

Prolactin-stimulated Growth of Cell Cultures Established from Malignant Nb Rat Lymphomas¹

Peter W. Gout, Charles T. Beer,² and Robert L. Noble

Department of Biochemistry, University of British Columbia, Vancouver V6T 1W5 [P. W. G., C. T. B.], and Department of Cancer Endocrinology, Cancer Control Agency of British Columbia, Vancouver V5Z 3J3 [R. L. N.], British Columbia, Canada

ABSTRACT

A malignant Nb rat lymphoma which *in vivo* is stimulated by estrogens has been established in suspension culture. The cultured cells grew readily in Fischer's medium supplemented with fetal calf serum (10%) and 2-mercaptoethanol (10^{-4} M). If horse serum was substituted for fetal calf serum, population growth ceased; *i.e.*, cultures became "stationary." Such stationary cultures could be induced to resume active growth by the addition of a pituitary hormone, prolactin (ovine, rat); concentrations as low as 10 pg/ml had a detectable effect. In contrast, other pituitary hormones or estrogens had little or no effect. The evidence in this and an accompanying paper suggests that prolactin (or related substances) has a role in the growth of some cancers of lymphoid origin in rats.

INTRODUCTION

A number of malignant lymphomas have arisen in the lymph nodes and thymus of Nb rats. Transplants of these lymphomas have shown varying degrees of dependency on estrogen for growth in the host (7, 10, 11), and in some cases they have been found to respond dramatically to treatment with *Vinca* alkaloids (9). In view of these properties, they appear to provide good models for study of the role of estrogens in the growth of lymphomas (4, 7, 8, 10) and the mechanism of oncolytic action of *Vinca* alkaloids (5, 9). Since many of the complexities inherent in studies with tumors *in vivo* may often be avoided by working with isolated cell systems, we have investigated methods for establishing permanent cultures of some of the Nb lymphoma lines. This paper describes a method for preparing continuous suspension cultures of cells isolated from one of the lymphoma lines, the Nb 2 node. It also describes how the cultures may be used for detecting "lymphoma cell growth-promoting activity" of sera, hormones, and other compounds. It has been found that prolactin, a pituitary hormone, was extremely active in stimulating the growth of the lymphoma cell cultures. A following paper (8) presents evidence that the blood of rats has lymphoma cell growth-promoting activity which is subject to control by a secretion from the pituitary gland.

MATERIALS AND METHODS

Tumor Line. Reticulum cell lymphomas occurring in Nb rats which involve only the lymph glands or thymus have been termed "node lymphomas." The lymphoma used in the present study, the Nb 2 node, arose in a lymph node of an estrogenized

male Nb rat and is described in greater detail elsewhere (8, 9). It has been maintained for over 7 years by serial transplantation into estrogenized rats using methods already described (10). In estrogenized males, the tumor grows approximately twice as rapidly as in untreated males; tumor growth is also accelerated in rats bearing transplanted pituitary tumors (8). The lymphoma is highly sensitive to treatment of the host with anticancer *Vinca* alkaloids (9).

Isolation and Culturing of Tumor Cells. Pieces of freshly excised tumor, free of necrotic and hemorrhagic areas, were minced in Fischer's medium, and the resulting suspension was filtered through stainless steel gauze (5). The cells in the filtrate were collected by centrifugation (3 min at $300 \times g$) and resuspended in Fischer's medium supplemented with 10% FCS,³ penicillin (50 units/ml), streptomycin (50 μ g/ml), and (10^{-4} M) 2-ME. The cell suspensions were incubated at 37° in plastic tissue culture flasks (Falcon) in an atmosphere of 5% CO₂-95% air. During incubation, essentially all the tumor cells remained in suspension. Population growth was monitored with a hemacytometer or an automatic cell counter (Coulter).

Preparation and Use of "Stationary" Cultures. The Nb 2 node lymphoma cell cultures could be induced to enter a phase of stationary growth by transferring the cells to medium supplemented with HS instead of FCS. Cultures of the cells in the logarithmic growth phase were centrifuged (3 min at about $300 \times g$). The packed cells were resuspended in Fischer's medium supplemented with 1% FCS, 10% HS, and 10^{-4} M 2-ME and incubated for about 20 hr at 37°, during which period there was a marked decline in the growth rate of the culture. The cells were then transferred to medium from which FCS had been omitted, *i.e.*, supplemented with 10% HS and 10^{-4} M 2-ME only (cell concentration, 2.5×10^5 cells/ml). In this medium, culture growth was essentially stationary. The "lymphoma cell growth-promoting activity" of hormones and other factors was assessed by measuring their ability to induce these stationary cultures to resume growth. Solutions of the hormones in Fischer's medium containing 10% HS (0.6 ml) were added to aliquots (5.4 ml) of the stationary cultures (25 sq cm culture flasks); this was usually done within 2 to 3 hr of transferring the cells to the medium supplemented only with HS and 2-ME. Triplicate cultures were incubated at 37° for 3 days; the cell population was determined daily.

