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THE ANTAGONISTIC EFFECT OF DEXAMETHASONE AND INSULIN ON α-FETOPROTEIN SECRETION BY CULTURED H4-II-E-C3 CELLS DERIVED FROM THE REUBER H-35 HEPATOMA

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SUMMARY: Non-confluent monolayers of H4-II-E-C3 cells were maintained in serum-free media. Dexamethasone alone (5×10^{-7}M) stimulated α-fetoprotein secretion 2- to 4-fold while insulin alone (8.7×10^{-8}M) inhibited α-fetoprotein secretion by 20%. When dexamethasone (5×10^{-7} to 5×10^{-9}M) and insulin (8.7×10^{-8} to 8.7×10^{-11}M) were added simultaneously, insulin diminished the stimulatory effect of dexamethasone. When α-fetoprotein secretion was elevated by dexamethasone and the medium was replaced by media containing either insulin or no hormones, the rate of α-fetoprotein secretion diminished more rapidly with the insulin-supplemented medium. Alone or in combination, insulin and dexamethasone had little effect on albumin secretion.

INTRODUCTION: Hepatoma cell lines are useful tools for the study of enzyme or protein induction by hormones and other substances (1-16). The cell line H4-II-E-C3, derived from the Reuber H-35 hepatoma, and cell strains derived from that line have proven valuable for studying regulation of the synthesis of enzymes (6-12), albumin (13-16) and other proteins (15,16). We presently report the effects of INS, DEX, and DEX + INS on the secretion of ALB and AFP by nonconfluent cultures of H4-II-E-C3(7) cells maintained on tissue culture plates in serum-free media.

MATERIALS AND METHODS: Cell Growth in S77 with Serum. After subculture by trypsin transfer (4), Reuber (H35) hepatoma cells of the H4-II-E-C3 line (7) were grown under an atmosphere of 5% CO_2 and 95% air as monolayers at 37°C in 55 mm diameter plastic tissue culture plates (Falcon Plastics, Oxnard, California) containing 4 ml of modified Swim's S77 medium with serum (6) hereafter referred to as S77 with serum. Powdered S77 medium and the sera were from GIBCO, Grand Island, N.Y. The inoculum was 2×10^5 cells per plate. The medium was replaced with fresh medium at 72 hr and every 24 hr thereafter (Fig. 1). The growth characteristics of this cell line, in this medium have been studied in detail (17).

Hormonal Additions to Cultures Maintained in S77. To assess the effect of INS and DEX on AFP and ALB secretion, the cells were grown for 72 hr in S77 with serum and then maintained in S77 for at least 24 hr prior to the first addition of hormones (Fig. 1). Hormones or their carriers were added to S77 just prior to the daily addition of the fresh media to cultures. Glucagon-free crystal-

Abbreviations: INS, insulin; DEX, dexamethasone; ALB, albumin; AFP, α-fetoprotein

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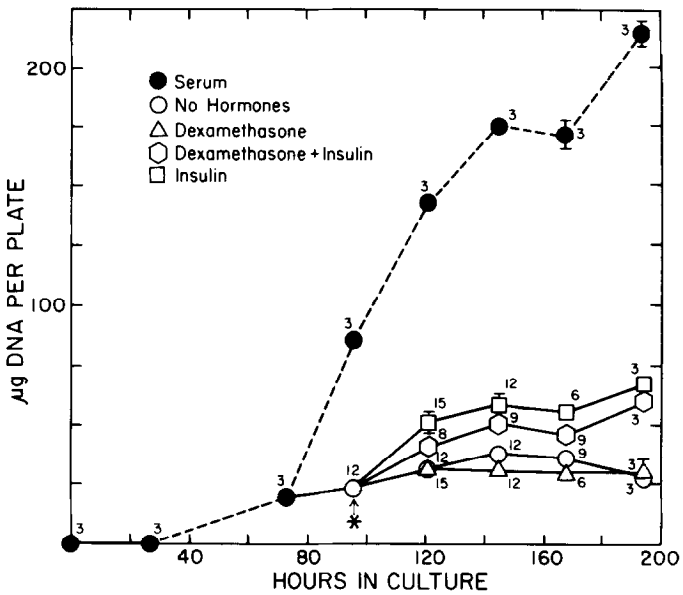


