Experimental Hematology Today—1985

Selected Papers from the 14th Annual Meeting
of the International Society for Experimental Hematology
July 14–18, 1985, Jerusalem, Israel

Editors
S.J. Baum D.H. Pluznik L.A. Rozenszajn

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Experimental Hematology Today

Preface

Experimental Hematology Today—1985 is a memento to the superb 14th Annual Meeting of the International Society for Experimental Hematology, held in Jerusalem, Israel in July 1985. It represents a selection of the best presentations at the meeting. The manuscripts were selected by the local scientific committee and carefully reviewed by the editors. The yearbook is divided into five parts and represents the most recent advances in the basic sciences and clinical applications.

Part I, under the leadership of Dr. L. A. Rozenszajn, is entitled "Hematopoietic Regulators." Papers in this section discuss the most recent discoveries on the physiological regulation of hematopoiesis. Part II, "Hematopoietic Microenvironment," introduced by Dr. J. S. Greenberger, deals with the involvement of the hematopoietic microenvironment in the control of hematopoiesis. Dr. M. Saito leads Part III, "Differentiation of Normal and Leukemic Cells," while Part IV, "Leukemic Cells in Leukemogenesis," is introduced by Dr. A. Raghavacher. The important discussions on recent advances in "Bone Marrow Transplantation," Part V, are headed by Dr. M. M. Bortin.

Recent findings in many disciplines in experimental and clinical hematology are presented in this yearbook. It should be of considerable value to experimental and clinical scientists.

The Editors

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Contents

Role of T-Lymphocyte Colony Enhancing Factor, TLCEF, in the Induction of CFU-TL

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ABSTRACT. T-lymphocyte colony enhancing factor (TLCEF) is a factor which is present in the conditioned medium of mononuclear cells stimulated with phytohemagglutinin (PHA). Using a preparation of partially purified TLCEF, which was devoid of other interleukin activities, it was possible to demonstrate that TLCEF was responsible for the enhancement of Type I colony formation in two-step cultures. On the other hand, interleukin-2 (IL-2), and not TLCEF, was shown to be able to induce proliferation of Type II colonies even in one-step cultures, i.e., under conditions which preclude formation of Type I colonies. Individual Type I and Type II colonies were expanded in long-term culture in the presence of IL-2-containing CM. Exogenous TLCEF, unlike IL-2, was unable to support growth and recolonization of cell lines derived from individual Type I colonies. The fact that each factor seems to support the formation of a different type of colony implies that each acts either on different CFU-TL or on CFU-TL at different stages of maturation.

INTRODUCTION

Ten years ago we developed in our laboratories cloning systems for lymphoid cells which have proved to be highly valuable for studying biological models of lymphocyte proliferation and differentiation in the immune system [1]. The basic protocol for these studies was to immobilize the seeded cells, usually peripheral blood mononuclear cells (MNC), bone marrow cells or lymph node cells [1-5]. The colony formation units of T-lymphocytes (CFU-TL) and B-lymphocytes (CFU-BL) were detected and monitored through their ability to proliferate in a semi-solid medium [6,7]. In this culture system containing mitogens, with or without conditioned medium (CM), CFU-TL and CFU-BL progenitor cells circulating in peripheral blood are able to undergo proliferation and subsequently to generate colonies containing cells bearing mature T and B cell surface phenotypes, respectively.

When MNC were seeded in a two-layer agar system, T-cell colonies developed both inside and on the surface of the upper agar layer. The lower colonies, which appeared after 3-5 days, were termed Type I, whereas the upper ones, which appeared 24-48 h later, were termed Type II.

