

# GLUCOCORTICOID-INDUCED ALTERATION OF THE SURFACE MEMBRANE OF CULTURED HEPATOMA CELLS

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## ABSTRACT

Glucocorticoids induce an alteration of the surface of hepatoma tissue culture (HTC) cells as expressed by changes in cell electrophoretic, antigenic, and adhesive properties. The alteration is assayed by the increased adhesiveness of induced cells for a glass surface. The induction process has a lag period of about 3 hr and attains a plateau level after 24–30 hr when 50–80% of the steroid-treated cells are firmly adhered. Less than 10% of untreated cells adhere under the same conditions. Induction is inhibited by actinomycin D and cycloheximide, demonstrates both pH and temperature dependence, and responds to changes in steroid concentration and structure. By contrast, the attachment per se of preinduced cells is not affected by inhibitors of RNA and protein synthesis, fluctuations of temperature and pH, and the presence or absence of the hormone. When the induction process is reversed by removal of steroid or addition of actinomycin D, preinduced adhesiveness is lost with a half-life of 13–24 hr, but in the presence of cycloheximide the loss is accelerated ( $t_{1/2}$  3–5.5 hr). These results suggest that glucocorticoids induce the biosynthesis of a protein which either modifies the cell surface (an enzyme) or is incorporated into surface structures (structural protein).

## INTRODUCTION

The synthesis of tyrosine aminotransferase in hepatoma tissue culture (HTC) cells is stimulated by adrenal steroid hormones (1–3). In this report we describe a second specific regulatory effect of the glucocorticoids in HTC cells: an induced alteration in the cell surface is expressed as changes in cell adhesive, electrophoretic, and antigenic properties. These inductions, both of which require new RNA and protein synthesis, occur without any detectable effect on the rates of total RNA

and protein synthesis (2). A preliminary report of this work has been published (4).

The mammalian cell surface may mediate such diverse biological phenomena as growth regulation (5, 6), transport (7), morphogenesis (8), and cell recognition and histocompatibility (9). Therefore the demonstration that specific surface factor(s) are under hormonal control may contribute to our understanding of both cell surface functions and control mechanisms for synthesis of specific proteins.

The hormone-induced alteration of the HTC cell surface was first detected as an increased adhesiveness of induced cells for a glass surface. As shown schematically in Fig. 1, the adhesion process can be visualized as three distinct reactions: (1) induction of surface adhesive factor(s) by the hormones, (2) the reversible attachment of induced cells to glass, and (3) flattening and coherence of cells which have been attached for prolonged periods. In the present communication various properties of reaction 2 (cell attachment) are presented first, and subsequently the induction process (reaction 1) is described.

#### MATERIALS AND METHODS

Dexamethasone (Dex),<sup>1</sup> a synthetic glucocorticoid, was a gift of Merck Chemical Company, Rahway, N. J.; other steroids were purchased from Mann Research Labs., Inc., New York. Stock solutions were prepared in absolute ethanol at a concentration of  $5 \times 10^{-3}$  M. Actinomycin D, puromycin, and cycloheximide, obtained from the Cancer Chemotherapy Section of the National Cancer Institute, Bethesda, Md., were dissolved in ethanol. Wheat germ lipase was obtained from Calbiochem, Los Angeles, Calif., and all other enzyme preparations were purchased from Worthington Biochemicals Corp., Frechold, N. J.

HTC cells, an established line of heteroploid cells derived from a Morris hepatoma (3), were grown in suspension culture at 37°C in Swim's S77 (Grand Island Biological Co., Grand Island, N. Y.) "growth medium" containing 10% serum (bovine and fetal calf) and modified as described elsewhere (10). Under these conditions the cells have a doubling time of 22–24 hr, remain in log phase growth between 50,000 and 700,000 cells/ml, and enter stationary phase at a cell density of approximately  $1 \times 10^6$ /ml. Detroit 6, Wistar Institute Susan Hayflick, and HeLa S-3 cell lines were kindly supplied by Dr. William Uhlenhof; Haman epithelial-2 and L-929 cell lines were obtained from Grand Island Biological Company; and Dr. E. Brad Thompson donated a line of Chinese Hamster epithelial cells (11). A second population of HeLa S-3 cells adapted for suspension culture was obtained through the courtesy of Dr. B. Moss. All cell lines grew readily in the standard tricine-buffered growth medium used for HTC cells. Cell densities were determined by hemocytometer counts and turbidity measurements (absorbance at 650 m $\mu$ ); a linear relationship existed between cell

<sup>1</sup>Free Dexamethasone, rather than Dexamethasone phosphate (1–3), was used in these experiments.

count and turbidity at all cell densities less than about  $1 \times 10^6$ /ml. Cell viability was established by exclusion of trypan blue stain (12). Cell electrophoresis was measured in a microelectrophoresis apparatus utilizing flat glass chambers and palladium electrodes (13).

Measurements of cell adhesion were carried out in serum-free "induction medium" which was otherwise identical to growth medium. HTC cells suspended in induction medium do not divide, but survive for at least 3 days (3). However, cell adhesiveness could be induced in either growth or induction medium. In growth medium, this was accomplished by growing cells for at least 2 days (to late log or early stationary phase) in the presence of  $10^{-6}$  M Dex. The cells were collected by centrifugation at room temperature for 5 min at 900 g, washed once or twice in induction medium, and resuspended in induction medium at the desired cell concentration (generally  $\sim 350,000$ /ml). 10-ml aliquots of the suspension were pipetted into sterile 125 ml glass bottles (Wheaton Glass and Plastics Co., Millville, N. J.) which were then rotated (preventing cell attachment) for variable periods at 100 rpm at 37°C in a Metabolyte gyrotatory water bath shaker (New Brunswick Scientific Co., New Brunswick, N. J.). The bottles were then incubated further at 37°C without shaking, and the ensuing adherence of the cells to glass was measured at desired intervals by the standard assay described below.

