The Effect of Cortisol on Viability and Glucose Uptake in Rat Thymocytes *in Vitro*[1] (35448)

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The lymphotoxic effects of the glucocorticoids have been well documented (9–12), but the mechanism of action of these steroids is not yet clear despite the increasing store of knowledge of their numerous, varied effects on lymphocyte metabolism. Most of the effects demonstrated have been inhibitory, including a decrease in the incorporation of precursors into DNA and RNA (1) and into protein (1–3), an inhibition of RNA polymerase activity (4–6), an inhibition of amino acid transport (1) and a decrease in glucose metabolism (2, 3, 7). In addition, an increase in DNase activity has been reported in thymocytes exposed to cortisol (8). The earliest inhibitory effect yet identified is on the uptake of glucose by rat thymocytes, an event which occurs within 15 to 20 min after exposure to cortisol *in vitro* (3, 7). It has been suggested, therefore, that an important effect of cortisol may be to produce a block at the level of glucose transport or phosphorylation (3, 7). Although a relationship between an initial block in glucose uptake and final cell injury and death seems likely in the lymphocyte, proof is lacking. The observation of Munck (7) that inhibition of glucose uptake by cortisol in the rat thymocyte does not occur under anaerobic conditions offers an opportunity to explore the relationship between the effects of cortisol on glucose metabolism and its effect on cellular integrity. If the two are related, anaerobic conditions should protect the thymocyte against injury by cortisol.

The effect of cortisol on cellular integrity has usually been measured by microscopic observation (9–12), an accurate but laborious method. In the present experiments, cellular integrity has been measured by the pronase-Cetrimide method (13), which depends upon the ability of the enzyme pronase to digest injured cells while sparing "viable" cells. The pronase-resistant cells are then treated with cetyltrimethylammonium bromide (Cetrimide)[2] which strips them of cytoplasm and reduces clumping, leaving a dispersion of single nuclei which can be enumerated in a Coulter counter.

Methods and Materials. Preparation of thymocyte suspensions. Thymocytes were isolated from male Sprague-Dawley rats weighing 125–250 g. The animals were killed by decapitation. The pooled thymus glands were placed on a fine, stainless steel screen and minced with scissors. The thymocytes were washed through the screen into a container with tissue culture medium 199 (TC 199).[3] The resulting suspension was centrifuged at room temperature at 800*g* for 10 min, and the supernatant was discarded. The thymocyte button was resuspended, either in TC 199 for experiments measuring glucose uptake, or in TC 199 plus 20% fetal calf serum (FCS)[4] for experiments measuring viability. The presence of FCS decreased the viability of thymocytes when used under the conditions of glucose-uptake experiments (dense suspension of thymocytes in 1-ml volume agitated in shaker). The addition of FCS was beneficial when a more dilute suspension of

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[2] Cetrimide, T5650, Eastman Organic Chemicals, Rochester, N.Y.

[3] Difco Laboratories, Detroit, Mich.

[4] Fetal Calf Serum, Hyland Laboratories, Los Angeles, Calif.

thymocytes was employed in experiments measuring viability. Sterile technique was employed in experiments which required overnight incubation and 100 U/ml of penicillin G and 100 μg/ml of streptomycin sulfate was added to the media. Cortisol (HC) was added as a solution of the hemisuccinate[5] in TC 199.

Preparation of human blood lymphocyte suspensions. Human blood lymphocytes were prepared by a published method (13) based on the separation of granulocytes from lymphocytes by gravity after the former have been permitted to phagocytize gum arabic-coated iron particles. Red blood cells were removed by dextran sedimentation. Whole blood from a healthy donor was used. Anticoagulation was achieved either by adding 7.5 U/ml of sodium heparin to the blood or by defibrination. The isolated lymphocytes had the same properties in relation to cortisol whether or not they had been exposed to heparin. The yield of lymphocytes was 25% when defibrination had been used and 50% when heparin anticoagulation had been employed. Isolated lymphocytes were washed once with TC 199 and resuspended in TC 199 plus 20% autologous serum.

