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GROWTH, DIFFERENTIATION AND THE β-ADRENERGIC SIGNAL SYSTEM OF HL-60 CELLS

CHARACTERIZATION IN A MEDIUM WITH INSULIN AS THE ONLY ADDED PROTEIN

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Abstract—The purpose of the present investigation was to define experimental conditions for studies on growth, differentiation and the β-adrenergic signal system of HL-60 cells. The cell medium was completely devoid of added proteins and hormones other than insulin. The HL-60 cell was able to grow and differentiate in this medium. The spontaneous differentiation along the granulocytic pathway after 72 hr, as assessed by the Nitro Blue tetrazolium test, increased by 400% compared to the serum supplemented medium, but the response to 1 μM retinoic acid was equal in the two media. Induction of monocytic differentiation by 0.16 μM phorbol-13-acetate-12-myristate, as determined by surface adherence after 24 hr, was lower in the absence than in the presence of serum. cAMP levels were elevated in response to (−)-isoproterenol. The EC_{50} was 0.36 ± 0.01 μM (mean ± SE, N = 3). The β-adrenergic ligand ^{3}H-CGP 12177 was specifically bound to 1 single class of binding sites (K_d: 0.15 ± 0.04 nM, B_{max}: 2220 ± 150, mean ± SE, N = 3). These data are comparable to our previously reported findings in serum supplemented medium. The present data show that HL-60 cells are able to grow and differentiate in the absence of serum proteins and hormones other than insulin. Under the present experimental conditions, these cells possessed functional β-adrenergic receptors.

The human leukemic progenitor cell line HL-60 is widely used in studies of *in vitro* regulation of growth and differentiation. This cell line is readily inducible by a variety of physiological and non-physiological differentiating agents [1]. Depending on the nature of the differentiating agent, the HL-60 cell can achieve phenotypes along the monocytic or granulocytic pathway.

Intercellular signal molecules participate in the control of normal cell growth and differentiation. Malignant transformation of cells may occur after oncogene-induced defects in signal systems [2]. The malignant phenotype may in some cases be reversed after pharmacological activation of signal systems [3]. The HL-60 cell reverses its malignant phenotype after activation of the cAMP dependent protein kinase (protein kinase A) and protein kinase C [4–6].

Formation of the intracellular second messenger cAMP is catalysed by membrane bound adenylate cyclase. The adenylate cyclase of HL-60 cells is responsive to several stimulators, including β-adrenergic agents [7, 8], prostaglandins [9] and H_2-receptor agonists [10].

The β-adrenergic signal system can be modulated by a number of agents (For review see Ref. 11). Prolonged exposure to β-adrenergic stimulators results in loss of responsiveness to further β-stimulation. Hormones and agents with receptors coupled to adenylate cyclase modulate the response of β-adrenergic stimulation. Hormones and agents whose effects are not mediated by cAMP, can also modulate these signal systems at different levels. The modulating actions of these different substances on the signal system may complicate *in vitro* studies.

Various serum proteins may also complicate signal system studies. The presence of albumin and other hormone and drug binding proteins reduces the free concentration of these biological active agents [12]. Serum proteins may also have effects themselves. The extracellular protein concentrations have been shown to influence the number of functional β-adrenergic receptors [13]. Batch-to-batch variation in the concentration and activity of these components of serum thus complicates the interpretation of data in signal system studies.

A number of approaches have been tried to replace serum in cell media. However, most of the media used up till now contain serum components with potentially complicating actions. Incomplete refinement of biologically active molecules results in unpredictable effects.

A stable synthetic serum replacement (SSR) has recently been developed (Dr Kjell Bertheussen, unpublished results). It contains the general foundation for growth and differentiation normally provided by serum. The composition of the metal ion buffer is based on a new principle: aurintricarboxylic acid is used as a replacement for the iron presenting ability of transferrin and the combination of non-protein chelators prohibits slow precipitation of trivalent metals. The surfactant polyether pluronic F68, which is a polyoxyethylene-polyoxypropylene-polyoxyethylene (ABA) type block copolymer is

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Table 1. Growth of HL-60 in serum-free (SSRM) and serum-supplemented (SSM) cell media

Cell medium	Doubling time (hr)	Maximal cell density (cells/ml × 10^5)	Viability (%)
SSRM	28.7 ± 1.8	5.10 ± 0.27	95.6 ± 1.6
SSM	19.7 ± 0.5	12.61 ± 0.94	97.6 ± 0.4

Doubling times and viability were determined during logarithmic cell growth. The cells were kept in an atmosphere of 5% (v/v) CO_2 in air at 37° The results are presented as mean values ± SE of 3 independent experiments.

