Requirement of Serum Pretreatment for Induction of Alkaline Phosphatase Activity with Prednisolone, Butyrate, Dibutylrly Cyclic Adenosine Monophosphate and NaCl in Human Liver Cells Continuously Cultured in Serum-Free Medium

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ABSTRACT. A cell line (HuL-1) derived from normal fetal human liver was adapted to grow continuously in a modified Eagle's minimum essential medium without serum or hormones. The population doubling time of this adapted cell line (HuL-1-317) was about 72 h and the modal number of chromosomes was 54. The morphology of HuL-1-317 cells was round in the absence of serum, but at 37°C with the addition of serum (1-10%), the cells flattened. HuL-1-317 cells had a low level of alkaline phosphatase activity. However the enzyme activity was slightly enhanced by the combination of prednisolone, butyrate, dibutylrly cyclic adenosine monophosphate and a hypertonic concentration of NaCl after 3 days of incubation at 37°C. The increase in alkaline phosphatase activity with the four agents was further amplified dose-dependently by the pretreatment of the cells with serum. The stimulatory effect of the serum was evident at concentrations as low as 1%, and was maximal at 20%. The half life of the effect of serum on alkaline phosphatase induction was 48 h at 37°C. Serum alone could not enhance the enzyme activity without the four agents. The present results indicate that serum contributes to the regulation of alkaline phosphatase induction by the combination of prednisolone, butyrate, dibutylrly cyclic adenosine monophosphate and NaCl in fetal human liver cells (HuL-1-317).

It is difficult to establish a cell line which grows in a protein-free culture medium. At present, investigators studying the effect of serum on cells use cells continuously cultured in the presence of serum and preincubate them with serum-free media for a certain period of time prior to use to cancel the endogenous effect of serum. Using this method, it may be presumed that the short term effect of serum disappears during the cultivation of the cells. However, it is likely that the long term effect of serum remains. Thus, cell lines which can be continuously grown in protein-free media are potentially useful in elucidating the effects of serum on cellular physiology, including enzyme regulation by hormones or other agents.

Recently, we showed that prednisolone, butyrate, dibutylrly cyclic adenosine monophosphate (Bt₂cAMP) and a hypertonic concentration of NaCl, increased alkaline phosphatase activity in human urinary bladder carcinoma cells (HUB-15,

Reg. No., JTC-32). A great increase in alkaline phosphatase activity was observed with the combination of the four agents in the presence of serum (10). However, since the cells could not grow in protein-free media, the effect of serum on the enzyme induction was investigated by increasing the concentration of serum from 1% to 10% (10). In the present study, a cell line (HuL-1-317), derived from fetal human liver cells (HuL-1) (3) and adapted to grow in protein-free media, was used to investigate the effects of serum on the induction of alkaline phosphatase activity with prednisolone, butyrate, Bt₂cAMP and a hypertonic concentration of NaCl, since the parental cell line (HuL-1) showed enhanced enzyme activity by the four aforementioned agents (12).

MATERIALS AND METHODS

Cell culture. HuL-1 cells were established from normal fetal human liver. The primary culture was carried out in DM-170 supplemented 10% fetal bovine serum on May 31, 1979 (3). The serum was removed from the culture medium on March 14, 1980 (HuL-1-P3). The cells were then cultured continuously for more than 4 years in a modified serum-free Eagle's minimum essential medium (EM # 317) in which glutamine was equimolarly replaced with glutamic acid and to which biotin (25 pg/ml) was added (16). The cells have been cultured in this medium which contained neither hormones nor growth factors since June 6, 1982 (Niwa, unpublished data). We named the cell line HuL-1-317. Stock cells were cultured in glass culture flasks, and for subsequent experiments the cells were inoculated in plastic culture dishes with a 35-mm diameter (Corning, N.Y., U.S.A.). The cells were cultured in an atmosphere of 5% CO₂ and 95% air at 37°C. Fetal bovine serum (Lot No. 29K5542) obtained from GIBCO Laboratories (Chagrin Falls, OH., U.S.A.) was used in the present study, unless otherwise stated.