Incubation of lymphocytes. Incubations in aerobic and anaerobic atmospheres were carried out in tightly stoppered, 50-ml serum bottles which were thoroughly gassed with 95% O_2:5% CO_2 and 95% N_2:5% CO_2, respectively. The bottles were regassed whenever a sample was taken. When an air:5% CO_2 atmosphere was employed, incubations were carried out in 125-ml narrow-mouthed bottles equipped with loosely fitting Bakelite caps. The bottles were placed in an air:CO_2 incubator.

Viable cell count. The viable cell count was determined by incubating an aliquot of thymocyte suspension with a quantity of pronase[6] solution (5 mg/ml in physiological saline) sufficient to give a resulting cell density no greater than 10^8 cells/ml. The pronase–thymocyte suspension was incubated for 75 min in an air:5% CO_2 incubator. A suitable aliquot was then added to a counting diluent consisting of physiological saline which was 0.012 *M* in HCl and contained 2.5 mg/ml of Cetrimide. The suspension of thymocyte nuclei thus produced was immediately counted in Model A Coulter counter.

TABLE I. Cell Count and Viability of Cultured Rat Thymocytes Treated with Cortisol.

Conc HC (moles/liter)	% of initial cell count[c]; time (hr): 0		24		48	
	C[a]	*V*[b]	*C*	*V*	*C*	*V*
0	100	89	89	64	79	27
10^{-7}	100	89	83	39	68	15
10^{-6}	100	89	85	47	73	17
10^{-5}	100	89	85	47	73	15
10^{-4}	100	89	85	53	67	17
10^{-3}	100	92	85	47	73	17

[a] *C* = total cell count.

[b] *V* = viable cell count (cell count after treatment with pronase).

[c] Cell count at time = 0 hr was 1.3×10^7/ml.

Assay for glucose. Glucose was determined by use of the Glucostat reagent.[7] Colorimetric measurements were made in a Beckman DU spectrophotometer at 410 mμ.

Results. Effect of cortisol on the cell count and viability of rat thymocytes. Table I demonstrates the effect of different concentrations of cortisol on the cell count and viability of rat thymocytes. The experiments were carried out at 37° in an air:5% CO_2 incubator. The incubation volume was 5 ml. The total cell count was determined by incubating the thymocytes with physiological saline rather than with pronase. In control samples the total cell count had decreased by about 20% at 48 hr of incubation, reflecting spontaneous lysis of a fraction of the thymocytes. Addition of cortisol resulted in a further small, but significant, decrease in total cell count. The most marked effect of cortisol was seen on the viable cell count which averaged only 16% at 48 hr in samples to which cortisol had been added. It is notable that the effects on total cell count and viable

[5] Cortisol-21-sodium succinate, Sigma Chemical Co., St. Louis, Mo.

[6] Pronase, B grade, Calbiochem, Spring Valley, N.J.

[7] Glucostat reagent, Worthington Biochemical Corporation, Freehold, N.J.

TABLE II. Effect of Cortisol on the Viability of Cultured Human Blood Lymphocytes.

Conc HC (moles/liter)	% Viable cells[b]; time 2 days	4 days	% Excess survival[a] at 4 days
0	98	85	0
10^{-7}	97	86	-4 ± 4.2
10^{-6}	94	80	-7 ± 1.6
10^{-5}	95	74	-9 ± 2.6
10^{-4}	94	76	-10 ± 4.1
10^{-3}	98	75	-10 ± 2.0

[a] Values given in this column represent the difference in percentage viability between the sample lacking cortisol and those containing the hormone. A negative sign means a decrease. Values are the mean of three experiments ± standard deviation.

[b] Values given as percentage of the viable cell count at time 0 (9.6×10^5/ml).

cell count occurred at a pharmacological concentration of cortisol (10^{-7} *M*) and were not increased by a concentration four orders of magnitude greater.

Other experiments (not shown) demonstrated that the earliest measurable effect of cortisol on cell viability, as determined by the pronase–Cetrimide method, occurred at 12 hr after exposure to the hormone.