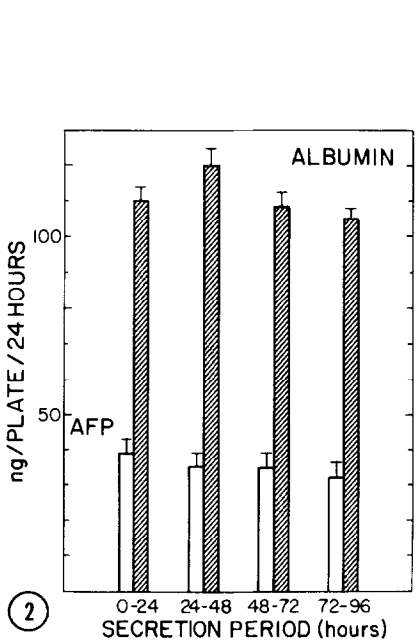
Figure 1: DNA content of H4-II-E-C3 cell monolayers maintained in S77 with serum (solid circles) or in serum-free S77 (open symbols). The medium was replaced at 72 hr and every 24 hr thereafter. Hormones (INS, 8.7 x 10^{-8}M; DEX, 5 x 10^{-7}M), if present, were first added at (*) and with each succeeding medium change. The number beside each symbol indicates the number of plates used to calculate the mean and S.E.M. (shown by vertical lines when extending beyond the symbol).

line porcine insulin (INS) and its carrier solution were gifts from Dr. Ronald E. Chance, Lilly Research Laboratories, Indianapolis, IN. Decadron (DEX) and its carrier solution (a gift) were from Merck, Sharp and Dohme, West Point, PA.

Collection of Media and Cell Harvest. Culture medium that had been with cells for 24 hr was removed, cooled to 2°C, and centrifuged for 5 min at 500 g before assay of AFP and ALB in supernatants. To estimate cell monolayer protein and DNA, the cells on the plates were rinsed twice with 0.85% NaCl before freezing on dry ice and storage at -20°C. The cells were then scraped from the plates with a rubber policeman, using 0.85% NaCl. Protein was determined with the Folin reagent (18). Bovine serum albumin served as the standard. DNA was extracted by the Schmidt-Thannhauser procedure followed by hot acid (0.5N $HClO_4$, 15 min, 90°C) hydrolysis (19) and DNA estimation by a fluorometric assay (20).

AFP and ALB Determination. AFP and ALB were determined either by Mancini's single radial immunodiffusion method (21) or by radioimmunoassay (22) as reported previously (23,24). Supernatants concentrated by Minicon B15 (Amicon Corp., Lexington, MA) ultrafiltration were used in assays by Mancini's method.

RESULTS: Fig. 1 shows that the shift from S77 with serum to the serum-free S77 medium essentially halted growth. When INS was added to this serum-free medium, the DNA per plate increased during the first 24 hr but thereafter remained at a rather constant plateau value. DEX partially inhibited the INS-mediated increase in DNA. Relative to cultures with no hormones added, DEX alone had little effect on DNA synthesis.