For optimal T-cell colony growth, and in particular for Type I colonies, it was necessary to presensitize the MNC with mitogen for 18 h in liquid phase and to seed the sensitized cells in the continuous presence of mitogen [1,6]. Moreover, it was found that the addition of CM from MNC stimulated with phytohemagglutinin (PHA), enhanced the formation of colonies. The factor present in this CM which is responsible for augmenting the number of colonies has been characterized and termed T-lymphocyte colony enhancing factor (TLCEF) [8-10]. In similarity to the situation in other lineages of the hemopoietic system, we assume that CFU-TL represent an early type of committed cell, which requires humoral regulatory factors to proliferate, differentiate and mature into T-cells. The aim of the present communication is to shed light on the nature of the interactions between CFU-TL and TLCEF, as well as on the influence of T-cell growth factor (TCGF), also termed interleukin-2 (IL-2), on T-cell colony formation.

METHODS

COLONY FORMATION

Isolation of seeded cells. The seeded cells were venous blood MNC obtained by Lymphoprep (sodium metrizoate/Ficoll, D=1.077) fractionation [11].

Two-step culture. This was performed essentially as originally described in a two-layer agar system [1], except that the technique was adapted to a semi-micro scale (Fig. 1). Briefly, cells were stimulated for 18-24 h in liquid phase with

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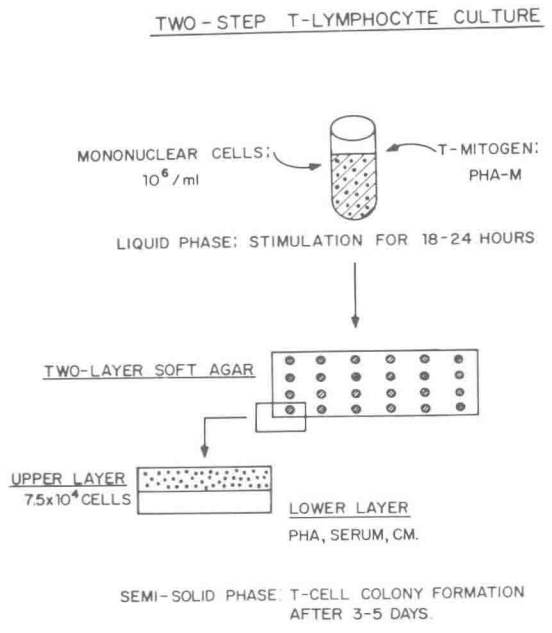

Fig. 1. A schematic diagram of the semi-micro technique used for two-step culture of T-lymphocytes.

125 ug/ml PHA-M (Difco) and 10% pooled human inactivated serum and thereafter seeded in the upper agar layer (75,000/well) in quadruplicates in 24-well multidishes (Nunc), in the continuous presence of 125 ug/ml PHA-M, and supplemented with 20% pooled human inactivated serum, without or with MNC-CM or a fraction purified from it (lower agar layer). After 3-5 days at 37°C in a fully humidified atmosphere containing CO_2 in air, Type I large colonies that had developed within the upper agar layer and had more than 50 cells were counted. Type II small and flat colonies were evaluated in some cases.

One-step culture. This was performed essentially as the two-step culture, except that the seeded cells were not stimulated with PHA in liquid phase prior to being plated in the two-layer agar system. The number of Type II colony cells was evaluated as follows: the upper agar layer on which Type II colonies had developed, was flooded with 0.5 ml of a trypsin (1:250) solution 0.25% Puck's Saline A containing EDTA (1:5000) - (Beth Haemek, Biological Industries, Israel). The trypsin solution apparently caused disintegration of the colonies, and the resulting cell suspension was passed several times through a 1 ml syringe before being counted. No development of Type I colonies was observed.

PREPARATION OF MNC-CM AND A PURIFIED TLCEF FRACTION

These were prepared essentially as previously reported [9,10]. Briefly, venous blood MNC ($1.5x10^6$/ml) were incubated for 72 h at 37°C in 5-7.5% CO_2 in air in the presence of 125 ug/ml PHA, 5 ng/ml phorbol 12-myristate-13-acetate (PMA) and 10% of a fraction of human serum, which was obtained as the precipitate of fractionation (2 cycles) with 40% saturation ammonium sulfate. The CM was purified 12-20-fold by treating it with the ammonium sulfate solution and the supernatant, the 40S fraction containing most of TLCEF activity, was used in the experiments described in this work.