Effect of cortisol on the viability of human blood lymphocytes. For the sake of comparison, studies were carried out on human blood lymphocytes incubated at 37° in an air:5% CO_2 atmosphere. The incubation medium contained 20% autologous serum, but the results were unchanged when 20% FCS was substituted. Table II shows these results. Human blood lymphocytes survived considerably longer in culture than did rat thymocytes. After 4 days of incubation the viable cell count in samples containing cortisol was lower than that in the control samples. The effect was significant ($p<0.05$) at concentrations of cortisol 10^{-6} *M* or greater and, as in the experiments with rat thymocytes, high concentrations of hormone did not significantly increase the effect above that observed with a pharmacological dose.

Effect of cortisol on viability of thymocytes in aerobic and anaerobic atmospheres. Incubations were carried out in a shaking incubator (90 cycles/min) at 37° in a volume of 3 ml. Table III shows the results. The thymocytes survived equally well after 24-hr incubation in either anaerobic or aerobic atmospheres. Cortisol decreased the viable cell count over and above the control in thymocyte suspensions exposed to either atmosphere, but manifested a greater injurious effect under aerobic conditions.

Effect of cortisol on glucose uptake by rat thymocytes under aerobic and anaerobic condition. Incubated volume was 2 ml. Aliquots were taken for glucose analysis at 1 and 2 hr. The aliquots were immediately placed in cracked ice in order to prevent further glucose utilization by the thymocytes, which were then sedimented by centrifugation at 500*g* at 10° for 10 min. The supernatant was analyzed for glucose content. The viable cell count was measured at the beginning and at the end of the incubation, at which time it had decreased uniformly about 10% in both the controls and in the samples containing cortisol. Differences in glucose uptake were not due, therefore, to decreased cell viability in the presence of cortisol. Table IV shows the result of these experiments, demonstrating that cortisol inhibited the uptake of glucose by rat thymocytes under both aerobic and anaerobic conditions. A marked Pasteur effect was seen when an oxygen atmosphere was present.

Discussion. The lytic effect of the glucocorticoids on thymus and other lymphatic tissue *in vivo* has been well established (9, 14). As Dougherty (15) has pointed out, the

TABLE III. Viability of Cultured Rat Thymocytes in the Absence and Presence of Cortisol (10^{-6} *M*) Under Aerobic and Anaerobic Conditions.[a]

Atm	Addition	% Viability at 24 hr[b]
95% O_2:5% CO_2	None	51 ± 2
	10^{-6} *M* HC	18 ± 2
95% N_2:5% CO_2	None	49 ± 4
	10^{-6} *M* HC	31 ± 2

[a] All experiments performed with triplicate samples. Values are given as means ± standard deviation.

[b] Initial viable cell count was 9.8×10^6/ml.

TABLE IV. Effect of Cortisol on Glucose Uptake in Cultured Rat Thymocytes Under Aerobic and Anaerobic Conditions.[a]

Expt. no.	Atm	Addition	Rate glucose uptake[b] (μmoles/10^8 cells/hr)
I	95% O_2:5% CO_2	None	131 ± 4
		10^{-6} *M* HC	93 ± 5
II	95% N_2:5% CO_2	None	510 ± 52
		10^{-6} *M* HC	265 ± 9

[a] Values given are means of triplicate samples ± standard deviation.

[b] Values are based on glucose remaining after 2 hr of incubation for Expt. I and after 1 hr for Expt. II. Initial viable cell count = 5.4×10^8 cells/ml in Expt. I and 2.5×10^8 cells/ml in Expt. II. Initial glucose concentration was 1 mg/ml.

