Transient Requirement for Prolactin as a Growth Initiator following Treatment of Autonomous Nb2 Node Rat Lymphoma Cell Cultures with Butyrate

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ABSTRACT

A previous study described the development of cultures of Nb2 node rat lymphoma cells which specifically require a lactogenic hormone, e.g., prolactin (PRL), as a growth factor. In the present study, sublines of these cultures have been established, including clones, which do not depend on exogenous lactogens for growth. The autonomous cells do not appear to produce autocrine, PRL-like substances. Although these autonomous cells grew readily in the complete absence of lactogens, their growth rate was stimulated (up to approximately 30%) by PRL concentrations >0.1 ng/ml. When cultures of the lactogen-dependent and of the cloned, autonomous lymphoma cells were incubated for 3 days with sodium n-butyrate (NaBT; 2 mM), cells were arrested in the G1 phase of the cell cycle, as shown by flow cytometric analysis. A substantial proportion of the G1-arrested cells was viable and readily resumed growth, within 1 day, when transferred to NaBT-free medium containing PRL (1–100 ng/ml). The resumption of growth of the cells from the lactogen-dependent cultures was critically dependent on PRL; in its absence cells lysed. Surprisingly, the rapid recovery of the cells from the lactogen-independent cultures also depended on the presence of PRL in the medium; in its absence growth did not resume for at least 2.3 days. The acquired need of the NaBT-treated, autonomous cells for PRL was only transient, since such cells reverted fully to the PRL-independent state within about 3 days of culturing in PRL-containing, NaBT-free medium. It is proposed, as a working hypothesis, that the autonomous lymphoma cells can be mitogenically activated by two different pathways, one requiring exogenous lactogens and another which is independent of lactogens; the latter pathway recovers more slowly from the treatment with NaBT than the lactogen-dependent pathway. This model could explain the sensitivity of the autonomous lymphoma cells to PRL and their transient dependency on exogenous lactogens during their recovery from NaBT treatment. NaBT would appear to be a useful agent for studying the mechanism(s) by which the autonomous lymphoma cells circumvent the need for mitogenic lactogens.

INTRODUCTION

The progression of cancers from a dependency on hormones for growth toward hormonal independence (autonomy), is a major problem in the clinical treatment of hormone-dependent malignancies. While ablative endocrine therapy, based on the depletion of essential, cancer growth-maintaining hormones in the circulation, can lead to long-lasting remissions, the malignancies frequently recur in forms which no longer depend on the hormones for growth and hence fail to respond to further endocrine therapy (1). In some cases, the growth of such recurrent, hormone-independent cancers is still hormone sensitive, i.e., can be accelerated by the hormone, as suggested by studies with experimental in vivo systems (2). The phenomenon of progression of cancers from hormonal dependency to autonomy is not fully understood, but may be related to the cellular heterogeneity of neoplasms (3, 4). For example, hormone-dependent cancers may contain minor subpopulations of autonomous cells in addition to the predominant hormone-dependent cells. When deprived of the mitogenic hormone, the dependent cells in the cancers would cease growth and eventually die; the autonomous cells on the other hand would continue to proliferate and give rise to hormone-independent cancers (5).

The present study describes the establishment of sublines of the Nb2 node lymphoma cell cultures which are completely independent of lactogens for growth. Cells of these autonomous cultures and of the parent, lactogen-dependent cultures have been treated with NaBT, a compound which has been reported to lead to changes in the expression of differentiated functions of cells, including hormone responsiveness (9, 10). While the treatment with NaBT did not lead to a change in the PRL requirements of the lactogen-dependent cells, the lactogen-independent cells were found to have acquired a transient dependency on PRL as an initiator of growth.

MATERIALS AND METHODS

Lactogen-dependent Lymphoma Cell Cultures. The cultured lactogen-dependent Nb2 node rat lymphoma cells (7) were maintained in suspension culture in 80-cm² tissue culture flasks (Nunc, Gibco Canada, Burlington, Ontario, Canada) in Fischer's medium supplemented with FCS (10%) as a source of lactogens, lactogen-deficient HS (10%), 2-ME (0.1 mM), penicillin (50 units/ml), and streptomycin (50 µg/ml) at 37°C in an atmosphere of 5% CO₂/95% air, as already described (11). Under these conditions the doubling time of logarithmically growing cultures was approximately 17 h. In experiments requiring precise amounts of lactogens, purified ovine PRL was used instead of FCS.

