

Production of erythropoietin-like activity by a murine erythroleukemia cell line

(erythropoiesis/growth factor/murine leukemia virus)

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ABSTRACT A transplantable murine leukemia, primarily induced by a biologically cloned Friend helper virus, was shown to induce polycythemia in recipient ICFW mice. A leukemia cell line (IW.32) was established *in vitro* from this transplantable leukemia. Sodium butyrate and hemin induced erythroid differentiation in these leukemia cells as has already been shown with other erythroleukemia cells. The supernatant of this cell line was devoid of spleen focus-forming virus activity. However, it induced the incorporation of ⁵⁹Fe in polycythemic mice and the *in vitro* differentiation of murine and human cfu-e into erythroid colonies. Therefore, these erythroleukemia cells produced a factor with all the biological properties of erythropoietin. The erythropoietic activity of IW.32 supernatant was higher *in vitro* [equivalent to 0.5–1 international unit (IU) of erythropoietin per ml] than *in vivo* (0.15–0.3 IU/ml). This erythropoietin-like activity was stable at 100°C for 3 min, which ruled out the possibility that a virus was responsible for these effects. Preliminary studies demonstrated that the biochemical properties of the IW.32 factor are strongly similar to those of Connaught step 3 erythropoietin, thus supporting the hypothesis that the IW.32 factor is indeed an erythropoietin.

Several leukemia cell lines produce growth factors or differentiation factors for hematopoietic cells of different lineages (1). The production of factors specific for the erythroid lineage appears, however, to be a rare event. Two human cell lines have been reported to synthesize factors capable of potentiating the differentiation of progenitors into erythroblasts (2, 3). These factors were efficient only in the presence of erythropoietin and were unable alone to induce the terminal steps of erythroid differentiation. An erythropoietin-like activity as defined by the induction of hemoglobin synthesis *in vivo* and *in vitro* has been found in the supernatant of *in vitro*-cultured murine macrophages (4). However, the production of erythropoietin-like activity by a continuous cell line has never been described. In the present paper we report experiments showing that a murine erythroleukemia cell line induced *in vivo* by a biologically cloned helper of Friend virus and established *in vitro* as a continuous cell line produces relatively large amounts of a protein with erythropoietin-like activity.

MATERIALS AND METHODS

Virus Leukemia and Cell Line. The I⁻⁵ virus stock has been described (5). This helper-independent ecotropic virus, Friend murine leukemia virus (F-MuLV), was isolated from the polycythemic Friend leukemia virus complex (Steeves-Lilly strain), free of any detectable spleen focus-forming virus (SFFV), xeno-

tropic, or mink cytopathogenic foci activities. It was shown to produce erythroid, myeloid, or lymphoid leukemias in mice resistant to or surviving the acute erythroproliferative disease induced by such F-MuLV (6, 7). To check for the presence of SFFV, 0.1 ml of plasma or culture supernatant was injected into 4- to 6-wk-old ICFW and BALB/c mice. Spleens were screened 10 days later for foci formation according to Axelrad and Steeves (8).

Establishment of the IW.32 Cell Line. The IW.32 leukemia was one of many leukemias induced in our laboratory in newborn ICFW mice infected with the I⁻⁵ virus stock. The initial *in vivo* evolution of this leukemia did not differ from the other leukemias induced by the same virus. One month after infection, the infected mice developed anemia and hepatosplenomegaly as found previously (6) in F-MuLV-infected mice. The infected mice completely recovered from this disease after transfusions. Two months later hepatosplenomegaly reappeared even though transfusions were maintained. The mice were then sacrificed. In the leukemic mouse that gave rise to the IW.32 leukemia cells, cytological studies of the spleen showed an infiltration of "undifferentiated" leukemia cells. These cells were serially transplantable in ICFW mice. The *in vivo* evolution of the particular IW.32 transplantable leukemia differed from that of other similar leukemias by the appearance of a polycythemia in graft recipients after seven *in vivo* passages. Mice with grafts appeared reddish and their hematocrit was found to increase by 60% one month after the leukemia cell inoculation. Spleen cells from the 13th *in vivo* passage were used to establish the cell line *in vitro*. Fragments from the tumoral spleen were carefully flushed with α -medium (Flow, Asnières, France) to obtain a single cell suspension. Cells ($1-5 \times 10^6$) were directly plated in 25-cm² culture flasks (Corning) with 10 ml of α -medium/10% fetal calf serum/20 mM glutamine. Successive passages of the nonadherent cells led to the establishment of a pure cell line in suspension. Leukemia cells were then passaged at 1:5 three times a week. Culture supernatant was collected after 48 hr in culture, filtered through 0.2- μ m filters (Millipore), and stored at -20°C until tested.

