IMMUNE REGULATION OF IN VITRO MURINE MEGAKARYOCYTE DEVELOPMENT

Role of T Lymphocytes and Ia Antigen Expression

By MICHAEL W. LONG and DAVID N. SHAPIRO

From the Division of Pediatric Hematology/Oncology, Mott Children's Hospital, University of Michigan, Ann Arbor, Michigan 48109

Thymus-derived lymphocytes are important regulatory cells in the lymphohematopoietic system. Subsets of T cells are crucial elements in both humoral and cellular immunity (1), as well as in the regulation of hematopoietic cell differentiation (2). Among the myeloid lineages, T cells or their products stimulate the early phases of erythroid differentiation (3), as well as influencing granulocyte (4) and eosinophil differentiation (5). Moreover, T cell subsets can inhibit erythroid progenitor cell development (6).

Antigens encoded by the I region of the murine major histocompatibility locus are known as Ia antigens. These cell surface proteins are important in immune modulation, as they regulate or restrict specific cellular interactions that occur during the immune response (7). Ia antigens exist on both murine and human hematopoietic cells. The expression of Ia antigens on these cells is transient, and occurs at specific early stages of hematopoietic differentiation (8). Thus, in the erythroid lineage, Ia is expressed on primitive progenitor cells (burst-forming unit of erythrocyte [BFU-E][1]), and is lost during differentiation to the colony-forming progenitor cell (CFU-E) (9). The colony-forming unit of granulocyte/macrophage (CFU-GM) also bears this antigen (10). During differentiation, Ia is lost in the granulocytic lineage, but is retained during differentiation of these progenitor cells to macrophages (11).

A relationship exists between the expression of Ia antigens and the stage of the cell cycle (12). Human multipotent (CFU of granulocytes, erythrocytes, macrophages, and megakaryocytes [CFU-GEMM]), erythroid (BFU-E), and granulocytic (CFU-GM) progenitor cells express Ia antigens transiently during phases of DNA synthesis (13, 14). Moreover, both Ia expression and colony formation

[1] Abbreviations used in this paper: BFU, burst-forming unit; CFU, colony-forming unit; CM, conditioned medium; Con A, concancavalin A; CSA, colony-stimulating activity; E, erythroid; FCS, fetal calf serum; GEMM, granulocyte/erythrocyte/macrophage/megakaryocyte; GM, granulocyte/macrophage; 5-HU, 5-hydroxyurea; IF, inducing factor; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; MAF, macrophage-activating factor; Mk, megakaryocyte; MLuCM, mouse lung–conditioned medium; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; RC' rabbit complement; TNC, total nucleated cells.

by CFU-GM are sensitive to in vitro hematopoietic regulatory activities such as acidic isoferritins (14) and prostaglandins of the E series (15).

Little information exists concerning the involvement of immunoregulatory cells in the regulation of megakaryocytopoiesis. The frequent observation of thrombocytosis secondary to infection or inflammatory response is suggestive of such interactions. We report that mitogen-activated murine T lymphocytes produce a factor(s) involved in megakaryocyte (Mk) differentiation. Using monoclonal antibodies (mAb), we show that a subpopulation of murine CFU-Mk express Ia antigens, and that this expression is related to cell cycle status (DNA synthesis). Finally, this T cell–derived regulatory activity is shown to concomitantly increase CFU-Mk Ia expression and DNA synthesis. These observations suggest that the immune system is capable of augmenting basal levels of thrombopoiesis.

Materials and Methods

Hematopoietic Progenitor Cell Assays.   Bone marrow cells obtained from femurs of specific pathogen–free, 4–8-wk-old male mice (C57BL/6, Charles River Laboratories, Inc., Wilmington, MA) were suspended in McCoy's 5A medium (Gibco Laboratories, Grand Island, NY) and monodispersed. Before plating, the cells were incubated at 37°C for 30–45 min at ~5 × 10^6 cells/ml in McCoy's 5A medium containing 10% fetal calf serum (FCS) (HyClone, Logan, UT). CFU-Mk assays were performed as previously described (16). Briefly, 3–5 × 10^4 total nucleated cells were cultivated in modified McCoy's 5A medium containing 10% fetal calf serum, 0.25% bacto agar (wt/vol) and 7.5–20% (vol/vol) stimulatory activities (see below). Following incubation (7 d, 7% CO_2, 100% humidity) cultures were removed from the incubator, air dried, stained in situ for acetylcholinesterase activity, and counterstained with hematoxylin (17). Colonies were counted at × 40.

Conditioned Media (CM).   Growth of murine Mk colonies requires two activities. Mk colony-stimulating activity (Mk-CSA) is found in CM from the WEHI-3 murine monomyelocytic leukemia line (18), or as an activity associated with the interleukin 3 (IL-3) molecule (19). Optimal growth of murine Mk colonies requires an additional activity (Mk-potentiator activity) found in mouse lung conditioned media (MLuCM) (20, 21), or can be substituted for by the active tumor-promoting phorbol diesters (phorbol myristate acetate [PMA]) (22).