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1 The abbreviations used are: PRL, prolactin; NaBT, sodium n-butyrate; FCS, fetal calf serum; HS, horse serum; 2-ME, 2-mercaptoethanol; PBS, phosphate-buffered saline.

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Cell populations were determined using an electronic counter (Coulter Electronics, Hialeah, FL). Fischer's medium, FCS, and antibiotics were obtained from Gibco Canada, and ovine PRL (31 units/mg) was from the Sigma Chemical Co. (St. Louis, MO). HS was purchased from M.A. Bioproducts (Walkersville, MD); its lactogen content was below the limit of detectability in the lymphoma cell bioassy (11), i.e., <10 pg/ml.

Ovine PRL and NaBT (Sigma) were added to cultures as solutions in Fischer's medium containing 10% HS. When it was necessary to wash cells free of extracellular lactogens and/or NaBT, cultures were centrifuged for 4 min at 350 × g, and the packed cells were resuspended in Fischer's medium supplemented with HS (10%), 2-ME (0.1 mM), and antibiotics; this procedure was repeated twice. The response of the cultures to ovine PRL, NaBT, and to the removal of extracellular NaBT was determined in 12-well Linbro tissue culture plates (Flow Laboratories, Inc., Mississauga, Ontario, Canada), using 2.0-ml portions of cell suspension.

Autonomous Lymphoma Cell Cultures. Cells from exponentially growing lactogen-dependent cultures were transferred to Fischer's medium supplemented with lactogen-deficient HS (10%), 2-ME (0.1 mM), and antibiotics for further incubation. The cultures that developed (see "Results") eventually grew in this lactogen-deficient medium with a doubling time of approximately 15 h.

**Serum-free, Chemically Defined Culture Medium.** A 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12, containing 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, penicillin (50 units/ml), and streptomycin (50 µg/ml) was supplemented with transferrin (40 µg/ml) and bovine insulin (20 µg/ml). This chemically defined, lactogen-free medium supported the growth of autonomous Nb2 node lymphoma cells but not that of the lactogen-dependent cells. Insulin (1882) was obtained from Sigma; Gibco Canada supplied the media and purified transferrin (680-3008). Culturing of cells in the chemically defined medium was done in 25-cm² plastic tissue culture flasks (Corning Canada, Toronto, Ontario, Canada).

**Preparation of Conditioned Medium and Cell-free Extract.** Autonomous Nb2 node lymphoma cells were grown in Fischer's medium supplemented with HS (10%), 2-ME (0.1 mM), and antibiotics to a concentration of 7 × 10⁶ cells/ml, i.e., when they were still in log phase. The culture was then centrifuged (5 min at 350 × g) and the supernatant was filtered through a 0.2 µm Nalgene membrane (BDH Canada Chemicals Ltd., Toronto, Ontario, Canada) for use as "conditioned medium." The packed cells (approximately 30 × 10⁸) were resuspended in 1 ml fresh, ice-cold medium, frozen and thawed three times, and disrupted in a Potter-Elvehjem homogenizer at 4°C; after checking for the absence of intact cells, the preparation was used as "cell-free extract."

Cloning of Autonomous Lymphoma Cells. Autonomous Nb2 node lymphoma cells (subline SF-14) were readily cloned, using the limiting dilution method (12), in multiwell plates and a 1:1 mixture of fresh medium and conditioned medium by SF-14 cells. The individual wells were examined by microscope to identify those in which initially only one cell was present. Colony growth could easily be followed since the proliferating cells had a tendency to remain in clusters. All studies with the cloned cell cultures were done within 3 weeks of their establishment.