***In Vivo* Assay for Erythropoietic Activity.** DBA₂ female adult mice were rendered polycythemic (hematocrit, >60%) by injecting intraperitoneal packed erythrocytes (1 ml on day -4 and 0.5 ml on day -1). Erythropoietin (Connaught Laboratories, Toronto), saline, and culture supernatant were injected subcutaneously on day 0 (five animals in each experimental group). On day 3, 1 μ Ci of [⁵⁹Fe]ferric citrate (Amersham; 1 Ci = 3.7 $\times 10^{10}$ Bq) diluted in 0.2 ml of modified Eagle's medium was

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Abbreviations: SFFV, spleen focus-forming virus; F-MuLV, Friend murine leukemia virus; cfu-e, colony-forming unit erythroid; IU, international unit(s).

injected intravenously. The ^{59}Fe incorporation into erythrocytes was measured on day 6.

In Vitro Assays for Normal Hematopoietic Progenitors. Colony-forming unit erythroid (cfu-e) assays were carried out in plasma clot cultures according to McLeod *et al.* (9) with few modifications (10). Cultures were set up either with normal mouse bone marrow cells (2×10^5 per ml) or with normal human bone marrow cells (5×10^5 per ml). Briefly, assays were carried out in 35×10 mm Petri dishes (Falcon 3001) containing 0.2 ml of serum (see below), 0.1 ml of detoxified bovine serum albumin, 0.1 ml of L-asparagine (Calbiochem) (0.2 mg/ml)/ α -medium/ CaCl_2 (28 mg/100 ml), 0.1 ml of cell suspension, 0.1 ml of erythropoietin step 3 (Connaught Laboratories), and 0.3 ml of α -medium. In some cultures erythropoietin was omitted and replaced either by α -medium or by 0.45–0.9 ml of culture supernatant from IW.32 cells. Duplicate cultures were allowed to clot after addition of 0.1 ml of citrated bovine plasma (Florio, Courbevoie, France). They were then incubated at 37°C in humidified 5% CO_2 /95% air. After incubation, clots were fixed with glutaraldehyde, stained with benzidine/hematoxylin, and scored for erythroid colonies. For murine cultures, fetal calf serum was used and incubation was for 60 hr. For human cultures, a pool of normal AB serum was used and incubation was for 7 days. Murine granulomacrophagic colony-forming unit assays were carried out in monolayer agar cultures as described by Metcalf (11). Mouse bone marrow cells (7×10^4) were cultured in 1 ml of 0.3% agar/ α -medium/10% fetal calf serum. Serum from mice previously injected with endotoxin (12) was used as a source of colony-stimulating factor. On day 7, cultures were scored for granulomonocytic colonies under an inverted microscope.

Induction of Differentiation. Hexamethylene-bis-acetamide (Serva), sodium butyrate (Sigma), dimethyl sulfoxide (Sigma), and hemin (Sigma) were tested for their ability to induce hemoglobin synthesis in IW.32 cells at concentrations indicated in Table 1. Hemoglobin synthesis was checked by the appearance of benzidine positive cells.