WEHI-3 cells were adapted to growth in suspension culture (23). Cells were grown in McCoy's 5A containing 5 × 10^{-5} M 2-mercaptoethanol and 2% fetal calf serum. The medium was harvested every 3–4 d, and concentrated fivefold by ultrafiltration (YM-10; Amicon Corp., Danvers, MA). MLuCM was prepared by coarsely cutting murine lungs and suspending one lung in 5 ml McCoy's 5A medium. The CM were collected after 3–4 d of incubation. Optimal concentrations of these CM were determined using reciprocal titrations (24). The concentrations used most typically were: 2.5–10% (vol/vol) WEHI-3-CM, 7.5% (vol/vol) MLuCM. (PMA) (Sigma Chemical Co., St. Louis, MO) was prepared as a stock solution of 10^{-5} M in dimethyl sulfoxide, and kept in the dark at −20°C until used. Fresh stock solutions were prepared every 3 wk. Immediately before use, a working solution was prepared using McCoy's 5A medium as the diluent. PMA serves as a powerful differentiation agent for murine Mk colonies (21). As such, it substitutes in these assays for MLuCM.

Preparation and Characterization of Murine T Cell Hybridomas.   T cell hybridoma FS7-20 and its subclones are the products of the fusion of normal B10.BR T cells to the AKR thymoma BW5147. This hybridoma was generously supplied by P. Marrack (National Jewish Hospital, Denver, CO). A subclone of this hybridoma (FS7-20.6.18) elaborates Ia-inducing factor (Ia-IF), macrophage activating factor (MAF), and γ interferon (IFN-γ) after stimulation with concanavalin A (Con A) (25). An independently derived T cell

hybridoma (FS7-6.18) is also inducible with Con A and produces IL-2 and B cell differentiating factor (26). Another T cell hybridoma (FS6-14.3) and gibbon T cell lymphoma line MLA-144 produce lectin-free IL-2 (27, 28).

Hybridoma cells (5×10^5 cells/ml) were grown in Iscove's modified Dulbecco's medium (Gibco Laboratories) containing 10% FCS and 4 μg/ml Con A. CM was harvested by centrifugation at 24 h, and 10 mg/ml α-methyl mannoside was added to inhibit Con A activity.

Hybridoma Antibody Reagents. To aid in determining the importance of the Ia expression during Mk growth and differentiation, a number of anti-Ia hybridoma reagents were used. Hybridomas 25-9-3 (I-A^b), 25-5-16 (I-A^b), and 25-9-17 (I-A^b) were obtained from the American Type Culture Collection (Bethesda, MD) (29). Hybridomas 14-4-4 (I-E^{k,d}) and 10-2-16 (I-A^{k,f,s}) were obtained from the Salk Institute (La Jolla, CA) (30). Hybridoma supernatants were used in cytotoxicity studies at 10–15% final concentration (vol/vol).

Cell Separation. Bone marrow adherent cells were collected by plastic adherence as described previously (22). Briefly, murine bone marrow cells were incubated (3×10^5 cells/ml) in McCoy's 5A medium containing 5% FCS for 1 h at 37°C, after which nonadherent cells were removed and subjected to a second adherent step. Adherent cells were detached using a rubber policeman. Adherent cell–depleted bone marrow cells were collected after the second depletion, and used as a source of progenitor cells in the overlayers of subsequent feeder-layer experiments (*vide infra*). Unless otherwise stated, all assays used adherent cell–depleted bone marrow as the target cell population. T cell–enriched populations were obtained by subjecting murine spleen cells to nylon wool adherence to deplete B lymphocytes and macrophages (32). Where necessary, nylon wool–depleted lymphocytes were subjected to a round of plastic adherence and immune depletion of T cells and B cells by complement-dependent mAb cytotoxicity (*vide infra*).

Preparation of Purified Cell Populations. Purified T cells (<1% contaminating macrophages) were prepared by passing a single-cell suspension of spleen cells over nylon wool columns. Cells collected in the first 15 ml effluent from the column were further depleted of macrophages by two 30-min incubations in the presence carbonyl iron (Atomergic Chemetals Corp., Plainview, NY) and phagocytic cells removed with a strong magnet. Cells incorporating latex particles and having morphologic characteristics of macrophages comprised <1% of this population. B cells were depleted by treatment of this macrophage-depleted population with rabbit anti–mouse IgG serum (1:100 final dilution) and rabbit complement (RC') as previously described (33). This B cell and macrophage–depleted population was used as a source of purified T cells. Purified T cells (5×10^5 cells/ml) were activated with 4 μg/ml Con A for 24 h, and used in feeder-layer experiments. For experiments requiring T cell depletion, these cells were treated with anti-Thy-1.2 serum (1:10 final dilution) and (RC').

Feeder-layer Experiments. The ability of nonadherent bone marrow cells to respond to T cell products was examined in feeder-layer experiments. Purified T cells were used as stimulator cells and examined for their ability to produce soluble factors in response to the T cell mitogen Con A. Mitogen-activated T cells were cultured at various cell concentrations ($3–10 \times 10^5$ cells/ml) suspended in McCoy's 5A medium containing 0.5% agar and plated as feeder layers. Adherent cell–depleted overlayers containing 5.0×10^4 bone marrow cells suspended in 0.3% agar were used as the target cell population. Relevant controls consisted of feeder layers without cells in the overlayer, thereby insuring that the adherent cells contained no progenitor cells, as well as cultures containing adherent cell–depleted marrow cells, to assess spontaneous Mk colony growth. Previous studies (22) have indicated that neither of these controls supports Mk or granulocyte colony development.