**Flow Cytometric Analysis of Cellular DNA Content.** Cells were stained for DNA with propidium iodide, following a method described by Clevenger et al. (13). As a routine, 3 × 10⁶ cells from log phase or NaBT-treated cultures were centrifuged (10 min at 200 × g) and fixed in 10 ml 0.5% paraformaldehyde (BDH, Poole, England) in PBS for 10 min at 4°C. The cells were again centrifuged and resuspended in ice-cold, 0.1% Triton X-100 (Sigma) in PBS for 3 min. Following centrifugation, the cells were then resuspended in 2 ml PBS containing RNase-A (Sigma; 1 mg/ml) and incubated for 20 min at 37°C. The cells were finally resuspended in 2 ml ice-cold PBS containing propidium iodide (Sigma; 50 µg/ml), and stored in the dark at 4°C for at least 1 h. Prior to flow cytometric analysis it was established, by microscopic examination, that over 95% of the cells were present as single cells. At least 30,000 cells per sample were analyzed for single parameter DNA fluorescence and forward angle light scatter on a 256-channel, Epics V flow cytometer (Coulter Electronics) equipped with a 5-W argon laser (Spectra-Physics, Mountain View, CA). Laser excitation was at 488 nm (500 mW); the red fluorescence of the cells (emission peak 590 nm) was monitored through a 515 nm interference and 630 nm long-pass filter. All samples were gated on both the fluorescence and light scatter signals to help distinguish cells from debris. "Full-bright" fluorescent microspheres (Lot 5870, Coulter Electronics) were used to aid instrument alignment and were run before and after analysis of the samples in duplicate. During the runs there was no significant shift in the position of any of the various G₁-peak channels (<2 channels). The percentage of cells which in a given sample was present in the G₉, S, and G₃ + M phases of the cell cycle, was estimated from the DNA histograms using a computer program "Parametric Analysis I" (Extended Analysis System, Coulter Electronics). For the flow cytometric analysis of mixtures of lactogen-dependent and autonomous lymphoma cells, preparations were used of cells which had been mixed prior to fixing and staining.

**RESULTS**

**Autonomous Nb2 Node Lymphoma Cell Cultures.** When cells from cultures of lactogen-dependent lymphoma cells were subcultured in 10% HS-containing medium, which was deficient in lactogens, the majority of the cells became smaller, lost their refractility, and eventually lysed. However, some cells survived and within 3–4 weeks gave rise to cultures which grew readily in the lactogen-deficient medium with a doubling time of approximately 15 h. When stained with Wright's stain, the microscopic appearance of these "autonomous" Nb2 node lymphoma cells was very similar to that of the lactogen-dependent cells (7).

The ability of the autonomous cells to proliferate in the complete absence of lactogens was demonstrated by culturing them in lactogen-free, chemically defined medium. The cells, washed free of HS, grew readily during a 14-day period in Dulbecco's modified Eagle's medium/F12 (1:1), supplemented with transferrin and insulin (average doubling time approximately 27 h). Transferrin was an essential growth factor for these cells and in its absence cells lysed within 2 days. Insulin was not needed for short-term culturing (e.g., 3 days) but enhanced the long-term survival of the cells. In view of the inability of lactogen-free medium to support the growth and maintain the viability of lactogen-dependent cells, it appeared highly probable that, after 14 days of subculturing in the chemically defined, lactogen-free medium, the cultures consisted only of lactogen-independent cells. The subline of autonomous Nb2 node lymphoma cells, developed in this manner, was designated "SF-14." Since these cells were rather fragile in serum-free medium, they were maintained in Fischer's medium supplemented with 10% HS.

Cloning of SF-14 cells led to the establishment of 3 lines of cloned, autonomous Nb2 node lymphoma cells, including a line designated "SFJCD1." These lines grew very readily in 10% HS-containing medium with a doubling time of approximately 15 h.

**Response of Nb2 Node Lymphoma Cell Cultures to PRL.** Fig. 1 shows the effect of PRL at various concentrations on the growth rates of the lactogen-dependent cell line and the various lines of autonomous cells, including the SF-14 subline and SFJCD1 cloned cell line. The lactogen-dependent cultures grew maximally at a PRL concentration of 10 ng/ml; there was no growth in the absence of the hormone. In contrast, all the autonomous lymphoma cell cultures grew readily in the absence of exogenous PRL. Their growth rates were, however, stimulated (up to approximately 30%) by PRL concentrations >0.1
ng/ml; at 0.01 ng/ml, PRL did not significantly stimulate the growth of the cultures. All 3 cloned autonomous cell lines showed similar sensitivity to PRL.

