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Continuous production of erythropoietin by an established human renal carcinoma cell line: Development of the cell line

(erythrocytosis/antiinterferon/athymic mice)

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ABSTRACT **Establishment of a stable, transformed human renal carcinoma cell line that produces erythropoietin *in vitro* and has maintained this function continuously since 1981 and for >150 passages in monolayer culture was accomplished by transplantation of human renal clear cell carcinoma tissue from a patient with erythrocytosis into an immunosuppressed athymic mouse. In addition to its immunocrossreactivity with native human urinary erythropoietin, the tumor erythropoietin demonstrates biological activity in the *in vitro* mouse erythroid colony-forming unit assay and in tumor-bearing nude mice. The cloned renal carcinoma cell line has an abnormal human karyotype and has ultrastructural features characteristic of human renal clear cell carcinoma. This cell line provides a reproducible model system for the production of an erythropoietin-like material and for the study of its synthesis and secretion.**

Several investigators have attempted to develop *in vitro* model systems that produce erythropoietin on a continuous and reproducible basis. These studies utilized either normal kidney (1) or renal carcinoma cells (2, 3), testicular germ cells (4), or mouse erythroleukemia cells (5). However, establishment of a stable transformed human cell line capable of continuous production of erythropoietin for several years has not yet been achieved.

We report the establishment of a human renal cell carcinoma cell line in culture that was generated by using an immunosuppressed nude mouse as a temporary host. These cells have been shown to produce erythropoietin on a continuous basis and have been maintained for >4 years and >150 passages in culture.

MATERIALS AND METHODS

Primary Tumor Cells. Tumor tissue was obtained in July 1981, from a female patient with renal cell carcinoma and erythrocytosis. Following nephrectomy, the tissue was immersed in ice-cold medium: Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture F-12 (Ham), 1:1 (vol/vol), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.10 mM nonessential amino acids, 100 units of penicillin/100 μg of streptomycin, and 2.5 μg of Fungizone (complete medium) per ml. All media components were obtained from GIBCO. A cell suspension was prepared by pressing the tissue through a Cellector (Bellco) followed by treatment with 0.1% trypsin/0.1 M EDTA for 10 min and inactivation of trypsin with complete medium. Cells were then resuspended either in serum-free DMEM/F-12 medium for injection into athymic mice or in complete medium for growth in culture.

Mice. Athymic female BALB/c *nu/nu* mice, 6- to 8-weeks old, were obtained from the Animal Institute of the Albert Einstein College of Medicine (Bronx, New York) and were maintained in laminar flow hoods (6). To increase the chance of tumor "take" (7), the mice were further immunosuppressed, either by irradiation from a ^{137}Cs source (Atomic Energy, Ottawa) 10 days before injection of tumor cells or by injection with 0.1 ml of rabbit anti-mouse lymphocyte serum (ALS) (M. A. Bioproducts, Walkersville, MD) 24 hr before and after inoculation of tumor cells and twice weekly thereafter for 4 weeks. One ALS-treated mouse received ALS and 0.1 ml of sheep anti-mouse α-interferon serum on the day of tumor cell injection.

Generation of Tumors in Nude Mice and Preparation of Cultures. Approximately 5×10^7 cells from the surgical specimen, suspended in 0.2–0.3 ml of serum-free medium, were injected s.c. into the flank region of six immunosuppressed and two untreated nude mice. The mouse tumor was excised when it weighed 150 mg. A cell suspension was prepared from the mouse tumor fragments and seeded in 30-mm tissue culture dishes in 2 ml of complete medium. Cells were incubated at 37°C in a 5% CO_2/95% air mixture. Part of the tumor was fixed in 10% buffered formalin (6) and histologic analysis was performed.

Cell Culture Methods. Tumor cells were routinely maintained in Corning (Corning) or Falcon (Becton-Dickinson, Cockeysville, MD) tissue culture flasks and plates in complete medium at 37°C in 5% CO_2/95% air. Confluent adherent cells were removed from the plate surface with 0.1% trypsin/0.1 M EDTA.