For induction in the nongrowing state, HTC cells, grown to the log phase in the absence of inducer, were washed and resuspended in serum-free induction medium at a concentration of 300,000–600,000/ml. Either ethanol (control) or inducer was added to aliquots of the cell suspensions which were then treated as described above.

We quantitated the adhesion of HTC cells to the glass surface by measuring the number of unattached cells in the incubation medium after a suspension was subjected to a standard shearing force consisting of shaking the vessels at 37°C in the gyrotatory water bath shaker for 40 sec at 100 rpm. Immediately thereafter a 1 ml aliquot of cell suspension was removed and the density of the unattached suspended cells was determined by measurement of turbidity at 650 m $\mu$  in a Gilford UV recording spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). The data are expressed either as per cent decrease in  $A_{650}$  from a zero time value (inversely proportional to adhesion), or as the percentage of total cells adhering. In experiments with several samplings the absorbance values were usually corrected to account for the decreasing volume of medium in each bottle. In routine kinetic experiments, however, the uncorrected absorbance values differed by less than 3% from corrected data. All the values reported represent the

average of duplicate experiments which varied less than 10%.

Fig. 2 shows the adherence of HTC cells after 6 hr of incubation with Dex. The cell concentrations in the medium, expressed as total units of absorbance ( $650\text{ m}\mu$ ) present, were measured after different intervals of shaking. As shown, there is a rapid increase in the number of unattached cells during the first 20–30 sec of the assay, followed by a very slow additional accumulation. A similar pattern was also observed on shaking cells which had been incubated 24 hr with steroid hormone. Therefore sampling cell suspensions after 40 sec of rotation gives a reproducible measurement of cells not attached to glass. The induction of adhesiveness by Dex is indicated in Fig. 2 by comparing absorbance values for control and Dex-treated systems. While essentially all of the con-

trol cells are unattached after 40 sec of agitation, cell suspensions exposed to Dex for only 6 hr contain a large population of firmly adherent cells.

## RESULTS

### *Properties of the Cell Adhesion Reaction (Reaction 2 of Fig. 1)*

Since the induction of adhesiveness in HTC cells (reaction 1) was usually measured in terms of cell adhesion (reaction 2), it is evident that properties of the latter process per se (reaction 2) can modify the results of induction experiments. Cells grown in suspension culture in the presence of Dex as described above readily adhere to glass

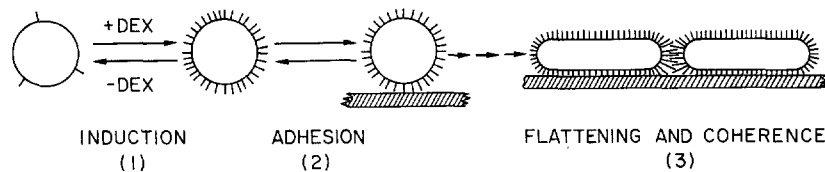


FIGURE 1 Schematic illustration of the inductive and adhesive processes in HTC cells. Uninduced cells, with a low basal level of the inducible surface factor, are represented by the circle containing three spikes. In the presence of Dexamethasone (Dex), a many-fold increase in the surface factor is induced, as illustrated by the other figures. Reaction 1 shows the reversible induction reaction, and reaction 2 depicts the adherence of induced cells to a glass surface. The development of firmer attachment which is seen in many cells is indicated in reaction 3.

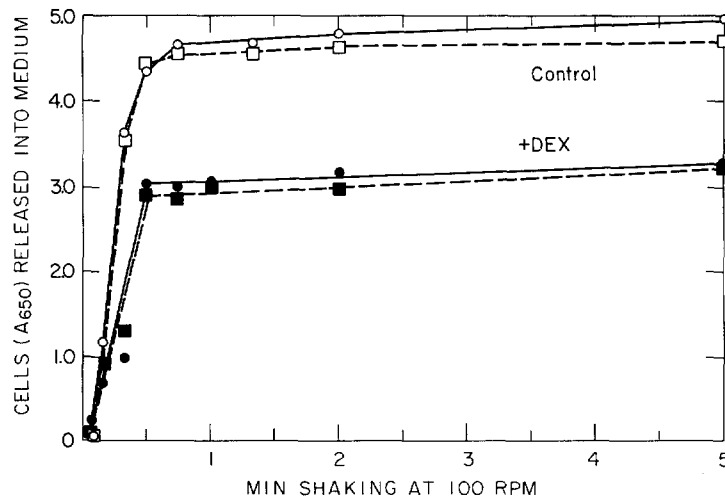


FIGURE 2 Release of HTC cells from glass as a function of time of shaking at 100 rpm. HTC cells were collected in log phase of growth and resuspended in induction medium at  $350,000/\text{ml}$ . 15-ml aliquots of cell suspension ( $5.0$  units of  $A_{650}$ ) were incubated without shaking at  $37^\circ\text{C}$  in the presence and absence of  $10^{-7}\text{ M}$  Dex. After 6 hr of incubation, vessels were rotated at 100 rpm and 1.5-ml aliquots of cell suspension were removed at various intervals for  $A_{650}$  measurements. The total number of cells (total amount of  $A_{650}$ ) released at each interval is shown for duplicate experiments.

both in the presence and in the absence of the hormone when the cells are resuspended in a serum-free medium. Thus, the attachment of cells to glass (reaction 2) can be investigated independent of the induction reaction. Maximal adhesion of preinduced cells occurs within 1–3 hr after they are transferred to induction medium and incubated without shaking, and the number of attached cells remains constant for about 6 hr.

The adhesion of preinduced cells was not affected by 0.5 to 10% serum (fetal calf and bovine) in the medium; however, control cells showed more attachment to glass at serum concentrations greater than 1%. With a serum concentration of 7%, for example, the difference in adhesion between an induced and control cell population was 21% compared with 72% in serum-free medium. For this reason, all measurements of cell adhesion were carried out in serum-free medium.

