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Leukemia inhibitory factor induces differentiation of pituitary corticotroph function: An immuno-neuroendocrine phenotypic switch

(cytokine/pro-opiomelanocortin/adrenocorticotrophin/cell cycle)

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ABSTRACT **Leukemia inhibitory factor (LIF) promotes differentiated cell function in several systems. We recently reported LIF and LIF receptor expression in human fetal pituitary corticotrophs *in vivo* and demonstrated LIF stimulation of adrenocorticotrophin (ACTH) transcription *in vitro*, suggesting a role for LIF in corticotroph development. We therefore assessed the action of LIF on proliferating murine corticotroph cells (AtT20). LIF impairs proliferation of AtT20 cells (25% reduction versus control, $P < 0.03$), while simultaneously enhancing ACTH secretion (2-fold, $P < 0.001$) and augmenting ACTH responsiveness to corticotrophin-releasing hormone (CRH) action (4-fold, $P < 0.001$). This attenuation of cell growth is due to a block of cell cycle progression from G_1 into S phase, as measured by flow cytometric analysis (24 ± 0.8 versus 11.57 ± 1.5, $P < 0.001$). Using bromodeoxyuridine incorporation assays, loss of cells in S phase was confirmed (25 ± 0.08 to 9.4 ± 1.4, $P < 0.008$). In contrast, CRH induced the G_2/M phase (3.6 ± 0.2 to 15.4 ± 3, $P < 0.001$). This effect was blunted by LIF ($P < 0.001$ versus CRH alone). Cyclin A mRNA levels, which decline in S phase, were stimulated 3.5-fold by LIF and markedly suppressed by CRH. These results indicate a LIF-induced cell cycle block occurring at G_1/S in corticotroph cells. Thus, LIF reduces proliferation, enhances ACTH secretion, and potentiates effects of CRH on ACTH secretion while blocking effects of CRH on the cell cycle. Responses of these three markers of differentiated corticotroph function indicate LIF to be a differentiation factor for pituitary corticotroph cells by preferential phenotypic switching from proliferative to synthetic.**

Several cytokines are important modulators of neuroendocrine functions and putative mediators of the immune and neuroendocrine system interaction (1–3). One of these intercellular messengers, leukemia inhibitory factor (LIF), was originally isolated as an inhibitor of mouse M1 myeloid leukemia cells (4) and subsequently isolated from bovine pituitary conditioned medium (5). LIF exerts pleiotropic effects on diverse tissues, either inhibiting differentiation and maintaining the developmental potential of embryonic stem cells (6) or stimulating proliferation of human erythroleukemia TF-1 cells (7). In the nervous system, LIF induces cholinergic switching of sympathetic neurons both *in vivo* and *in vitro* (8). LIF acts through a specific receptor subunit forming a heterodimeric complex with gp130 (9–10), a signal transduction molecule shared with oncostatin M (OSM) and interleukin 6.

Corticotrophin-releasing hormone (CRH) is an important regulator of the hypothalamic–pituitary–adrenal axis during development and also in response to stress (11–12). This hypothalamic peptide induces adenylate cyclase activity and stimulates pro-opiomelanocortin (POMC) gene transcription (13) and adrenocorticotrophin (ACTH) release (14), and may be mitogenic for pituitary corticotroph cells *in vivo* (15) and *in vitro* (16).

We have previously shown LIF expression in both normal human fetal pituitary corticotroph cells and in pituitary corticotroph adenomas and have demonstrated LIF to induce ACTH secretion from the AtT20 corticotroph cell line (17) and to stimulate POMC gene transcription (18). We now examine LIF effects on corticotroph cell proliferation, both alone and together with CRH.

MATERIALS AND METHODS

Cells and Cell Culture. AtT20 D16:16 monolayer mouse anterior pituitary cells obtained from the American Type Culture Collection were grown as described (18). 3T3 F442A fibroblasts were cultivated as described (19). Mouse pituitary glands were obtained from B6SJ1 mice within minutes of decapitation (17). Materials and reagents were obtained from Sigma; LIF and OSM were purchased from R&D; CRH was obtained from American Peptide Company (Santa Clara, CA).

Proliferation Assays. *Cell number.* Cell numbers were determined directly in a Coulter counter after trypsinization and dispersion (Fig. 1*a*).