Table 2. Growth inhibition and differentiation of HL-60 cells in serum-free (SSRM) and serum-supplemented (SSM) cell media after exposure to differentiation inducing agents

Cell medium	Treatment	Cell growth (% increase)	Differentiation (% positive)
SSRM	C-1	247 ± 2.2	21.8 ± 2.15
	RA (1 μM)	16 ± 1.8	83.7 ± 5.46
	C-2	71 ± 3.0	4.5 ± 1.39
	PMA (0.16 μM)	47 ± 6.2	57.2 ± 3.13
SSM	C-1	877 ± 6.3	4.4 ± 1.97
	RA (1 μM)	489 ± 20.9	81.3 ± 4.25
	C-2	152 ± 1.1	5.8 ± 1.66
	PMA (0.16 μM)	27 ± 10.1	90.7 ± 2.15

Differentiation after 72 hr exposure to RA was determined by the Nitro Blue tetrazolium test. Differentiation after 24 hr PMA-treatment was determined by adherence to the culture flask surface. C-1 and C-2 are untreated parallels to RA and PMA treated cells, respectively. Results are presented as mean values ± SE of 5 independent experiments.

added as a replacement for the surfactant action of some proteins.

We wanted to investigate the HL-60 cells' ability to proliferate and differentiate in the absence of identified and unidentified serum components. The second task was to characterize the HL-60 β-adrenergic signal system in this completely defined environment. The data show that the present system is useful for studies of signal systems central to proliferation and differentiation of a leukemic cell line.

MATERIALS AND METHODS

Chemicals. The following chemicals were employed in the study: (^{3}H)-(−)-CGP-12177 (sp. act. 53.1 Ci/mmol), (5,8-^{3}H)-cyclic 3′,5′-adenosine monophosphate, (sp. act. 52 Ci/mmol) from Amersham International plc (Buckinghamshire, U.K.), cyclic 3′,5′-adenosine monophosphate, (−)-alprenolol (+)-bitartrate, (−)-isoproterenol (+)-bitartrate, natrium pyruvate, L-glutamine, phorbol 12-myristate 13-acetate, retinoic acid and Nitro Blue tetrazolium from Sigma (St Louis, MO) and theophylline from the Norwegian Medicinal Depot (Harstad, Norway). All other chemicals were of analytical grade.

Incubation buffer for β-receptor studies. The composition of the incubation buffer was: NaCl 122 mM, KCl 4.9 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.3 mM, Na_2HPO_4 15.9 mM and *d*-Glucose 5 mM. The pH was 7.4.

Cell media. Serum supplemented medium (SSM): RPMI 1640 (Gibco Ltd, Paisley, U.K.) supplemented with 10% horse serum, 1 mM Na pyruvate, 1% MEM—non essential amino acids, 0.5 mM L-glutamine, 0.1 mg/ml streptomycin and 100 IU/ml penicillin.

Synthetic serum replacement supplemented medium (SSRM): RPMI 1640 powder (Gibco Ltd) dissolved in sterile water (Travenol Laboratories Inc., Deerfield, U.S.A.), supplemented with 0.1% Medicult® Synthetic Serum Replacement 2 (SSR 2) (Gea Ltd, Biotech, Hvidovre, Denmark), 15 mM Hepes, 3.6 g/l glucose (total concentration), 2.2 g/l $NaHCO_3$ (total concentration), 0.8 mM Na pyruvate, 3.3 mM L-glutamine (total concentration), 50 μg/ml streptomycin and 50 IU/ml penicillin. Osmolarity: 280 mOsm.

