Cyclic AMP-Mediated Stimulation of Thymocyte Proliferation by Low Concentrations of Cortisol[1] (34967)

J. F. WHITFIELD, J. P. MACMANUS, AND R. H. RIXON
(Introduced by Helen J. Morton)
Division of Biology, National Research Council of Canada, Ottawa 7, Canada

Thymic lymphocytes (thymocytes) lose their characteristic nuclear morphology and then die soon after exposure to relatively high concentrations of cortisol either *in vivo* or *in vitro* (1, 2). In marked contrast to its lethal action on thymocytes, cortisol initiates specific nucleic acid and protein syntheses in cells of tissues such as liver and mammary gland (3–10). It has been suggested (2) that the irreversible dispersal of the heterochromatin structures in the cortisol-treated thymocyte nucleus and the stimulation (or derepression) by cortisol of synthetic activities in the liver cell are both ultimately due to a dissociation of deoxyribonucleoprotein complexes (2–4, 10). If this be true, exposure of thymocytes to very low, nonlethal concentrations of cortisol might cause only small nucleoprotein changes which would stimulate some of their metabolic processes and even increase their proliferation.

In this communication, we show that low concentrations of cortisol do stimulate the proliferation of rat thymocytes maintained *in vitro*. Furthermore, evidence is presented which indicates that cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) participates in the steroid's mitogenic action.

Methods. Thymus tissue was removed from albino, male, specific pathogen-free rats and thymocyte suspensions (containing about 10^8 cells/ml) were prepared as previously described (11). The cells were suspended in serum-free MAC-1 medium (at 37°) and contained in roller tubes revolving *horizontally* at 40 rpm to ensure adequate aeration. It should be noted that none of the treatments described below caused a lowering of the cell concentration in thymocyte suspensions as determined with a Coulter model B electronic cell counter (Coulter Electronics, Hialeah, Fla.).

MAC-1 medium consisted of the glucose–salts (GS) medium described previously (11) plus all of the amino acids, vitamins, nitrogenous bases and supplementary growth factors contained in the tissue culture medium 199 (12). The GS medium contained 5.5 m*M* glucose, 5.0 m*M* KCl, 1.0 m*M* $MgSO_4$, 0.6 m*M* $CaCl_2$, 120 m*M* NaCl, 5.0 m*M* Na_2HPO_4, and 5.0 m*M* *tris* (hydroxymethyl) aminomethane buffer (pH 7.2). Medium 199 minus its glucose and salts was obtained from Difco Laboratories (Detroit) and we then combined this partial medium with the GS solution to produce MAC-1 medium.

Only a small part (up to a maximum of 20%) of a thymocyte populatiion is capable of proliferating and rapidly (with 2 to 4 hr) responding to stimulation by a variety of mitogenic agents (13). Therefore, the effect of cortisol on the proliferation of these mitotically competent thymocytes cannot be accurately assessed by simply measuring the total cell concentration. However, when the MAC-1 medium contained 0.06 m*M* colchicine, the cells of the proliferating subpopulation were able to flow into mitosis, but could not progress beyond metaphase. The rate of progression of these cells through their growth–division cycle into mitosis was then accurately measurable by plotting the progressive accumulation in the population of cells in colchicine metaphase. This was done by removing samples from the cell suspension after various times of incubation, fixing the cells in phosphate-buffered neutral formalin and staining them with Harris' hematoxylin.

Cortisol (hydrocortisone) was obtained

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from Nutritional Biochemicals Corp. (Cleveland). The hormone was dissolved in absolute ethanol to produce a concentrated (1.0 m*M*) stock solution which was then diluted with MAC-1 medium to give the appropriate experimental concentrations. The small amounts of ethanol which were introduced into the cell suspensions with the cortisol did not affect cell proliferation. Cyclic adenosine 3′, 5′-monophosphate was obtained from Sigma Chemical Co. (St. Louis).