3-(4,5-Dimethyl)-thyazol-2,5-diphenyltetrazolium bromide (MTT). Cell growth was also indirectly determined by a MTT colorimetric assay (Promega) (20). Briefly, mitochondria in living cells metabolize MTT to a formazan derivative, measured in an ELISA plate reader (Fig. 1*b*).

Growth stimulation ratios (GSR) were calculated using the following equation, where A is absorbance at 550 nm: GSR (% control) = $(A_{\text{sample}} - A_{\text{blank}}/A_{\text{control}} - A_{\text{blank}}) \times 100$.

Bromodeoxyuridine (BrdU) assay. BrdU incorporation was assayed using an enzymoimmunoassay (EIA) system (21, 22), as recommended by the manufacturer (Amersham). BrdU incorporation was also analyzed by flow cytometric analysis.

Cell cycle analysis. A quantitative measure of cell cycle distribution was obtained by flow cytometric analysis of DNA histograms. Cells were stained with propidium iodide (Sigma) in the presence of ribonuclease (Promega), and cell fluorescence was measured in a FACScan flow cytometer (Becton Dickinson) by argon ion laser at 488 nm for excitation. A minimum of 10^4 cells per sample were analyzed.

Northern Blot. Cells were synchronized by a thymidine block (23) and treated for 6 h, and total RNA was harvested as described (17). RNA (10 μg) was fractionated in 1% agarose alkaline-denaturating gel and transferred as described (17).

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Abbreviations: LIF, leukemia inhibitory factor; CRH, corticotrophin-releasing hormone; OSM, oncostatin M; POMC, pro-opiomelanocortin; ACTH, adrenocorticotrophin; BrdU, bromodeoxyuridine; MTT, 3-(4,5-dimethyl)-thyazol-2,5-diphenyltetrazolium bromide; EIA, enzymoimmunoassay; FACS, fluorescence-activated cell sorting.

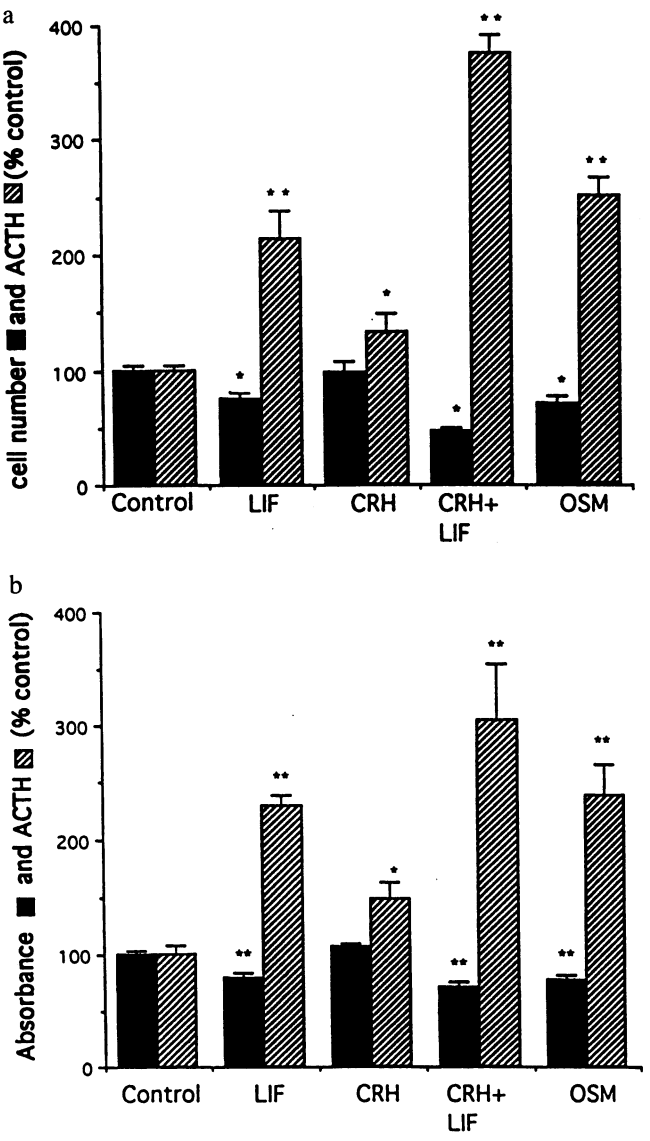

FIG. 1. (*a*) ACTH secretion (hatched bars) and cell number (shaded bars) of AtT20 pituitary cells after treatment with 10 nM LIF, 10 nM CRH, 10 nM LIF + 10 nM CRH, or 10 nM OSM for 72 h in serum-free medium (mean ± SEM of triplicate wells from a representative experiment repeated more than six times). *, $P < 0.05$ versus control. **, $P < 0.001$ versus control. (*b*) Mitogenic activity, expressed as MTT colorimetry, and ACTH secretion after treatment with 10 nM LIF, 10 nM CRH, 10 nM LIF + 10 nM CRH, or 10 nM OSM for 72 h in serum-free medium. Three independent experiments were performed. Values are mean ± SEM obtained from six to eight wells from a representative experiment. *, $P < 0.05$ versus control; **, $P < 0.001$ versus control.