Enzyme assay. The cells were harvested with a silicone-rubber policeman and the cell suspension was centrifuged at 2,000 rpm for 15 min. The cell pellets were frozen at -20°C before use. They were homogenized in a Teflon-glass Potter-Elvehjem type homogenizer in H₂O at 0°C.

Alkaline phosphatase activity in cell homogenates was measured according to the method of Watanabe *et al.* (15), with a slight modification. These procedures have been described in detail in a previous paper (9). The protein concentration of cell homogenates was estimated according to the method of Lowry *et al.* (4) with bovine serum albumin as a standard.

Chromosome analysis. Specimens for the chromosome analysis of cultured cells were prepared by the dropping and flaming method (17).

Chemicals. Prednisolone, sodium butyrate and disodium phenylphosphate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bt₂cAMP was obtained from Yamasa Shoyu Co., Ltd. (Choshi, Chiba, Japan). Other chemicals were commercial products of reagent grade.

RESULTS

Cell growth. Figure 1 shows the growth curves of fetal human liver cells (HuL-1-317) in the presence and absence of serum. The population doubling time based on protein contents was about 72 h in the absence and presence of serum. The stimulatory effect of the serum on cell growth was very small.

Chromosome number. Based on chromosome analysis, the karyotypes distinctly showed that HuL-1-317 cells were of human origin. The distribution of

Fig. 1. Growth curves of HuL-1-317 cells in the presence and absence of serum. Stock cells, continuously cultured in the absence of serum, were inoculated in plastic culture dishes. After one day of cultivation, the cells were divided into the following two groups; one was cultured in serum-free medium (○—○) and the other was cultured in a 10% serum supplemented medium (●—●). The values are the means for 2–4 culture dishes.

chromosome numbers is shown in Fig. 2. The modal number was 54 chromosomes. In the parental cells (HuL-1), the number was 57 (3).

Changes in cell morphology by serum. The cell morphology of HuL-1-P3 cells, which are parental cells of HuL-1-317 cells, was flat in DM-170 without serum. However, the morphology of HuL-1-317 cells was round in the absence of serum (Fig. 3, A). The cells rounded during adaptation to a modified serum-free Eagle's

Fig. 2. Distribution of chromosome number in HuL-1-317 cells.

TABLE 1. DOSE-DEPENDENT EFFECT OF SERUM ON ALKALINE PHOSPHATASE ACTIVITY INDUCTION IN HuL-1-317 CELLS

Serum concentration (%, v/v)		Alkaline phosphatase activity (10 ⁻⁸ mol/min/mg protein)	
		Without induction	With induction
Experiment I	0	0.65 ± 0.14	3.23 ± 0.06
	1		7.29 ± 0.63
	2		8.84 ± 0.79
	5		12.0 ± 0.3
	10		15.6 ± 1.1
Experiment II	0	1.03 ± 0.12	3.28 ± 0.29
	10	1.25 ± 0.09	14.3 ± 1.4
	20	1.05 ± 0.03	18.4 ± 1.8
	50	0.87 ± 0.02	17.8 ± 1.3

Cells were pretreated with serum for 3 days, and then incubated with and without Bt_2cAMP (1 mM), prednisolone (0.5 $\mu\text{g}/\text{ml}$), butyrate (1 mM) plus NaCl (final 0.166 M) for 3 days. The optimal concentrations of the agents were determined according to the results obtained in our previous study (10). The values are the means $\pm$ S.D. for 3 or 4 culture dishes.

minimum essential medium. The addition of serum to the culture medium induced the cells to flatten (Fig. 3, B, C and D). The effect of 10% serum on shape appeared significant after 1 h of incubation at 37°C. The serum's effect was not observed at 4°C. Cell flattening took place at serum levels as low as 1%. When serum was removed from the culture medium, the cell morphology did not change to a round shape until the 8th day of culture.