Biochemical Studies. Trypsin digestions were performed as follows: 1 ml of sample was incubated with 0.2 or 0.4 mg of trypsin L-1-tosylamide-2-phenylmethyl chloromethyl ketone (227 units/mg) (Worthington) diluted in 1 ml of Tris-buffered saline (0.02 M Tris-HCl, pH 7.4/0.5 M NaCl). The incubation was at 37°C for 1 or 6 hr. The digestion was stopped by addition of 0.4 or 0.8 mg of soybean trypsin inhibitor (Sigma). Controls were incubated at 37°C with trypsin L-1-tosylamide-2-phenylmethyl chloromethyl ketone/soybean trypsin inhibitor or with soybean trypsin inhibitor alone. Chromatography using concanavalin A-Sepharose (Pharmacia) was carried out with a column equilibrated in Tris-buffered saline. The bound proteins were eluted in the same buffer containing 0.2 and 0.5 M methyl mannopyranoside. Ammonium sulfate precipitations were performed at 0°C by addition of sufficient solid ammonium sulfate to achieve 50%, 60%, 70%, 80%, and 90% saturation. Successive protein precipitates were dissolved in Tris-buffered saline (pH 7.4) and dialyzed for 2 days against the same buffer and 1 day against α -medium.

RESULTS

The IW.32 cell line growing in suspension looked like round nonadherent blastoid cells with a basophilic cytoplasm containing numerous vacuoles. Myeloperoxidase and esterase staining were negative as was benzidine reaction. After incubation with butyrate and hemin, numerous benzidine-positive cells appeared. Dimethyl sulfoxide and hexamethylene-bis-acetamide were ineffective (Table 1). A preliminary cytogenetic

Table 1. Effect of HMBA, sodium butyrate, dimethyl sulfoxide, and hemin on induction of benzidine-positive cells in the IW.32 cell line

Treatment	% of benzidine-positive cells		
	24 hr	72 hr	96 hr
None	0	0	0
6–15 mM HMBA	0	0	0
0.5 mM sodium butyrate	0	0	≥ 70
0.1–1% dimethyl sulfoxide	0	0	0
50 μM hemin	0	≥ 95	≥ 95

HMBA, hexamethylene-bis-acetamide.

study showed an abnormal karyotype with 70 chromosomes. All ICFW mice inoculated subcutaneously or intraperitoneally with IW.32 cells developed a local tumor or a splenomegaly (or both) within 2–4 wk. At the same time, they developed a polycythemia as shown by their increasing hematocrit value. To determine whether this polycythemic disease differed from a Friend virus polycythemic strain-induced polycythemic Friend disease, we looked for erythropoietin-independent cfu-e in recipient mouse bone marrow cells. Bone marrow mononuclear cells were cultured with and without added erythropoietin. cfu-e-derived colonies were observed only in the presence of added erythropoietin. IW.32 supernatant and plasmid from tumor-bearing mice were devoid of any SFFV activity because the inoculation of ICFW and BALB/c adult mice did not result in the formation of spleen foci after 10 days. This was confirmed by the lack of splenomegaly and polycythemia at day 28 after inoculation of IW.32 supernatant.

Erythropoietic Activity of IW.32 Supernatant. *In vivo*, the inoculation of 1 ml of crude or 0.5 ml of concentrated ($\times 10$) IW.32 supernatant in hypertransfused polycythemic mice induced a significant incorporation of ^{59}Fe into circulating erythrocytes (Fig. 1). The erythropoietin equivalent activity could