In experiments designed to determine whether or not mitogen-stimulated T cells were capable of elaborating Mk regulatory activities, each of the two activities required for optimal Mk colony formation were added individually to the cultures. Thus, a source of Mk-CSA (WEHI-3-CM) was added to determine whether or not the Con A–stimulated T cells produced Mk potentiator activity. Conversely, a source of Mk potentiator activity

(either 10^{-6} M PMA or MLuCM) was added to determine whether or not the mitogen-activated T cells could produce Mk-CSA.

Antibody-dependent Cellular Cytotoxicity.   The presence of Ia antigen on murine megakaryocyte progenitor (CFU-Mk) cells was determined using anti-Ia mAb in complement-dependent cytotoxicity assays. Murine bone marrow cells (7.0×10^6 cells/ml) were treated with anti-Ia mAb diluted 1:10 in serum-free McCoy's medium containing RC' (diluted 1:20). This suspension was incubated at 37°C for 30 min, the cells were washed three times in serum-free McCoy's 5A, and were resuspended in 1–2 ml of McCoy's 5A containing 10% FCS. Controls for these experiments were bone marrow cells incubated in RC' only, as well as cells incubated in the presence of RC' and an inappropriate anti-Ia mAb (secreted by hybridomas 14-4-4 and 10-1-16, see above). The antibody-treated cells or controls were then assayed for CFU-Mk as described above.

The percentage of CFU-Mk expressing Ia antigen was determined by the percent of cytotoxicity occurring in anti-Ia–treated bone marrow cells as compared to the cells treated with RC' alone. Treatment with RC' alone resulted in 2.6 ± 10.0% nonspecific killing of CFU-Mk (n = 6 experiments of three to five replicate cultures per experiment).

Sensitivity of CFU-Mk to 5-Hydroxyurea (5-HU) (Cell Cycle Status).   The proportion of CFU-Mk synthesizing DNA in vivo was determined by injection of 5-HU (900 mg/kg i.p.), as described previously (34). The mice were killed 3 h after injection, and the femurs were excised and flushed into McCoy's 5A medium. CFU-Mk assays were performed as described above. Control animals received an equal volume of phosphate-buffered saline (PBS). 5-HU-mediated progenitor cell cytotoxicity was determined by comparison of the total number of CFU-Mk per femur between control and 5-HU–treated animals.

To determine the in vitro cell cycle status of CFU-Mk, murine bone marrow suspensions were treated in vitro with 2×10^{-3} M 5-HU (35). Murine bone marrow cells were incubated in the presence or absence of 5-HU for 30 min at 37°C, and assayed for CFU-Mk as described. In certain experiments, C57BL/6 mice were treated with 5-HU in vivo (to remove cells synthesizing DNA); the femurs were excised, flushed, an aliquot was cultured for CFU-Mk and the remainder were tested for Ia expression using in vitro antibody-dependent cytotoxicity assays.

Effects of T Cell Products on CFU-Mk.   In testing the actions of T cell products on both Ia expression and cell cycle status, adherent cell–depleted, T cell–depleted murine bone marrow cells were incubated in the presence or absence of the appropriate T cell–conditioned medium (i.e. the T cell–CM known to contain Mk-potentiator activity; see Results) at 37°C for 60 min. Following incubation, these cells were washed three times in serum-free McCoy's 5A, resuspended in supplemented McCoy's 5A medium, assayed for Mk progenitor cell numbers (input), and subsequently tested for either Ia expression or 5-HU sensitivity, as described. Finally, to determine the relationship between Ia expression and cell cycle status, studies were performed in which adherent cell–depleted, B cell–depleted, and T cell–depleted murine bone marrow cells were first incubated (with or without) T cell–CM, treated in an antibody-dependent anti-Ia cytotoxicity assay, and subsequently subjected to in vitro 5-HU cytotoxicity. In all of these experiments, the appropriate controls were subjected to 5-HU sensitivity as well.