Results and Discussion. During the first 6 hr after suspension in colchicine-containing MAC-1 medium, cells belonging to the actively "cycling" segment of a thymocyte population continued to enter mitosis and accumulate at metaphase (Fig. 1). When the medium also contained 10^{-9} or 10^{-8} *M* cortisol, the flow of cells into mitosis was the same as in untreated populations for the first 4 hr, but thereafter it very sharply increased (Fig. 1). However, when the cortisol concentration exceeded 10^{-7} *M*, the flow of cells into mitosis during the 6-hr incubation period was markedly lower than in normal populations (Fig. 1). The overall relation between the cortisol concentration in the medium and the level of cellular proliferation is illustrated in Fig. 2, which shows that the maximally mitogenic hormonal concentration was 10^{-8} *M*.

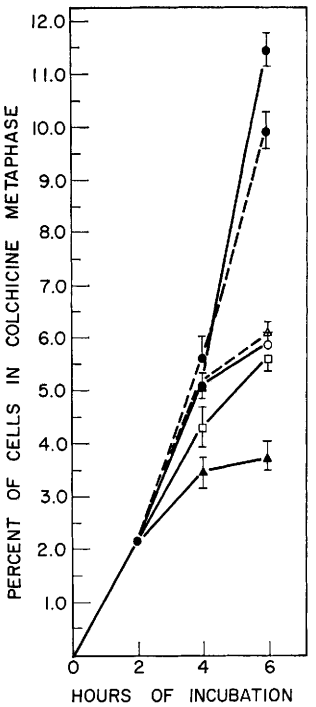

FIG. 1. The effects of several concentrations of cortisol on the flow of cells into mitosis during 6 hr of incubation of thymocyte populations in colchicine-containing MAC-1 medium: (○), untreated cells; (△), cells suspended in medium containing 10^{-10} *M* cortisol; (● - - -), cells exposed to 10^{-9} *M* cortisol; (●—), cells exposed to 10^{-8} *M* cortisol; (□), cells exposed to 10^{-7} *M* cortisol; (▲), cells exposed to 5×10^{-7} *M* cortisol. Each point is the mean ± SEM of 8 to 27 separate experiments.

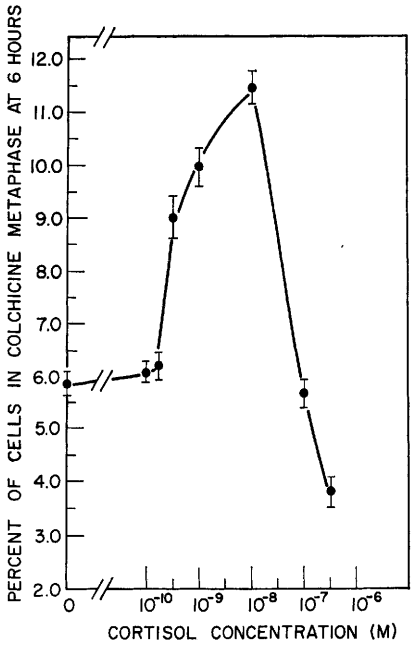

FIG. 2. The relation between the cortisol concentration in the medium and the level of cell proliferation in rat thymocyte populations maintained *in vitro*. Cells were suspended in MAC-1 medium containing colchicine and various cortisol levels. After 6 hr of incubation, the total proportion of cells which had accumulated at metaphase was determined. Each point is the mean ± SEM of 5 to 13 separate determinations.