Effects of serum on alkaline phosphatase induction. In order to check whether serum regulates the induction of alkaline phosphatase activity with prednisolone, butyrate, Bt_2cAMP and a hypertonic concentration of NaCl (10), enzyme induction was compared between serum-treated cells at different concentrations and untreated cells. When HuL-1-317 cells continuously cultured in a serum-free medium, were incubated with a combination of the four agents for 3 days at 37°C, alkaline phosphatase activity increased 3- to 5-fold of the control level (Table 1). In order to separate the effects of serum from those of the other agents, the cells were pretreated with serum alone, and then incubated with the four agents in the absence of serum. Serum alone did not increase the enzyme activity in the present study, which corroborates the finding of an earlier study in human cell cultures (2). In other cases, however, serum increased alkaline phosphatase activity in rat hepatoma cells (12, 13), chimpanzee liver cells (14) and in tissue culture of embryonic femora (5). When the serum-treated HuL-1-317 cells were incubated with the four agents, increases in enzyme activity were observed. The effect of serum increased in a dose-dependent manner. A significant effect was observed at the 1% level and higher effects were observed at 5–20% (Table 1). Therefore, the treatment of HuL-1-317 cells with serum was carried out at the 10% level throughout subsequent

Fig. 3. Phase-contrast photomicrographs of HuL-1-317 cells. $\times 200$. A, cultured in a serum-free medium; B, C and D, cultured in a 10% serum-supplemented medium for 5, 22 h and 2 days, respectively.

experiments. When the serum-untreated cells were simultaneously treated with serum and the four agents, the serum showed no stimulatory effect on alkaline phosphatase induction. In human urinary bladder carcinoma cells (HUB-15), the treatment of the cells with the combination of the four agents reduced the rates of cell growth and protein synthesis (10).

Time course of serum effects. The cells (HuL-1-317) were pretreated with 10% serum for various periods at 37°C, and then transferred to a serum-free environment for enzyme induction. The cells were treated with the four agents and alkaline phosphatase activity was measured. A substantial effect on alkaline phosphatase induction was observed for pretreatment periods as low as 2 days in duration (Fig. 4). The effect of serum pretreatment increased along with pretreatment time. After 2 days pretreatment there was a slight activity increase daily. Therefore, the cells were pretreated for 3 days in the present study.

Effects of different serum preparations. The effects of serum on alkaline phosphatase induction were investigated with various serum preparations (Table 2). Both fetal bovine serum and calf serum increased enzyme activity, although fetal bovine serum was more effective than calf serum. The dialysis of sera slightly decreased the effect of sera on the enzyme induction with the four agents. These results suggest that at least two serum components, one dialyzable and the other not, contribute to the regulation of alkaline phosphatase activity in HuL-1-317 cells with the contribution of the undialyzable components being larger than that of the dialyzable ones.

Induction of alkaline phosphatase. The effect of each agent on alkaline phosphatase activity was investigated with various combinations of the four agents (Table 3). Butyrate and NaCl slightly increased enzyme activity. Prednisolone and Bt₂cAMP alone had a negligible effect. The more the agents in combination, the

Fig. 4. Effect of serum on alkaline phosphatase induction with prednisolone, butyrate, Bt₂cAMP and NaCl in HuL-1-317 cells. Cells were cultured in the presence (●—●) and absence (○—○) of 10% serum for the stated periods, and then incubated with the combination of the four agents for 3 days at 37°C in the absence of serum. The concentrations of the agents were described in the legend to Table 1. The values are the means for 3 or 4 culture dishes, with S.D. within 5% of the mean values.

TABLE 2. EFFECTS OF DIALYZED AND UNDIALYZED SERA ON ALKALINE PHOSPHATASE ACTIVITY INDUCTION IN HuL-1-317 CELLS

Serum preparation	Alkaline phosphatase activity (10^{-8} mol/min/mg protein)
None	3.11 ± 0.21
Fetal bovine serum (1)	
Undialyzed	12.8 ± 0.4
Dialyzed	12.1 ± 0.9
Fetal bovine serum (2)	
Undialyzed	14.8 ± 0.8
Dialyzed	11.3 ± 0.6
Calf serum (1)	
Undialyzed	11.0 ± 0.5
Dialyzed	8.9 ± 0.4
Calf serum (2)	
Undialyzed	9.64 ± 0.4
Dialyzed	7.53 ± 0.3

Dialyzed sera; sera were dialyzed against phosphate buffered saline, pH 7.4, overnight at 4°C. Cells were pretreated with and without sera at 10% for 3 days, and then incubated with the four agents for 3 days, as described in the legend to Table 1. The values are the means $\pm$ S.D. for 3 culture dishes.