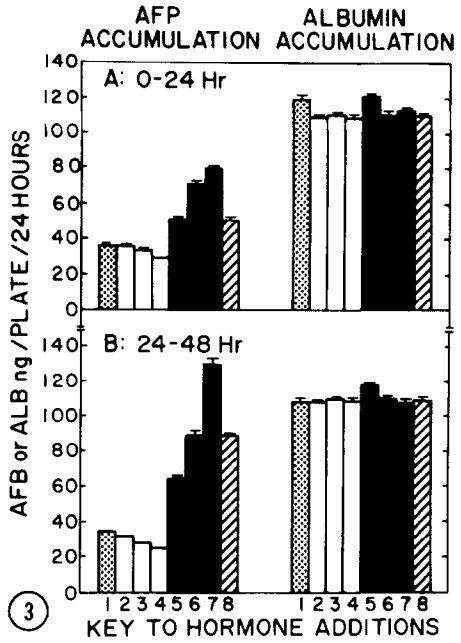
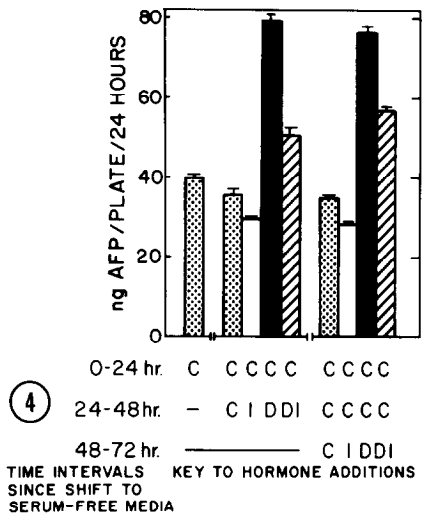


Figure 2: Secretion of ALB (shaded bars) and AFP (open bars) during 24-hr intervals by H4-II-E-C3 cells maintained in serum-free S77 medium without hormonal supplements. Vertical lines indicate the S.E.M. for 3 plates per secretion period. The medium was changed every 24 hr.

Figure 3: ALB and AFP secretion by H4-II-E-C3 cells treated with DEX, INS, or DEX + INS. A. First 24-hr secretion period, 24-48 hr after shift to serum-free S-77. B. Second 24-hr secretion period, 48-72 hr after shift to serum-free S-77. Supplements were first added 24 hr after shift to serum-free S77. Media, with supplements indicated below, were replaced every 24 hr. 1. control; 2. 8.7×10^{-10}M INS; 3. 8.7×10^{-9}M INS; 4. 8.7×10^{-8}M INS; 5. 5×10^{-9}M DEX; 6. 5×10^{-8}M DEX; 7. 5×10^{-7}M DEX; 8. 8.7×10^{-8}M INS and 5×10^{-7}M DEX. Each bar is the mean for 3 plates. S.E.M. is shown by vertical lines.

In the absence of hormones, the accumulation of ALB and of AFP in the medium during each 24-hr interval studied was constant within experimental error (Fig. 2).

When DEX was first added to cultures at 24 hr after the shift to serum-free S77 (Fig. 1), it stimulated AFP secretion during both the first (Fig. 3A) and second (Fig. 3B) 24-hr periods of AFP accumulation. The degree of stimulation was directly related to the DEX concentration. INS partially inhibited (32-37%) the DEX-mediated increase in AFP secretion and INS alone, at the highest concentration (8.7×10^{-8}M), inhibited AFP secretion by 20% relative to cultures with no hormones. INS alone, DEX alone, or INS + DEX had little effect on ALB secretion (Fig. 3A,B). The carrier solutions for INS and DEX had no detectable effect on the secretion of ALB or AFP. The absolute amount of AFP and ALB secreted per 24 hr (ng/plate/24 hr) was little affected by INS,



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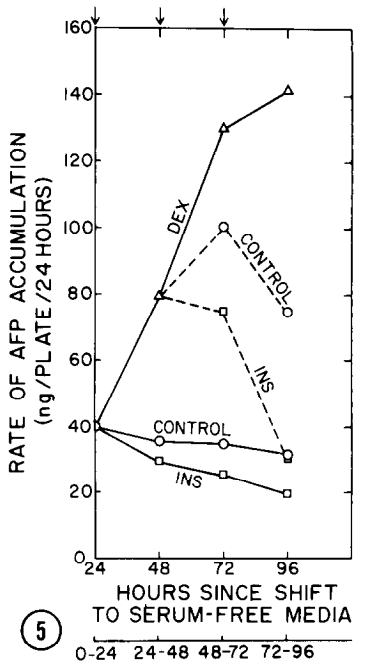

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Figure 4: Comparison of hormone effects on AFP secretion by H4-II-E-C3 cells when hormones were first added at 24 hr or at 48 hr after the shift to serum-free media. C, control; I, 8.7×10^{-8}M INS; D, 5×10^{-7}M DEX. Media were replaced every 24 hr. Each bar is the mean for 3 plates. S.E.M. is shown by vertical lines.