Composition of SSR 2 after dilution in final medium: 0.5 μg/ml insulin, 20.0 μg/ml Pluronic F 68, 4.0 μM EDTA, 40.0 μM sodium citrate, 3.0 μM aurintricarboxylic acid, 3.0 μM Fe, 1.0 nM Mn, 1.0 nM Cr, 0.1 μM Zn, 0.2 nM Ni, 0.2 nM Co, 20.0 nM Cu, 2.0 nM Al and 10.0 nM Se.

Cells. The human promyelocytic leukemia cell line HL-60 was grown at 37° in an atmosphere of 5% CO_2 and 95% air. Cell counts were determined by the use of a hemocytometer and cell viability was assessed by exclusion of Trypan Blue.

For β-adrenergic receptor studies, cells grown in SSRM for a minimum of 58 hr were washed twice in

Table 3. Characterization of the β-adrenergic signal system of intact HL-60 cells grown in serumfree medium

Maximal saturable binding sites (sites/cell)	2220 ± 150 (3)
K_d (nM)	0.15 ± 0.04 (3)
Basal cAMP level (pmol cAMP/10^6 cells)	6.8 ± 0.7 (6)
Maximal cAMP level (pmol cAMP/10^6 cells)	91.5 ± 24.1 (3)
EC_{50} (μM)	0.36 ± 0.01 (3)

Maximal binding and equilibrium dissociation constants were determined by the specific binding of ^{3}H-CGP 12177. Specific binding was measured in presence of 0.1 μM (−)-alprenolol. Basal cAMP levels were measured after 30 min preincubation with 4 mM theophylline at 37°. Maximal cAMP levels and EC_{50} were determined after stimulation with various concentrations of (−)-isoproterenol for 3 min. The results are presented as mean values ± SE, number in parenthesis = number of independent experiments.

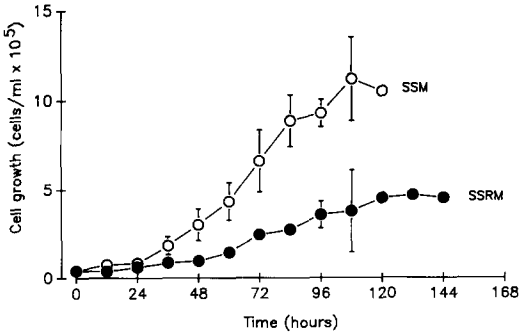

Fig. 1. Growth of HL-60 cells in serum-free (SSRM) and serum-supplemented (SSM) RPMI 1640 cell media. To obtain serum-free grown cells, HL-60 grown in SSM were washed twice in SSRM and went through at least two cell doublings before starting the experiment. The cells were kept in an atmosphere of 5% CO_2 and 95% air at 37°. Cell number was determined in a hemocytometer. The results are presented as mean values ± SE of 3 independent experiments.

ice-cold incubation buffer before final resuspension in ice-cold buffer to the desired cell concentration. Viability after this treatment was 93 ± 1% (mean ± SE, N = 9).

Differentiation. Differentiation along the granulocytic pathway was assessed by the Nitro Blue tetrazolium test, essentially as described [14], the number of positive cells being determined in a hemocytometer in unstained preparation [15].

Monocytic differentiation was measured by an adherence test. After removal of the medium, adherent cells were incubated for 10 min with 0.25% trypsin (Gibco Ltd) with 0.05% EDTA in PBS buffer at 37°. The cells lost their adherence after violet shaking of the culture flask. SSM was added in ratio 4 to 1 respective to trypsin, and the cells were counted in a hemocytometer. Viability was unaffected by this treatment (data not shown).

Radioligand binding. Intact HL-60 cells, 2.16 ± 0.20 × 10^6 (mean ± SE, N = 3) cells in a total volume of 500 μl, were incubated at gentle shaking for 30 min at 37°. Radioligand binding was performed as described previously [8].

Cyclic AMP determination. Intact HL-60 cells, 9.93 ± 0.04 × 10^5 cells (mean ± SE, N = 6) were incubated by gentle shaking at 37° in a total volume of 1 ml. After 30 min preincubation with 4 mM theophylline, the cells were stimulated with (−)-isoproterenol. The reaction was terminated by addition of ice-cold trichloroacetic acid to a final concentration of 10% (v/v). The concentrations of cAMP were determined by radioimmuno assay [16], with antibody raised in our laboratory.

