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Independence of the Lethal Actions of Glucocorticoids on Lymphoid Cells From Possible Hormone Effects on Calcium Uptake

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We have examined the possibility that hormone-induced increases in calcium uptake might initiate the lethal actions of glucocorticoids in two types of lymphoid cells. Hormone-induced increases in nuclear fragility are used as the measure of hormone action, since in both rat thymus cells and in mouse P1798 lymphosarcoma cells increased nuclear fragility (the inability of nuclei to survive lysis of the cells by hypotonic shock) precedes other indices of cellular deterioration by several hours.

In the case of the tumor cells, those from corticosteroid-sensitive lines are less able to withstand incubation in vitro than resistant cells. Such differences in cell survival are predicted both by earlier changes in nuclear fragility and also by differences in calcium uptake. However, there is no detectable early glucocorticoid effect on calcium uptake that precedes or coincides with the substantial hormone-induced increases in nuclear fragility that develop in the sensitive cells by 2 h.

In rat thymus cells the absence of calcium in the medium does prevent some of the increase in nuclear fragility and cell disintegration that occurs spontaneously during incubation in vitro. Nevertheless, when cells are exposed to hormones the glucocorticoid effect on nuclear fragility develops in the absence of calcium and is similar in magnitude to that seen in the presence of calcium.

We conclude that calcium seems to enhance the spontaneous deterioration of lymphoid cells, and there is a large increase in calcium uptake that occurs as cells deteriorate. It nevertheless seems unlikely that hormone-induced changes in calcium uptake *initiate* the lethal actions of glucocorticoids. The data also support a proposal made earlier [2] that resistance to glucocorticoids in tumor cells may develop by the selection of cells with hardier membranes.

Key words: glucocorticoids, calcium, thymus, lymphosarcoma

Previous studies from this laboratory of the adverse effects of incubation conditions and of adrenal steroid hormones on thymus [1] and lymphosarcoma cells [2] show that

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the earliest structural evidence of cellular deterioration is an increase in nuclear fragility (the inability of nuclei to survive the lysis of whole cells by hypotonic shock). Changes in this parameter generally precede other indices of deterioration (spontaneous release of DNA or inability of the cells to exclude dye) by several to many hours. For example, the eventual cytolytic actions of glucocorticoids observed at 8–12 h in thymus cells are foretold by increases in nuclear fragility that are evident within 0.5–2 h.

The present study examines the possibility that glucocorticoid-induced increases in nuclear fragility may be initiated by hormone-induced increases in calcium uptake. It attempts to differentiate possible early changes in calcium uptake that might be present prior to, and possibly be the cause of, cellular deterioration from later increases in calcium uptake that may result from a loss of cellular integrity. This portion of our work was prompted by an association seen between the late stages of hormone-induced cellular deterioration (inability to exclude dyes) and a large increase in the uptake of calcium that raised the possibility of increased calcium influx as an initiator of the lethal actions [3].

In studies on corticosteroid-sensitive and -resistant strains of P1798 lymphosarcoma cells that exhibit differences in rates of spontaneous deterioration we find parallel differences in calcium uptake. However, other results demonstrate that early hormone-induced increases in nuclear fragility are not preceded, nor accompanied by, hormone-induced increases in calcium uptake. Moreover, studies on rat thymus cells demonstrate hormone-induced increases in nuclear fragility in the absence of extracellular calcium. It thus seems unlikely that hormone-induced changes in calcium uptake *initiate* the lethal actions of glucocorticoids.

These comparisons between sensitive and resistant lymphosarcoma cells in the absence of hormones also offer new insights into possible mechanisms of the progression from hormone sensitivity to resistance. The tendency for decreased nuclear fragility suggests that the development of hardier membranes underlies both the decreased sensitivity to hormones and the increased virulence that occurs in the resistant cell line [2]. Also, the demonstration that deteriorated resistant cells also exhibit hormone-induced increases in nuclear fragility (and later lysis) make it quite unlikely that resistance has developed as a consequence of the absence of hormone receptors, as had been previously proposed [4].