The filter was probed for cyclin A and after high stringency wash was exposed to film overnight at −70°C.

Hormone Quantification. ACTH was assayed using a double-antibody radioimmunoassay kit from Diagnostic Products (Los Angeles). After dilution testing, all samples from the same experiment were analyzed in duplicate in the same radioimmunoassay. Intra-assay coefficient of variation at the mean of 152 pg/ml was 4.9%, and inter-assay coefficient of variation at the mean of 134 pg/ml was 6.4%. Sensitivity was 8 pg/ml.

Statistics. For the MTT and EIA assay, 6–8 wells per experimental group were assayed in a given experiment. The mean of each experiment is reported ± SEM. Data were automatically calculated by the microplate reader software (SOFTMAX, Molecular Devices). For cell cycle analysis, values represent the mean ± SEM of triplicate dishes.

Differences were assessed by one way analysis of variance, in combination with the Bonferroni *t* test.

RESULTS

Cell Number. Concentrations of LIF greater than 1 nM consistently inhibited AtT20 cell proliferation. LIF (10 nM) reduced cell number to 74 ± 7% of control wells ($P < 0.03$) (Fig. 1*a*). This was accompanied by increased ACTH release to 223 ± 25% of control ($P < 0.001$). OSM, a related peptide, also suppressed cell proliferation to 71 ± 6% ($P < 0.05$) and induced ACTH secretion to 259 ± 18% of control. This effect was confirmed using primary mouse pituitary cells, where 1 nM LIF was sufficient to enhance ACTH secretion (143 ± 15% of control, $P < 0.02$). Because primary pituitary cells do not replicate *in vitro*, their antiproliferative response to LIF was not measurable. In human corticotroph tumor cells, the ACTH response to LIF treatment was heterogeneous (data not shown). To further examine LIF-mediated inhibition of AtT20 cell proliferation, viable mitochondrial function was tested using the MTT assay (Fig. 1*b*). LIF treatment reduced MTT from 100 ± 3.4% to 78.5 ± 5.2% ($P < 0.001$ versus control).

Cell Cycle Analysis. To identify a mechanism for LIF-induced attenuation of cell proliferation, cell cycle analysis was performed using propidium iodide stained cells and FACS (Fig. 2*a* and Table 1). The fibroblast cell line 3T3 F442A is known to be LIF-responsive, and LIF effects on this line were therefore also tested. LIF (1 nM) appeared to block cell entry into the S-phase, with a significant decrease in cell number to 66 ± 4.6% of control ($P < 0.009$). When AtT20 cells were analyzed, the proportion of cells at the pre-G_0 phase was similar in control, LIF, and OSM-tested wells. Surprisingly, CRH increased the number of pre-G_0 or apoptotic cells (from 11.5 ± 1.6 to 27 ± 3, $P < 0.003$). This population of dead cells was therefore excluded from subsequent cell-cycle phase analysis. Significantly, more cells were at G_1 in the LIF-treated cells as compared with the other three groups, and LIF treatment reduced the number of cells in S-phase from 24 ± 0.8 to 11.57 ± 1.5% ($P < 0.001$). CRH treatment predominantly increased the numbers of gated cells in the G_2/M phase from 3.62 ± 0.2% to 15.4 ± 3% ($P < 0.001$).

Cyclin A mRNA analysis. Cyclin A mRNA declines in the S phase (24, 25). LIF treatment of synchronized cells resulted in higher levels of cyclin A mRNA than control, supporting inhibition of S phase entry. CRH markedly enhanced cyclin A reduction, an effect blunted by LIF (Fig. 2*b*).