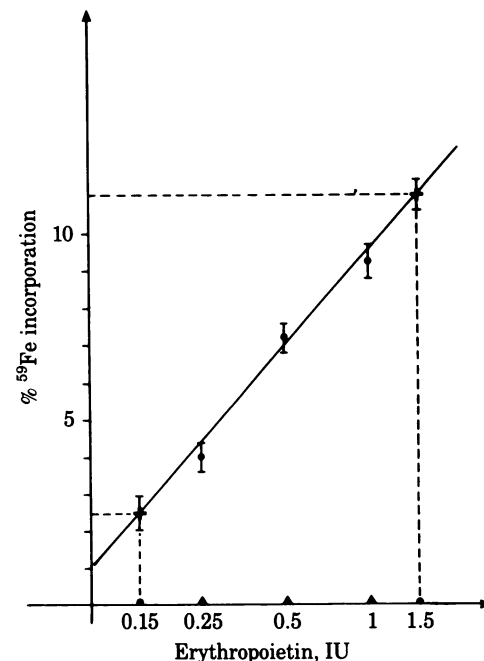


Fig. 1. *In vivo* titration curve of IW.32 supernatant. According to this dose-response curve, as shown with dotted lines, 1 ml of crude supernatant corresponded to 0.15 international unit (IU) of erythropoietin and 0.5 ml of concentrated ($\times 10$) supernatant corresponded to 1.5 IU of erythropoietin.

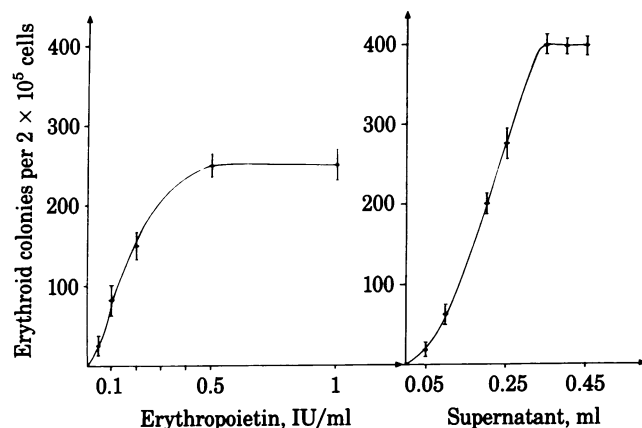


FIG. 2. Dose-response curves of murine bone marrow cfu-e to erythropoietin step 3 Connaught (*Left*) and to IW.32 supernatant (*Right*). Erythropoietin colonies were scored after 60 hr of culture.

be estimated between 0.15 and 0.30 IU/ml of supernatant.

In vitro, IW.32 supernatant was added to the culture of normal murine bone marrow mononuclear cells instead of erythropoietin. It induced characteristic benzidine-positive erythropoietin colonies in plasma clot cultures after 60 hr (cfu-e derived) and 7 days (burst-forming unit erythropoietin derived). As little as 10 μ l of supernatant per culture was sufficient to induce such colonies. Dose-response curves of normal murine bone marrow cfu-e to IW.32 supernatant and erythropoietin step 3 were strikingly similar, showing a linear increase in the number of cfu-e-derived colonies, followed by a plateau (Fig. 2). However, the number of erythropoietin colonies at the plateau was significantly higher in the presence of IW.32 supernatant than with erythropoietin step 3. The erythropoietin equivalent activity could be estimated to range between 0.5 and 1 IU/ml of supernatant. IW.32 supernatant exhibited no granulomacrophage colony-stimulating factor activity when added to normal mouse bone marrow cells in agar culture (data not shown). The *in vitro* erythropoietic activity of IW.32 was not species specific because IW.32 supernatant induced typical cfu-e-derived ery-

thropoietin colonies at 7 days when added to human bone marrow mononuclear cells in plasma clot culture (use of 0.5 ml of IW.32 supernatant instead of erythropoietin induced 510 ± 30 erythropoietin colonies per 5×10^5 bone marrow cells).