For determination of growth curves, either 1×10^5 or 2×10^5 cells were seeded on 60-mm tissue culture dishes in 5 ml of complete medium and incubated as above (6). Cell counts were determined on triplicate dishes on day 1 and every 48 hr thereafter for 18 days (6), with replacement of medium in the remaining dishes. Attachment efficiency, doubling time, and saturation density for renal carcinoma cells were determined (6).

Ultrastructure Analysis of Renal Carcinoma Cells. Cells grown on plastic coverslips were fixed with 3% phosphate-buffered gluteraldehyde, postfixed with 2% osmium tetroxide, stained *en bloc* with 0.5% uranyl acetate, and dehydrated with a graded series of ethanols. Epon 812 (Polysciences, Warrington, PA) was used as the embedding medium. Ultrathin sections were obtained with an LKB II ultramicrotome and examined with a JEOL 100CX transmission electron microscope (8, 9).

Chromosome Analysis. Chromosome analysis was determined as described by Worton and Duff (10). Cells at the peak of logarithmic growth were treated with a hypotonic 0.075 M potassium chloride solution and with deacetylmethyl colchicine (Colcemid, GIBCO, 0.05 μg/ml). Chromosome G banding was performed by using a modification of the technique described by Klinger (11).

Preparation of Samples for Detection of Erythropoietin. Culture supernates were centrifuged for 10 min at 800 rpm

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Abbreviations: CFU-E, erythroid colony-forming unit(s); ALS, anti-mouse lymphocyte serum; mU, milliunits.

(4°C) and the pellets were discarded. The supernates were stored at −70°C. To obtain intracellular erythropoietin, cells were harvested from cultures with 0.1% trypsin/0.1 M EDTA, washed with Earle's balanced salt solution, and extracted in 0.1 M phosphate buffer by homogenization with a Polytron (Brinkmann) as described by Sherwood and Goldwasser (12). The cell extracts were frozen at −70°C.

RIA of Erythropoietin. The erythropoietin levels in the samples were determined in the RIA described by Sherwood and Goldwasser (13). The pure human urinary erythropoietin used as the ^{125}I tracer has been described by Miyake *et al.* (14) and was generously supplied by Eugene Goldwasser (University of Chicago) and by the Blood Diseases Branch, Division of Blood Diseases and Resources of the National Heart, Lung, and Blood Institute, National Institutes of Health. All erythropoietin standards and unknown samples were assayed in triplicate. Medium with and without additives, incubated without cells, served as assay controls.

Assay of Biological Activity. Erythropoietic activity in the culture medium was determined by the *in vitro* erythroid colony-forming unit (CFU-E) assay. For the *in vitro* assay, mouse bone marrow cells were established in culture by using the methylcellulose technique described by Iscove *et al.* (15) and erythropoietin standard or samples were added. Erythroid colonies (benzidine-positive) were counted after 48 hr of culture.

RESULTS

Nude Mouse Tumor. Only the ALS/anti-interferon-treated mouse developed a tumor. The morphology of the nude mouse tumor did not differ significantly from that of the human tumor obtained during nephrectomy. Both tumors had large vacuolated cells arranged in glandular type structures surrounded by fibrous septae, features characteristic of clear cell carcinoma. The cells were periodic acid/Schiff reagent (PAS) positive; however, diastase digestion eliminated the PAS staining in the vacuoles.

Development of Renal Carcinoma Cell Line. Cells obtained from the nude mouse tumor were established in culture dishes in complete medium. When several million cells were obtained, cells were cloned twice by the limiting dilution technique. Following recloning, two cell lines were established in culture and were designated RC-1 and RC-2. The present communication describes the RC-1 cell line.