Fetal bovine serum (1) (Lot No. 79801), Microbiological Associates Inc., Bethesda, MD.; fetal bovine serum (2) (Lot No. 29K5542), GIBCO Laboratories, OH.; calf serum (1) (Lot No. R168125), GIBCO; calf serum (2) (Lot No. R639125), GIBCO.

TABLE 3. INDUCTION OF ALKALINE PHOSPHATASE ACTIVITY UNDER VARIOUS CONDITIONS IN HuL-1-317 CELLS

Condition	Enzyme activity (10^{-8} mol/min/mg protein)
None	1.32 ± 0.08
Prednisolone	1.66 ± 0.10
Butyrate	2.44 ± 0.07
Bt ₂ cAMP	1.42 ± 0.12
NaCl	1.98 ± 0.20
Prednisolone + Butyrate	2.72 ± 0.24
Prednisolone + Bt ₂ cAMP	1.58 ± 0.08
Prednisolone + NaCl	3.93 ± 0.25
Butyrate + Bt ₂ cAMP	1.79 ± 0.10
Butyrate + NaCl	8.24 ± 0.36
Bt ₂ cAMP + NaCl	2.90 ± 0.15
Prednisolone + Butyrate + Bt ₂ cAMP	3.49 ± 0.10
Prednisolone + Butyrate + NaCl	17.7 ± 0.7
Prednisolone + Bt ₂ cAMP + NaCl	5.01 ± 0.50
Butyrate + Bt ₂ cAMP + NaCl	8.73 ± 0.82
Prednisolone + Butyrate + Bt ₂ cAMP + NaCl	19.7 ± 2.0

Cells were preincubated with 10% serum for 5 days at 37°C, and then incubated with various agents for 3 days at 37°C in the absence of serum. The values are the means $\pm$ S.D. for 3 or 4 culture dishes.

TABLE 4. EFFECTS OF CYCLOHEXIMIDE AND ACTINOMYCIN D ON THE INCREASE IN ALKALINE PHOSPHATASE ACTIVITY BY PREDNISOLONE, BUTYRATE, Bt₂cAMP AND NaCl IN HuL-1-317 CELLS

Condition	Enzyme activity (10 ⁻⁸ mol/min/mg protein)	
	Without agents	With agents
Serum-free	0.45 ± 0.05	1.27 ± 0.02
+ Cycloheximide	0.85 ± 0.08	1.08 ± 0.06
+ Actinomycin D	0.59 ± 0.04	0.63 ± 0.01
Serum-treated	0.56 ± 0.04	5.97 ± 0.10
+ Cycloheximide	0.82 ± 0.04	1.42 ± 0.08
+ Actinomycin D	0.98 ± 0.08	0.97 ± 0.09

Serum-treated cells, the cells were pretreated with 10% serum for 3 days. The cells were incubated with and without the four agents for 3 days (see the legend to Table 1). The concentrations of cycloheximide and actinomycin D were 10 µg/ml and 5 µg/ml, respectively. The values are the means ± S.D. for 3 culture dishes.

higher the induction observed. The largest increase was observed with the combination of all four agents, although the stimulatory effect of Bt₂cAMP was very small. However, the effect of Bt₂cAMP on enzyme induction was significantly observed in human urinary bladder carcinoma cells (HUB-15) (10).

Effects of cycloheximide and actinomycin D on the enzyme induction. In order to check whether the increase in alkaline phosphatase activity by the four agents involves a new enzyme synthesis, the cells were incubated with the four agents in the presence and absence of cycloheximide or actinomycin D. Both cycloheximide and actinomycin D inhibited the increase in the enzyme activity with the four agents

Fig. 5. Decrease in the effect of serum on alkaline phosphatase induction during cultivation of the cells in the absence of serum. Cells cultured at 10% serum for 3 days at 37°C, were then cultured in the absence of serum. The concentrations of the four agents were as described in the legend to Table 1. The values are the means for 3 culture dishes.

