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Colony-Stimulating Activity in Cultures of Granulocytosis-Inducing Tumor[1, 2] (19610)

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Granulocytosis is sometimes associated with malignant tumors arising in other than hemopoietic tissue. A "leukemoid" syndrome including splenomegaly, extreme leukocytosis, reversal of the granulocyte-lymphocyte ratio, and a mild anemia was described by Bateman (1) as an accompaniment to certain transplantable myoepitheliomas of mice. Earlier history and current status of the field have been reviewed recently (2, 3). Mechanisms underlying the syndrome remain unexplained but in recent years attention has been directed to possible involvement of granulopoietic factors elaborated by the tumors (4–7).

We have studied a granulocytosis-inducing tumor which occurred in a Swiss albino mouse of the Brookhaven (Hale/Stoner) strain and has been carried through 38 animal passages over a period of 25 months. The tumor is a mammary adenocarcinoma, the most commonly occurring spontaneous tumor in this strain (8). Our interest has focused on hematopoietic cell kinetics in host mice, to be reported elsewhere (unpublished data), and features of cell cultures of tumor tissue and the products elaborated by these cultures.

Colony-stimulating activity (CSA) is produced in high amounts over extended time periods in these cultures, making the system especially interesting and potentially valuable because, unlike other sources of CSA, these cells have the capacity to evoke, *in vivo*, gross changes in granulopoiesis. In our view, this considerably enhances the likelihood that the culture-derived products are physiologically important regulators.

Methods. Tumor is passaged every 20–22 days in 3- to 4-month-old Brookhaven strain mice by sc inoculation of 0.5 ml of a 10% (w/v) tumor cell mince in McCoy's modified medium. Hosts in passages 1–4 received 350 rad whole body irradiation before tumor inoculation. Subsequent passages (41 to date) have been carried in unirradiated hosts inasmuch as growth characteristics and hemopoietic response were found to be no different in irradiated or nonirradiated hosts.

Cell cultures, established from either tumor mince or trypsinized tumor cell suspensions, are grown in McCoy's medium plus 10% fetal calf serum (FCS) as monolayers or in suspension in spinner medium containing 2–5% FCS. Once the cultures are established, medium is collected every 3 to 8 days and stored at −20° for later treatment and assay.

Colony-stimulating activity is assayed by the agar plate method as utilized by Robinson *et al.* (9). Sample or standard stimulus (L-cell conditioned medium) is added along with 10^5 nucleated mouse marrow cells in 1 ml of McCoy's agar to 35-mm culture dishes and incubated for 7 days at 37° in a humidified 7.5% CO_2 atmosphere. Groups of 50 or more cells are scored as colonies. Samples were assayed in three to five dishes. Inhibitor to CSA was measured in cultures supplied with standard stimulus and test sample. The decrease in colonies as compared to standard stimulus alone indicated the degree of colony inhibition by the test sample.

Preparation and assay of antibody to col-

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[2] The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[3] Scholar of the Leukemia Society of America, Inc.