Biochemical Studies. Biochemical characteristics of IW.32 supernatant and erythropoietin step 3 were compared by testing the ability to induce murine bone marrow cfu-e-derived colonies after similar biochemical treatments. The biological activities of both erythropoietin step 3 and IW.32 supernatants were unaffected or only partially decreased by heating at 56°C for 30 min, by boiling at 100°C for 3 min, by freeze-drying and dialysis, or by treatment with trypsin (100 μ g/ml) for 1 hr at 37°C. They were abolished by trypsin at 200 μ g/ml for 6 hr at 37°C (Table 2). IW.32 erythropoietic stimulating factor was precipitated by ammonium sulfate between 50% and 80% saturation (Table 3). After concanavalin A-Sepharose chromatography the activities of both factors were eluted immediately after the void volume (Fig. 3).

DISCUSSION

In this paper we describe the production of an erythropoietic factor by a murine erythroleukemia cell line. The erythropoietic differentiation ability of the leukemia cell line was shown by the induction of hemoglobin synthesis (checked by benzidine staining) after incubation with some biochemical compounds (butyrate and hemin) known to induce the *in vitro* differentiation of other murine erythroleukemia cell lines. No erythropoietic differentiation was induced by the autologous supernatant or by erythropoietin. The IW.32 leukemia cell line was derived from a leukemia induced in mice infected with a biologically cloned ecotropic F-MuLV isolated from the polycythemia-inducing Friend virus complex. Although the virus stock used was free of any detectable SFFV, the first hypothesis to rule out was the presence in the cell line of a polycythemia-inducing virus. This hypothesis was ruled out for the following reasons. (i) No SFFV could be detected in IW.32 supernatants or in the plasma of polycythemic tumor-bearing mice by the classical *in vivo* assay. (ii) Polycythemic mice bearing an IW.32 tumor lacked spontaneous cfu-e progenitors capable of forming erythropoietin

Table 2. Effect of physicochemical and enzymatic treatments on erythropoietin colony-stimulating activity

Exp.	Treatment	Erythropoietin colonies per 10^5 cells		% recovery of activity	
		IW.32	Erythropoietin	Proteins of IW.32 conditioned medium	Erythropoietin
1	None	504	397	100	100
	56°C, 10 min	565	463	112	117
	56°C, 30 min	454	521	90	131
	100°C, 1 min	388	318	77	80
	100°C, 3 min	474	238	94	60
2	None	319	259	100	100
	Freeze-drying and dialysis	188	201	59	78
3	Control, 37°C, 1 hr	407	478	100	100
	Trypsin (100 μ g/ml), 37°C, 1 hr	466	392	114	82
	Trypsin (100 μ g/ml)/TI (200 μ g/ml), 37°C, 1 hr	749	558	184	117
4	Control, 37°C, 6 hr	271	133	100	100
	Trypsin (200 μ g/ml), 37°C, 6 hr	18	13	7	10
	Trypsin (200 μ g/ml)/TI (400 μ g/ml), 37°C, 6 hr	324	292	120	220
	TI (400 μ g/ml), 37°C, 6 hr	163	174	60	131

TI, soybean trypsin inhibitor.

Table 3. Ammonium sulfate fractionation of proteins of IW.32-conditioned medium

Fraction	A ₂₈₀	Erythroid colonies per 10 ⁵ cells
50% precipitate	7.80	21
50-60% precipitate	3.37	341
60-70% precipitate	6.37	216
70-80% precipitate	8.97	103
80-90% precipitate	1.42	0

Proteins of IW.32-conditioned medium (200 ml) were precipitated at successive 50%, 60%, 70%, 80%, and 90% saturation. After centrifugation at 35,000 × g for 30 min, precipitates were dissolved in Tris-buffered saline and dialyzed against Tris-buffered saline (2 days) and α-medium (1 day) before assay.

in vitro in the absence of erythropoietin, whereas such colonies were always found in bone marrow of the mice rendered polycythemic by the Friend virus polycythemic strain as described (13, 14). (iii) The erythropoietic activity of IW.32 supernatants was stable at 100°C, a temperature known to destroy the infectivity of any type C virus.