Results

Stimulatory Activities Found in T Cell Hybridoma–CM.   CM from three T cell hybridomas were analyzed for their solitary ability to support Mk colony growth (i.e., the ability to function as an IL-3-like molecule or source of Mk-CSA), or for their ability to augment Mk colony formation when cocultured with a known source of Mk-CSA, such as WEHI-3-CM, (i.e., Could these CM function as a source of Mk potentiator activity?). CM from one of the three T cell hybridomas (FS7-20.6.18) was found to augment Mk colony formation, but lacked the sole ability to support Mk colony development (Fig. 1). Culture of murine bone marrow cells in the presence of WEHI-3-CM alone generated 10.0 ± 3.0 Mk
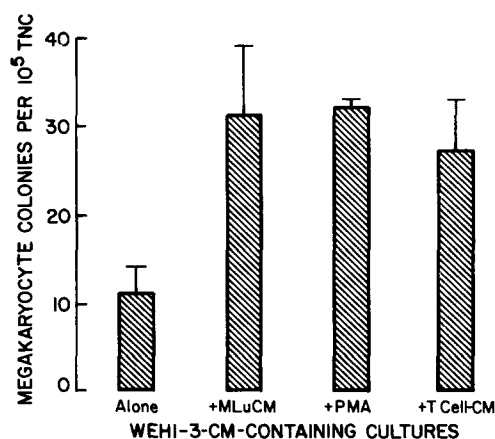
FIGURE 1.   Augmentation of murine Mk colony formation by various CM. Mk colonies grown as in Materials and Methods. Adherent cell–depleted bone marrow cells (50,000 cells/ml) cultured in the presence of WEHI-3-CM (7.5% vol/vol) only (*Alone*) or cocultured with WEHI-3-CM and MLuCM, PMA, or T cell hybridoma (FS7-20.6.18)–CM. Colony numbers grown in the presence of MLuCM, PMA, or T cell–CM are significantly greater than colonies formed in the presence of WEHI-3-CM only ($p \leq 0.05$, Student's t test). These three activities (PMA, MLuCM, and T cell–CM) lack the sole ability to stimulate colony formation (see Table 2).

colonies per 10^5 total nucleated cells (10^5 TNC). When cultured with optimal concentrations of WEHI-3-CM (2.5% final concentration), CM from this T cell hybridoma resulted in 25.7 ± 6.7 Mk colonies from 10^5 TNC. This T cell–CM augmented Mk colony development to a degree similar to that of MLuCM or 10^{-6} M PMA. MLuCM is a standard biological source of Mk potentiator activity (16, 24), and PMA is capable of substituting for this activity in vitro (22). The effect(s) of this Con A–stimulated T cell hybridoma–CM were not eliminated in the presence of 10 mg/ml of α-methyl mannoside, the competing sugar for the Con A lectin (data not shown). Conversely, CM from two other T cell hybridomas (FS7-6.18, FS6-14.13), or gibbon T cell lymphoma line MLA-144 (a source of lectin-free IL-2) were unable to support Mk colony formation (data not shown), nor could they augment colony formation in the presence of a source of Mk-CSA (WEHI-3-CM) (Fig. 2).

The responsiveness of murine CFU-Mk to varying concentrations of T cell hybridoma–CM is indicated in Fig. 2. The active hybridoma (FS7-20.6.18) augmented Mk colony formation from ~0.15–20%, (vol/vol), with optimal activities detected at 2.5–5.0%, final concentration. No activity was seen with the other CM.

Con A–activated T Cells Produce Mk Regulatory Activities.   To establish whether this stimulatory activity was an intrinsic activity of the T hybridoma, or whether it could also be produced by syngeneic murine T cells, T cells were purified, stimulated with Con A, and established as feeder layers at varying cell concentrations using adherent cell–depleted bone marrow as target cells. These feeder layers were examined for their ability to augment Mk colony formation. Syngeneic T cells were found to produce Mk potentiator–like activity only when stimulated with the T cell mitogen Con A (Table I). The ability of T cell feeder layers to augment Mk colony formation varied with the concentration of T cells
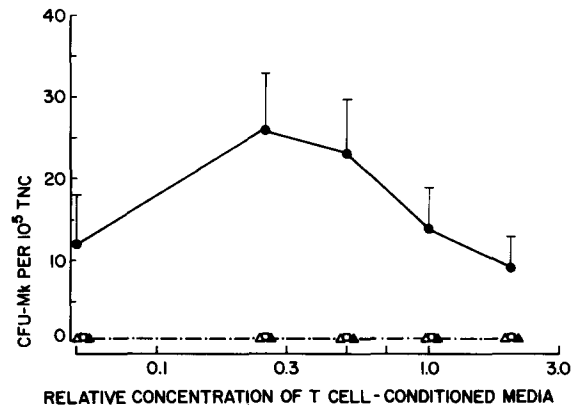
FIGURE 2. Responsiveness of murine CFU-Mk to varying concentrations of T cell–CM. Progenitor cell colonies grown as in Materials and Methods. Adherent cell–depleted bone marrow cells (50,000 cell/ml) cultured in the presence of constant amounts of WEHI-3-CM (7.5% vol/vol) and varying concentrations of T cell–CM. Filled circles, T cell hybridoma FS7-20.6.18–CM; Open triangles, CM from T cell hybridoma FS7-6.18; Closed triangles, T cell–CM from hybridoma FS6-14.3; Open squares, CM from gibbon T cell lymphoma line MLA-144. On the abscissa, relative concentration of 1.0, 10% (vol/vol) final concentration.