Calculations. The binding data were analysed using the IBM-PC version of the radioligand binding programs EBDA and LIGAND (Elsevier Biosoft, Cambridge, U.K.) [17]. The concentration–response data were analysed by the IBM-PC-program "Meancurv" [18].

RESULTS

Cell growth

The HL-60 cells grew logarithmically in both media. The logarithmic phase doubling times were 28.7 ± 1.8 and 19.7 ± 0.5 hr (mean value ± SE, N = 3) in SSRM and SSM, respectively. The maximal cell density was lower in the synthetic serum replaced medium. In both media the viability was >94%.

Differentiation

Addition of RA and PMA caused inhibition of HL-60 cell growth in SSRM as well as in SSM. One μM RA very effectively blocked the proliferation of the HL-60 cells in serum-free medium (Table 2). In serum-supplemented medium the cell growth was reduced. The growth inhibition after treatment with 0.16 μM PMA was stronger in SSM than in SSRM ($P < 0.01$).

The spontaneous differentiation along the granulocytic pathway after 72 hr, was increased by 400% compared to the serum-supplemented medium. One μM RA caused >80% differentiation after 72 hr in both media. The degree of RA-induced differentiation was not different in the two media.

Differentiation along the monocytic pathway took place in both media in response to 0.16 μM PMA. After 24 hr exposure to PMA the cells grown in SSRM showed less adherence than the cells grown in SSM ($P < 0.01$). In SSM 91% of the cells were adherent after PMA treatment, while 57% of the cells in SSRM adhered to the culture flask surface.

β-receptor studies

The hydrophilic radioligand ^{3}H-CGP 12177 showed low non saturable binding to the HL-60 cells (Fig. 2). Analysis of concentration saturation experiments by the computer program EBDA and LIGAND revealed a single saturable binding site with $K_d = 0.15 \pm 0.04$ nM and $B_{max} = 2220 \pm 150$ sites per cell (mean value ± SE, N = 3).

The cAMP levels increased linearly in the presence of theophylline (results not shown). The unstimulated cAMP levels after 30-min preincubation was 6.8 ± 0.7 pmol/10^6 cells (mean value ± SE, N = 6). (−)-Isoproterenol stimulation caused a rapid accumulation of cAMP with maximal levels after 3

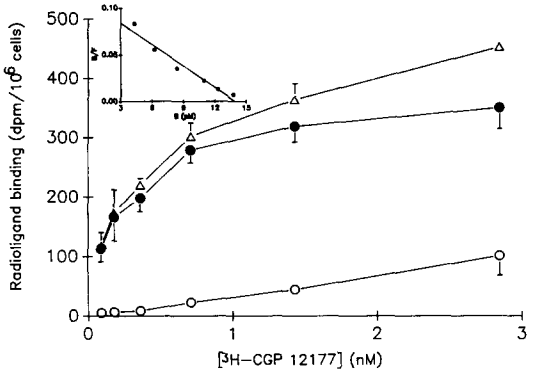

Fig. 2. Binding of ^{3}H-CGP 12177 to intact HL-60 cells grown in SSRM. The cells were resuspended in incubation buffer and allowed to achieve equilibrium with the added ligands at 37°. Total binding (△—△) and non-saturable (○—○) binding represents binding in absence and presence of 0.1 μM (−)-alprenolol, respectively. Saturable ^{3}H-CGP 12177 binding (●—●) is given as the difference between total and non-saturable binding. Inset: Saturable binding plotted according to Scatchard [19] (r = 0.98). The results are presented as mean values ± SE of 3 independent experiments.