Absence of "PRL-like" Activity in Medium Conditioned by Autonomous Cells and in Extracts of Autonomous Cells. Medium conditioned by autonomous SF-14 cells did not support the growth of lactogen-dependent Nb2 node lymphoma cells, even when used at a concentration of 50%. Similarly, cell-free extracts of autonomous cells had no mitogenic activity. The lack of mitogenic activity was not due to the presence of growth-inhibitory factors in either the extracts or media, since neither interfered with the proliferation of the lactogen-dependent cells in medium containing PRL (0.2 ng/ml).

Response of Lymphoma Cell Cultures to Treatment with NaBT. Fig. 2 shows the population growth of lactogen-dependent and autonomous (SF-14) lymphoma cell cultures as affected by incubation with NaBT at various concentrations. The growth of cultures of both types was arrested by NaBT concentrations of 1–2 mm; higher NaBT concentrations resulted in cell lysis. A substantial proportion of the cells in NaBT-arrested cultures remained viable. Thus, even after a 3-day incubation with 2 mm NaBT, cultures resumed growth, within 1 day, when restored to NaBT-free medium (Fig. 3). The resumption of growth of the lactogen-dependent cultures was critically dependent on the presence of PRL in the medium; in the absence of the hormone cells lysed (Fig. 3A). The cloned, lactogen-independent cultures resumed growth in the absence of PRL but only after a lag period of at least 2.3 days. This lag period was markedly shortened, however, if PRL (e.g., 1–100 ng/ml) was present in the medium. For example, whereas in the absence of PRL there was only a minor increase in cell numbers after the first 65 h of recovery (76%), in the presence of PRL there was an early increase in cell population, i.e., 42% at 26 h, rising to 822% at 65 h (Fig. 3B). The resumption of growth of the autonomous cell cultures in the presence of PRL was very similar to that of the lactogen-dependent cultures (Fig. 3A). The results suggest that, as a consequence of the treatment with NaBT, the cloned, autonomous lymphoma cell cultures had acquired a need for PRL as a growth initiator. This requirement for PRL was not permanent, however, since the cells after growing in the presence of PRL for about 77 h (see Fig. 3B, arrow), were once more able to grow readily in lactogen-deficient medium (doubling time approximately 18 h). The results in Fig. 3 were independent of the inoculum size in the range 5 x 10^4–10^5 cells/ml (data not shown).

DNA Content of Cells. The relative DNA content of lactogen-dependent and SF-14 lymphoma cells from log phase cultures, and from cultures which had been incubated with 2 mm NaBT for 3 days, was determined by flow cytometric analysis. The distribution of the cells over the various phases of the cell cycle was very similar for the 2 log phase cultures: about 50% of the cells were in the G_0 period, 30% in the S period, and 20% in the G_1 + M period. In contrast, the cells from the NaBT-treated cultures were concentrated in the G_1 period, which in both cases contained about 90% of the cells (data not shown). The results suggest that treatment with NaBT leads to the arrest of the lymphoma cells in the G_1 phase of the cell cycle. The DNA histograms of the lactogen-dependent lymphoma cells and cloned SF-14 cells (subline SFJCD1) were both completely superimposable with DNA histograms obtained from 1:1 mixtures of the 2 types of cell, indicating that the DNA content of the autonomous cells was essentially identical to that of the lactogen-dependent cells (data not shown).
DISCUSSION

The autonomous Nb2 node lymphoma cell lines developed in this study were able to grow in the complete absence of lactogens, as shown by their proliferation in chemically defined, lactogen-free medium. The acceleration of the growth of cloned, autonomous cell cultures by PRL at concentrations $\geq 0.1$ ng/ml (Fig. 1), indicates that the cloned cells, although lactogen independent, were nevertheless intrinsically sensitive to PRL. Since all 3 of the cloned cell cultures were similarly sensitive to PRL, it appears likely that this sensitivity is a general property of the autonomous lymphoma cells which can explain the PRL sensitivity of the autonomous cultures prior to cloning (Fig. 1).

The development of autonomy in the lactogen-dependent lymphoma cell cultures, which followed the reduction of extracellular lactogen levels, could be related to cellular heterogeneity in these cultures. The extensive cell lysis, which was associated with this process, is consistent with the loss of a major subpopulation of cells which critically depend on lactogens for growth. It is not known whether the cells which survived were already present in the initial lactogen-dependent culture as a minor subpopulation of autonomous cells, or whether they became lactogen independent as a consequence of the removal of the extracellular lactogens. It appears likely that the autonomous, PRL-sensitive cells stem from lactogen-dependent cells which managed to circumvent their need for mitogenic lactogens. The emergence of autonomous but hormone-sensitive cancer cells in this study is very similar to the hormone-sensitive growth of cancers occurring after endocrine therapy of certain experimental hormone-dependent malignancies (2).