TABLE I

Augmentation of Mk Colony Formation by T Cell Feeder Layers

Stimuli	Concentration of T cells in feeder layer (TNC/ml × 10⁻⁵)				
	0.5	1.0	2.5	5.0	10.0
Con A + WEHI-3-CM*	36.7 ± 6.0‡	50.0 ± 2.1	62.0 ± 6.1	42.6 ± 7.6	35.0 ± 8.8
Con A§	0	0	0	0	0
WEHI-3-CM‖	13.5 ± 4.9	14.0 ± 2.8	9.2 ± 4.7	9.0 ± 4.4	7.0 ± 2.6
Media¶	0	0	0	0	0

Syngeneic splenic T cells (prepared by plastic and nylon wool adherence) were established at the above concentrations in feeder layers of McCoy's 5A media containing 0.5% agar. Overlayers contained 10^5 adherent cell–depleted bone marrow cells in McCoy's 5A containing 0.3% agar. Values are mean ± SD based on two experiments of 3–5 replicate cultures per condition.

* Con A–activated T cells in feeder layer in the presence of a source of Mk-CSA (WEHI-3-CM [5% vol/vol]).
‡ $p \leq 0.05$ comparison is to WEHI-3-CM at each given T cell concentration.
§ Con A–stimulated T cells cultured without WEHI-3-CM.
‖ Cultures containing quiescent T cells in the feeder layer plus WEHI-3-CM.
¶ Cultures containing overlayer and feeder layer cells without stimuli.

in the feeder layer, whereas quiescent (nonstimulated) T cells were incapable of producing this activity. Maximal stimulation occurred with 2.5×10^5 T cells within the feeder layer. Con A–stimulated T cells, established as feeder layers in the absence of WEHI-3-CM, did not support Mk colony formation, thus indicating that Con A could not directly stimulate CFU-Mk. In addition, Con A could not stimulate residual adherent cells to produce activities necessary for Mk colony formation. Cultures established in the absence of any stimulatory activity (i.e., without Con A or WEHI-3-CM) ruled out the presence of residual or spontaneous CFU-Mk present in these feeder layers, leading to false positive results. Finally, cultures stimulated with WEHI-3-CM alone eliminated the possibility of sponta-

neous production of Mk potentiator activity by either quiescent T cells (in the feeder layer), or by residual adherent cells in the overlayer.

Actions of Recombinant IFN-γ on CFU-Mk. Con A stimulation of this T cell hybridoma (FS7-20.6.18) has been reported to result in elaboration of IFN-γ, MAF, and IA-IF (25). The action of recombinant IFN-γ on murine CFU-Mk was examined to determine if this purified lymphokine was responsible for the Mk potentiating activity observed in T cell hybridoma–CM. Murine IFN-γ lacked the solitary ability to support Mk colony formation over a range of 10–5,000 U/ml (Table II). This indicated that IFN-γ did not act as an Mk-CSA. Also, IFN-γ failed to synergize with crude Mk-CSA (WEHI-3-CM) over the same range of concentrations, showing that it lacked the ability to augment Mk colony development. In comparison, purified IL-3 (over a range of 2–40 ng/ml) was capable of solely supporting Mk colony formation, and this colony development was enhanced by the addition of 10^{-6} M PMA. Purified human platelet-derived growth factor, murine nerve growth factor, and murine epidermal growth factor (Sigma Chemical Co.), were used as specificity controls, and had no effects in this assay system.

Ia Expression on Murine CFU-Mk: Relation to Cell Cycle Status. Based on the ability of T cells to interact with cells expressing major histocompatibility complex antigens, CFU-Mk were studied for the expression of Ia antigens and the relation of Ia expression to CFU-Mk cell cycle status. In vitro cytotoxicity experiments using anti-Ia mAb indicated that $24.3 \pm 6.0\%$ (mean $\pm$ SD; $n = 5$) of the CFU-Mk expressed Ia antigen on their surface (Table III). Additionally, in vivo

TABLE II
Responsiveness of Murine CFU-Mk to Various Regulatory Factors

Factor	CFU-Mk/10^5 TNC		
	Alone*	With WEHI-3-CM[‡]	With 10^{-6} M PMA[§]
IL-3	22.8 ± 5.2	NT[‖]	48.0 ± 13.0[¶]
WEHI-3-CM	10.0 ± 3.0	NT	31.0 ± 1.5[¶]
T cell–CM	0	25.6 ± 1.4[¶]	1.0 ± 1.0
20-6-18-CM	0	23.5 ± 3.6[¶]	0
Murine IFN-γ	0	0	0
PDGF	0	0	0
NGF	0	0	0
EGF	0	0	0

Murine CFU-Mk cultured as described in Materials and Methods. Purified IL-3 cultured at 10 ng/ml, WEHI-3-CM at 2.5% (vol/vol), MLuCM at 15% (vol/vol), 20-6-18-CM at 2.5% (vol/vol), IFN-γ at 10–5,000 U/ml, PDGF at 20–400 U/ml, NGF and EGF at 10 and 100 ng/ml. Values are mean $\pm$ SD for 2–5 separate experiments of 3–5 replicate cultures per point, except for PDGF, NGF, and EGF, which are single experiments.
* Factors cultured alone to determine their solitary ability to support Mk colony formation.
[‡] Factors cocultured with WEHI-3-CM (a source of Mk-CSA) to test for the presence of Mk potentiator activity.
[§] Factors cocultured with 10^{-6} M PMA (which augments Mk colony development) to test for the presence of Mk-CSA.
[‖] NT, not tested.
[¶] $p \leq 0.05$ compared to WEHI-3-CM alone, except for IL-3 plus PMA, which is contrasted to IL-3, alone.