The polycythemia observed in graft recipients could therefore be due to the secretion of an erythropoietic factor by the malignant IW.32 cells. This was further demonstrated by studying the properties of the supernatant of the IW.32 cell line permanently established *in vitro*. This supernatant possessed two

of the major properties of erythropoietin: (i) it induced a wave of erythroid differentiation *in vivo* as judged by the incorporation of ⁵⁹Fe into reticulocytes of mice rendered polycythemic by transfusions, and (ii) it allowed the *in vitro* differentiation of murine cfu-e into mature erythroblastic colonies. Moreover, its activity was not species specific because cfu-e-derived erythroid colonies were also obtained from human bone marrow cells as described for genuine erythropoietin (15). This suggests that the factor produced by IW.32 cells is similar or identical to erythropoietin.

Several biochemical methods have been used to distinguish the properties of the IW.32 factor from those of erythropoietin step 3. They have been unsuccessful so far. Further biochemical and immunological characteristics are needed to determine whether the IW.32 cell line produces an erythropoietic activity indistinguishable from erythropoietin obtained from *in vivo* sources.

The only difference found until now between erythropoietin step 3 and the IW.32 factor was the discrepancy between the *in vivo* and the *in vitro* activities. Indeed, IW.32 supernatant was more active *in vitro* than *in vivo* compared to erythropoietin step 3 as shown by the estimated erythropoietin-equivalent activity of IW.32 supernatant: 0.15 to 0.3 IU/ml *in vivo* and 0.5 to 1 IU/ml *in vitro*. A difference in the glycosylation of IW.32 erythropoietic factor and sheep step 3 erythropoietin might account for this discrepancy because erythropoietin lost its activ-