Sera and Hormones. FCS, calf serum, and HS were obtained from GIBCO Canada, Calgary, Alberta, Canada. Prolactin (ovine, "highly purified by exclusion chromatography," NIH-P-S13), growth hormone (ovine, NIH-GH-S11), luteinizing hormone (bovine, NIH-LH-B2), and follicle-stimulating hormone (ovine, NIH-FSH-S2) were gifts from the NIAMDD. Rat prolac-

¹ Supported by grants from the National Cancer Institute of Canada, the Medical Research Council of Canada, and Eli Lilly and Co., Indianapolis, Ind.

² Career Investigator of the Medical Research Council of Canada.

Received December 19, 1979; accepted April 14, 1980.

³ The abbreviations used are: FCS, fetal calf serum; 2-ME, 2-mercaptoethanol; HS, horse serum; NIAMDD, National Institute of Arthritis Metabolism and Digestive Diseases; ACTH, adrenocorticotrophic hormone.

tin-B-2 was kindly supplied by Dr. A. F. Parlow of the NIAMDD Rat Pituitary Hormone Distribution Program. The following compounds were obtained from the Sigma Chemical Company, St. Louis, Mo.: ACTH (porcine, Sigma A6002); thyroid-stimulating hormone (bovine, Sigma TS-10); α -melanocyte-stimulating hormone (Sigma M4135); insulin (bovine pancreatic, Sigma I5500); fetuin (from FCS, Sigma F2379); vasopressin (Sigma V6752); oxytocin (Sigma O4250); estrone (Sigma E9750); and 17β -estradiol (Sigma E8875). 2-ME (Aldrich M 370-1) was purchased from the Aldrich Chemical Company, Milwaukee, Wis.; stock solutions (5 mM) in 0.9% NaCl solution were stored frozen (-20°). 2-ME was added to the culture medium immediately before use.

RESULTS AND DISCUSSION

Establishment of Nb 2 Node Lymphoma Cell Cultures.

Permanent cultures of Nb 2 node lymphoma cells in Fischer's medium supplemented with 10% FCS and 10^{-4} M 2-ME were readily established by following the procedure described above. The cultures consisted of suspensions of single cells, which microscopically appeared as large, well-differentiated mononuclear cells (8). The preference of the cells to remain in suspension instead of attaching to the substrate was of considerable advantage both in monitoring population growth and in freeing the freshly isolated tumor cells from contaminating fibroblasts. The doubling time of newly established cultures was about 60 hr; the growth rate steadily increased over a period of several months to reach a constant doubling time of about 20 hr (Chart 1, 10% FCS). Cultures have now been maintained continuously by twice-weekly subculturing for over 15 months. Cells frozen and stored in medium containing dimethyl sulfoxide (8%) could be readily revived. Even after

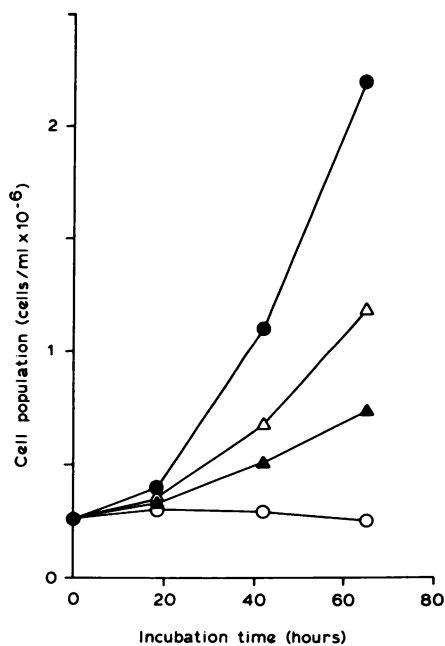


Chart 1. Effect of FCS and HS on growth of cultured Nb 2 node lymphoma cells. Cells were transferred from propagation medium, as described in "Materials and Methods," to Fischer's medium supplemented with FCS and/or HS, plus 2-ME (10^{-4} M) and antibiotics for incubation at 37° . Cell populations were determined at intervals using a Coulter electronic cell counter. Points, mean values of triplicate flasks (S.D., $\pm 3\%$ of mean). ●, FCS (10%); △, FCS (1%) and HS (10%); ▲, FCS (0.5%) and HS (10%); ○, HS (2.5, 5, 10, or 20%).