TABLE III

Ia Expression by Murine CFU-Mk and Its Abrogation by In Vivo 5-HU Treatment

Anti-Ia–mediated cytotoxicity when treated with:	CFU-Mk/10⁵ TNC	Reduction (%)
RC'	43.4 ± 4.5	3.0 ± 10
Inappropriate* mAb + RC'	41.4 ± 6.8	3.6 ± 4.2
Anti-Ia mAb + RC'	$32.7 \pm 2.6^{\ddagger}$	24.3 ± 6.0

5-HU Sensitivity

In vitro treatment	In vivo treatment	
	PBS (CFU-Mk/10⁵ TNC)	5-HU
RC'	$43.9 \pm 7.0^{\ddagger}$	44.7 ± 6.6
Inappropriate* mAb	38.3 ± 8.0	40.3 ± 5.0
Anti-Ia mAb + RC'	31.3 ± 6.0	40.2 ± 6.0
	Reduction with anti-Ia mAb (%):	
	29.0 ± 3.5	10.9 ± 15.3

Cytotoxicity assays performed as in Materials and Methods. For 5-HU experiments, animals were treated for 3 h with 5-HU (900 mg/kg, i.p.), or with an equivalent volume of PBS (controls). After treatment the animals were killed, and their bone marrow cells were assayed for Ia expression by antibody-mediated cytotoxicity. Values are mean ± SD for three experiments of 3–5 replicate cultures per point.

* Inappropriate mAb was either 14-4-4 or 10-2-16.

$\ddagger$ $p \leq 0.05$, comparing RC' vs. mAb + RC'.

treatment of murine bone marrow with 5-HU resulted in an 18.3 ± 10.7 ($n = 6$) reduction in CFU-Mk number (data not shown).

To determine whether the progenitor cells expressing Ia were those cells that were actively synthesizing DNA, mice were treated in vivo with 5-HU (to eliminate cells in S phase) (34). Bone marrow cells isolated from these animals were then examined for Ia expression using an in vitro cytotoxicity assay. Control animals (injected with PBS) showed the same proportion of anti-Ia–mediated reduction in CFU-Mk number ($29.0 \pm 3.5\%$; $n = 3$ experiments of three animals each) (Table III) as did the above studies on Ia expression. Pretreatment of animals in vivo with the cell cycle–specific agent 5-HU abrogated the anti-Ia–mediated cytotoxicity, suggesting that the cells in the S phase of cell cycle were those cells that expressed Ia antigen.

T Cell Factors Regulate CFU-Mk Ia Expression and Cell Cycle Status. Further studies were undertaken to determine whether or not the association between Ia expression and cell cycle status could be mediated by T cell factors. Murine bone marrow cells preincubated in CM from the active T cell hybridoma (FS7-20.6.18), significantly increased expression of the Ia antigen from $20.5 \pm 4.5\%$ of CFU-Mk expressing this antigen to $39.3 \pm 6.3\%$ (Table IV). In separate studies, preincubation of the CFU-Mk in this CM increased the number of cells in DNA synthesis from 14.3 ± 3.5 to $45.6 \pm 3.5\%$ (Table IV).

To more firmly establish the relationship between T cell factors, Ia expression, and cell cycle status, the two previous observations were combined in a single

Table IV
T Cell–CM Increases In Vitro CFU-Mk Ia Expression and the Number of CFU-Mk in S Phase

Anti-Ia–mediated cytotoxicity when treated with:	CFU-Mk/10^5 TNC after incubation in:	
	T cell–CM	Plain medium
Inappropriate mAb	51.3 ± 8.9	55.2 ± 12.5
Anti-Ia mAb	31.3 ± 7.5	43.0 ± 9.7
Reduction (%):	39.3 ± 6.3*	20.5 ± 4.5
5-HU sensitivity		
Before incubation with 5-HU	43.7 ± 6.6	52.8 ± 21.2
After incubation with 5-HU	25.8 ± 10.9	43.4 ± 12.8
Reduction (%):	45.6 ± 3.5*	14.3 ± 12.8

Cytotoxicity assays performed as in Materials and Methods. Values are mean ± SD for 3–4 separate experiments (except two experiments for anti-Ia treatment of T cell–CM–incubated cells) of 3–5 replicate cultures per condition.
* $p \leq 0.05$ comparing T cell–CM with plain medium for percent reduction.

experiment. Murine CFU-Mk were preincubated in CM from T cell hybridoma a FS7-20.6.18, subjected to anti-Ia cytotoxicity, and subsequently incubated in the presence of 5-HU to determine cell cycle status. These experiments indicated, again, that Ia expression was linked to cell cycle status. Ia expression on progenitor cells not exposed to this active CM was 27.5%. Following exposure to T cell hybridoma CM, the proportion of cells in S phase increased to 69% (Table V). Subsequently, treatment of T cell hybridoma–CM–exposed progenitor cells with anti-Ia mAb abolished the 5-HU-related cytotoxicity (Table V). Therefore, removal of Ia$^+$ cells before 5-HU cytotoxicity removes target cells for 5-HU (i.e., cells in active DNA synthesis). This observation confirmed the previous in vivo and in vitro results showing that CFU-Mk in active phases of DNA synthesis express Ia.