The percentage of fully preinduced cells which adhered to glass in routine experiments ranged from 50 to 80%. This fraction was influenced by the density of cells in the incubation vessel, with maximal levels of adhesion observed at cell concentrations less than about  $2.5 \times 10^5$ /ml. In one experiment, for example, induced cells were assayed at densities of (eg)  $8.5 \times 10^5$ /ml,  $5.5 \times 10^5$ /ml,  $3.8 \times 10^5$ /ml,  $2.7 \times 10^5$ /ml, and  $0.5 \times 10^5$ /ml, and the levels of adhesion were 40, 53, 59, 68, and 70%, respectively. These results suggested that individual induced HTC cells adhered readily to glass, but that the total number of attached cells was influenced by the available surface area.

During the first hours of incubation, adherent cells were spherical and were attached firmly to the glass apparently only at a small portion of their surface (Fig. 3). After approximately 24 hr of incubation in Wheaton bottles, however, many cells had flattened and cohered with their neighbors (see reaction 3 of Fig. 1) to form areas of confluence typical of cell growth in monolayer cultures.

Those induced cells which were not adherent during the assay, approximately 30% of the cell population in a typical experiment, were tested for potential adhesiveness by transferring them to a new vessel. Again 70–80% of the cells readily adhered to the new surface. The majority of non-adhering cells after the transfer were viable. Since their failure to adhere could not result from a lack of available glass surface, this observation suggests

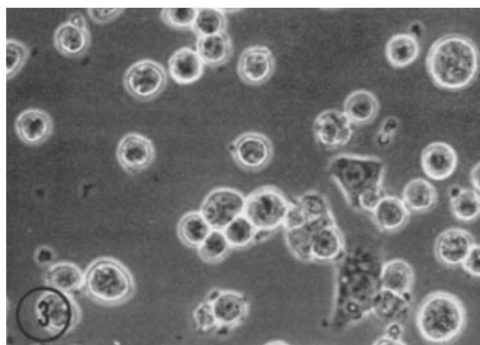


FIGURE 3 Phase-contrast micrograph of preinduced cells attached to a Pyrex glass surface after 4 hr of incubation in serum-free medium. Most of the cells appear spherical, but flattening of two cells may be observed.  $\times 200$ .

that under the assay conditions used, preinduced HTC cells establish an equilibrium between the free and attached state. This equilibrium presumably would be influenced by the number of adhesive sites on the cell as well as the number of available and occupied binding sites on the glass surface. We conclude, however, that virtually all hormone-treated cells exhibit induced levels of adhesiveness and are capable of firm attachment to glass.

Induced, but not uninduced, HTC cells will adhere to a variety of surfaces, including borosilicate glasses, polypropylene, polyethylene, and Falcon treated polystyrene. Induced cells attach with reduced affinity to untreated polystyrene dishes and do not adhere to siliconized borosilicate glass. Approximately 50% of induced cells attached to a borosilicate glass surface are removed after 2 hr of shaking at 100 rpm.

Preinduced cells attached to glass for 7 hr were released after 10 min of shaking at 37°C with trypsin (0.001%) or papain (35  $\mu$ g/ml), or after 60 min of exposure to a commercial collagenase preparation (10  $\mu$ g/ml) containing protease activity. Similarly, when preinduced cells were shaken for 30 min at 37°C with trypsin (0.001%), collagenase (50  $\mu$ g/ml), or chymotrypsin (10  $\mu$ g/ml), washed in induction medium and then assayed 1.5 hr later, adhesiveness was completely abolished. The attachment of preinduced cells was not affected by treatment with DNase (10  $\mu$ g/ml), RNase (10  $\mu$ g/ml), lysozyme (10  $\mu$ g/ml), elastase (10  $\mu$ g/ml), hyaluronidase (50  $\mu$ g/ml), or wheat germ lipase (50  $\mu$ g/ml). Cell viability, measured

by exclusion of trypan blue, was not altered by any of the above enzyme treatments. Incubation of induced cells with 10  $\mu\text{g/ml}$  of neuraminidase for at least 1 hr at 37°C, however, released cells which were nonviable.

The effect of various other treatments of the cells on their ability to attach to glass was examined and the results are presented in Table I. Neither actinomycin D, at a concentration known to inhibit RNA synthesis by more than 90% (14), cycloheximide, nor 2,4-dinitrophenol affected cell adhesion. Disodium ethylenediamine tetracetate (EDTA), at a concentration adequate to complex all available magnesium ions, inhibited reaction 2, and heat treatment abolished the difference between control and induced levels of adhesion. None of the compounds altered the adhesive behavior of control cells.

The role of divalent cations, particularly  $\text{Ca}^{++}$ , in cell-cell and cell-glass interaction is well documented (15). Calcium was routinely absent from both the growth and induction media used for HTC cells, while magnesium was present at 0.83 mM. The addition of 0.9 mM calcium chloride to induction medium increased the adhesiveness of both control cells and cells treated with steroid hormone. However, the difference between induced and control adhesion values was similar to the differences seen without calcium. Thus the induction of adhesiveness can be demonstrated

even when control levels of adhesion are elevated by addition of calcium. The requirement for divalent cations for the attachment of HTC cells, as of other cells (15), is apparent since adhesion is increased in the presence of calcium and inhibited by EDTA.

The temperature and pH dependence of reaction 2 was also investigated with fully preinduced cells. Between 24° and 37.5°C there was little difference in the maximal level of attachment of induced cells to glass. Induced cells attached equally well over the pH range from 6.45 to 7.5. However, at pH values of 7.85 and 8.35, the adhesion of cells was inhibited 25 and 53%, respectively.

These combined observations suggest that the attachment of induced cells does not depend on synthesis of a macromolecule or enzymic catalysis of an attachment reaction. Rather it appears that HTC cells attach via electrostatic and other non-covalent forces operating between the glass and specific portions of the cell surface (15).