ASSAYS

IL-2 assay. IL-2 was determined in a microassay using an IL-2-dependent rat cytotoxic T-lymphocyte line [12]. The samples containing activity were tested at several dilutions and the activity (in U/ml) was determined by logarithmically plotting the cpm of tritiated thymidine uptake against the logarithmic dilution of the sample [12] or by probit analysis [13]. The assay was standardized with a sample of IL-2 purified from a gibbon T-cell line, MLA-144 (a gift from Dr. H. Rabin, NCI, Frederick, MD).

IL-1 assay. Interleukin-1 was determined using murine thymocytes as responder cells [14].

IL-3 assay. Interleukin-3 was determined according to Greenberger et al. [15], using the murine interleukin-3-dependent line SD.

CELL LINES DERIVED FROM LYMPHOCYTE COLONIES (TYPE I AND TYPE II)

Expansion and maintenance. Individual Type I and Type II colonies were expanded and maintained in long-term cultures. Type I colonies were picked from the agar with a capillary tube. Type II colonies, from the surface of the agar layer, were collected by flooding the agar with RPMI-1640 medium. The colonies were transferred, 1 colony/well, to flat-bottomed microtiter plates (Nunc), in 0.2 ml complete RPMI-1640 culture medium (RPMI-1640, supplemented with 100 U/ml penicillin, 10 ug/ml streptomycin, 1% 200 mM glutamine, 1% 100 mM sodium pyruvate, 1% non-essential amino-acids and $5x10^{-5}$ M 2-mercapto-ethanol) containing 10% inactivated pooled human serum and 20% MNC-CM. The cultures were incubated at 37°C in a fully humidified atmosphere containing 7.5% CO_2 in air. One half of the culture medium was replaced with fresh medium twice a week. Once a month, irradiated (3000 R) peripheral blood MNC from healthy donors were added as feeder cells at a ratio of 1 irradiated cell/6 cultured cells. For further maintenance, cells were transferred to 24-well tissue dishes and the cell lines expanded under the same conditions as described above.

Phenotypic analysis of cell lines. T-cell subsets were determined by an indirect immunofluorescence method according to their surface antigen specificity using monoclonal antibodies [16].

RESULTS

In the one-step cultures, colony formation took place only when CM containing growth factors was added to the lower agar layer and essentially only Type II colonies developed [6]. In two-step cultures, the development of both Type I and Type II colonies was not entirely dependent on the addition of CM to the lower agar layer. However, the number and size of colonies was enhanced by the CM. The characteristics of the two types of

Table 1. Characteristics of Type I and Type II T- cell colonies growth in two layer soft agar culture

Colony characteristics	Type I	Type II
Development in culture	after 3-5 days	after 5-7 days
Cell content	200 - 500	50 - 150
Morphology	large, with a compact center	small, roundish and flat
Location in agar	within the upper layer	on the surface of the upper layer

Table 2. Purification of TLCEF[a]

Step	Purification method	Fraction	Degree of purification
I	40% ammonium sulfate fractionation	40S	12 - 20
II	Phenyl-Sepharose chromatography	peak II	200 - 400
III	Gel filtration	peak I	13,000 - 20,000

a. Refs 9, 10.

colonies are summarized in Table 1. Since the kinetics of appearance, the plating efficiency, the size and shape of the two types of colonies are different, it is possible that they originate from CFU-TL in different stages of maturation, and are therefore able to respond to different humoral factors.

We have attempted to identify the active substances that trigger the formation and development of Type I and Type II colonies. The purification of TLCEF is summarized in Table 2. TLCEF was purified up to 20,000 fold from a 3 days-CM of MNC under the synergistic stimulation of PHA and PMA [9,10]. Purified TLCEF was found to be devoid of other lymphokine activities (Table 3).