long periods in culture, the cells, when injected into Nb rats, gave rise to tumors identical to the original lymphoma in virulence, stimulation by estrogen (8), and inhibition by *Vinca* alkaloids (9). Cells from these tumors could be reestablished in culture. The culturing procedure has been used successfully not only for the Nb 2 node lymphoma but also to establish lines from 7 other lymphomas.

2-ME, a thiol already reported to be useful in establishing cultures of other lymphomas (1), was an essential component of the medium used to establish the Nb 2 node lymphoma cell cultures. In the absence of 2-ME, cells died after 2 to 3 days; they grew most rapidly at a 2-ME concentration of 10^{-4} M. After a few months in culture, the cells showed a diminished requirement for 2-ME and could eventually be maintained in its absence. These cells, when injected into rats, gave rise to tumors which again were sensitive to estrogen. To reestablish cultures from these tumors, the presence of 2-ME was again required.

FCS was found to be satisfactory as a medium supplement for the maintenance of the cultures; maximum growth rate was obtained at FCS concentrations greater than 5%. Calf serum could be used instead of FCS.

Effect of Hormones on Stationary Lymphoma Cell Cultures. In contrast to medium supplemented with FCS, medium supplemented with HS (up to 20%) did not support the growth of the cultures. As shown in Chart 1, population growth ceased if the cells were transferred to medium containing HS instead of FCS; *i.e.*, the cultures became stationary. On continued incubation, the cells gradually diminished in size and eventually died. Five different batches of HS have all given a similar result. However, medium containing HS (10%) would support growth if in addition low concentrations of FCS (*e.g.*, 0.5%) were present (Chart 1). This observation indicated that HS was not inhibitory or cytotoxic but was instead deficient in essential growth factors which were present in FCS. This raised the possibility that the stationary cultures could be used for the detection of specific factors promoting the growth of the lymphoma cells.

Table 1 and Chart 2 show the effects of adding a variety of specific compounds, including estrogens and pituitary hormones, to stationary lymphoma cell cultures. The hormones were chosen on the basis of evidence that estrogens and pituitary hormones might play a role in the growth of the lymphomas (4, 7, 8). Prolactin (ovine, rat) was the only hormone found to be highly active in stimulating population growth. The ovine hormone (Chart 2) induced active growth at a concentration as low as 0.05 ng/ml; there was a detectable effect at 0.01 ng/ml. Stimulation of culture growth was a function of the ovine prolactin concentration in the range of about 0.01 to 10 ng/ml. At 10 ng/ml, the doubling time of the cultures was about 20 hr, a time similar to that of cultures containing 10% FCS (Chart 1); this appears to be the maximum growth rate of the cultures. Higher prolactin concentrations (*e.g.*, up to 10^4 ng/ml) had no additional stimulatory effect on growth rate, nor were they inhibitory. There was excellent agreement between replicate flasks and from experiment to experiment. A preparation of rat prolactin has given results of the same magnitude as those obtained with the ovine hormone (Table 1). Subcultures which had become independent of 2-ME for growth continued to respond typically to prolactin in the absence of 2-ME. The stimulation of culture growth by prolactin did not

Table 1

Effect of hormones on growth of stationary Nb 2 node lymphoma cell cultures

Hormones, dissolved in Fischer's medium containing HS (10%), were added at the concentrations shown to stationary cultures of Nb 2 node lymphoma cells as described in "Materials and Methods." The effect of the hormones on culture growth is expressed as the percentage change in the population after incubation for 65 hr at 37°. Estrone, 17 β -estradiol (10⁻⁵ to 10⁻¹³ M) and vasopressin, and oxytocin (10⁻⁷ to 10⁻¹² M) did not stimulate culture growth.

Hormones	% increase in cell population after 65 hr incubation at following hormone concentrations ^a			
	10 ³ ng/ml	10 ² ng/ml	10 ng/ml	1 ng/ml
Prolactin (ovine)	741	726	759	666
Prolactin (rat)	791	787	707	518
Growth hormone (ovine)	678	524	135	22
ACTH (porcine)	510	135	4	0
Luteinizing hormone (bovine)	}	}	}	}
Follicle-stimulating hormone (ovine)				
Thyroid-stimulating hormone (bovine)				
α -Melanocyte-stimulating hormone				
Insulin (bovine pancreatic)				
Fetuin (from FCS)	0	0	0	0

^a Results are the averages of 2 to 5 experiments (S.D., \pm 4% of mean).