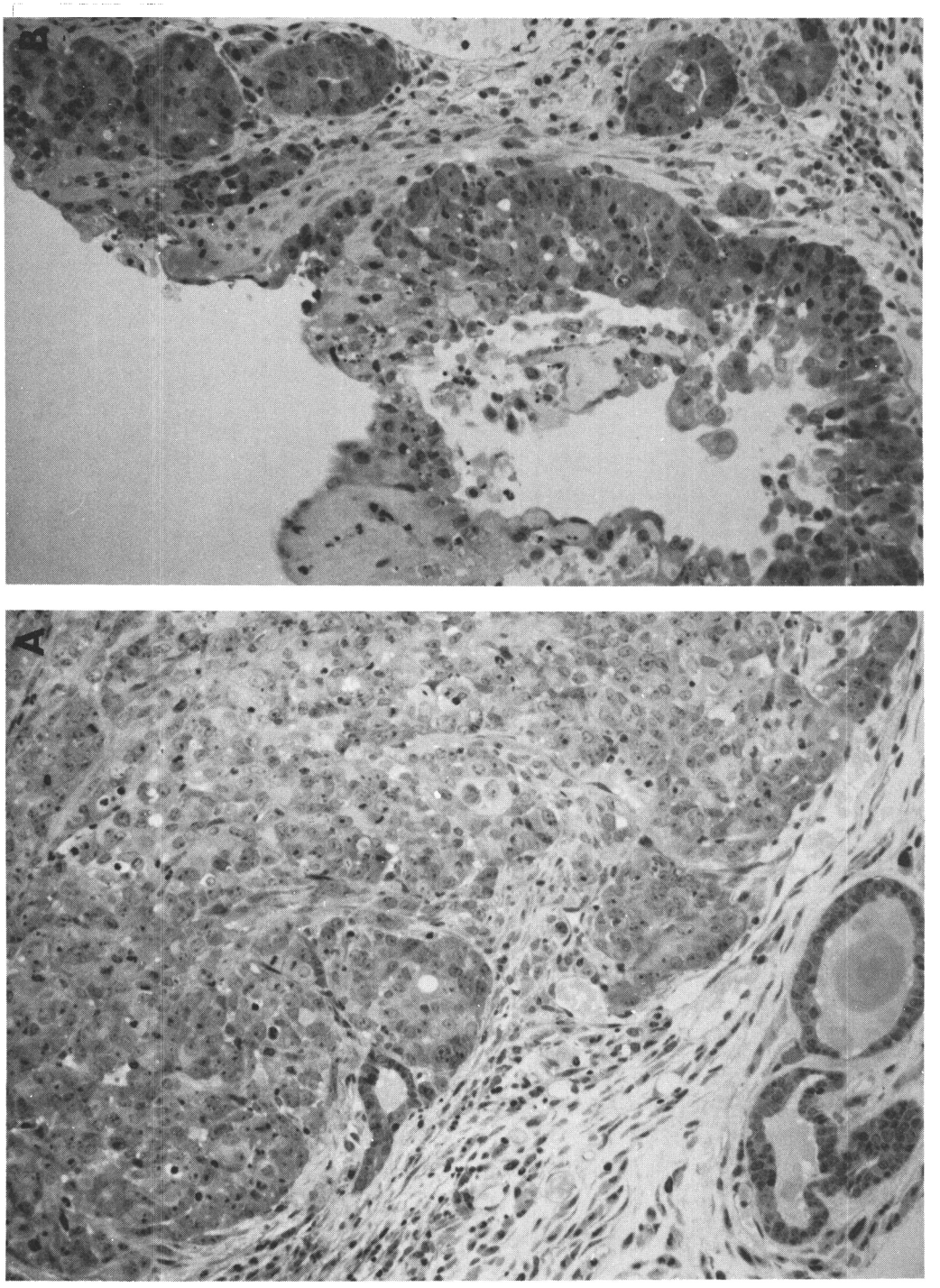

FIG. 1. Mammary adenocarcinoma; original tumor. (A) Predominantly solid components. (B) Cystic area. Hematoxylin and eosin, 140 ×.

ony-stimulating factor (CSF) was as previously described (10). Briefly, partially purified L-cell CSF was emulsified with complete Freund's adjuvant and used for immunization of rabbits. The resultant antiserum was absorbed four times with mouse red cells to remove nonspecific cross-species antibodies. The neutralizing capacity using various dilutions of 0.1 ml of antiserum was measured in the CSF assay.

Results and Discussion. Tumor characteristics. The tumor is a mammary carcinoma which has remained unchanged in morphology throughout 41 passages (Fig. 1). It bears close resemblance to the adenocarcinoma type A (11) and is composed of epithelial cells arranged in solid sheets, tubular, and cribiform structures. Some areas are cystic. The stroma is usually scarce. The core is frequently necrotic and hemorrhagic. The host may show metastatic involvement of lymph nodes and spleen. Etiology is unknown. Electron micrographs of tumor tissue failed to reveal presence of viral particles. Furthermore, ultrafiltrates of tumor homogenates did not result in tumor growth when inoculated into new-born hosts, suggesting a nonviral origin.

Subcutaneous inoculation of 0.5 ml of a 10% tumor cell mince produces palpable tumors in at least 90% of recipients in 5–7 days. By 10–14 days, the diameter is 10–20 mm. Recipients usually succumb 21–30 days postinoculation bearing ulcerated tumors up to 50 mm in diameter. Host age or sex has no appreciable influence on tumor characteristics or the ensuing hematopoietic syndrome.