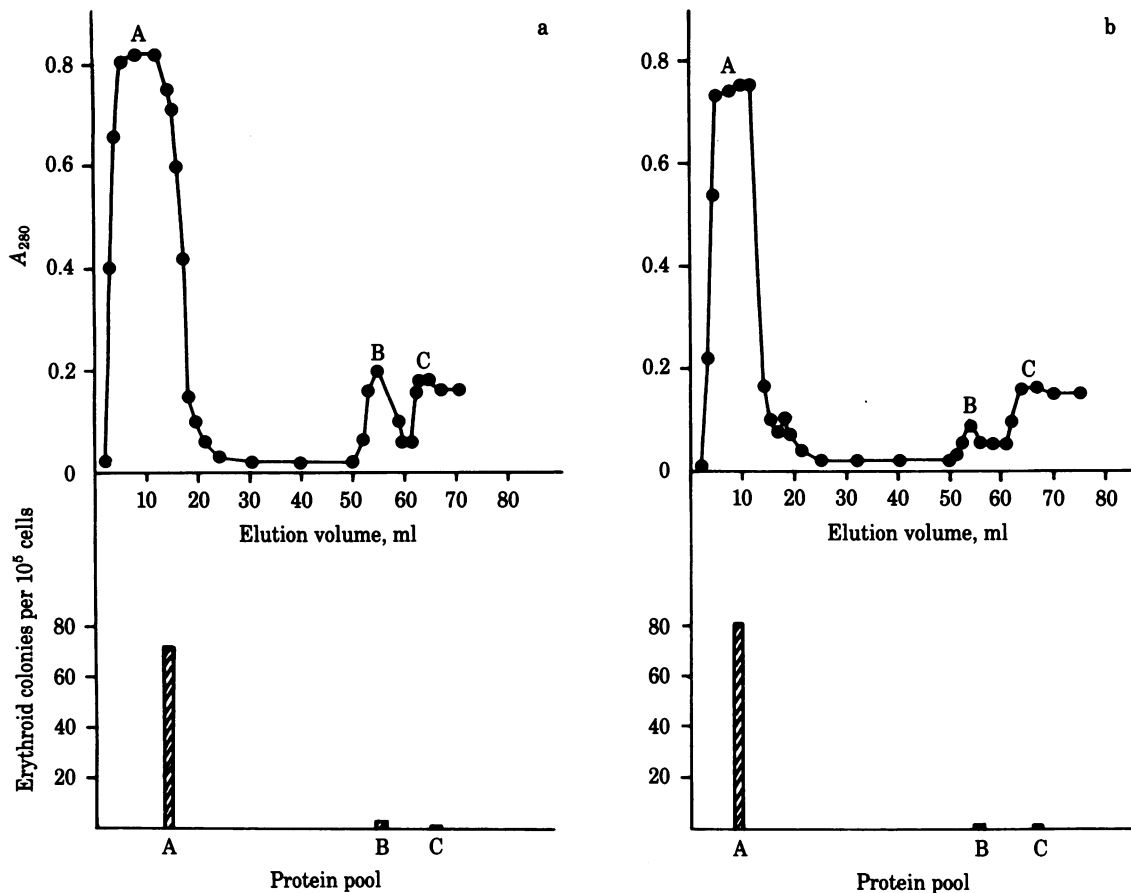


FIG. 3. Erythroid colony-stimulating activity in fractions obtained by chromatography on concanavalin A-Sepharose of IW.32-CM (a) and erythropoietin (b). IW.32 conditioned medium (20 ml) and erythropoietin (4 IU) diluted in α-medium/5% fetal calf serum (20 ml) were freeze-dried and dialyzed against starting buffer (Tris-buffered saline). Samples were applied to a concanavalin A-Sepharose column (0.9 × 2 cm) equilibrated in starting buffer (flow rate, 10 ml/hr). Unretained proteins constituted pool A. Columns were washed with 35 ml of starting buffer; the pool B proteins were removed by elution with 15 ml of 0.2 M α-methyl mannoside in starting buffer and the pool C proteins were removed by elution with 15 ml of 0.5 M α-methyl mannoside in starting buffer. Samples were dialyzed against Tris-buffered saline (pH 7.4) before assay.

ity *in vivo* after treatment with neuraminidase (16).

The IW.32 supernatant does not contain granulomacrophagic colony-stimulating factor activity. The production of other growth factors by this cell line is currently being investigated. It is already clear that the erythropoietic activity produced by the IW.32 cell line differed from erythropoietic factors that have been previously distinguished from erythropoietin. The erythropoietic burst-promoting activity has been defined as a substance necessary for the differentiation of early progenitors into cfu-e (17). It was unable to trigger the differentiation of cfu-e into erythroblasts. In contrast, erythropoietin and the IW.32 factor induced *in vitro* mature erythroblastic colonies from murine bone marrow cells in 2 days. This does not rule out the presence in IW.32 supernatant of a burst-promoting activity together with the erythropoietin-like activity. The factor described by Johnson and Metcalf (18) can be ruled out because it is active only on hepatic erythroid progenitors of fetal mice and is inactive on adult bone marrow cells. Recently an erythropoietic factor has been described in the supernatant of splenocytes stimulated by allogeneic cells (19). However, this factor was shown to differ from erythropoietin because it was retained on a concanavalin A-Sepharose column. This type of chromatography did not allow the distinction between erythropoietin and IW.32 erythropoietic activity.

In conclusion, the properties of the IW.32 cell lines are of importance for at least two reasons. (i) This is an example of a malignant cell line producing *in vitro* large amounts of a soluble factor very similar to erythropoietin. It will be necessary to determine whether or not leukemia cell growth is dependent on this factor. Indeed these malignant cells were shown to belong to the erythroid lineage, and other examples have already been described of malignant hematopoietic cell lines in which growth is autostimulated by factors similar to physiological growth factors (20, 21). However, IW.32 is an example of an erythroid cell line producing an erythropoietic factor. We have been unable to find such an activity in three supernatants of erythroleukemia cell lines induced by the Friend virus complex. (ii) IW.32 cells appear as a potent source of an erythropoietin-like activity, which might be used, at least *in vitro*, to replace erythropoietin if its similarity to currently used erythropoietin is confirmed.

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