Cloned lines consist of large vacuolated cells characteristic of human renal clear cell carcinoma. Cell size appeared to decrease with increasing passage number: cells from passage 37 had a saturation density of 2.95×10^6 cells in 60-mm culture dishes and those from passage 53, 6×10^6 cells. The cells grow as a homogeneous monolayer with loss of contact inhibition, which causes piling up of cells. Additional evidence for the neoplastic nature of RC-1 cells is supported by the observation that these cells can be cloned and grown in soft agar (16) (data not shown). These cells are free of mycoplasma contamination, as determined by Hoechst fluorescence stain (6).

Chromosome Analysis. Ten metaphases were counted from the RC-1 cell line at passage 61. The karyotype of the RC-1 cell line is of a human female, is compatible with that of a malignant, predominantly triploid cell, and includes two

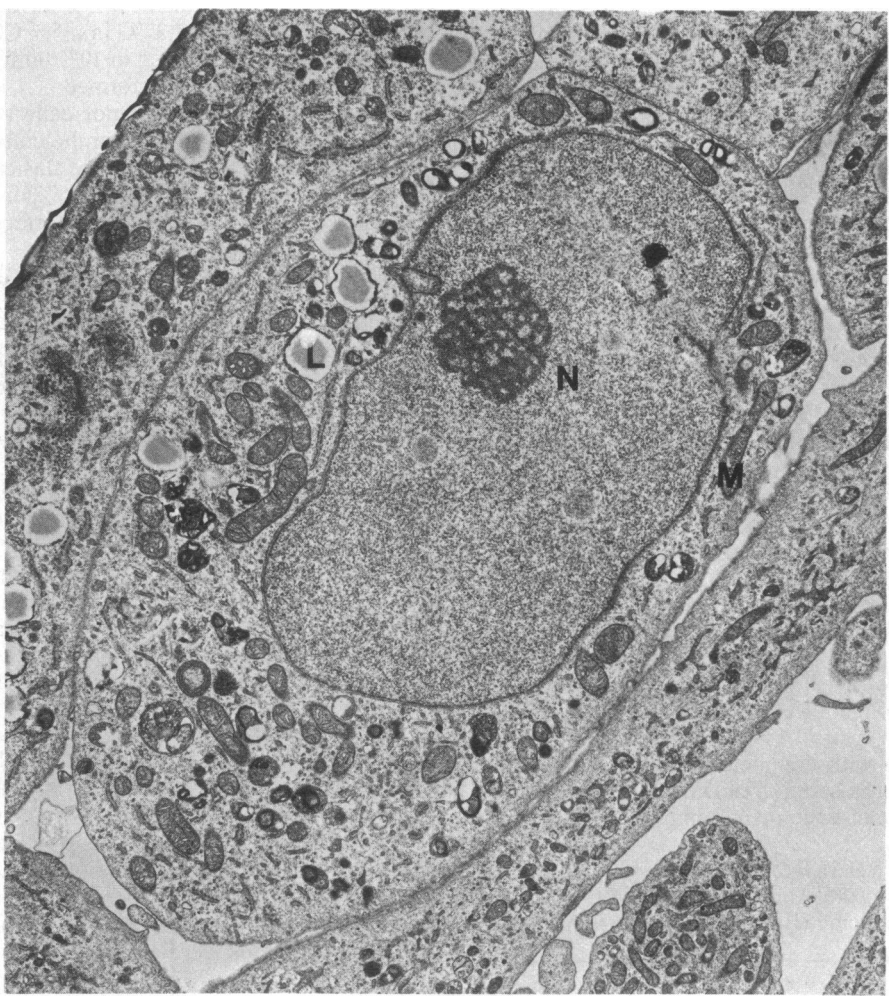

FIG. 1. Electron micrograph: RC-1 cell line from passage 35. N, nucleolus; L, lipoid droplets; M, mitochondrion. (×9900.)

marker chromosomes. The prevalent chromosome number per cell was 69. Structural anomalies such as translocations and deletions were observed. No evidence of HeLa cell contamination was present.