#### *Induction of Adhesiveness in HTC Cells (Reaction 1)*

In this section, we describe the induction by glucocorticoids of the specific surface factor(s) required for the attachment of HTC cells to glass. Fig. 4 illustrates the time course for adhesion of

TABLE I  
*Properties of HTC Cell Adhesion to Glass (Reaction 2)*

Exp. No.	Addition	Inhibitor concentration	Adhesion	
			Control cells	Induced cells
			%	
1	None	—	0	81
	Dex	—	0	82
	Dex + actinomycin D	0.15 $\mu\text{g/ml}$ .	0	83
	Dex + cycloheximide	0.2 mM	0	75
	Heated cells	—	18	15
2	None	—	9	81
	EDTA	2.9 mM	8	26
	2,4-dinitrophenol	1 mM	5	77

HTC cells were grown in the presence or absence (control) of  $10^{-6}$  M Dex for 4 days, collected in early stationary phase, washed, and resuspended in induction medium. At time zero, inhibitors were added to the final concentrations noted, and Dex ( $10^{-7}$  M) was added as indicated. Heated cells were maintained at 60°C for 20 min. After 5 min of shaking and 1 hr (experiment 1) or 2 hr (experiment 2) of stationary incubation, adhesion was measured as described in Materials and Methods.

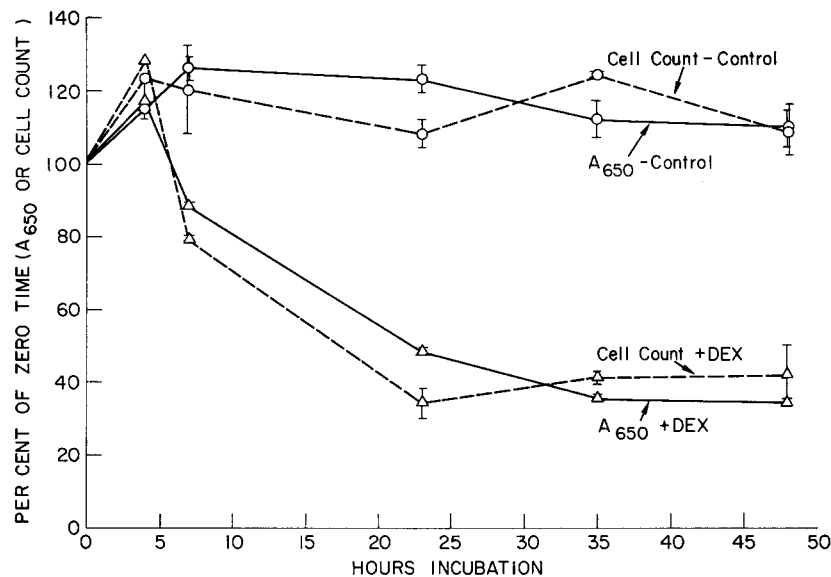


FIGURE 4 Time course of induction of cell adhesiveness: determination of cell density by both cell counts and light scattering. HTC cells were collected in log phase growth, resuspended in induction medium, and aliquots were incubated in the presence or absence of  $10^{-7}$  M Dex. Vessels were incubated with shaking for 4 hr and then left without shaking at  $37^{\circ}\text{C}$ . At the intervals shown, adhesion of cells was assayed by determining the number of cells removed from the glass after a standardized shaking interval (Materials and Methods). Cell density was measured by light scattering ( $A_{650}$ ) and by cell counts in a hemocytometer (count). The range for duplicate determinations is indicated.

HTC cells to glass in the presence and absence of  $10^{-7}$  M Dex suspended in medium without serum. As shown, in the absence of Dex (control) less than 10% of the cells became attached in 48 hr, as shown by the small decrease in both the  $A_{650}$ <sup>2</sup> and cell count values. (In other experiments, levels of adhesion in untreated cell populations varied between 0 and 12%). Addition of Dex to cell suspensions, however, results in the adhesion of about 65% of the cells by 24 hr. Typically, induction of cell adherence has a lag period of about 3 hr, is half-maximal at about 7 hr, and attains a plateau after 24–30 hr of incubation. Fig. 4 also illustrates the correlation between cell count, by hemocytometer, and the absorbance values determined by light scattering at 650  $m\mu$ . Parallel results are obtained by the two methods of determining cell density, indicating that the light-scattering prop-

<sup>2</sup> A slight increase in  $A_{650}$  and cell count was usually observed 1–3 hr after transfer of cells to serum-free medium, and resulted from both continued cell division (completion of mitosis) and some formation of cellular debris.

erties of the cells are relatively constant during the incubation in serum-free medium.

As previously reported (4), requirements for RNA and protein synthesis in the induction process were demonstrated with actinomycin D, puromycin, and cycloheximide. If any of the inhibitors were added at the same time as the inducing steroid, there was no subsequent induction (4). When actinomycin D (which does not inhibit attachment itself) was added to cells during the induction reaction, further adhesion of cells was inhibited after a lag period of several hours. By contrast, addition of either cycloheximide or puromycin rapidly terminated the induction process.

In Fig. 5 the plateau level of cell adhesion, measured after 48 hr of incubation, is plotted as a function of the concentration of Dex in the cell suspension. Induction is first detected at a Dex concentration of about  $2 \times 10^{-9}$  M, half maximal adhesion occurs at  $\sim 4 \times 10^{-9}$  M, and saturation at  $2.5 \times 10^{-8}$  M, increasing no further between  $2.5 \times 10^{-8}$  and  $10^{-5}$  M Dex. Thus induction

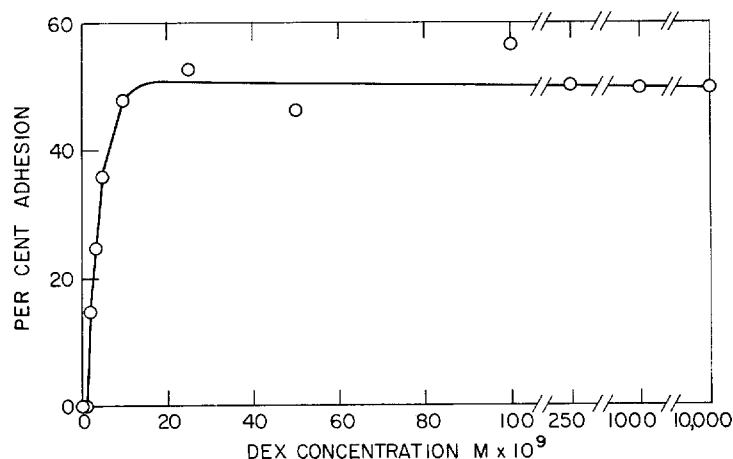


FIGURE 5 Dependence of cell adhesion on concentration of Dex. HTC cells were incubated in the presence of ethanol or various concentrations of Dex from  $10^{-9}$  to  $10^{-5}$  M. Cell adhesion was measured at intervals as described in Materials and Methods. Plateau levels of adhesion at each concentration of Dex are presented.