BrdU Incorporation. As LIF appeared to act at the G_1/S boundary, its effects on the S-phase was examined in more detail. Cells were treated with LIF, pulse-labeled with BrdU, fixed, and immunolabeled and then sorted by FACS (Fig. 3). The attenuation of S-phase entry by LIF was confirmed by observing a reduction in labeled cells from 25 ± 0.2 to 9 ± 1.4% ($P < 0.008$). In addition, cells in S-phase were identified using an EIA for BrdU. CRH caused an increase of BrdU to 225 ± 17% ($P < 0.001$). The conditioned medium from these cells was assayed for ACTH, and the results are depicted in Fig. 4. Notably, addition of LIF to CRH blocked the proliferative response induced by CRH alone, while further stimulating

Table 1. Percentage of cells in cell cycle phases

Phase	Control	LIF	CRH
G_0/G_1	72.37 ± 0.7	81 ± 1.4	69 ± 0.7
S	24 ± 0.8	11.57 ± 1.5	15.4 ± 2
G_2/M	3.62 ± 0.2	7.3 ± 0.1	15.4 ± 3

Each value represents mean ± SEM of triplicate wells.

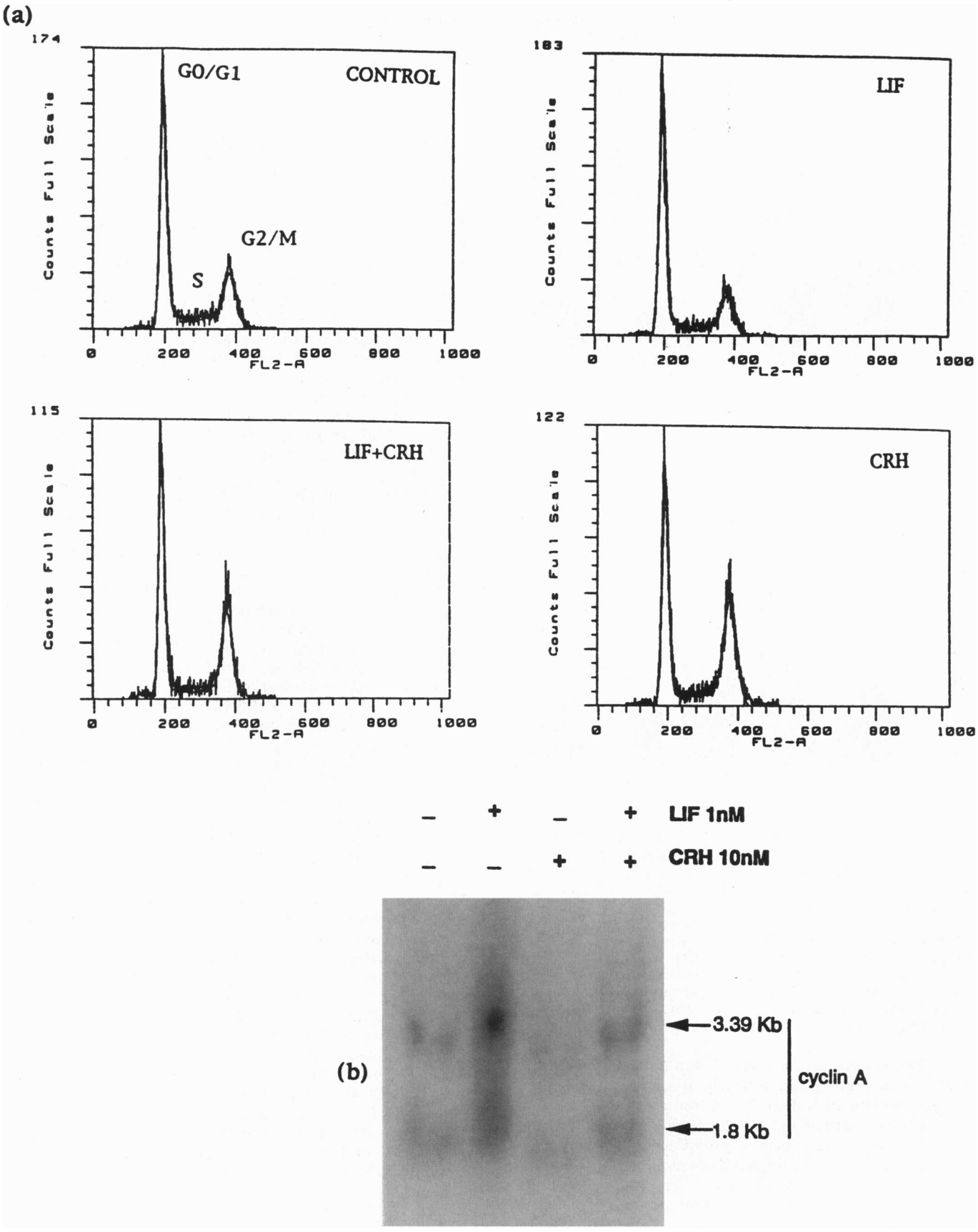

FIG. 2. (*a*) Flow cytometric DNA histograms of AtT20 cells. DNA content (FL-2A) is depicted on the *x* axis, and number of cells after staining with propidium iodide is depicted on the *y* axis. Synchronized cells were stimulated with 1 nM LIF, 10 nM CRH, or 1 nM LIF + 10 nM CRH for 72 h. Results depicted are from a single