There is evidence that glucocorticoids sensitize liver cells to the gluconeogenic action of cyclic AMP (14) and act synergistically with the cyclic nucleotide to cause the induction of several enzymes in this tissue (15). Since we have repeatedly demonstrated that

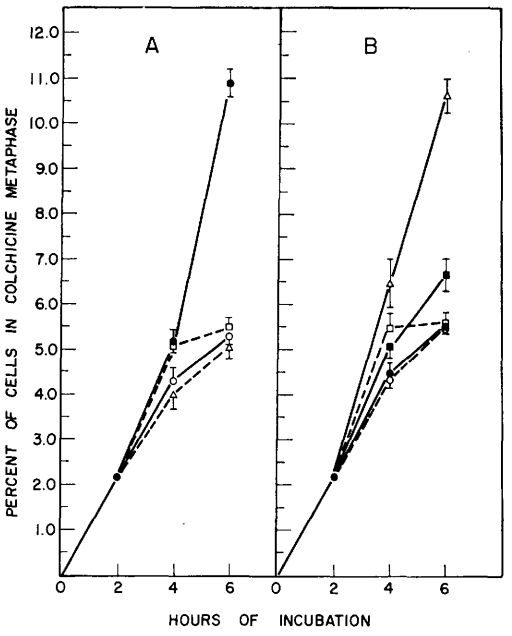

FIG. 3. A. The effect of imidazole on the ability of cortisol to stimulate the flow of thymocytes into mitosis (□), untreated cells suspended in colchicine-containing MAC-1 medium; (●), cells exposed to 10^{-8} *M* cortisol; (Δ), cells suspended in medium containing 4.0 m*M* imidazole; (○), cells exposed to 10^{-8} *M* cortisol and 4.0 m*M* imidazole. B. The ability of caffeine to increase the mitogenic effectiveness of cortisol: (□), cells exposed to 0.4 m*M* caffeine in colchicine-containing MAC-1 medium; (○), cells exposed to 4.0 μ*M* caffeine; (●), cells exposed to 10^{-10} *M* cortisol; (Δ), cells exposed to 10^{-10} *M* cortisol and 4.0 μ*M* caffeine; (■), cells exposed to 10^{-10} *M* cortisol and 0.4 m*M* caffeine. Each point is the mean ± SEM of 6 to 14 separate determinations.

this cyclic nucleotide is also a powerful stimulator of thymocyte proliferation *in vitro* (16–19), it might be involved in the cortisol-induced mitotic stimulation. If this be true, the steroid stimulatory action should be inhibited by imidazole, a compound which increases intracellular cyclic AMP degradation by specifically raising the activity of the enzyme cyclic nucleotide phosphodiesterase (16–21). On the other hand, the hormone mitogenic effectiveness should be increased by caffeine, a compound which raises the intracellular level of cyclic AMP by specifically inhibiting phosphodiesterase activity (16–20).

In the presence of 4.0 m*M* imidazole, the normally strongly mitogenic 10^{-8} *M* cortisol did not stimulate the flow of cells into mitosis (Fig. 3A). On the other hand, in the presence of 4.0 μ*M* caffeine, the normally nonmitogenic 10^{-10} *M* cortisol (Figs. 1 and 2) strongly stimulated thymocyte proliferation (Fig. 3B). Surprisingly, 10^{-10} *M* cortisol was much less effective in the presence of 0.4 m*M* caffeine (Fig. 3B). It should be noted that this higher caffeine level by itself has no effect whatsoever on mitotic activity, but has been repeatedly shown to facilitate the cyclic AMP-mediated actions of the parathyroid and growth hormones as well as bradykinin (16, 17, 19, 21). These results could be explained if the higher caffeine concentration increased 10^{-10} *M* cortisol activity to give an excessive, and therefore inhibitory, effect. Since caffeine and imidazole also affect the mitogenic action of cyclic AMP itself in exactly the same ways as they affect cortisol action (16), it would be reasonable to suspect that this cyclic nucleotide might participate in the mitogenic process initiated by cortisol.