occurs within a concentration range equivalent to physiologic glucocorticoid levels (16, 17) and exhibits a dose-response pattern not unlike those observed for other steroid-inducible functions in mammalian cells (18-22).

Other steroids were tested for the ability to induce adhesiveness in HTC cells, and results have been published (4). Of the various steroid compounds examined, maximal levels of adhesiveness were induced by corticosterone, cortisol, 21-deoxycortisol, aldosterone, and Dexamethasone (optimal inducers) (22). Other compounds exhibited decreased activity (suboptimal inducers) (22), or were completely inactive (noninducing). Thus, the HTC cell responds to different steroids which induce characteristic levels of adhesiveness, apparently reflecting differences in the interaction of various steroids with a receptor system (22). Strikingly similar results of steroid specificity are found in induction of tyrosine aminotransferase in HTC cells (22), induction of glutamine synthetase in embryonic retina (18), and steroid-induced involution of lymphoid cells (20).

The effects of incubation temperature and medium pH on the induction process were also investigated. Induction was maximal when carried out at  $37.5^{\circ}\text{C}$ , and was completely inhibited at  $19^{\circ}\text{C}$ . Induction was suboptimal at intermediate temperatures and at  $42^{\circ}\text{C}$ . An approximate  $Q_{10}$  of 2.5 for induction was calculated for the effect of temperature between  $27.5^{\circ}$  and  $37.5^{\circ}\text{C}$ .

Decreasing the pH of the induction medium

from pH 7.5 to 7.0 and 6.7 inhibited induction 21 and 29%, respectively. At pH 7.85 induced adhesion was reduced by an amount equivalent only to the inhibition of the adhesion itself (reaction 2), suggesting that induction at that pH proceeded at optimal levels.

The results discussed thus far indicate only that certain steroid hormones induce a change in the adhesive properties of HTC cells which requires both RNA and protein synthesis. We next investigated the possibility that this hormone-induced adhesiveness might not be specific, but rather might be secondary to other more general effects of steroids on the cells. Therefore the viability of HTC cells after a 24 hr incubation in induction medium, without shaking, in the presence and absence of Dex was examined; no differences were seen in the exclusion of trypan blue or in the capacity of cells for growth on transfer to serum-containing medium. When metabolic activity was judged by the fall of pH in the incubation medium after 48 hr, no significant difference between control and induced cells was found. Similarly, control and induced cell populations incubated 24 hr without shaking showed comparable rates of labeled amino acid incorporation into acid-precipitable material, consistent with earlier studies (2). Since steroid hormones do not appear to alter adhesiveness by affecting general metabolic activity, cell viability, or over-all rates of synthesis of RNA (2) or protein, the hormonal induction of adhesiveness, like that of tyrosine

aminotransferase (2), is a specific process regulated by glucocorticoids.

It has been previously noted that prolonged treatment of HeLa cells (23), RPM-41 and H.ep. cells (24), Chang's human conjunctival cells (25), KB cells (26), and amnion cell lines (27) with glucocorticoids promotes the viability of the cultured cells and their attachment in monolayer (termed "sustaining effect"). However, in these studies it has not been clear whether the steroids affected primarily the cell surface, or rather maintained attachment secondary to enhancing cell viability. In this regard, we assayed other established cell lines for steroid-induced changes in adhesiveness under conditions used for HTC cells. Various cell lines were grown in suspension culture, with the growth medium developed for HTC cells, and collected during the log phase of growth. As shown in Table II, no other cell line demonstrated a significant level of inducible adhesiveness when assayed under conditions optimal for induction of HTC cells. Of interest, however, was the observation that, compared with uninduced HTC cells, other cell lines showed considerably more adherence to glass as well as increased cell-cell interactions during growth in suspension culture (cell clumping, aggregation, or sheet formation). HTC cells exhibit such clumping only during growth in the presence of Dex. Certain cell lines were also examined for inducible adhesiveness after growing for 3 days in the presence of Dex. The results (not shown) also indicated no increased cell adhesiveness.

It would seem from these observations that most cell lines adhere readily to glass, and that this property is not altered by exposure to steroid hormone. Thus, the reported effects of steroid on cell maintenance are probably not the result of specific membrane changes. By contrast, HTC cells appear deficient in the ability for both cell-glass and cell-cell interactions. However, these properties may be induced by glucocorticoid hormones.

The reversibility of induction was examined with HTC cells grown for 2-3 days to late log phase in the presence of  $10^{-6}$  M Dex. The cells were collected by centrifugation, washed, and resuspended in fresh induction medium in the presence or absence of  $10^{-7}$  M Dex, incubated further without agitation, and subsequently assayed for adhesion. Within 3 hr, as shown in Fig. 6, the preinduced cells adhered maximally to

TABLE II  
*Adhesion of Various Cell Lines to Glass*

Cell line	Cell-cell interaction*	Maximal adhesion	
		Control	Dex added
		%	
H.ep.-2	C, S	56	59
Detroit 6	C	36	16
WISH	C	67	77
HeLa S-3			
Clone 1	C	50	64
Clone 2	C	64	56
Chinese Hamster	C	68	68
L-929	A, C	38	37
HTC		0-12	75

Cells were cultured in the standard modified Swim's S77 growth medium and collected during logarithmic growth for assay of adhesiveness. Cells were washed and suspended in induction medium, and either ethanol (control) or Dex ( $10^{-7}$  M) was added. After an initial shaking period of 4 hr, adhesiveness was followed by the standard assay described in Materials and Methods. Maximal adhesion refers to the greatest amount of adhesion noted at any of four samplings during 24 hr of incubation.