MATERIALS AND METHODS

Tumors were grown and passaged into male BALB/c mice, and glucocorticoid sensitivity was tested as described in Kaiser et al [4]. Cell suspensions (4.5×10^8 cells per milliliter) were prepared and incubated in RPMI-1640 that was equilibriated with 95% O_2, 5% CO_2 throughout the incubation and subsequent procedures. Aliquots (1 ml) of this suspension were added to 10-ml Erlenmeyer flasks and incubated with and without glucocorticoids for 1–9 h in a Dubnoff metabolic shaking incubator (100 cycles per minute) at 37° C. Aliquots of cell suspension (0.15 ml) were removed and added to separate vials containing $^{45}CaCl$ (0.06 μCi, 0.028 μg). Calcium uptake was then measured by two separate methods. In the first method (Ficoll method) 50-μl aliquots were removed at various times and layered over 200 μl of Ficoll solution (9% Ficoll, 0.9% NaCl). This Ficoll solution is isotonic to the cells but has a density between that of the cells and the medium. Cells were then spun through the Ficoll in a microfuge centrifuge for 15 sec. Tips of the microfuge tubes were cut off and cells were dissociated in 0.5 ml water and counted after the addition of 5 ml Aqueous Counting Scintillant (ACS) counting fluid. In the second method (saline method) 25-μl aliquots were removed from the small vials (containing 0.15 ml cell suspension) at various times, added to 1 ml saline (0°), and centrifuged for 20 sec in an Eppendorf

zentrifuge. This washing step was repeated three more times, and cell pellets were dissolved in 0.5 ml water and counted after the addition of 5 ml ACS counting fluid. Inulin space was determined by the addition at 3 h of incubations of 1.5 mg cold inulin with ^{3}H-inulin (0.6 μCi, 0.324 mg) to 600 μl of cell suspension. Cells were then incubated for an additional 10 min or 60 min; then radioactivity was determined either by the saline or Ficoll method and compared to ^{3}H-inulin present in the supernatant of the cell suspension.

The Ficoll method was used as a rapid assay for total calcium uptake, including calcium associated with the cell surface, while the saline method with its extensive washings probably measured to a larger extent calcium uptake into the cells. Neither method extrapolates to zero at the start of the labeling period, suggesting that in both methods a considerable amount of the calcium being measured is attached to the cell surface. The amount of trapped extracellular water, as determined by inulin associated with the cell pellet, could account for no more than 10% of the calcium (Ficoll method) or 20% of the calcium (saline method) associated with the cells.

Stock solutions of hormones were made up at 10^{-4} M aqueous solution by measuring optical absorbance at absorbance maximum (cortisol at A_{240}, ϵ = 16,300; dexamethasone at A_{238}, ϵ = 15,400) the day of, or the day preceding, the experiment, and this stock was stored at room temperature. This stock was diluted with water to a concentration appropriate for addition to the cell suspension at 1% of the total volume. The same volume of water was added to the controls.

The method of measuring the degree of nuclear fragility in thymus cells is essentially that of Giddings and Young [1]; however, the quantity of DNA released into the medium prior to hypotonic lysis of whole cells (DNA in a 20-μl-aliquot diluted 50-fold into Krebs-Ringer bicarbonate buffer, KRB, at 3°C) was subtracted from measurements of DNA release post-lysis (DNA in a 20 μl-aliquot diluted 50-fold into 1.5 mM MgCl at 3°C) to obtain a true measure of nuclear fragility. For lymphosarcoma cells incubated in RPMI-1640, two 50-μl aliquots were diluted 20-fold into either 1.5 mM MgCl or RPMI-1640 at 3°C and the degree of nuclear fragility was calculated as described with thymus cells.

Cortisol (chromatographically pure) was purchased from Calbiochem, dexamethasone and Ficoll from Sigma Chemical Co. RPMI-1640 was purchased from Grand Island Biological Company. Hydrocortisone acetate for in vivo sensitivity tests was obtained from the Upjohn Company. ^{45}CaCl (1.58 Ci/mM) and ^{3}H-inulin (1.30 Ci/mmole) were obtained from New England Nuclear. ACS was purchased from Amersham/Searle Corp. Other chemicals were reagent grade, purchased from Fisher Scientific Co. Statistical significance was determined by the Student's t test.