representative experiment. (*b*) Northern blot analysis of cyclin A mRNA in synchronized AtT20 cells. Cells grown in serum-free medium were treated with 1 nM LIF, 10 nM CRH, 1 nM LIF + 10 nM CRH for 6 h, total RNA extracted and blot probed with ^{32}P-labeled cyclin A (8×10^8 Bq/μg).

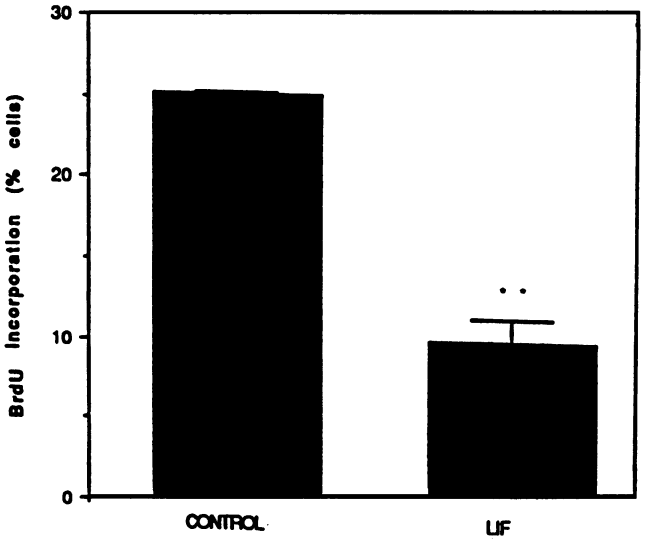

FIG. 3. Effect of LIF (1 nM) on the cell cycle distribution determined by BrdU incorporation. Asynchronous AtT20 cells were treated with 1 nM LIF for 72 h. Cells were pulse-labeled with BrdU (1 h) and analyzed for DNA content by FACS analysis. BrdU incorporation during the S-phase is expressed as percent of stained cells (mean ± SEM of duplicate wells from a representative experiment). **, $P <$ 0.008 versus control.

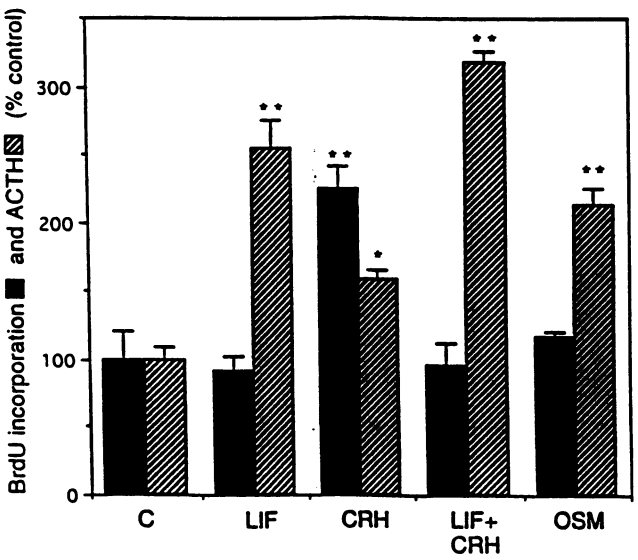

FIG. 4. EIA for BrdU incorporation was performed with asynchronous AtT20 cells. Cells were treated for 48 h with 10 nM LIF, 10 nM CRH, 10 nM LIF + 10 nM CRH, or 10 nM OSM in serum-free medium. Cells were then pulse-labeled for 16 h and analyzed for BrdU incorporation. ACTH secretion was measured in conditioned medium. Results are expressed as mean ± SEM of a representative experiment repeated three times. Four to six wells were analyzed in each experiment. *, $P < 0.006$ versus control; **, $P < 0.001$ versus control.