\* Clumping (C), aggregation (A), and sheet formation (S) were observed in a qualitative manner during growth of the cell lines in suspension culture.

glass. During the first 6 hr of stationary culture, preinduced cells adhered equally well both in the presence and in the absence of Dex. In the absence of hormone, however, preinduced cells were slowly released from the surface, beginning about 6 hr after removal of the inducer. Release of all adhered cells occurs after approximately 48 hr of incubation at 37°C. Since over 95% of the cells which became detached were viable by trypan blue exclusion, release of preinduced cells from glass after the removal of hormone is not secondary to cell death. These observations suggest that the inducible adhesive factor of the cell surface slowly disappears when its synthesis is arrested by removal of the hormone as well as by addition of inhibitors of RNA or protein synthesis (4).

To gain further insight into the mechanism of steroid action the effects of interrupting induction by various means were compared. Cells, preinduced overnight in induction medium, were divided and then washed in and resuspended in induction medium either in the presence or in the



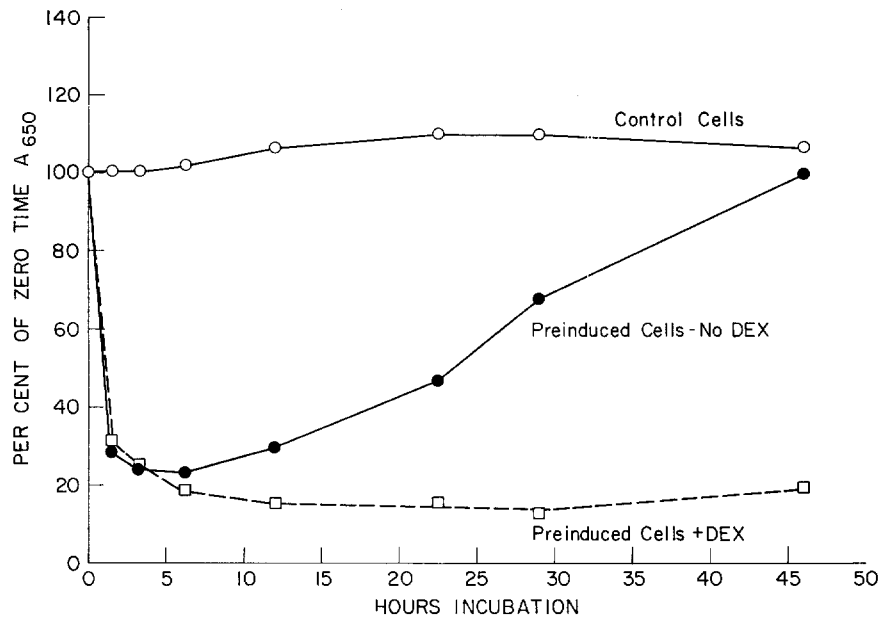


FIGURE 6 Adhesion of preinduced cells in the presence and absence of Dex. HTC cells were grown for 4 days in  $10^{-6}$  M Dex, washed free of inducer, and resuspended in induction medium with and without  $10^{-7}$  M Dex. At the times shown, adhesion of cell suspensions in stationary incubation were assayed as described in Materials and Methods.  $A_{650}$  values were adjusted to compensate for decreasing volume of medium.

absence of Dex. Those cells resuspended in Dex-containing medium received either actinomycin D, cycloheximide, or ethanol (as a control), while cells washed in inducer-free medium received ethanol (i.e., steroid removal only). The cell suspensions were agitated for various intervals before arresting the motion and assaying the ability retained by cells to attach to glass. It is seen in Table III that the level of adhesiveness decreases progressively with time after removing the inducer or adding the inhibitors. In the presence of cycloheximide and Dex, the level of induced adhesiveness declines at a rapid rate ( $t_{1/2} \sim 4$  hr). By contrast, systems either without Dex or with actinomycin D plus Dex both demonstrate a slow loss of adhesiveness. These data indicate that adhesiveness decays at a similar rate on either removing the inducer or inhibiting RNA synthesis (with actinomycin D), and that the loss is considerably more rapid on inhibiting protein synthesis (with cycloheximide).

Qualitatively similar results were obtained in other experiments where the release of attached preinduced cells was followed in systems receiving no additions (steroid wash-out only), actinomycin

D, Dex plus actinomycin D, or Dex plus cycloheximide. As shown in Fig. 7, release of previously induced HTC cells from glass proceeds at a similar rate both in the presence and in the absence of actinomycin D. Cells reexposed to Dex remain adherent, while systems receiving Dex plus actinomycin D release cells at the same rate as those with the inducer removed. By contrast, the addition of cycloheximide under similar conditions (not shown) causes a rapid release of cells (4). These observations support the conclusion that preinduced cells slowly lose their adhesive properties when steroid is removed, and that the rate of decline is not influenced by actinomycin D. Inhibition of protein synthesis with cycloheximide, however, accelerates the loss of adhesiveness. This would suggest that removal of steroid hormone does not inhibit translation of preformed mRNA.