(Table 4). These results suggest that the increase in alkaline phosphatase activity is a consequence of greater biosynthesis of the alkaline phosphatase molecules with the four agents. More direct evidence for this conclusion will be obtained by the quantitative measurement of alkaline phosphatase mRNA under various conditions. It was reported that actinomycin D increased alkaline phosphatase activity *in vivo* (7, 8), and that actinomycin D as well as cycloheximide increased enzyme activity in rat hepatoma cells (11, 13). In the present study, however, these drugs did not increase enzyme activity.

Effective length of serum. In order to investigate the effective length of the serum, the cells, preincubated with 10% serum for 3 days were further cultured in the absence of serum for the stated periods, and then incubated with the four agents for 3 days in the absence of serum. The effect of serum on alkaline phosphatase induction decreased exponentially, as shown in Fig. 5. The half life of the serum effect was 48 h at 37°C. The effect of serum on alkaline phosphatase induction was observed to be significant, even after 5 days of culture in the absence of serum. It takes about one week to reduce the effect of serum on alkaline phosphatase induction to less than 10% of the initial serum effect.

DISCUSSION

Fibronectin caused flattening of round BHK cells (1), derived from hamster kidney. On the other hand, Michl *et al.* (6) recently showed that serum components, except for fibronectin, caused HeLa cells to flatten. When HuL-1-317 cells were incubated with fibronectin at 2–10 µg/ml at 37°C, the cellular morphology did not change. However, the round HuL-1-317 cells was flattened by serum passed through a gelatin-Sepharose column, in which fibronectin was adsorbed (unpublished data). Judging from these results, the cell flattening phenomena of HuL-1-317 cells may be induced by other substances such as vitronectin and laminin.

Evidently, serum caused the cells to flatten, and serum treatment was required for the induction of alkaline phosphatase with prednisolone, butyrate, Bt₂cAMP and a hypertonic concentration of NaCl. After removal of serum from the culture medium, the effect of serum on cell morphology of HuL-1-317 cells did not change until the 8th day, whereas the effect of serum on alkaline phosphatase induction decreased with increasing culture period in the absence of serum. In addition, the effective concentration of serum required to induce cell morphological changes differed from that required to induce alkaline phosphatase induction. Thus, the morphological change of the cells with serum might not be directly linked to the regulatory mechanism of alkaline phosphatase induction with hormones or other agents.

The present results (Table 1 and Fig. 4) indicate that, regarding the four agents tested, serum treatment of the cells was required for alkaline phosphatase induction to occur. The effect of serum on alkaline phosphatase induction did not disappear immediately from serum-treated HuL-1-317 cells after removal of the serum (Fig. 5). Evidently, the effect of serum remained significantly, even though the cells were cultured in the absence of serum for a few days. Therefore, a clear result of the effect of serum was not obtained with the parental cells (HuL-1) continuously cultured on 10% serum, even when the cells were preincubated in a serum-free medium. The long half life of serum effects should be taken into consideration if cells continuously cultured in the presence of serum are used for an investigation of

the effect of serum.

The present results suggest that various agents including hormones and serum protein(s) mutually affect the physiology of living cells. Therefore, the synergistic effect of various agents can be observed in several cellular phenomena. The present study confirmed that a combination of the four agents, prednisolone, butyrate, Bt₂cAMP and a hypertonic concentration of NaCl, provides an enhanced condition for alkaline phosphatase induction. In the present study, the increase in alkaline phosphatase activity with the four agents was completely inhibited by either cycloheximide or actinomycin D in HuL-1-317 cells (Table 4). Similar data were obtained in the previous study (10). It seems, therefore, that the serum constituents affect the synthesis of the alkaline phosphatase molecule in HuL-1-317 cells. This cell line should be useful for further investigations on the effect of serum on enzyme regulations as well as on the regulatory mechanism of cell flattening.

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