
Thy-1^{-}†	24 ± 3
Unseparated	28 ± 3

Lethally irradiated (850 rads) CBA mice were injected with various cell fractions.

* No colonies were formed in any of a group of eight uninjected irradiated mice.

† Thy-1-positive and Thy-1 negative fractions were those assayed in Exp. 2B of Table 3.

1 has been a major factor in assigning a given cell to the T-cell lineage. These include studies in which the appearance of Thy-1 has been a major factor in concluding that differentiation to a "prothymocyte" or T cell has been induced *in vitro* (e.g., see refs. 20–22). Although in some cases additional T-cell markers (e.g., TL or Lyt) have been monitored, the possibility that these antigens too occur on activated hemopoietic progenitor cells should be formally excluded. The presence of Thy-1 on natural killer cells (23) likewise now seems less compelling as evidence that these cells are members of the T-cell lineage. Certainly Thy-1 can no longer be regarded as the best routine early marker for commitment to the T-cell lineage (22).

The observation that the factor involved in the appearance of Thy-1-positive cells from bone marrow is produced by mitogen-activated T cells and T-cell hybridomas (Fig. 3) is of some practical importance. In our experience, all sources of murine TCGF are contaminated by this activity; thus, it is hazardous to conclude that one is inducing the differentiation of pre-T cells to T cells with TCGF, if one is monitoring solely the appearance of Thy-1-positive cells in the presence of crude conditioned medium. Biochemical analyses indicate that the factor that is involved in the present phenomenon, to which we have given the operational title Thy-1-inducing factor (TIF), copurifies on hydrophobic chromatography with PSF and thus can be conveniently separated from TCGF (6, 14). It has an apparent molecular weight on gel filtration of $\approx$30,000 (unpublished data).

It should be noted that, under appropriate conditions, the use of TCGF can result in the growth of T cells from bone marrow (5, 24). Furthermore, we cannot exclude the possibility that a fraction of the Thy-1-positive cells we have studied could, under certain conditions, be shown to be T-cell precursors. The point we stress is that, when dealing with cell populations containing hemopoietic stem or progenitor cells, Thy-1 can no longer be regarded as an exclusive marker for cells of the T-cell lineage.

The present observations are relevant to a number of previous reports. In the rat, Thy-1 occurs on B cells and both immature and mature nonlymphoid hemopoietic cells (25). Ritter *et al.* (26) observed that previous treatment of mouse bone marrow with a rabbit anti-Thy-1 antiserum (but not an alloantiserum) reduced cfu-s numbers, although they could not exclude that the effect was on an accessory cell. A number of factor-dependent lines of myeloid cells generated from long-term mouse bone marrow cultures are Thy-1 positive (27). Ihle and co-workers (28) observed that a factor distinct from TCGF and present in both CAS and WEHI-3B-conditioned medium supported initiation of lines of Thy-1-positive cells that they classified as T cells but, as yet, there is no evidence that this factor acted directly on these cells or their precursors. Interestingly, the line WEHI-3B, originally isolated from a myelomonocytic leukemia, is Thy-1 positive (29).

In conclusion, we have shown that, in the mouse, at least *in vitro* in the presence of appropriate growth factors, Thy-1 can occur on members of the hemopoietic differentiation pathway that are unequivocally distinct from T cells. It will be important to determine whether an analogous situation exists *in vivo*. If so, Thy-1 may prove useful as a marker for the *in vivo* stimulation of hemopoietic cells by regulatory molecules derived from activated T lymphocytes.

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