Figure 5: Change in AFP secretion rate after shift from a DEX-supplemented to a control or INS-supplemented medium. Hormones were first added 24 hr after the shift to serum-free media. Arrows indicate medium changes. ○, control; △, 5×10^{-7}M DEX; □, 8.7×10^{-8}M INS. Each point represents the mean for 3 plates. S.E.M.'s fall within the symbols.

even though INS (8.7×10^{-8}M) induced a doubling of monolayer DNA during the first 24 hr with little increase thereafter. We present our data in absolute terms (ng/plate/24 hr) but have commented wherever secretion patterns differ markedly if expressed as ng/mg protein/24 hr.

Any residual effects of serum on AFP secretion had apparently subsided within the first 24 hr that the cells were in the serum-free S77 since the response of AFP secretion to INS, DEX, or INS + DEX was the same whether hormones were first added at 24 hr or at 48 hr after the shift to the serum-free medium (Fig. 4).

When DEX had increased the AFP secretion rate to twice the basal rate and the DEX was then replaced by INS or no hormones (Fig. 5), the rate of AFP secretion declined more rapidly with INS than with no hormones in the medium. INS, DEX, or INS + DEX caused no detectable change in the intracellular accumulation of AFP (data not shown).

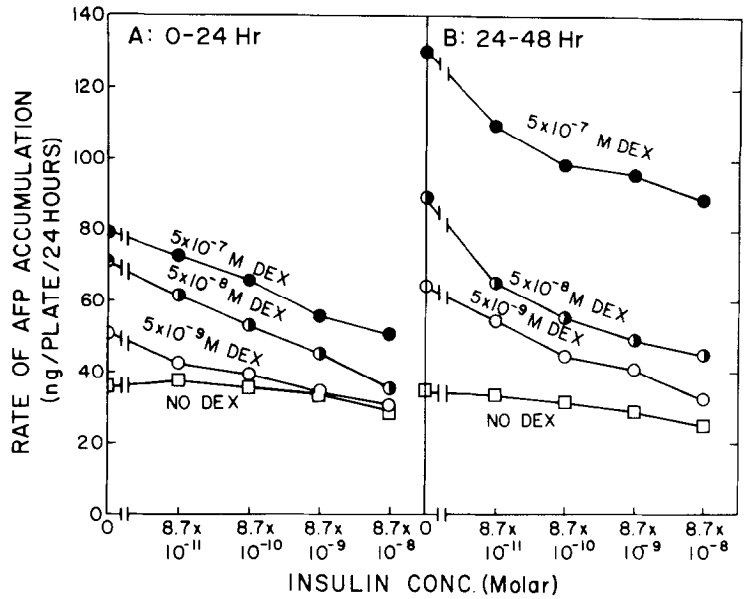

Figure 6: The effect of various INS and DEX concentration combinations on AFP secretion by H4-II-E-C3 cells. Hormones were first added 24 hr after the shift to serum-free media. A, first 24-hr secretion period, 24-48 hr after shift to serum-free S77. B. second 24-hr secretion period, 48-72 hr after shift to serum-free S77. Media were replaced every 24 hr. Each point represents the mean for 3 plates. S.E.M.'s fall within the symbols.

At all DEX concentrations tested (5×10^{-7} to 5×10^{-9}M), INS (8.7×10^{-8} to 8.7×10^{-11}M) partially or completely inhibited the DEX-mediated stimulation of AFP secretion (Fig. 6). An increasing concentration of INS had little effect on AFP secretion in the absence of DEX but had a direct concentration dependent effect in diminishing the DEX-stimulated component of AFP secretion. When these results were plotted on the basis of ng AFP/mg protein/24 hr the plots differed in two minor ways. a. INS, at the lower concentrations, appeared slightly more effective in antagonizing the DEX-mediated effect. b. The antagonism between DEX and INS was diminished during the 24-48 hr period of AFP secretion in the continued presence of the highest concentration of DEX and the two highest concentrations of INS.