The results of one-step experiments (Table 4) show that purified IL-2, but not the fraction 40S which contains TLCEF and is IL-2 free, is able to support the formation of Type II colonies. The reverse is true for Type I colonies obtained in two-step cultures (Table 5): in this case TLCEF, and not IL-2, is capable of enhancing the formation of Type I colonies. It should be emphasized that under the conditions of the two-step culture, endogenous IL-2 and TLCEF are both secreted in the semi-solid medium, resulting in the formation of both Type I and Type II colonies.

Individual Type I and Type II colonies were expanded in long-term culture in the presence of IL-2-containing CM. When cell lines derived from individual Type I colonies were recolonized in agar, it was found that in the presence of fraction 40S, which contains TLCEF and is free of IL-2, no colony formation took place, whereas in the presence of CM, which contains both IL-2

Table 3. Interleukin activities of partially purified TLCEF

Sample	TLCEF 10^{-3}x U/ml	IL-1[a] 10^{-3}x cpm	IL-2 10^{-2}x U/ml	IL-3[b] 10^{-3}x cpm
CM	2.43	194.5	61.5	< 5
Fraction 40S	2.03	18.5	0.6	< 5
Phenyl-Sepharose, peak II[c]	0.82	3.1	0	0

a. At a 1:8 dilution; b. At a 1:16 dilution; c. Ref 10.

Table 4. Effect of exogenous active factors on T-cell colony formation -- one step culture[a]

Active factor	Exp't No.	Type I No. of colonies	Type II[b] No. of colony cells
Control	1	None	None
	2	None	15,000
IL-2, 50 U	1	None	600,000
	2	None	210,000
IL-2, 25 U	1	None	400,000
	2	None	195,000
CM (containing	1	None	600,000
IL-2 and TLCEF)	2	None	55,000
Fraction 40S	1	None	None
(containing TLCEF)	2	None	17,000

a. $3x10^5$ cells seeded; b. Cells of pooled colonies were scored after flooding colonies with trypsin solution.

Table 5. Enhancement effect of exogenous active factors on T-cell colony formation (Type I)

Active factors	No. of colonies/ $3x10^5$ cells plated
Control	41.0 ± 10.8
CM (containing IL-2 and TLCEF)	98.7 ± 37.3*
Fraction 40S (containing TLCEF)	90.0 ± 31.8*

Results represent the mean number of colonies ± SE of 8 separate experiments. *$p < 0.05$, relative to the control.

and TLCEF, only flat Type II colonies developed. Surface marker analysis revealed that most of the cell lines derived from Type I colonies had a heterogeneous phenotypic pattern (Fig. 2A), whereas those derived from Type II colonies were mainly either OKT 4 positive or OKT 8 positive cells (Fig. 2B).