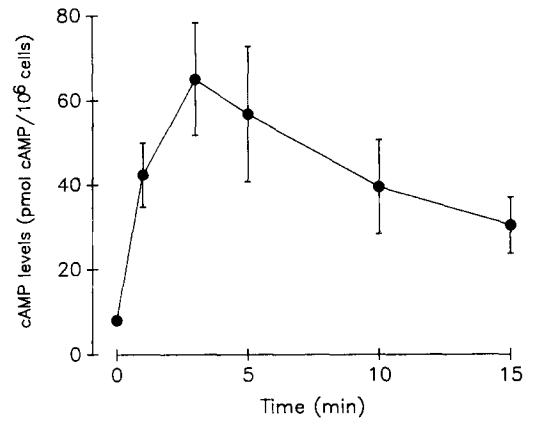

Fig. 3. cAMP accumulation in intact HL-60 grown in SSRM after stimulation by (−)-isoproterenol. The cells were resuspended in incubation buffer prior to the experiment. After 30-min preincubation with theophylline at 37° the cells were exposed to 10 μM (−)-isoproterenol for 0–15 min. The results are presented as mean values ± SE of 3 independent experiments.

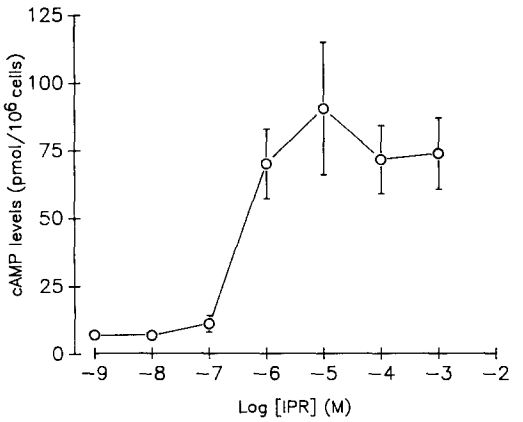

Fig. 4. cAMP accumulation in intact HL-60 cells grown in SSRM, as a function of (−)-isoproterenol concentration. The cells were resuspended in incubation buffer prior to the experiment. After 30-min preincubation with theophylline at 37°, the cells were exposed to the indicated (−)-isoproterenol concentrations for 3 min. The results are presented as mean values ± SE of 3 independent experiments.

to 5 min (Figs 3 and 4). The cAMP response was concentration dependent with half maximal stimulation (EC_{50}) at 0.36 ± 0.01 μM (mean value ± SE, N = 3). The range of maximal cAMP levels was 65 to 140 pmol/10^6 cells.

DISCUSSION

In this study we used a synthetic serum replacement medium completely devoid of added proteins and hormones other than insulin. Previous attempts to grow cells in serum-free environment have been limited by the apparent absolute requirements of some serum proteins, growth promoting factors and hormones. The last few years research in this area have made it possible to exclude, one by one, each of these serum components, substituting them with well-defined chemical substances. This has been possible as a better understanding of the primary needs of the cells is achieved. The absolute requirement for transferrin reported for many cell types [20] has been difficult to overcome for two main reasons: (1) The presence of physiological amounts of biocarbonate and Ca^{2+}/Mg^{2+} ions will precipitate iron and trivalent trace metals from almost any known chelator system (except transferrin) at 37° and pH 7–8 (Dr Kjell Bertheussen, unpublished results). (2) The ability of cells to utilize iron bound to non-protein chelators is limited. The medium used in this study overcomes these problems by a unique combination of the chelators EDTA and citrate, and aurintricarboxylic acid as an iron presenting substance (Dr Kjell Bertheussen, unpublished results). We are now left with insulin as the only biological molecule with multiple cellular effects. This hormone has so far not been possible to substitute with non-hormonal agents.

Primary cultures of murine hematopoietic progenitor cells (CFU-GEMM) grow in the absence of serum, but the presence of two growth factors (interleukin 3 and hemin) seems to be crucial [21]. Addition of bovine serum albumin, cholesterol, transferrin and insulin was essential for growth of human acute myeloblastic leukemia (AML) cells [22]. HL-60 cells have previously been shown to grow in serum-free culture, but Breitman *et al.* [23] found an absolute requirement for both insulin and transferrin. Our findings indicate that the addition of transferrin is not required for HL-60 growth.

The growth rate of HL-60 in SSRM was 69% and the saturation density was 41% compared to cells grown in SSM. This is slightly lower than the findings in insulin and transferrin supplemented contra calf serum supplemented medium [24]. The removal of transferrin from the serum-free medium may contribute to the difference. Addition of bovine serum

albumin to serum-free medium reduces the lag period and increases the overall cell growth (data not shown) [23]. The binding of breakdown products from dead cells and medium components to serum albumin may produce more favorable growth conditions. A protein-rich environment in addition to hormones and other growth promoting agents present in serum may thus contribute to optimal cell growth.