destructive effect of the glucocorticoids on the individual lymphyoyte is primarily karyorrhectic rather than lytic. Nevertheless, the loss of weight and cellularity of the thymus (so-called thymic involution) observed in animals treated with corticosteroids and the dramatic decrease in the size of lymphoid organs seen in the leukemic patient who responds to steroid treatment (16) testify that the end result of the action of the corticosteroids can be absolute destruction of the lymphocyte. The dissolution of the corticosteroid-sensitive lymphocyte may occur largely via phagocytosis of the injured cell rather than by spontaneous lysis. If so, the absence of a dramatic lytic effect of the corticosteroids *in vitro* is understandable. What has usually been observed in *in vitro* experiments involving the action of cortisol and cortisol-like compounds on lymphocytes and thymocytes is microscopic evidence of cell injury, manifested first on the nucleus and progressing to pyknosis of the cell (10, 12, 17). In addition, a decrease in cell count at higher concentrations of hormone has been reported (12). The results of our studies are in agreement with those cited above. The most pronounced effect of cortisol on the rat thymocyte is one of gradually-increasing injury as measured by the pronase-Cetrimide method. Lysis of the cell, resulting in a decreased total cell count, occurred to a lesser but significant extent. The earliest time at which evidence of cortisol-induced injury could be seen was 12 hr after the initial exposure to cortisol. A study (12) employing electron microscopic observation of rat thymocytes exposed to 2.7×10^{-7} *M* cortisol reported that injurious effects on the cell nucleus could be seen as early as 2 hr after the start of incubation. In *in vivo* experiments, the degeneration of thymocytes has been observed as early as 1 hr after the injection of adrenal cortical extracts into the mouse (9). The later onset of effect in our experiments is probably due to the greater degree of cell injury required to produce susceptibility to digestion by pronase.

Comparison of human blood lymphocytes with rat thymocytes showed that the former survived considerably longer in tissue culture suspension and were relatively resistant to injury by cortisol. Similar results have been reported in another study whose criteria of effect were cytological changes observed by conventional microscopy of cultured lymphocytes (11).

Because of the results of Munck (7), showing that an inhibition of glucose uptake in the presence of cortisol fails to occur under anaerobic conditions, it was expected that under anaerobic conditions thymocytes would also be protected against cell injury by cortisol. Our results failed to confirm this expectation. Instead, unmistakable cell injury had occurred after 24 hr of incubation in the presence of cortisol under anaerobic conditions. There was evidence, however, that rat thymocytes were more susceptible to cortisol under aerobic conditions, since cell viability was lowest in those samples containing the hormone and exposed to an oxygen atmosphere (see Table III).

In our hands, glucose uptake by rat thymocytes was inhibited to approximately the same extent whether the cells were in an aerobic or an anaerobic atmosphere (see Table IV). In this respect our results differ from those of Munck (7) who found no inhibition under anaerobic conditions. Our experiments differed from his in that we used TC 199 as a suspending medium instead of Krebs–Ringer–bicarbonate buffer. Other than this difference there is no obvious explanation

for the discrepancy between our results and his.

From the heuristic point of view, our findings that cortisol effects on the thymocyte are similar under both aerobic and anaerobic conditions is a disappointing one. If the case were otherwise, the key to the mechanism of action of the hormone on lymphoid tissue might lie in one of the differences between aerobic and anaerobic metabolism. The only difference found between aerobic and anaerobic conditions in our experiments was an increased toxicity of cortisol when oxygen was present. The significance of this finding is not yet clear, but it merits further investigation.

Despite the experimental discrepancy described above, in so far as we have not been able to separate the inhibitory effect of cortisol on glucose uptake from its later toxic effect, the latter always following the former in our experiments, our results are consistent with the hypothesis of Munck (7) that a block in glucose uptake is an early event which is responsible for later decreases in RNA and protein synthesis, and eventual cell death. On the basis of the decreased levels of ATP found in rat thymocytes exposed to cortisol, Young (18) has hypothesized that the cortisol-induced block in glucose metabolism interferes with the synthesis of macromolecules by altering adenine nucleotide metabolism. As Munck has pointed out, the inhibition of glucose uptake may not be the primary effect of cortisol on the lymphocyte, although it may be the mediator of later effects. The lag in time which occurs between exposure of the thymocyte to cortisol and the manifestation of its effects on glucose metabolism (3, 7) suggests an earlier primary action, which remains undiscovered.

Summary. Cortisol decreased cell viability and inhibited glucose uptake by cultured rat thymocytes. The survival of human blood lymphocytes was also affected adversely by cortisol. The effects of the steroid on rat thymocytes occurred under anaerobic as well as under aerobic conditions although toxicity to the cell was more profound when oxygen was present. These results are consistent with the idea that a block in glucose uptake is an important link in the chain of events which results eventually in irreversible cellular injury in lymphocytes which have been exposed to pharmacological doses of the glucocorticoids.

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