Hematopoietic response. Granulocytosis develops progressively with tumor, reaching counts greater than 100,000/mm^3 in some mice (Fig. 2). Differential white cell counts show granulocytes constituting 70–80% of the total, with mainly lymphocytes making up the remainder. Banded granulocytes are also increased and no cells less mature than band forms appear in peripheral blood. Extirpation of 80% or more of tumor results in a return of white cell number toward normal but regrowth provokes return of leukocytosis (Table I). The spleen, although grossly enlarged and remarkably granulopoietic (Fig. 3), appears not to be essential to development of the granulocytosis (unpublished data). Anemia is also a consistent component of the response (Fig. 4).

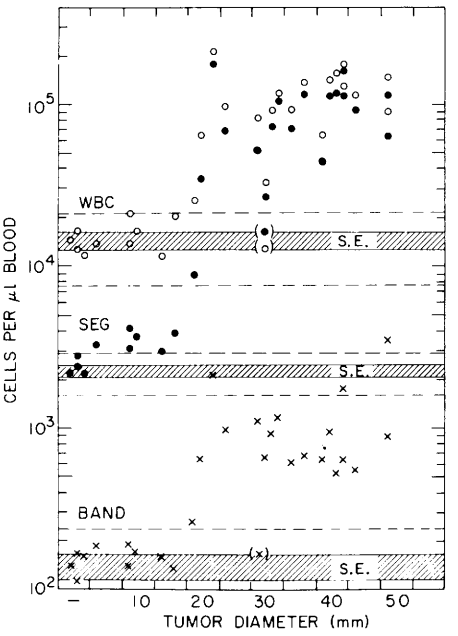

FIG. 2. Granulocytosis during tumor growth. Total white blood cells (open circles), segmented granulocytes (closed circles), and banded granulocytes (crosses) are plotted against the largest tumor diameter. The graph contains data of three experiments (passages 4, 13, 17). Each tumor mouse is represented once. The pooled values of medium-injected control mice are shown as shaded bands (mean ± standard error) and hatched lines (mean ± standard deviation).

TABLE I. INFLUENCE OF TUMOR REMOVAL AND RECURRENCE ON WHITE BLOOD COUNTS.

Mouse no.	Tumor diameter (mm)	Treatment	Blood counts/μl WBC	Blood counts/μl RBC (million)
1	30	Extirpation	68,000	6.55
	20	5 Days later	32,000	5.76
2	25	Extirpation	78,800	6.45
	>20	5 Days later	30,600	5.64
	30	9 Days later	67,000	6.94
3	25	Extirpation	92,000	5.77
	–	5 Days later	18,800	5.52
	15	9 Days later	56,200	6.34

Tumor in culture. Monolayers originating from either tumor mince or trysinized suspensions consist of mixed epithelial-like and