Discussion

The in vitro development of murine CFU-Mk requires the presence of two regulatory activities: (a) Mk-CSA, an obligate factor for the proliferation of the CFU in vitro (16), which is produced by WEHI-3 cells, a murine monomyelocytic leukemia line (23), or as an activity of the purified lymphokine IL-3 (19); and (b) Mk potentiator activity, produced by bone, bone marrow, spleen, and lung cells (20, 36). Mk potentiator activity increases the DNA content of colony-derived murine Mk (36), and also directly stimulates the differentiation of immature murine Mk into single, mature cells (37). The role of T cells, or T cell products, as regulators of in vitro megakaryocytopoiesis is shown by the ability of T cell lymphokines to mimic potentiator activity by augmenting Mk colony formation. As well, T cell products increase CFU-Mk Ia expression, and regulate the number of CFU in active DNA synthesis.

Mitogen-activated T lymphocytes produce an activity that augments Mk colony formation when cocultured with a source of Mk-CSA (WEHI-3-CM). T cells or

TABLE V

Abrogation of 5-HU Sensitivity by Prior Removal of Ia⁺ CFU-Mk

Anti-Ia-mediated cytotoxicity when preincubated with T cell–CM and:	CFU-Mk/10^5 TNC	Reduction (%)
RC'	76.0 ± 15.8	0
Inappropriate mAb + RC'	118.0 ± 19.4	0
Anti-Ia mAb	36.6 ± 10.4	69.0
Preincubation with plain medium and:		
RC'	35.0 ± 7.0	0
Inappropriate mAb + RC'	64.6 ± 8.6	0
Anti-Ia + RC'	47.0 ± 5.2	27.5

5-HU sensitivity

	CFU-Mk/10^5 TNC		
Primary incubation	Before incubation with 5-HU*	After incubation with 5-HU	Reduction (%)
RC'	76.0 ± 15.8	56.6 ± 4.4	25.5
Inappropriate mAb	118.0 ± 19.4	47.0 ± 9.6	60.0
Anti-Ia mAb	36.6 ± 8.6	49.0 ± 5.8	0

Cytotoxicity assays performed as in Materials and Methods. In anti-Ia-mediated cytotoxicity, T–, B–, and adherent cell–depleted marrow cells were incubated (37°C for 1 h) in the presence of T cell (20-6-18)–CM, or in plain medium as a control. Following immunocytotoxicity, an aliquot of cells was removed for culture; the remaining cells were washed and subjected to 5-HU sensitivity assays.

* Values reported (mean ± SD for a single representative experiment) are those for cells previously incubated in T cell–CM and then subjected to anti-Ia–mediated cytotoxicity. 5-HU media controls (i.e., cells incubated in media and then subjected to anti-Ia and 5-HU cytotoxicity) showed no differences from Ia-mediated cytotoxicity alone (data not shown).

their products lacked the solitary ability to substitute for WEHI-3-CM or IL-3 in stimulating CFU-Mk. These observations indicate that active T cells produce Mk potentiator activity but not Mk-CSA. The production of such a hematopoietic activity by T cells is consistent with reports showing that T cell subpopulations produce activities that effect granulocytopoiesis (3), as well as early phases of erythroid development (4, 5). However, the restriction of Mk potentiator activity production to mitogen-activated T lymphocytes is of interest. Hematopoietic factor production in response to immunological stimulation is variably reported (4, 38) in studies of granulocytic or erythrocytic progenitor cells. Other studies (39, 40) suggest that synergistic actions of macrophages and T cells are responsible for causing the production of hematopoietic factors. Such cell-cell interactions may be responsible for the variability noted in the relationship between antigen activation and T cell factor production. We observe that bone marrow cells exhaustively depleted of macrophages, B cells, and T cells retain responsiveness to both T cell–CM, MLuCM, and PMA (the latter substitutes for Mk potentiator activity and is used as an internal standard in these assays). This suggests that T cell factors work directly on CFU-Mk. However, these studies were carried out on enriched but not purified cell populations, and used, with

few exceptions, CM rather than purified biological activities. These limitations make direct assessment of the actions of T cell products on CFU-Mk difficult.

Complement-dependent cytotoxicity assays using mAb directed against appropriate Ia determinants demonstrate that a subpopulation of CFU-Mk bears Ia antigens. This observation extends the role of Ia as a hematopoietic differentiation antigen to cells of the Mk lineage. Previous studies (41, 42) indicate that the murine pluripotent spleen CFU lack Ia antigen, but that CFU-GM possess this determinant (43). Studies (13, 14) of human hematopoietic cells confirm these observations, and show the presence of Ia antigen on multipotent cells (CFU-GEMM), which are capable of trilineal expression in vitro (44), and on the BFU-E (9). The Ia antigen is apparently lost at the promyelocyte level in the granulocytic lineage (45), and at the CFU-E level during erythroid development (9). Interestingly, Rabellino et al. (46) show that ~15% of mature human Mk retain Ia-like antigenic determinants. This suggests that this antigen may also mark a functional subpopulation of mature Mk.