From these and other data it appears that the induced alteration in the cell surface, reflected by an increased adhesiveness, has a half-life of 3–5.5 hr (five experiments) in the presence of cycloheximide and a longer half-life on removal of the inducer (19–24 hr), or after inhibition of RNA synthesis (13–20 hr). These observations are

TABLE III  
Loss of Adhesiveness in Preinduced Cells after  
Removing Inducer or Adding Inhibitors

Time after treatment	Wash out Dex	Adhesiveness remaining	
		Actinomycin D + Dex	Cycloheximide + Dex
Hour		%	
0	100	100	100
2	92	90	61
5	91	87	36
8.5	81	76	14
20.5	57	48	3
28	39	29	0

Cells were collected during the log phase of growth, washed, and resuspended in induction medium at  $1.1 \times 10^6$ /ml, and incubated with stirring for 18 hr in the presence of  $10^{-7}$  M Dex. The suspension of induced cells was divided, washed in, and resuspended in fresh induction medium either with or without  $10^{-7}$  M Dex. At time zero, aliquots of the cell suspension in Dex-containing medium received either ethanol (as a control), actinomycin D (0.15  $\mu$ g/ml), or cycloheximide (0.2 mM). Those cells washed in inducer-free medium received only ethanol. After shaking the cells for various intervals (to prevent adhesion), stationary incubation was started (to allow attachment) and the adhesion of cells was measured 45 min and 1.5 hr later as described in Materials and Methods. The level of adhesion at each time interval was compared to the adhesion value for preinduced cells continually exposed to Dex.

consistent with the hormonal induction of a relatively stable surface factor. This factor decays slowly after removal of hormone or addition of actinomycin D, presumably reflecting a relatively long-lived mRNA and continued synthesis of the specific protein(s) involved in adhesiveness.

#### Modification of the Cell Surface

The development of adhesive properties in HTC cells after exposure to glucocorticoid hormone presumably involves an alteration of the cell surface. In this regard HTC cells were examined by electrophoresis to evaluate the net surface charge on induced and noninduced cells. Induction was carried out for 22 hr in the presence of  $10^{-7}$  M Dex and the induced cells were electrophoresed both in the presence and in the absence of inducer. Noninduced control cells were ex-

amined similarly after the addition of ethanol. The results of mobility determinations are shown in Table IV. It is seen that the mobility of induced cells is approximately 11% less than the mean value for control cells, reflecting a decrease in the total net negative charge of the induced cell surface (28). Although electrophoresis does not distinguish between the loss of negatively charged components, addition of positively charged groups, or relocation of charged moieties, the difference in mobilities observed does indicate an alteration of the cell surface after exposure to steroid hormone.

To investigate further the surface changes, antisera were prepared from rabbits receiving either untreated or fully induced ("Dex antiserum") HTC cells. Both antisera contained agglutinating antibody against both uninduced and induced cells, whereas serum from unimmunized rabbits, or from rabbits receiving injections of purified tyrosine aminotransferase, did not cause cell agglutination at any concentration.

To remove antibodies present in Dex antiserum directed against surface antigens common to both uninduced and induced HTC cells, Dex antiserum was absorbed with untreated cells. Table V shows that after three such treatments, the Dex antiserum did not agglutinate uninduced cells at a 1:20 dilution, while induced cells were agglutinated at a serum dilution of 1:160. Further absorptions of the Dex antiserum lowered the titer for agglutination of induced cells. In parallel experiments antiserum prepared against uninduced cells was absorbed under conditions similar to those shown in Table V. As expected, there was a parallel decrease in the titer of this antiserum for both control and induced cells.

These results indicate that induced cells possess a higher level of certain surface antigens than uninduced cells. By exposing Dex antiserum to uninduced cells, antiserum relatively specific for the induced surface factor(s) is obtained. Basal levels of this factor apparently exist in uninduced cells, since continued absorption of Dex antiserum removes the agglutinating activity.

#### DISCUSSION

Hormonal modification of the cell surface measured by the induction of adhesive properties in HTC cells has been described. As uninduced HTC cells demonstrated negligible attachment to a glass surface under the conditions of the standard

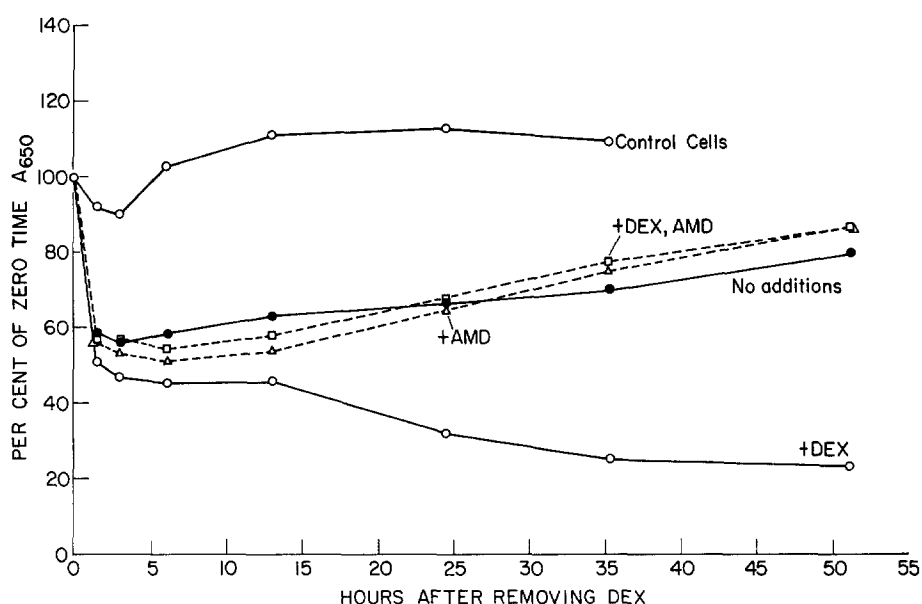


FIGURE 7 Adhesion of preinduced HTC cells in the presence and absence of Dex and actinomycin D. HTC cells grown for 3 days in the presence and absence (control) of  $10^{-6}$  M Dex were washed and resuspended in induction medium. At time zero, 15-ml suspensions of cells received no additions ( $-$ Dex),  $10^{-7}$  M Dex, or actinomycin D (AMD),  $0.15 \mu\text{g/ml}$ . Vessels were incubated without shaking at  $37^\circ\text{C}$ , and cell adhesion was assayed at the intervals shown as described in Materials and Methods.  $A_{650}$  values were adjusted to compensate for decreasing volume of medium.