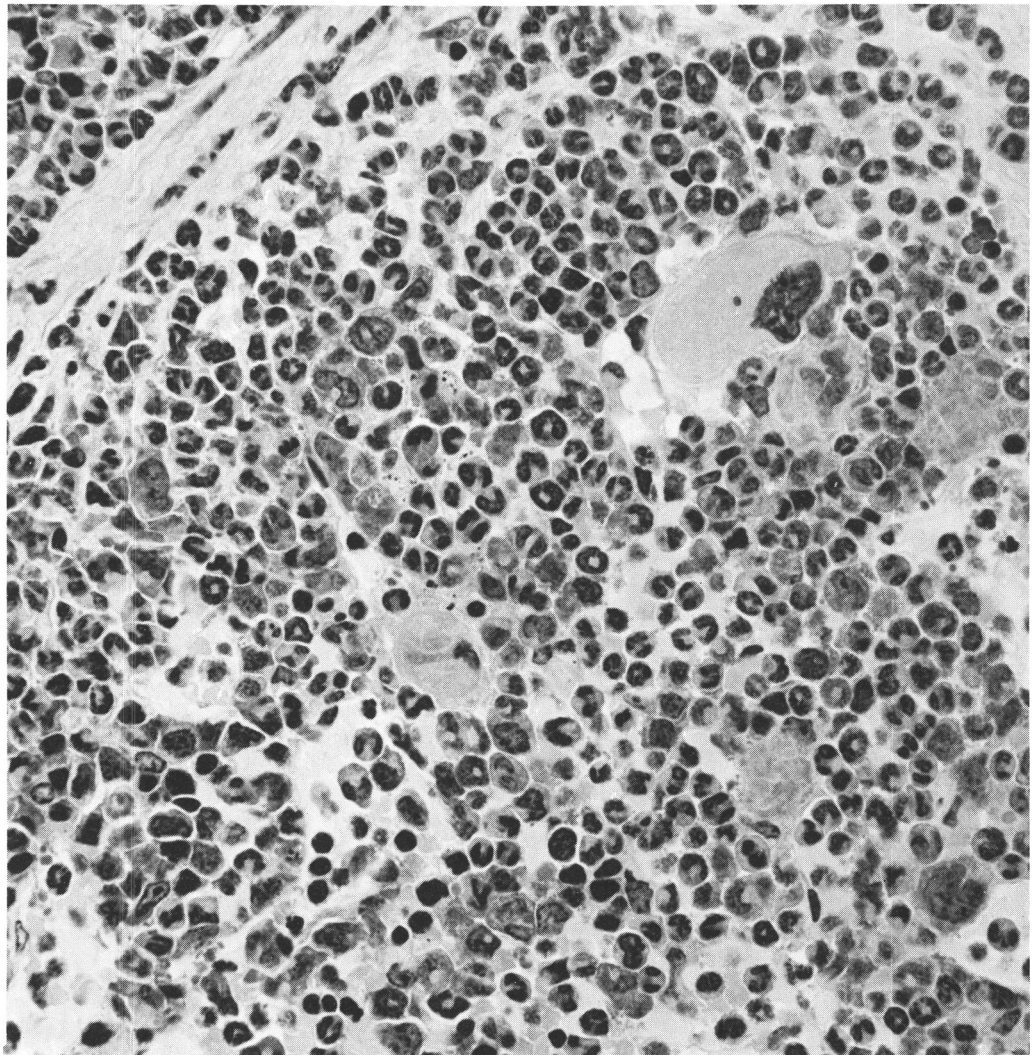

FIG. 3. Spleen of original tumor host, red pulp: granulopoiesis predominates. Small erythropoietic foci are occasionally seen (Bottom). Hematoxylin and eosin, 500 ×.

fibroblastic cells (Fig. 5). Typically the cells display lack of contact inhibition, a high rate of glycolytic and protein synthetic activity, and a capacity for continued propagation when passaged repeatedly. Suspension cultures can be established from monolayers or from fresh trypsinized tumor. They have been maintained for periods up to 75 days yielding high levels of CSA at all stages of culture.

Subcutaneous injection of the cultured cells generates mammary adenocarcinoma with unchanged host response after multiple *in vitro* passages. Similarly, cultured cells which had been frozen and stored for 17 months displayed unaltered tumorigenicity and capacity for CSA production *in vitro*.

CSA production in culture. Both monolayer and suspension cultures consistently yield CSA to the culture medium. Activity is present in successive media collections made at 3- to 8-day intervals over periods extending to 75 days. Table II presents typical findings from two cultures. It should be noted that heat (90°, 15 min), in addition to abolishing CSA, appears to unmask or generate inhibitor activity. Media conditioned by primary cultures of mouse lung and kid-

ney and a cell line derived from rat prostatic tumor associated with leukemia had negligible CSA when assayed simultaneously as controls for mammary tumor culture media.

Characteristics of culture-derived CSA. Heat lability is a property of CSF which is presumed to be a large molecular weight glycoprotein (12). The CSA associated with tumor culture, is also inactivated by high temperature, is nondialyzable, and, from results of studies utilizing ultrafiltration membranes, the activity is associated with a fraction having a molecular weight > 30,000. This would distinguish the factor produced by our tumor cell cultures from the material studied by Delmonte and Liebelt extracted from a transplantable mouse mammary carcinoma (4). The extractable granulocytosis-producing factor was initially stated by these authors to possess physicochemical features comparable to those of erythropoietin, e.g., thermostability, pH stability, and nondialyzability. However, modification of the extraction procedure later yielded a product which the authors now characterize as a protein-free, thermostable, dialyzable (M.W. < 2000) micromolecule.