Ultrastructure of Cell Line. Cells from passage 37 of the RC-1 line were analyzed by transmission electron microscopy. Fig. 1 (9900× magnification) shows the prominent nucleolus and large lipoid droplets characteristic of these cells. Fig. 2 is a 78,000× magnification of two contiguous cells. Ultrastructural features characteristic of clear cell carcinoma can be clearly seen—i.e., the lamellar inclusion bodies, usually associated with the cellular secretion of lipid material, the elongated mitochondria, and the scattered glycogen granules. It has been suggested that the origin of these cells is the proximal convoluted tubule (17) (Fig. 2).

Growth Properties. Growth curve studies showed that the doubling time at the peak of the logarithmic growth slope was 48 hr for passage 37 cells, 29 hr for passage 40 cells, and 24 hr for passage 53 cells when complete medium was used. The doubling time for passage 53 cells grown in RPMI 1640 medium with 10% fetal bovine serum was ≈36 hr. Attachment efficiency at passage 53 was 80%.

Erythropoietin Production. Erythropoietin, determined in the RIA (13), was not detectable in the supernatant medium of cells in the exponential phase of the growth curve (performed at passage 37). In the plateau phase of the curve, 82 milliunits (mU) of erythropoietin was found per ml of unconcentrated medium.

When increasing amounts of concentrated culture supernatant medium were assayed, the displacement curves obtained were parallel to the curve produced by the pure erythropoietin standard, suggesting that the material produced by these cells behaved immunologically like human erythropoietin (Fig. 3).

Table 1 shows the immunoreactive erythropoietin activity of the culture medium obtained from several passages of the cells of the RC-1 line. Erythropoietin activity increased with passage number (Table 1), a phenomenon that we continue to observe. In comparison, cultures of normal mouse kidney and human kidney fibroblasts did not contain detectable erythropoietin levels (<5 mU/ml).

In the mouse CFU-E assay, unconcentrated culture supernatant fluids produced an increase in colony number that was significantly higher than that produced by human kidney fibroblast supernatant medium and control culture medium incubated without cells. In these studies the carcinoma line and control cells were maintained for 14 days without a medium change, with the proportion of cells to medium ≈10^6 cells per ml of supernatant medium. Table 2 shows that a dose-dependent stimulation of colony number occurred when the supernatant fluid (from passage 37 cells) was assayed in increasing amounts, with a 9.9-fold increase over background at 50 μl of supernate and a 16-fold increase at 100 μl. In comparison, the supernatant media from human kidney fibroblast cultures did not give an increased colony count (same as background). Although the control culture medium