TABLE IV  
*Electrophoresis of HTC Cells*

Buffer	Cells	Average time	Mobility	"P" value
		<i>sec</i>	$\mu/\text{sec per v per cm}$	
Induction medium	Control	10.4 (8.5-12.3)	-1.20	<0.005
Induction medium	Induced	11.4 (10.3-14.4)	-1.09	
Phosphate buffered saline	Control	12.9 (10.8-14.4)	-1.21	
Phosphate buffered saline	Induced	15.2 (12.4-16.6)	-1.03	<0.001

HTC cells in log phase growth were washed and resuspended in induction medium. Either ethanol or Dex (final concentration  $10^{-7}$  M) was added and the cells were rotated at 100 rpm for 22 hr at  $37^\circ\text{C}$ . For electrophoresis measurements, cells were examined either in induction medium ( $R = 75.3$  ohms) or after washing and resuspending in steroid-free isotonic, phosphate-buffered saline solution ( $R = 60.3$  ohms). Mobility determinations were made at  $20^\circ\text{C}$ , and each time value represents the average of 20 measurements with the range of values shown.

assay, the adhesiveness of induced cells provided a relatively simple and quantitative measure of the induction process. The attachment reaction per se was found to be relatively insensitive to various

inhibitors, fluctuations in temperature and pH, and the presence or absence of the steroid hormone. The induction process, on the other hand, was sensitive to inhibitors of RNA and protein

TABLE V  
*Agglutination of HTC Cells by Absorbed Antiserum*

Number of absorptions	Agglutination titer	
	With control cells	With induced cells
0	320	640
1	320	640
2	80	320
3	40	320
4	<20	160
5	<20	<20

HTC cells were induced during growth as described in the text, washed, and resuspended in buffered saline. Adult female New Zealand White rabbits received five injections of  $2.5 \times 10^6$  cells in each footpad at 3- to 6-day intervals, and antiserum was obtained from bleedings via the marginal ear vein 7 days after the final injection. Serial dilutions of serum with induction medium were prepared and an aliquot was mixed with an equal amount of a cell suspension (either untreated or induced cells) at  $2 \times 10^6$  cells/ml. The systems were incubated at 37°C with gentle shaking for 45 min when aliquots were examined microscopically for cell agglutination. 2 ml of antiserum was absorbed each time with  $1 \times 10^8$  uninduced HTC cells for 45 min at 37°C.

synthesis, demonstrated a temperature and pH dependence, and responded to changes in steroid concentration and structure.

Because steroids are known to interact with various membrane structures (29, 30), we considered the possibility that direct interaction of inducer with the HTC cell surface might explain the altered adhesive properties of hormone-treated cells. Such a mechanism of hormone action is unlikely, however, in view of the kinetics of induction and the requirement of RNA and protein synthesis. In addition, as noted earlier, preinduced cells remained adhesive after two washes in steroid-free medium, conditions which have been shown to rapidly remove labeled steroid from cells (31).

In other cultured cell lines glucocorticoids have an effect on surface properties as suggested by reports of increased cell viability and attachment to glass (23-27, 32), and resistance to various lytic agents (26, 27, 33). However, in these systems it has not been demonstrated that glucocorticoids induce the biosynthesis of a specific surface factor, such as observed in HTC cells, and properties of the induction process are not well defined. In our experiments with various cell lines, we did not detect inducible adhesiveness similar to that ob-

served in HTC cells. This suggested that the reported effects of steroids on cell maintenance were probably not the result of a specific membrane change affecting adhesiveness. While a specific inductive process may occur in other cell lines, it also appears possible that steroid hormones primarily sustain or stimulate cell metabolism and viability, or inhibit growth (34) and secondarily maintain surface membrane integrity or alter its structure in a nonspecific manner.

Thus glucocorticoids appear to regulate the synthesis of at least two macromolecules in HTC cells; a cytoplasmic enzyme, tyrosine aminotransferase, and a surface substance. The two inducible systems respond similarly with regard to steroid dose effect and the specificity of active steroids (4, 22). In earlier studies (4) competitive effects of various steroids were compared in the two induction processes; similar responses were detected in both systems. These observations strongly suggested that the steroid hormones interact with a single type of receptor system in HTC cells and thereby initiate at least two separate synthetic responses. Specific binding of steroids, exhibiting properties consistent with those of induction, is present in HTC cells (31), and a model system for steroid-receptor interaction has been discussed elsewhere (22).

Previous studies from this laboratory have provided evidence that steroid hormones control tyrosine aminotransferase synthesis in HTC cells via a posttranscriptional mechanism (2, 10, 35). In part, this conclusion stems from the observation that the effect of removing inducer differs greatly from the effects of adding actinomycin D. Removal of inducer by wash-out or dilution techniques quickly stops synthesis of transaminase enzyme, while addition of actinomycin D in the presence of inducer permits enzyme synthesis to continue for many hours. In contrast with these findings, the induced surface factor responds similarly to either removal of inducer or addition of actinomycin D. This observation is most compatible with hormonal control at a transcriptional level, although, because the adhesion assay may not accurately reflect rates of synthesis, regulation at a posttranscriptional site cannot be excluded. Thus it might be that although the steroids bind to a single type of receptor in HTC cells, effects of the hormone may be expressed at both transcriptional (surface factor) and posttranscriptional (tyrosine aminotransferase) sites.

The nature and function of the inducible surface factor on HTC cells is not yet known. The requirements for RNA synthesis and protein synthesis suggest that steroid hormones induce a protein which either modifies the surface (an enzyme) or is incorporated into the surface structure (structural protein). The inducible substance on the cell surface of HTC cells may also be present in adult or fetal mammalian tissues, as is tyrosine aminotransferase. In this event, the function of the altered surface membrane would be of particular interest in relation to embryogenesis and histocompatibility. Alternatively, the occurrence or inducibility of this surface factor may be restricted to the particular hepatoma from which HTC cells are derived. This would provide a unique example of a glucocorticoid-inducible, tumor-specific surface antigen, which might be involved in phenomena such as loss of contact inhibition, metastasizing, and invasive growth.

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