RESULTS

Lymphosarcoma cells do not withstand longterm incubation in vitro. While cellular integrity appears to be unimpaired for about 3 h, afterwards a progressive deterioration becomes evident, as measured by the gradual release of DNA from the cells as they lyse. By 6 or 9 h, at least 15% of the cells are lysed in the corticosteroid-sensitive and -resistant lines respectively. Figure 1 demonstrates the time course of this spontaneous lysis, which is different in the sensitive and resistant cell lines; it is evident that the steroid-sensitive cells are considerably less hardy. It can also be seen that deterioration in both cell lines is enhanced by glucocorticoids, but the hormone effect appears earlier in those cells that are sensitive to steroid killing in vivo.

The data in Figure 2 show that the changes in cell survival (as seen in Fig. 1) are predicted by earlier changes in nuclear fragility. In both lines the cortisol effect on nuclear

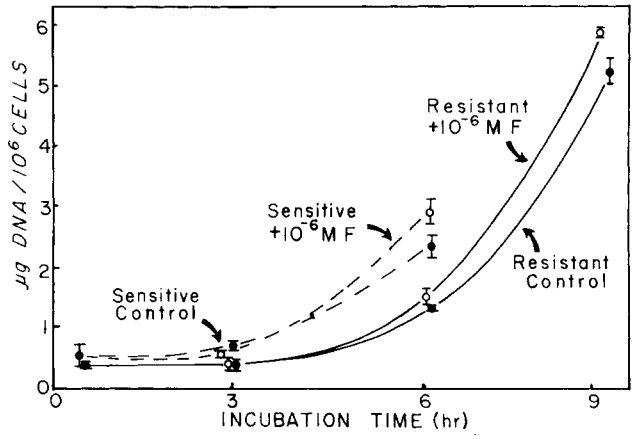

Fig. 1. Time course of the development of the cortisol effect on DNA released spontaneously from P1798 lymphosarcoma cells into the incubation medium. Cell suspensions were incubated in RPMI-1640 with or without cortisol (F) (10^{-6} M) for up to 9 h. (Flasks were gased with 95% O_2, 5% CO_2 every 0.5 h). Aliquots (50 μl) were taken for determination of prelysis DNA by 20-fold dilution into RPMI-1640 as described in Materials and Methods. The data, presented as μg DNA released per 10^6 cells present at the beginning of the experiment, are the means of determinations from 5–6 flasks ± SEM. The difference between cortisol-treated and control for the sensitive line is significant ($P < 0.02$) only at 6 h, and for the resistant line only at 9 h ($P < 0.05$).

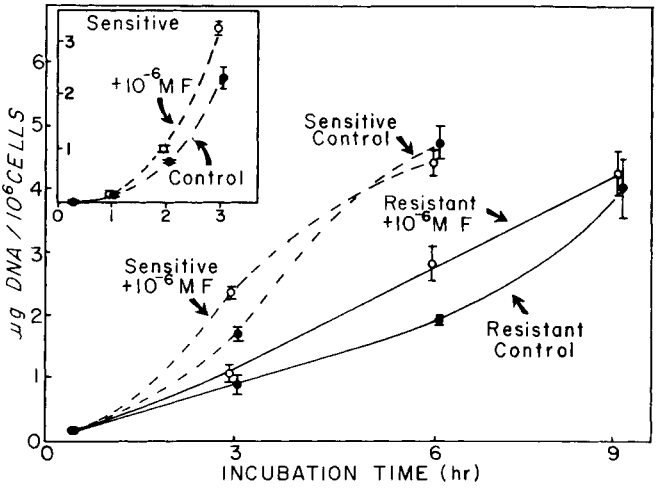

Fig. 2. Time course of the development of the cortisol effect on nuclear fragility of the P1798 lymphosarcoma cells. Experimental details are identical to those described for Figure 1. Concurrent with the determination of prelysis DNA, a 50-μl aliquot was taken for the determination of postlysis DNA, where cells were diluted in 1.5 mM MgCl and processed as described in Materials and Methods. Prelysis DNA was subtracted from total postlysis DNA to obtain the quantity of DNA released as a result of the rupture of nuclei as cells are lysed. The insert is a typical experiment demonstrating the onset of the cortisol effect on nuclear fragility in the sensitive line. The data represent the means of determinations for 5–6 flasks ± SEM. The difference between the cortisol-treated and control for the sensitive line is significant ($P < 0.001$) only at the 2-h and 3-h points, and for the resistant line it is significant ($P < 0.006$) only at the 6-h point.