DISCUSSION: This is the first time that AFP production has been demonstrated in the H4-II-E-C3 cell line. The secretion of AFP by this line was, however, three orders of magnitude less than that by the McA-RH7777 and McA-RH8994 hepatoma cell lines (3,4).

In the present study, the production of AFP was compared with that of ALB, another secreted product of H4-II-E-C3 cells (13-16). This line, in the absence of serum and added hormones, produced AFP and ALB at 0.32:1 while the

ratio was 600:1 for McA-RH7777 hepatoma cells (4). In H4-II-E-C3 cells, INS (8.7×10^{-11} to 8.7×10^{-8}M) had little effect on AFP production while DEX (5×10^{-9} to 5×10^{-7}M) stimulated AFP production and INS antagonized the effect of DEX. In contrast, ALB production was negligibly affected by either DEX, INS, or DEX + INS. A similar differential stimulation of the production of secreted proteins was seen in a study of ALB and retinol binding protein (RBP) secretion by H4-II-E-C3 cells (16) in which retinol (0.35 or 3.5 nmole/ml) stimulated the synthesis and secretion of RBP without affecting the basal level of ALB synthesis and secretion. Differential control of ALB and AFP synthesis was also found in studies of secretion during liver development (25,26), during the growth phase of fetal liver cells (27) and during the cell cycle of synchronized hepatoma cell cultures (23).

INS, even at the lowest concentration tested (8.7×10^{-11}M), partially inhibited the DEX-mediated increase in AFP secretion by H4-II-E-C3 cells. Others have observed that INS (5×10^{-10} to 1.8×10^{-6}M) can inhibit DEX-mediated (2×10^{-8} to 10^{-5}M) increases of phosphoenolpyruvate carboxykinase activity (8) and synthesis (9,10) in H4-II-E-C3 cells and of argininosuccinate synthetase and argininosuccinate lyase in fetal rat liver explants in organ culture (28). The mechanism for this antagonism remains obscure (8-10,29).

Changes in ALB and AFP phenotypic expression associated with liver tumorigenesis apparently do not involve permanent changes in gene number or rearrangement of ALB and AFP genes (30). The level of ALB and AFP synthesis in liver and Morris hepatoma 7777 was mainly regulated by modulating the steady-state concentration of the corresponding functional mRNA's and translational control did not appear to be a major factor (31).

In H4-II-E-C3 cells and other hepatoma cell lines (4), there was an inverse relationship between the response of cell multiplication and of AFP synthesis to DEX addition. Thus, DEX slightly inhibited DNA synthesis or cell multipliciation and stimulated AFP secretion in H4-II-E-C3 and McA-RH8994 (4) cells while DEX stimulated cell multiplication and depressed AFP production by McA-RH7777 cells (4). Similarly, INS both increased DNA synthesis slightly and reduced AFP secretion when antagonizing DEX-stimulated AFP secretion in H4-II-E-C3 cells. The relationship between AFP synthesis and DNA synthesis may be permissive or temporal rather than causal since AFP can be synthesized before or without detectable DNA synthesis (23,24,32,33). AFP was produced during only part of the cell cycle in hepatoma clones (23,24) and fetal liver hepatocytes (34) while ALB was produced throughout the cell cycle in a clone of the H4-II-E-C3 line (13). DEX and INS could, then, affect AFP synthesis without affecting ALB synthesis if these hormones changed cell cycle parameters (35) or the balance among cycling and noncycling cell phenotypes (26,36). Cell

populations differing with respect to AFP and ALB synthesis may exist even in a synchronously-growing hepatoma (AH-66) cell line. Double immunofluorescent staining at late S phase, where both AFP and ALB synthesis were nearly maximal, showed that AFP and ALB were probably synthesized by different cells (23). Also, in nonsynchronized cells, the percent of AFP-synthesizing McA-RH7777 hepatoma cells varied with position in the cell growth curve (37). Further studies of AFP and ALB synthesis, using DEX and INS as tools, may be useful for determining whether changes in the cell cycle or in the balance among cycling and noncycling cell phenotypes are related to the appearance of AFP-producing and ALB-producing hepatoma cell populations.

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