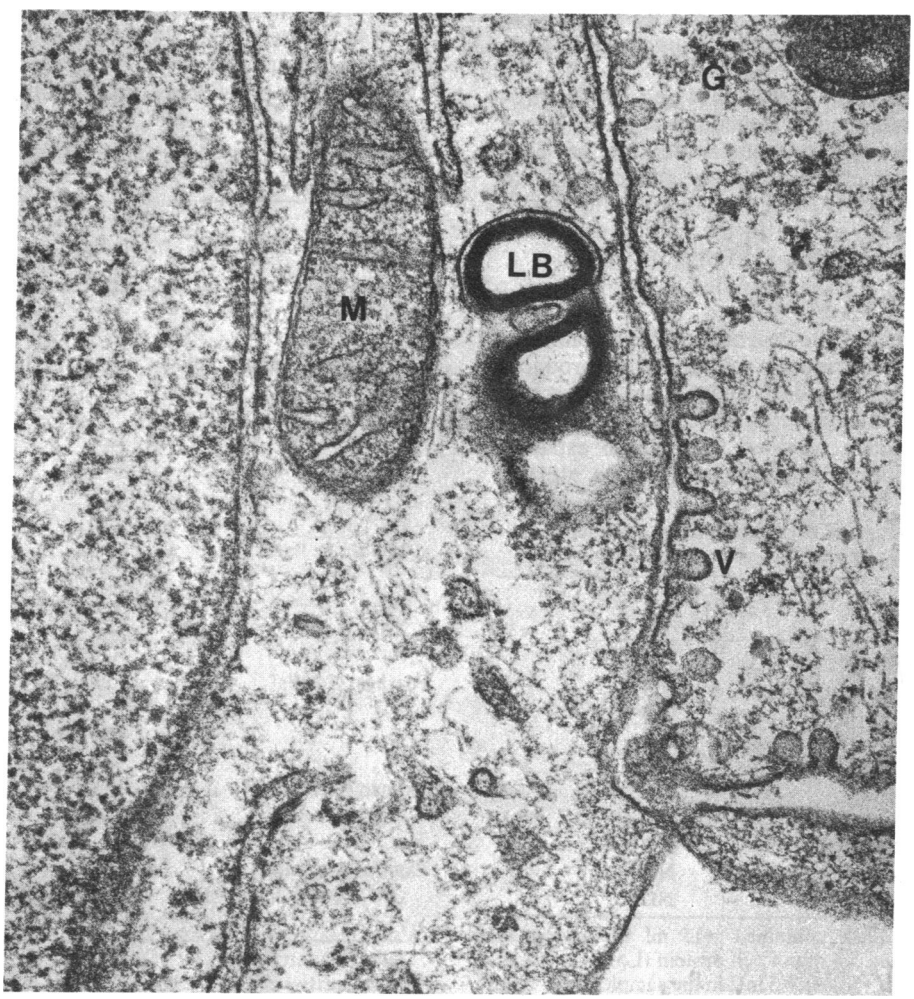

FIG. 2. Electron micrograph: Two contiguous cells of the RC-1 cell line from passage 35. LB, lamellar inclusion body; M, mitochondrion; G, glycogen granules; V, pinocytotic vesicle. (×78,000.)

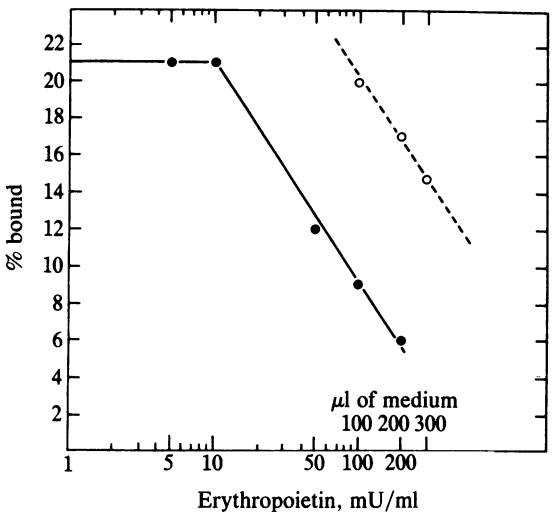

FIG. 3. RIA dose–response curves of human urinary erythropoietin (●) and culture supernatant medium from renal carcinoma cell lines (○).

with 10% fetal calf serum gave a 2-fold stimulation, a dose-related response was not observed.

Measurement of immunoreactive and biologically active erythropoietin levels in the supernatant fluids from passage 40 cells demonstrated the presence of ≈400 mU of biologically active (CFU-E assay) and 500 mU of immunoreactive (RIA) erythropoietin per ml of medium. In both assays, these values were significantly higher than those for the control preparations (human kidney fibroblast supernatants and control culture medium).

In studies using cells from passages 34 and 35, erythropoietin in unconcentrated culture medium was compared with intracellular erythropoietin obtained by extraction of the cells (12). The erythropoietin levels in the supernates were higher than in the cell pellets, with a ratio of erythropoietin (per flask) in the medium to that in the cells = 1.76:1 for passage 34 and 6.5:1 for passage 35 (Table 3).