fragility appears about 3 h earlier than the effect on spontaneous DNA release (compare Figs. 1 and 2). Similarly the basal nuclear fragility is significantly higher in the sensitivity line than in the resistant.

Experiments such as that in Figure 3 demonstrate that the difference in cell survival between the corticosteroid-sensitive and -resistant lines is also reflected by earlier changes in calcium uptake. Calcium uptake was measured here using the "Ficoll method" (see Methods). As can be seen in Figure 3 after 3 h of incubation the sensitive cells have considerably more ^{45}Ca associated with them than the resistant cells. Nonetheless in accumulated data from several experiments we have found that while the cells do take up calcium at a steady rate, incubation with cortisol from 45 min to 3.5 h does not at any time produce a consistent difference in calcium uptake when compared to control values (for example, see Fig. 4).

A second method for determining calcium uptake has also been used – one that is designed to measure calcium uptake into cells that cannot be removed by extensive saline washing (see Methods). A smaller quantity of ^{45}Ca is found associated with the cells when measured by this method. However, similarly to the experiments that utilized the Ficoll method, the sensitive cells demonstrated an increase in the uptake of calcium with time, yet cortisol did not produce a consistent change in calcium uptake over the 6-h incubation period (see Fig. 5). It is possible that a hormone-induced increase in calcium uptake may have occurred that is too small to be detected by these methods; however, when data from similar experiments are combined they rule out the possibility of a hormone-induced change larger than 5%. Thus by the use of two separate methods (Ficoll and saline) we are unable to detect cortisol-induced increases in calcium uptake in lymphosarcoma cells at times up to 4.5 h after addition of the hormone; whereas, we do observe the cortisol-induced increases in nuclear fragility (of about 20%) by 2–3 h of incubation.

In rat thymus cells very large changes in calcium uptake (twofold to sevenfold) are associated with glucocorticoid-induced cell death [3]. We therefore chose to look at the

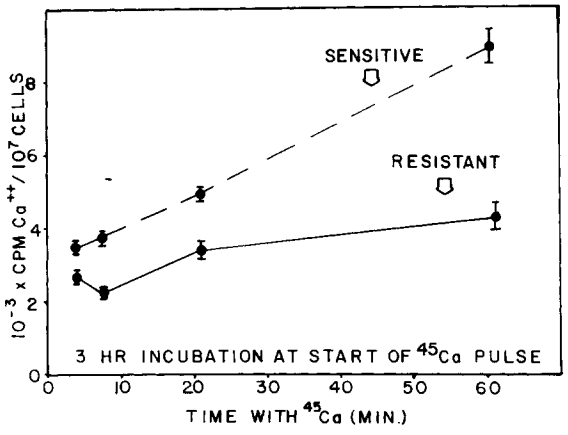

Fig. 3. Time course of calcium uptake in the P1798-sensitive and -resistant tumor cells by the Ficoll method. Cell suspensions were incubated in RPMI-1640 for 3 h. Cells were added to ^{45}Ca at this time and calcium uptake was determined at various time intervals (up to 1 h) as described in Materials and Methods. The data, presented as cpm $^{45}Ca/10^7$ cells, are the means of determinations from four flasks ± SEM. The difference between uptake in sensitive and resistant cells is significant ($P < 0.005$) at 7, 20, and 60 min following the calcium pulse.