It was then found that cortisol strongly increased the sensitivity of cells to exogenous cyclic AMP. In agreement with previous observations (16, 18), exposure of thymocyte populations to 10^{-9} *M* cyclic AMP did not appreciably affect the flow of cells into mitosis (Fig. 4); the cyclic nucleotide is mitogenic only at concentrations between 10^{-8} and 10^{-6} *M* (16, 17, 19, 21). However, when thymocyte populations were exposed to 10^{-9} *M* cyclic AMP in the presence of the nonmitogenic 2.5×10^{-10} *M* cortisol (Fig. 2), the progression of cells into mitosis was strongly stimulated (Fig. 4). In this case, the stimulated cells began to arrive at metaphase about 2 hr earlier than they would have done if stimulated by cortisol alone (Figs. 1 and 4). This more rapid arrival of stimulated cells at metaphase is characteristic of the mitotic response to either higher levels of exogenous cyclic AMP or hormones which stimulate intracellular cyclic AMP formation (16–19, 21).

These observations suggest that low concentrations of cortisol stimulate thymocyte

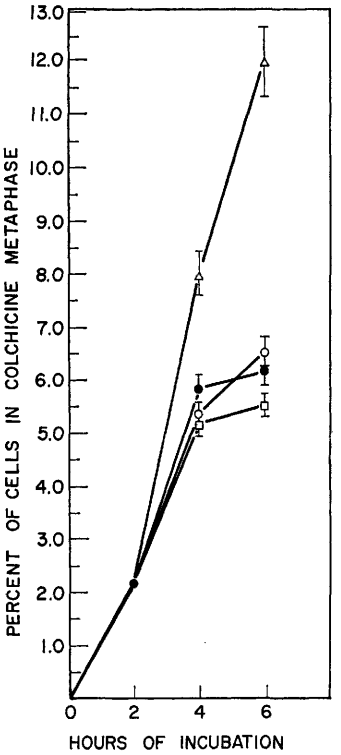

FIG. 4. Facilitation of the mitogenic action of cyclic AMP on thymocyte populations by a nonmitogenic concentration of cortisol: (□), cells suspended in colchicine-containing MAC-1 medium; (○), cells exposed to 10^{-9} *M* cyclic AMP; (●), cells exposed to 2.5×10^{-10} *M* cortisol; (△), cells exposed to 10^{-9} *M* cyclic AMP and 2.5×10^{-10} *M* cortisol. Each point is the mean ± SEM of 5 to 16 separate determinations.

proliferation by a process which is some way involves cyclic AMP. Cortisol certainly need not act by raising the intracellular level of cyclic AMP and there is no evidence to suggest that it does so (14, 15, 20). However, the steroid could act by sensitizing a mitogenic enzyme system to the existing intracellular cyclic nucleotide as has been suggested by Friedmann *et al.* (14) to explain the ability of glucocorticoids to facilitate the cyclic AMP-mediated gluconeogenic action of glucagon on liver. Such an explanation is supported by the present observation that cortisol strongly sensitized the thymocyte mitogenic system to the action of a very low, and normally nonmitogenic, level of cyclic AMP.

The mitotic inhibition and destruction of thymocytes following exposure to higher cortisol concentrations could be explained in the same way. As is the case with cortisol, very low concentrations of exogenous cyclic AMP stimulate thymocyte proliferation, but higher concentrations of the nucleotide inhibit proliferation (18). Furthermore, experiments still in progress have shown that the mitotically inhibitory levels of exogenous cyclic AMP, like the lethal higher cortisol concentrations (2), rapidly cause the dissolution of thymocyte nuclear structure and then death of the cell. Thus, an excessive sensitization of thymocytes to the action of preexisting intracellular cyclic AMP by higher cortisol concentrations could convert a potentially mitogenic process into a lethal reaction.

Summary. Low concentrations (10^{-9} to 10^{-8} *M*) of cortisol strongly stimulate the proliferation of rat thymocytes maintained *in vitro* by a process which involves cyclic AMP. On the other hand, cortisol concentrations greater than 10^{-7} *M* inhibit cell proliferation. The steroid's stimulatory effect may be due to sensitization of the cells to the mitogenic action of cyclic AMP.

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