DISCUSSION

In the studies reported here, we describe the development of a stable human renal carcinoma cell line that produces erythropoietin on a continuous basis and that has been grown in culture since 1981 and has been passaged >150 times. Several investigators have reported attempts to establish systems for the production of erythropoietin *in vitro*, utilizing both normal kidney cells (18–21) and transformed cells (2–5). In all human transformed cells and in systems utilizing normal cells, this capacity has been lost within a few passages from the primary cultures. One exception might be the human testicular germ cell line reported by Ascensao *et al.* (4), for which only a preliminary report exists with no long-term observations reported.

Therefore, in the present study, the nude mouse has been chosen as a temporary host for the primary human renal tumor in order to provide the best available physiologic conditions for selection and survival of the desired cells. Indeed, the use of the athymic mouse for selection of a stable renal carcinoma cell line that was later propagated independently in culture turned out to be invaluable. Samples of the primary tumors from the patient that were maintained in culture without initial passage through the nude mouse were lost within 3–6 weeks of primary culture. The rationale for the use of immunosuppressed nude mice transplanted with the primary tumor is based on a previous observation of Shouval *et al.* (7) that suppression of natural killer (NK) cell activity and of residual B cells in nude mice may contribute to significant augmentation of "take" rates and tumorigenicity of established human carcinoma cell lines. Therefore, primary renal tumor tissue was injected into athymic mice either untreated or immunosuppressed with three different protocols to maximize the chance of primary tumor growth. Only one tumor developed within 54 days of injection in an athymic mouse that was immunosuppressed by a combination of antiinterferon globulin and ALS. None of the other mice, regardless of treatment, produced a tumor. The tumor that was excised from the nude mouse adapted easily to growth in culture and gave rise to our cell line.

Several lines of evidence support our conclusion that this

Table 1. *In vitro* production of erythropoietin by a human renal carcinoma cell line

Cell type	Erythropoietin, mU/ml*
RC-1 line at passage	
1	7.4
14	10.0
16	12.7
20	12.5
27	24.0
31	26.0
Normal mouse kidney	ND
Human kidney fibroblasts	ND
Culture medium control	ND

Each 75-cm² culture flask contained ≈15 ml of supernatant medium, 5 × 10⁶ cells, and 5.4 mg of cell protein (Lowry method). In this RIA, not detectable (ND) is <5 mU in the sample. Incubation periods were 14 days.
*mU of erythropoietin in supernatant per ml of unconcentrated medium.

Table 2. Mouse CFU-E assay of erythropoietin produced by RC-1 cells in culture

Sample	Sample volume, μl	CFU-E per 5 × 10⁵ cells*	Mean % of control
Control (saline)	—	68	100
		72	
EPO standard, units			
0.01	—	88	120
		80	
0.05	—	116	167
		120	
0.10	—	124	183
		132	
0.50	—	192	286
		208	
1.0	—	232	331
2.0	—	240	351
		252	
Passage 37 cells†	50	692	991
		696	
	100	1144	1634
		1143	
Human fibroblasts‡	50	64	91
(control cells)	100	76	103
		68	
Control medium	50	192	274
(10% fetal calf serum)	100	164	237
		168	

EPO, erythropoietin.
*Bone marrow cells from BALB/c male mouse in Iscove's medium with methyl-cellulose and 10% fetal calf serum.
†Maintained in DMEM/F-12 medium with 5% fetal calf serum.
‡Maintained in DMEM/F-12 medium with 10% fetal calf serum.