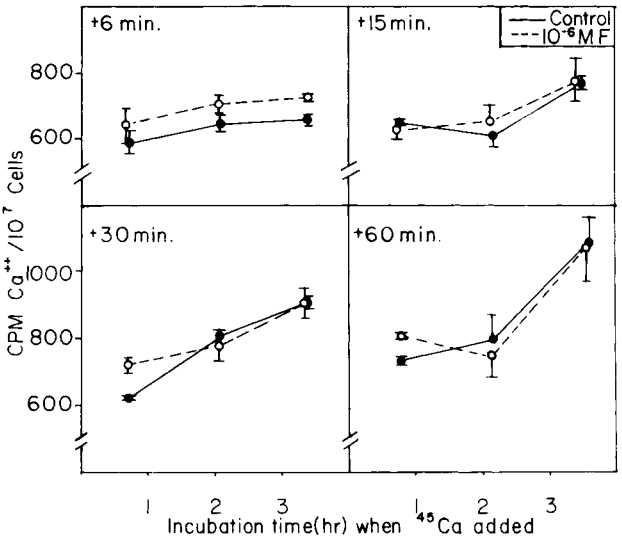

Fig. 4. Effect of glucocorticoids on calcium associated with P1798-sensitive cells by the Ficoll method. Cell suspensions were incubated in RPMI-1640 with or without cortisol (10^{-6} M) for up to 4.5 h. At 45 minutes, 2 h, and 3.5 h after the start of the incubation ^{45}Ca was added. ^{45}Ca associated with cells was determined by the Ficoll method after 6-, 15-, 30-, and 60-min pulses. The data are presented as a means of determinations from three flasks ± SEM.

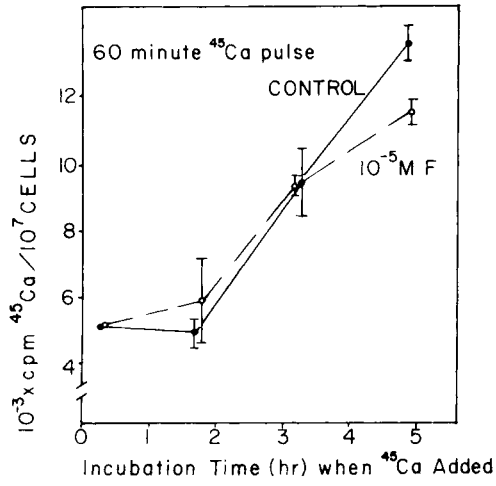

Fig. 5. Effect of glucocorticoids on calcium uptake in P1798-sensitive cells using the saline method. Cell suspensions were incubated in RPMI-1640 with or without 10^{-6} M cortisol for up to 5 h prior to the addition of ^{45}Ca. Calcium uptake was measured by the saline method, as described in Materials and Methods, following a 60-min pulse with ^{45}Ca. The data are the means of determinations from three flasks ± SEM. The difference between cortisol-treated and control is significant ($P < 0.02$) only at 5 h.

effect of calcium in the medium on the degree of nuclear fragility and on spontaneous DNA release in thymus cells. Results, as shown in Figure 6, indicate that an absence of calcium in the medium does, to some degree, reduce basal nuclear fragility and also some of the spontaneous cellular disintegration. There is a further slight decrease in both nuclear fragility and spontaneous DNA release from whole cells when [Ethylenebis (oxyethelene-nitrilo)] tetraacetic acid (EGTA) is added to the calcium-free medium. This is presumably due to EGTA binding of that calcium released by cells during the incubation. It should also be noted that a lack of magnesium in the medium, while preventing the spontaneous release of DNA from whole cells, does not appreciably change the amount of basal nuclear fragility.

Finally we determined whether the glucocorticoid effect on nuclear fragility was apparent when cells were incubated without calcium in the medium. In Figure 7 the effect of glucocorticoids was compared in cells incubated in either Krebs-Ringer Bicarbonate buffer (KRB), or KRB minus calcium plus 0.5 mM EGTA. The magnitude of the glucocorticoid effect on nuclear fragility in the absence of calcium is similar to the effect in the presence of calcium. Dexamethasone (Dex), which in vitro is about ten times more active as a glucocorticoid than cortisol, was used here (at 1/10 the concentration) to eliminate the possibility of nonspecific, surface-active steroid effects.