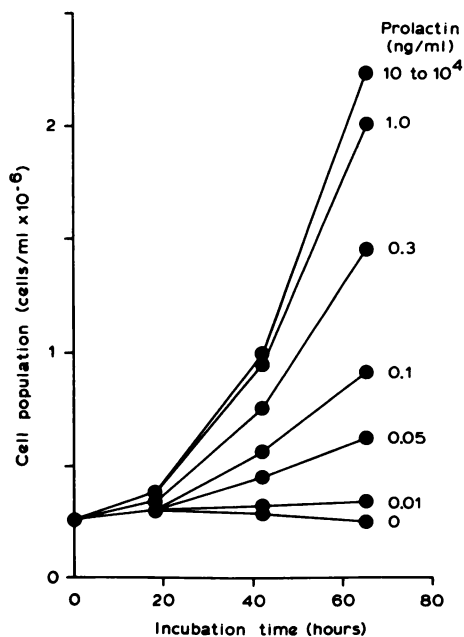


Chart 2. Stimulation of growth of stationary Nb 2 node lymphoma cell cultures by prolactin. Ovine prolactin, at the concentrations indicated, was added (0 hr) to stationary cultures of Nb 2 node cells (see "Materials and Methods"). The cultures were incubated at 37° and the cell populations were determined at daily intervals. Points, the mean values of triplicate flasks (S.D., \pm 3% of mean).

therefore depend on the presence of 2-ME in the medium.

Of the other pituitary hormones examined, only growth hormone (ovine), and to a lesser extent ACTH (porcine), had any growth-stimulating activity (Table 1). On a weight basis, however, growth hormone and ACTH were both much less active than prolactin, having, respectively, less than one-hundredth and one-thousandth of the activity shown by the prolactin preparation. These low activities may be due to the presence in the samples of small amounts of prolactin as an impurity or may possibly be intrinsic properties of the hormones.

The ovine prolactin preparation (NIH-P-S13) used in this

study was a highly purified preparation supplied by the NIAMDD, and its very high culture growth-promoting activity relative to the other 8 pituitary hormones examined strongly suggests that the growth-stimulatory effects observed are due to prolactin *per se*.⁴ The present evidence does not exclude the possibility that the prolactin sample contains trace amounts of some other, presently unknown, lymphoma growth-promoting factor. It has been found, however, that the activity of the prolactin preparation survives treatments which inactivate the pituitary fibroblast growth factor, incubation with 1% formic acid or 1 N acetic acid for 20 hr at 4° (2).

It may be noted (Table 1) that insulin, which has been reported to have growth-promoting activity in a number of cell systems (3), and fetuin, a major component of fetal calf serum, did not stimulate the growth of the stationary lymphoma cell cultures. It is of particular interest that, in contrast to the acceleration of lymphoma growth by estrogens *in vivo*, neither estrone nor estradiol, added to the medium over a wide concentration range, had any effect on the growth of the cultured lymphoma cells (Table 1).

The cultured Nb 2 node lymphoma cells have retained important characteristics of the lymphoma from which they were derived. In view of this, they appear to be good tools for studying various aspects of the growth of the malignant lymphoma *in vivo*, including its response to hormonal stimuli. Stationary lymphoma cell cultures have been found to be particularly useful for studies of this type as shown in a following paper (8) in which evidence is presented that the growth of the Nb 2 node lymphoma *in vivo* is promoted by factor(s) in the blood which are of pituitary origin. It appears probable, in the light of the findings in this communication, that these lymphoma growth-promoting factors include prolactin or prolactin-like compounds.

The evidence that prolactin may be essential for the growth of the Nb 2 node lymphoma cells may have a bearing on the remarkable sensitivity of the lymphoma in the rat to treatment with the oncolytic alkaloids vinblastine and vincristine (9). The well-known antimetabolic effects of the alkaloids are almost certainly a major factor in the regression of the tumor. The alkaloids, however, have also been shown to interfere with the glandular secretion of hormones as reviewed in Ref. 12. It therefore seems possible that alkaloid administration could affect the blood levels of prolactin or similar lymphoma growth-promoting factors and perhaps thereby contribute indirectly to the oncolytic effects of the alkaloids. It is of considerable interest that the alkaloids have been found to be markedly less effective against the node lymphoma in estrogenized or pituitary tumor-bearing rats.⁵ It may also be relevant that the acute toxicity of vinblastine is reduced in similarly treated animals (6).