Serum is not required for the onset of differentiation of a number of cell types [21, 23–27]. Increased expression of differentiated functions have been shown in the absence of serum. Progesterone production in response to FSH is higher in porcine granulosa cells cultured in serum-free medium than in medium supplemented with 10% fetal calf serum [28, 29]. We report here that the spontaneous differentiation of HL-60 cells along the granulocytic pathway was increased by 400% in the absence of serum.

Increased cellular responsiveness to differentiation-inducing agents has been shown in serum-free media. An increased sensitivity to the colony growth promoting factors interleukin 3, erythropoietin and hemin is shown for CFU–GEMM when the cells are allowed to make differentiated colonies in serum-free as compared to serum-supplemented medium [21]. U937 [24] and HL-60 [25] show differentiated morphology after much lower doses of calcitriol in the absence than in the presence of serum. RA requirement for differentiation of embryonal carcinoma cells is also decreased in the absence of serum [26].

In conclusion, these observations suggest that some components of serum may inhibit the expression of differentiated functions of different cell types. However, serum may have the opposite effect on some hematopoietic cell types. Erythroid differentiation of the multipotent cell line K562 is higher in the presence than in the absence of serum [30].

With 1 μM RA we found >80% differentiation in both media. This is in agreement with the findings of Breitman *et al.* [23] who reported no difference between RA-induced differentiation in serum-free and serum-supplemented media. The extent of HL-60 differentiation in response to 1 μM RA is thus not influenced by the high binding capacity of serum albumin to RA (>99%) [31].

Lower adherence of macrophages have been demonstrated in serum-free medium [27]. This may be due to the absence of cell adhesion proteins provided by serum. Other differentiated characteristics, that is, antiviral activity, Fc mediated phagocytosis and Ia antigen expression were comparable after interferon-induced differentiation of murine macrophages in serum-free and serum-supplemented media [27]. The lower portion of adherent HL-60 cells after PMA treatment in serum-free medium, may thus be due to failure to adhere of the differentiated phenotypes rather than lower degree of differentiation. However the lower growth inhibiting effect of PMA in SSRM than in SSM suggests a lower differentiation in the absence of serum.

Components of serum modulates cAMP utilizing signal pathways. Rat brain astrocytes [32], human erythrocytes [32] and human mononuclear leukocytes [13, 34, 35] show increased β-adrenergic receptors and responsiveness in the presence of serum. In murine S49 lymfoma cells, horse serum caused an acute (within 1 hr) increase in basal and hormone stimulated cAMP accumulation [36].

On the other hand, the chronic exposure of S49 lymphoma cells to horse serum lead to a loss of hormone and cholera-toxin-stimulated cAMP accumulation [36]. The long-term effects of serum was due to a reduction of functional β-receptors and an induction of phosphodiesterase activity. A decrease in number of functional β-receptors on rat C6 glioma cells in the presence of serum was explained by receptor downregulation caused by serum chatecholamines [37].

This shows that serum may have diverse effects on the cAMP pathway of different cell types. The need of a more defined culture system in the study of these signal systems is thus evident. However most of the serum-free media still contain potentially modulating agents [20]. The presence of hormones, prostaglandins and proteins in serum-free media may affect the β-adrenergic system. Hydrocortisone [38], testosterone [39] and insulin (40) is shown to influence cAMP utilizing signal systems at different levels. Some of the prostaglandins occasionally added to serum-free media are potent stimulators of adenylate cyclase [41–43]. The number of β-adrenergic receptors may also be dependent on extracellular protein concentration [13].

We have characterized the β-adrenergic signal system in a medium completely devoid of added proteins and hormones other than insulin. The basal cAMP level, isoproterenol stimulation, number of CGP 12177 binding sites and dissociation constants were comparable to our previous findings in serum-supplemented medium [8]. The ability of the HL-60 cell to grow and differentiate in the absence of all proteins and hormones other than insulin, gives us the opportunity to fully control experimental conditions. This will be of great value in further studies on the role of this cAMP utilizing signal system in the process of differentiation.

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