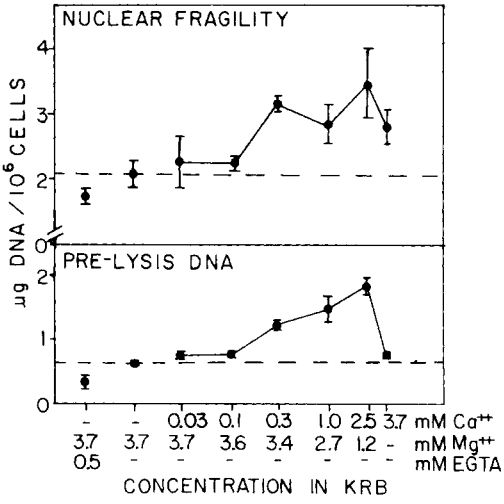

Fig. 6. Effect of calcium concentration on basal nuclear fragility and on the spontaneous disintegration of rat thymus cells. Cell suspensions were incubated in KRB with varying concentrations of calcium for 2 h. Magnesium was substituted for calcium to keep the ion concentrations constant. Aliquots (20 μl) were taken for the determination of prelysis DNA (in KRB) and postlysis DNA (in 1.5 mM MgCl), as described in Materials and Methods. Nuclear fragility was calculated as described. The data, presented as μg DNA released per 10^6 cells, are the means of determinations from six flasks ± SEM. There is a significant ($P < 0.05$) difference in nuclear fragility between zero calcium and those concentrations higher than 0.3 mM calcium. There are also significant differences in DNA released spontaneously between zero calcium and a) zero calcium + 0.5 mM EGTA, b) 0.3 mM calcium, c) 1 mM calcium, and d) 2.5 mM calcium.

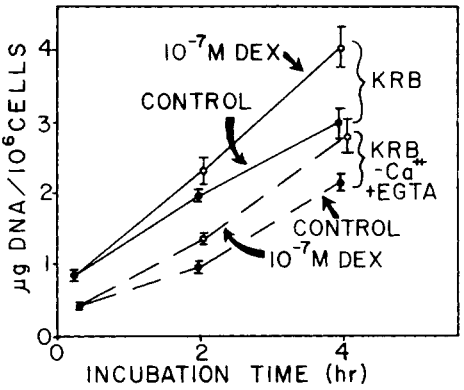

Fig. 7. Calcium independence of the glucocorticoid effect on nuclear fragility in rat thymus cells. Cell suspensions were prepared in KRB (2.5 mM Ca, 1.2 mM Mg) and cells were resuspended in either KRB, or KRB – calcium + EGTA (0 mM Ca, 3.7 mM Mg, 0.5 mM EGTA). Cell suspensions were added to flasks containing dexamethasone (final concentration 10^{-7} M) or water and incubated for 15 min, 2 h, or 4 h. Aliquots (20 μl) were taken for determination of prelysis and postlysis DNA at the times indicated in the figure. The data are presented as micrograms DNA released per 10^6 cells and are the means of determinations from 5 to 6 flasks ± SEM (2–3 flasks for 15 min incubation time). Dexamethasone-treated (compared to control) represents an increase of 18% and 35% at 2 and 4 h, respectively. The difference between dexamethasone-treated and control is significant at 2 h (KRB – Ca + EGTA, $P < 0.002$) and at 4 h (KRB – Ca + EGTA, $P < 0.01$; KRB, $P < 0.009$).

DISCUSSION

Previous research has shown that an increase in nuclear fragility represents the most rapidly appearing structural evidence of hormone-induced cellular deterioration, and therefore could represent the initial manifestation of intracellular events that ultimately lead to cell lysis [1, 2]. In thymus cells increased nuclear fragility precedes hormone-induced changes in the pattern of chromatin and nuclear edema observed via electron microscopy [5]. It is probable that those intracellular hormone effects that eventually lead to nuclear edema also increase nuclear susceptibility to lysis when whole cells are lysed. In both rat thymus and mouse lymphosarcoma cells the increase in nuclear fragility is a "specific" glucocorticoid effect, as shown by structural specificity and dose-response studies and by the ability of an excess of cortexolone (11-deoxycortisol) to protect cells, presumably by blocking the occupancy of glucocorticoid receptors [6]. Also, in a manner similar to other specific hormone actions, protein synthesis is required for the generation of this effect [1, 2]. Our earlier work suggests rather strongly that this destructive action of glucocorticoids is not a consequence of the small steady-state changes in adenine nucleotide ratios (reduction in energy charge, secondary to decreased mitochondrial and glycolytic energy production) that seems to account for some other hormone actions (eg, reductions in uridine uptake and protein biosynthesis) [7–9, 12]. The data presented here are also compatible with the working hypothesis that hormone-induced increases in nuclear fragility represent changes in the properties of the nuclear membrane.