The great sensitivity and apparent specificity of the response of the cultured cells to prolactin suggest that the cultures could form the basis of a new method for the bioassay of this hormone. The cultures would also appear to be very useful models for biochemical studies of prolactin-target cell interactions.

⁴ Additional evidence that the observed growth-stimulatory effects are due to prolactin *per se* has come from experiments which showed that the stimulatory effects of the ovine prolactin preparation were completely blocked by addition to the cultures of anti-ovine prolactin rabbit antiserum, as distinct from normal rabbit serum (unpublished observation).

⁵ R. L. Noble, unpublished observations.

ACKNOWLEDGMENTS

We are grateful to Dr. H. G. Friesen (Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada) for helpful discussion. Kay Chen is thanked for technical assistance.

Note Added in Proof

Dr. A. H. Pontifex of the Cancer Control Agency of British Columbia has reported on the cultured lymphoma cells as follows:

The Wright's stain revealed large numbers of relatively monomorphous cells with a fine chromatin pattern, basophilic cytoplasm and numerous mitoses. The acid phosphatase stain was strongly positive in a stippled fashion with the granules distributed in a random fashion throughout the cytoplasm. The nonspecific esterase stain was also strongly positive and distributed as large aggregates adjacent to the nucleus. Terminal transferase reaction was negative. Using data derived from human sources, the special stains are more suggestive of a T cell origin rather than a B cell origin for this lymphoma.

REFERENCES

1. Broome, J. D., and Jeng, M. W. Promotion of replication in lymphoid cells by specific thiols and disulfides *in vitro*. *J. Exp. Med.*, 138: 574-592, 1973.
2. Gospodarowicz, D., Bialecki, H., and Greenburg, G. Purification of the fibroblast growth factor activity from bovine brain. *J. Biol. Chem.*, 253: 3736-3743, 1978.
3. Gospodarowicz, D., and Moran, J. S. Growth factors in mammalian cell culture. *Annu. Rev. Biochem.* 45: 531-558, 1976.
4. Gout, P. W., Beer, C. T., and Noble, R. L. Pituitary secretion stimulates growth of malignant lymphomas in Nb rats—evidence *in vivo* and *in vitro*. *Proc. Am. Assoc. Cancer Res.*, 20: 27, 1979.
5. Gout, P. W., Wijcik, L. L., and Beer, C. T. Differences between vinblastine and vincristine in distribution in the blood of rats and binding by platelets and malignant cells. *Eur. J. Cancer*, 14: 1167-1178, 1978.
6. Noble, R. L. Anti-cancer alkaloids of *Vinca rosea*. In: K. K. Chen and B. M. Mukerji (eds.), *Pharmacology of Oriental Plants. Proceedings of the Second International Pharmacological Meeting*, Vol. 7, pp. 61-78. Oxford, England: Pergamon Press, 1965.
7. Noble, R. L. Activation of growth of autonomous lymphoma cells following pulses of estrogen acting through the pituitary gland in Nb rats. *Proc. Am. Assoc. Cancer Res.*, 19: 216, 1978.
8. Noble, R. L., Beer, C. T., and Gout, P. W. Evidence *in vivo* and *in vitro* of a role for the pituitary in the growth of malignant lymphomas in Nb rats. *Cancer Res.*, 40: 2437-2440, 1980.
9. Noble, R. L., Gout, P. W., Wijcik, L. L., Hebden, H. F., and Beer, C. T. The distribution of [³H]vinblastine in tumor and host tissues of Nb rats bearing a transplantable lymphoma which is highly sensitive to the alkaloid. *Cancer Res.*, 37: 1455-1460, 1977.
10. Noble, R. L., Hochachka, B. C., and King, D. Spontaneous and estrogen-produced tumors in Nb rats and their behavior after transplantation. *Cancer Res.*, 35: 766-780, 1975.
11. Noble, R. L., and Hoover, L. A classification of transplanted tumors in Nb rats controlled by estrogen from dormancy to autonomy. *Cancer Res.*, 35: 2935-2941, 1975.
12. Wolff, J., and Williams, J. A. The role of microtubules and microfilaments in thyroid secretion. In: R. O. Greep (ed.), *Recent Prog. Horm. Res.*, 29: 229-285, 1973.