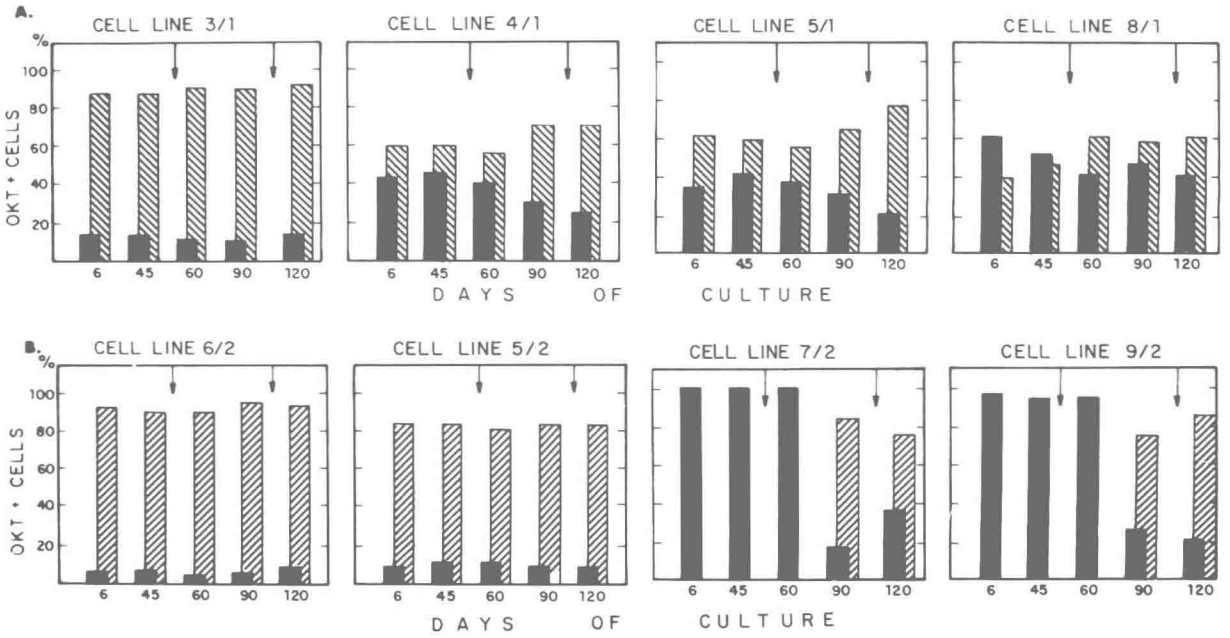

Fig. 2. Phenotypic analysis of 8 typical cell lines derived from the 2 types of individual T-cell colonies: A. Cell lines derived from Type I colonies; B. Cell lines derived from Type II colonies. ▨ OKT 8 positive cells; ▬ OKT 4 positive cells. At times indicated by arrows the cultures were supplemented with irradiated MNC (3000 R) and PHA.

DISCUSSION

In this work we show that in the continuous presence of a T-lymphocyte mitogen, such as PHA, CFU-TL can be induced to proliferate in semi-solid medium in response to stimulation by endogenous as well as exogenous growth factors present in the added CM. There seems to be no doubt that TLCEF, a factor isolated and purified from MNC-CM, is distinct from IL-2 as well as other interleukin activities (Tables 2 and 3; refs. 8-10). Other workers also have recently postulated that factors other than IL-2 may be required for in vitro proliferation, differentiation and maturation of human T-colony forming cells [22-24]. The differences between TLCEF and IL-2 are summarized in Table 6. The fact that each factor seems to support the formation of a different type of colony implies that each acts either on different CFU-TL or on CFU-TL in different stages of maturation [21]. Purified IL-2 is able to induce proliferation of Type II colony-forming cells, even in the one-step culture, i.e., under conditions which preclude formation of Type I colonies (Table 4). On the other hand, enhancement of Type I colony formation is promoted by partially purified TLCEF and not by IL-2 (Table 5). However, under all our culture conditions, in the presence of PHA, both IL-2 and TLCEF are produced endogenously. Thus, more experimental evidence is required to elucidate the exact nature of TLCEF action, in particular with respect to its ability to induce self-renewal of the CFU-TL compartment on which it acts. At present, we may only speculate that TLCEF can be a differentiation and maturation factor for a population of immature IL-2-refractive T-cells. By influencing the expression of IL-2 receptors, TLCEF would render these cells responsive to the proliferative signal of endogenous or exogenous IL-2. Exogenous TLCEF, unlike IL-2, was unable to support growth and recolonization of cell lines derived from individual Type I colonies. The lack of success in finding a population of T-cell precursors that

Table 6. Comparison between characteristics of human TLCEF and IL-2

Property	TLCEF	IL-2
Optimal time for production by MNC	48 - 72 hr	24 hr[a]
Additive required for stability at low protein concentration	None	Albumin or polyethylene glycol (PEG)[b]
Molecular weight (from gel filtration)	100,000-130,000	20,000-25,000
pH stability	up to 12	2 - 10[c]
Type of T-cell colonies supported	I	II[d]

a. Ref. 12; b. Ref. 17; c. Ref. 18; d. Refs. 19, 20, and Table 4, this work.