The frequency of CFU-Mk bearing Ia suggests that these cells are also the CFU-Mk that are in active phases of the cell cycle. The relation between Ia expression on murine CFU-Mk and the mitotic cycle is shown by the loss of CFU-Mk sensitivity to Ia antibody treatment in animals treated in vivo with 5-HU. The linkage between Ia expression and DNA synthesis is also demonstrated by in vitro studies, in which removal of Ia^+ progenitor cells by antibody-mediated cell lysis also removed those cells that were sensitive in vitro to 5-HU. Extensive studies (13, 15, 47, 48) of human hematopoietic progenitor cells indicate that CFU-GM, BFU-E, and CFU-GEMM all express Ia antigenic determinants, and that the expression of Ia is associated with the S phase of the mitotic cycle. Moreover, the expression of these Ia-like molecules is associated with the responsiveness of these progenitor cells to known in vitro hematopoietic regulatory activities such as acidic isoferritins and prostaglandins of the E series (14, 15, 17).

We hypothesize that the presence of Ia molecules on the surface of cycling populations of CFU-Mk makes these cells important targets for immune regulation of megakaryocytopoiesis. Studies in which incubation of CFU-Mk in the presence of T cell hybridoma (FS7-20.6.18)–CM increased both Ia expression and the percent of CFU-Mk actively synthesizing DNA suggests that this hypothesis is correct. Studies by Torok-Storb et al. (49) and Lipton et al. (50) suggest that regulation of erythropoiesis by T cell subsets in humans is genetically restricted to cell-cell interaction mediated through recognition of Ia-like molecules. Moreover, Torok-Storb et al. (49) further suggest that the Ia molecule itself may function as a cell receptor in immunoregulatory interactions. Thus, the apparent role of Ia antigenic determinants on the surface of CFU-Mk may be to serve as a determinant for immune recognition.

T lymphocyte–derived Mk potentiator activity seems to function by recruiting CFU-Mk into active DNA synthesis. Thus, ~10–25% of steady-state CFU-Mk are in S phase (24, 50). Optimal concentrations of T hybridoma–CM elevates this resting population to ~70 %, and concomitantly increases Ia expression to a similar level. Williams et al. (51) have suggested that in vitro Mk colony development is regulated, in part, by the cell cycle status of the progenitor cells. The

mechanism by which such modulation occurs may be through regulatory molecules interacting with and stimulating Ia^+ progenitor cells to increase DNA synthesis.

The exact nature of T cell–derived Mk potentiator activity is unknown. We do not believe that the activity is IFN-γ, because recombinant IFN-γ is incapable of stimulating in vitro megakaryocytopoiesis. Williams et al. (51) have shown that a murine macrophage line (P388.D1) also produces Mk potentiating activity (50). Both this macrophage line and the T cell hybridoma (FS7-20.6.18) produce Ia-inducing activity that is distinct from IFN-γ (52). Whether Ia-inducing activity is the same as Mk potentiating activity, or whether the various sources of potentiating activity (e.g. T cells and macrophages) produce the same molecule must await purification of these activities.

We report that antigen-activated murine T lymphocytes produce an activity that meets the functional definition of a known in vitro regulator of Mk development (Mk potentiator activity). Additionally, murine CFU-Mk are shown to bear Ia determinants, and the expression of this antigen is related to active DNA synthesis. Finally, the actions of T cell–derived Mk potentiator activity appears to involve the recruitment of quiescent CFU-Mk into actively cycling cells. These observations strongly suggest that the immune system can amplify basal levels of thrombocytopoiesis in response to immunological stimulation such as inflammation or infection.

Summary

Mitogen-activated murine T lymphocytes or T cell hybridomas produce an activity (megakaryocyte [Mk] potentiator activity) that enhances the in vitro growth and development of Mk colonies. This activity was found in optimal concentrations (2.5%) in T cell hybridoma–conditioned medium, and was also produced by feeder layers of concanavalin A–activated T cells. A subpopulation of murine Mk progenitor cells (colony-forming units; CFU-Mk) bears the Ia antigen. Separate experiments indicated that T cell products stimulate CFU-Mk by increasing their basal levels of Ia expression as well as the frequency of cells actively synthesizing DNA. The hypothesis that the expression of this antigen was related to the cell cycle status of these progenitor cells was confirmed in studies that indicated that ablation of actively cycling cells in vivo abrogated the cytotoxic effects of anti-Ia monoclonal antibodies. The interdependence of T cell lymphokine regulation of both Ia expression and cell cycle status was also seen in in vitro experiments in which Ia^+ progenitor cells were eliminated by complement-dependent cytotoxicity. The removal of Ia^+ cells prevented 5-hydroxyurea-mediated inhibition of cells in S phase. We hypothesize that immune modulation of megakaryocytopoiesis occurs via soluble T cell products that augment Mk differentiation. Further, the mechanism of immune recognition/modulation may occur via Ia antigens present on the surface of these progenitor cells.

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