ACTH production in a synergistic manner (317 ± 16.2%, $P <$ 0.001) (Fig. 4).

DISCUSSION

LIF exerts profound effects on cell behavior, including the cholinergic switch of sympathetic neurons (8) and inhibition of embryonic stem cell differentiation (6). Circulating LIF rises acutely in endotoxemic shock (26), as do hypothalamic and pituitary LIF mRNA levels (27). Furthermore, administration of LIF to endotoxemic animals enhances their survival rate (28). Activation of the hypothalamic–pituitary–adrenal axis accompanies endotoxemia, and exogenous glucocorticoid administration also increases survival rate. CRH is critical to mounting an effective stress response, as demonstrated in the CRH knock-out mouse model (12), but CRH cannot stimulate ACTH levels to those observed during stress, suggesting a role for additional corticotropic factors.

As CRH may also be a mitogen for pituitary corticotrophs both *in vivo* and *in vitro*, the influence of LIF on CRH-mediated proliferation was examined. As primary pituitary cultures represent a mixed cell population with complex paracrine interaction (29), and as primary cultures do not proliferate even under optimal growth conditions, we chose to use the AtT20 cell line to address this question. Nevertheless, we observed that physiologic concentrations of LIF induced ACTH from normal murine pituitary cultures.

Cell number as measured both by direct counting and, using an MTT assay, was inhibited by LIF, while the cytokine simultaneously enhanced ACTH secretion. This cell loss may reflect either increased cell death or inhibition of division. We have little evidence, however, for LIF induction of cell death. FACS analysis was undertaken to count cells in different phases of the cell cycle and to estimate numbers of apoptotic cells. These results suggest that LIF induced a block in cell cycle progression from G_1 to S phase. CRH, in contrast, enhanced the proportion of proliferating cells (G_2/M phase), and, surprisingly, enhanced the rate of apoptosis. This explains the weak effects of CRH on cell accumulation observed after prolonged incubation (72 h) (Fig. 1*a*).

To examine the influence of LIF on entry of cells to S phase, bromodeoxyuridine incorporation was measured (21, 22), using both a FACS method and an EIA. These two approaches yielded complementary results, with a significant reduction in BrdU incorporation induced by LIF. In the EIA the basal proliferation (control) was at the lower level of assay sensitivity, therefore basal LIF effects may have been masked. Although, after induction with CRH, LIF inhibitory effect of BrdU incorporation was markedly evident. A direct toxic effect of LIF was effectively excluded as ACTH was induced. CRH strongly enhanced S-phase entry, in contrast to its weak effects on cell proliferation. This effect was opposed by LIF, concurrent with LIF/CRH synergy to stimulate ACTH secretion.

Cyclin A mRNA expression, which is suppressed in S-phase (24, 25), was also increased by LIF treatment of synchronized cells, while CRH markedly suppressed cyclin A mRNA levels, suggesting promotion of cell entry into S phase. LIF attenuated this effect, and, taken together with inhibition by LIF of CRH stimulation of BrdU incorporation, suggests an important attenuating effect of LIF on growth-promoting CRH action.

The observation that LIF stimulates ACTH production, an index of corticotroph differentiated function, while inhibiting cell proliferation suggests a LIF-induced switch in cell phenotype from proliferative to biosynthetic. Interestingly, although interleukin 6 has been shown to alter rat pituitary thymidine incorporation *in vitro*, the specific cell type responding to the cytokine was not delineated (30). We previously described detection of LIF in human fetal pituitary corticotroph cells, suggesting a possible role for LIF in development and also in pituitary adenomas (17). Interestingly LIF and CRH exert synergistic effects on hormone production, acting at the level of POMC transcription (18) but are here shown to have antagonistic effects on cell proliferation. LIF blunted the mitogenic effects of CRH. This mechanism may be important physiologically to limit potentially proliferative effects of CRH on pituitary corticotrophs under conditions of stress when enhanced ACTH production is required to confer beneficial effects to the host.

In conclusion, LIF, a potent stimulator of ACTH expression by corticotroph cells, is now shown to inhibit S-phase entry. Although, LIF effectively synergizes with CRH to potentiate hormone production, in contrast to CRH it inhibits corticotroph proliferation. LIF appears to block cell cycle progression at a point in G_1, suggesting a subtle interplay between central (CRH) endocrine and peripheral immune responses, resulting in phenotypic maturation of corticotroph function.

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