The lethal actions of glucocorticoids that were measured by Kaiser and Edelman [3] in rat thymus cells after 12 h exposure to the hormone were associated with large (twofold to sevenfold) increases in calcium uptake. In that study it was unclear whether the increased uptake occurred in the surviving cells. The evidence did suggest that an

increase in calcium uptake is required for the later stages of cellular deterioration, which were measured by the inability of the cells to exclude dye. However, the authors did not differentiate hormone-induced changes in calcium uptake that might occur *as the result of* a loss of cellular integrity, at or near the time of cell death, from hormone-induced uptake that might initiate the lethal actions. The present studies were accordingly undertaken to further investigate whether glucocorticoid-induced changes in calcium uptake might initiate the lethal actions in two responsive cells lines, using those hormone-induced changes in nuclear fragility that occur during the first few hours to measure the onset of the destructive hormone actions.

The studies on mouse lymphosarcoma cells presented here lead to several interesting conclusions. The differences in the time of survival of the corticosteroid-sensitive and -resistant lines (measured by spontaneous release of DNA; as seen in Fig. 1) are foreshadowed by differences in nuclear fragility (compare Figs. 1 and 2), and also by differences in rates of calcium uptake (Fig. 3). If, as we suspect, increased calcium uptake occurs only in severely compromised cells, these results suggest either that some of the sensitive cells are damaged at the beginning of the incubation or, more likely, that they rapidly degenerate under the in vitro conditions used. This appears to be the case; within 6 h a significant portion of the sensitive cells have lysed even in the absence of hormone.

While such studies do suggest that increased calcium uptake appears to be a characteristic of compromised cells, we have nevertheless been unable to detect an additional increment in calcium uptake in glucocorticoid-treated cells prior to or coincident with the appearance of a fairly substantial (20%) increment in nuclear fragility (Figs. 4 and 5). This indicates that calcium uptake is the result of rather than a cause of cellular deterioration.

It has previously been proposed that the emergence of resistance to glucocorticoids in lymphosarcoma cells is due to the lack of hormone receptors [4]. Yet it is evident here that, while they take longer to develop, substantial glucocorticoid effects on nuclear fragility do appear in the resistant cells. Also, one can differentiate between sensitive and resistant cells on the basis of differences in cell survival during incubation (spontaneous release of DNA), differences in calcium uptake, and differences in nuclear fragility. These findings are in accord with a working hypothesis that we have advanced previously, specifically, that resistance emerges via the selection of cells with hardier membranes, those that are better able to withstand the intracellular changes initiated by the hormone. According to this model, when the resistant cells reach a sufficient state of deterioration they also become susceptible to the nucleolytic actions of glucocorticoids (see Nicholson and Young [2] for further discussion). The observations by Behrens and co-workers [10, 11], who found differences between cell surface characteristics of resistant and sensitive cells, suggest that the membrane changes may be widespread, as opposed to being confined solely to nuclear membranes.

As with the lymphosarcoma cells our studies with rat thymus cells also demonstrate an increasing tendency of cells to take up calcium with time of incubation. They do not, however, show an additional hormone-induced increase in calcium uptake concurrent with or preceding the development of effects on nuclear fragility. While extracellular calcium does appear to increase basal nuclear fragility, the increment of the hormone-induced increase in nuclear fragility is of the same magnitude whether the cells are incubated in the presence or absence of calcium in the medium. Although these results do not exclude possible changes in intracellular compartmentalization as a mediator of glucocorticoid effects, they do seem to rule out a role for hormone-induced increases in calcium uptake as an initiator of cellular deterioration.

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