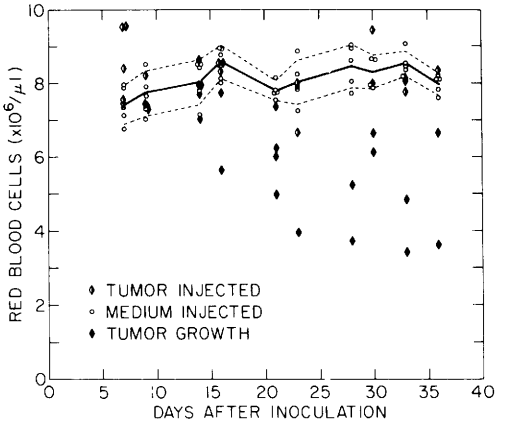

FIG. 4. Tumor-associated anemia. These data were obtained from early passages in which host mice received 350 rad whole body irradiation before tumor injection. The irradiation had no effect upon red or white cell count. It was found that preirradiation of hosts is unnecessary and unirradiated hosts have been used since passage 4.

Further characterization of the culture-derived CSA is needed before it can be equated to CSF. One strong evidence that the material is actually CSF comes from the results shown in Table III. Antibody to L-cell CSF completely neutralized the activity of tumor conditioned medium at dilutions up to 1:64. Antibody dilution (1:128), which neutralized only 20% of CSF of L-cell-conditioned medium, still abolished 88 and 72% of activity in tumor culture-condi-

TABLE II. COLONY-STIMULATING ACTIVITY IN MEDIA OF TUMOR CELL CULTURES.

Culture type	Culture age (days)	Collection interval (days)	Average number of colonies[a]		Inhibitor activity[b] (%)	
			Untreated[c]	90°, 15 min	Before 90°	After 90°
Suspension culture of mouse mammary tumor	25	3	64.7	0	11	n.d.
	33	8	72.3	0	19	n.d.
	53	7	95.3	0	5	34
Monolayer culture of mouse mammary tumor	26	5	149.3	0	0	35
	59	7	59.3	0	0	25
	75	2	36.3	0	37	n.d.
Monolayer culture of rat prostatic tumor cells (control)[d]	20	6	20.3	0	18	n.d.
	39	3	15.7	0	17	n.d.
	50	3	12.7	0	7	n.d.
Standard[e] (1) 0.03 ml			138.5			

[a] Colony = group of 50 or more cells. Average of counts in three agar plates containing 0.15 ml of tumor cell-conditioned culture medium in a total volume of 1 ml. Each plate contained 10^5 normal mouse marrow cells.
[b] Activity in 0.1 ml of tumor cell culture medium which inhibits the expected response to standard stimulus.
[c] All samples dialyzed against distilled water at 4° for 72 hr.
[d] Rat prostatic tumor cells associated with a leukocytosis.
[e] L-cell-conditioned medium.

FIG. 5. Tumor-derived cells in monolayer culture (10 days). Hematoxylin and eosin, 250 ×.

tioned medium, further suggesting that the observed colony-stimulating activity is probably CSF.

The cell culture system reported here appears to have special value in studies of regulation of granulopoiesis. By virtue of originating from a tumor which evokes massive hematopoietic perturbations in host an-

TABLE III. NEUTRALIZATION OF COLONY-STIMULATING ACTIVITY IN TUMOR-CONDITIONED MEDIUM BY ANTIBODY TO CSF.[a]

Antibody dilution	Mixture: 0.1 ml of antibody to CSF, plus: 0.05 ml of L-cell-conditioned medium		0.05 ml of tumor-conditioned medium	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
1:2	0[a]	0	0	0
1:4	0	0	0	0
1:8	0	0	0	0
1:16	0	0	0	0
1:32	4	5	1	0
1:64	17	23	0	3.5
1:128	82	87	9.5	22
1:256	n.d.	n.d.	n.d.	60
1:512	n.d.	n.d.	n.d.	68
Medium without antibody	104	105	n.d.	76

[a] Values represent the average number of colonies in three to five culture plates.

imals the culture-generated activity is more apt to be specifically related to physiologically important *in vivo* regulators of granulopoiesis than are other culture-derived factors. However, direct evidence awaits availability of enough purified material for testing in animals.

Summary. A transplantable mouse mammary carcinoma-producing granulocytosis in host mice has been established in cell cultures. Colony-stimulating activity (CSA) is released to the medium by both monolayers and suspension cultures over sustained periods. The CSA is heat labile and nondialyzable with an apparent molecular weight > 30,000. It is neutralized by antibody to colony-stimulating factor (CSF), suggesting that the material produced in tumor cell cultures is in fact CSF.

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