It has previously been shown that the action of PRL on the lactogen-dependent Nb2 node lymphoma cells is mediated by specific cell surface receptors (14). It has also been found that the autonomous (noncloned) Nb2 node lymphoma cells have high numbers of lactogen receptors (approximately 7000/cell), and the PRL-sensitivity of the cloned, autonomous SF-14 cells (Fig. 1) indicates that these cells also have lactogen receptors. The findings raised the possibility that the autonomous lymphoma cells might be capable of producing autocrine substances with PRL-like, mitogenic activity and thereby stimulate their own proliferation even in the absence of exogenous lactogens. However, neither medium conditioned by autonomous cells, nor extracts of these cells, had any mitogenic, PRL-like activity when assayed by the Nb2 node lymphoma cell bioassay (11), indicating that the growth of the autonomous cells in lactogen-free medium is not due to the production of autocrine, PRL-like factors. The molecular basis of the different hormonal requirements of the lactogen-dependent and autonomous lymphoma cells is not known, but does not appear to involve significant differences in the DNA content of the cells, as indicated by the flow cytometric analyses.

NaBT has been reported to affect a wide range of processes in mammalian cells, including gene expression, proliferation, and responsiveness to hormones (9, 10). The inhibitory effect of NaBT on the growth of the lactogen-dependent and autonomous lymphoma cell cultures was very similar (Fig. 2), and resembled that observed in other mammalian cell systems (9, 15). Furthermore, NaBT arrested both types of cells in the G1 phase of the cell cycle, as also found in other systems (15). Whereas treatment with NaBT did not lead to a loss of lactogen dependency of the hormone-dependent lymphoma cells (Fig. 3), it markedly affected the hormone requirements of the autonomous lymphoma cells. Thus although the cloned SF-14 cells normally grow very readily in the absence of lactogens (Fig. 1), they had, following treatment with NaBT, acquired a need for PRL as a growth initiator (Fig. 3B). This acquired dependency on PRL was only transient, since the cells were found to have reverted to the PRL-independent state within about 3 days of culturing in PRL-containing, NaBT-free medium.

The mechanisms by which NaBT acts on cells are not completely understood, but in some systems the effects of this agent have been related to its inhibitory action on histone deacetylase, leading to hyperacetylation of chromatin-associated histones and thereby to changes in gene expression (9, 16). It is also not known why treatment of the autonomous Nb2 node lymphoma cells with NaBT leads to a transient dependency on PRL as a growth initiator. It appears from the mitogenic response of the NaBT-treated autonomous cells to PRL (Fig. 3B), and from the sensitivity of the untreated autonomous cells to the hormone (Fig. 1), that these cells have a mitogenic activation pathway which is sensitive to exogenous lactogens, similar to that operative in the lactogen-dependent lymphoma cells. In addition, the autonomous cells must have another mitogenic pathway which is independent of lactogens, as indicated by the finding that the cells are able to replicate in the absence of exogenous lactogens and autocrine, PRL-like substances. Using this dual pathway model (Fig. 4), a tentative explanation may be advanced for the transient PRL dependency observed in the NaBT-treated autonomous cell cultures. It is proposed that the lactogen-dependent pathway in the autonomous cells recovers quite quickly from treatment with NaBT, as indicated by the ready resumption of growth of the G1-arrested cells in the presence of PRL (Fig. 3B). In contrast, the lactogen-independent pathway would appear to recover only slowly, as suggested by the sluggish resumption of growth of the NaBT-treated cells in the absence of PRL (Fig. 3B). The transient PRL dependency following NaBT treatment would result from the temporary impairment of the lactogen-independent pathway, obliging the cells, during the recovering period, to rely solely on the lactogen-dependent pathway.

The Nb2 node lymphoma cell cultures described in this study appear to provide good models for investigating the biological and biochemical processes by which cancer cells, which initially depend on a polypeptide hormone for growth, can circumvent the need for the hormone. A better understanding of such progression-related processes could have important implications for the clinical therapy of hormone-dependent malignancies. NaBT may be a useful tool in such studies.

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