Table 3. Erythropoietin in incubation medium and tissue during 14-day culture period

Culture	Erythropoietin per flask, mU		Ratio*
	Medium	Cells	
Passage 34	114 ± 20	1943 ± 201	1.76:1
Passage 35	420 ± 143	1943 ± 752	6.48:1

Each 150-cm^2 culture flask contained ≈30 ml of supernatant medium and 9.8 × 10^6 cells. The incubation period with the medium was 14 days. Erythropoietin levels were measured in the RIA. Each value represents the mean ± SD of two flasks.
*Ratio of erythropoietin in medium to erythropoietin in cells.

cloned renal carcinoma cell line is of human origin rather than derived from the nude mouse host: (*i*) RC-1 cloned cells have a human female karyotype; (*ii*) electron microscopic analysis of cloned cells revealed features that are characteristic of human renal clear cell carcinoma; (*iii*) DNA extracted from the cell line (passage 37) hybridized with a ^{32}P-labeled human genomic probe (second intervening sequence of human gamma globin gene). In studies using the same stringent hybridization conditions (incubation at 65°C overnight in low-salt buffer), mouse cellular DNA did not hybridize with this human probe (22) (data not shown).

The erythropoietic factor produced by this cell line is detectable in our RIA. Displacement curves produced by increasing amounts of RC-1 culture medium were parallel to that produced by human urinary erythropoietin used as standard, suggesting that the tumor erythropoietin shares some structural similarities with the native human hormone.

Although values found by CFU-E assay and RIA for passage 40 fluids show reasonable correspondence, the slightly higher erythropoietin level found in the RIA (500 versus 400 mU per ml of medium) may reflect the presence of hormone that is immunologically but not biologically active, as described by Sherwood and Goldwasser (23) for circulating human erythropoietin, or may simply reflect differences in the assay systems. Although other factors have been shown to affect CFU-E growth in this assay (24), the agreement with RIA values suggests that the CFU-E assay is measuring erythropoietin in these experiments.

The erythropoietic factor produced by this cell line appears to be biologically active *in vitro* and *in vivo*. In the *in vitro* mouse CFU-E assay (15), media from RC-1 cultures produced a dose-dependent increase in CFU-E number, in contrast to control samples. In studies to be reported elsewhere, RC-1 tumor-bearing nude mice developed significant splenomegaly and erythrocytosis with increased erythrocyte mass and volume, with no thrombocytosis or elevated leukocyte count. Nontumor-bearing mice and mice bearing human hepatoma and the RC-2 cloned line did not develop erythrocytosis.

Our observation that erythropoietin was detectable only in the supernatant medium of cells in the plateau phase of the growth curve—i.e., at confluence—and not in the logarithmic phase is in agreement with observations reported by Ascensao *et al.* (4) and Hagiwara *et al.* (3) for other erythropoietin-producing cells and may reflect terminal differentiation.

The significantly higher levels of erythropoietin in the incubation medium as compared with intracellular hormone levels suggest active synthesis of erythropoietin and secretion of this material during maintenance in tissue culture. Viability of the cultured cells was demonstrated by attachment to the substratum (flask surface) and by exclusion of trypan blue dye. The erythropoietin present in the cell pellet may represent storage pools of hormone.

Preliminary studies suggest that certain physiologic regulatory processes with respect to erythropoietin production appear to be present in the RC-1 cells. When the RC-1 cells from passage 72 were incubated under 3%, 18%, and 95% O_2, an inverse correlation between oxygen concentration and erythropoietin levels in the medium was observed (1240 ± 100 mU of erythropoietin per ml of medium for 3% O_2, 871 ± 134 mU/ml for 18% O_2, and 355 ± 17 mU/ml for 98% O_2). Addition of 10 mM cobaltous chloride to passage 75 cells was associated with an erythropoietin level of 486 ± 50 mU/ml of medium as compared with 89 ± 31 mU/ml for unstimulated cells. A secretory response to cAMP has been observed (25), studies that we will report elsewhere.

The RC-1 line has now been passaged >150 times and continues to synthesize and release erythropoietic factor into the culture medium. We therefore have a cell line that provides an *in vitro* model system to study the regulation of erythropoietin synthesis and secretion. This system provides considerable advantages over *in vivo* systems in terms of elimination of cellular heterogeneity and ability to define and vary conditions, such as oxygen tension and additives, in a controlled manner.

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