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Embryonic mouse thymocyte development

ENHANCING EFFECT OF CORTICOSTERONE AT PHYSIOLOGICAL LEVELS

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Summary. The action of corticosterone on embryonic mouse thymus development has been studied *in vitro*. Effects of the hormone on lymphoid differentiation varied according to the dose. 0·2–1·0 μg/ml had an enhancing effect, resulting in an increase in the proportion of Thy-1.2 positive cells as compared with control cultures. Two to fifty micrograms per millilitre had an inhibitory effect, appearing to selectively kill small lymphocytes while leaving large and medium lymphocytes intact. At higher doses these immature cells were also killed.

INTRODUCTION

Corticosteroids have a marked effect upon the lymphoid system of some species ('sensitive' species: mouse, rat, rabbit, hamster), but not of others ('resistant' species: man, monkey, guinea-pig, chicken). In sensitive hosts these hormones have been shown to induce lymphocytolysis in the thymic cortex, cell migration and redistribution within lymphoid tissues, and, at low doses, proliferation and activation of adult lymphocytes in *in vitro* cell suspensions (Dougherty, Berliner & Berliner, 1962; Ishidate & Metcalf, 1963; Burton, Storr & Dunn, 1967; Cohen, 1972; Moorhead & Claman, 1972; Zaiz, 1975; Whitfield, MacManus & Rixon, 1970; Smith, Sherman & Middleton, 1972). However, little is known of the action of corticosteroids in the ontogeny of the lymphoid system.

The experiments described in this paper examine the effects of corticosterone on the lymphoid development of embryonic mouse thymus. An *in vitro* system was selected in order to minimize secondary effects and to remove the uncontrollable and as yet not clearly defined contribution of cell migration to the action of the hormone on thymic tissue.

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MATERIALS AND METHODS

Embryonic thymus

Thymic rudiments were removed from 13-day CBA/H embryos (appearance of vaginal plug = day 0) using a Wild stereomicroscope and fine cataract knives.

Tissue culture system

Tissues were cultured in a modified filter well assembly (Ball & Auerbach, 1960), using Falcon organ culture dishes. The absorbent ring in the outer part of the culture dish was saturated with sterile distilled water to provide a humid atmosphere. Cultures were gassed in 5% CO_2 in air at 37°.

Tissue culture medium

Dulbecco's modified minimal essential (Flow Laboratories) was supplemented with 10% heat-inactivated

foetal calf serum, 500 iu/ml penicillin and streptomycin and 2·0 ml/100 ml of 200 mM L-glutamine.

Addition of hormone

Corticosterone (Organon) was dissolved in 70% ethanol, and diluted to the appropriate concentration with culture medium (0·2, 1, 2, 5, 10, 50, and 250 μg/ml). The final concentration of ethanol per ml was no more than 0·5%. For each experimental hormone concentration, control cultures were set up containing no corticosterone but the equivalent concentration of ethanol.

Histology

Sections. Some control and experimental thymic rudiments were sampled after 4 and 6 days in culture, fixed in Bouin's, blocked in polyester wax (British Drug Houses), sectioned at 7μm and stained in Giemsa R66 (G.T. Gurr).

Smears. Other control and experimental rudiments were sampled at 4 days. Tissues were gently teased apart in medium containing 10% foetal calf serum, and the cell concentration adjusted to $1–2 \times 10^6$/ml. Smears were prepared in a cytocentrifuge by spinning 0·1-ml aliquots at 500 r.p.m. for 10 min. Slides were air dried and stained in MacNeil's stain.

Preparation of anti- Thy-1·2 antiserum

Antiserum was raised in AKR female hosts using CBA/H thymocytes according to the method of Reif & Allen (1966). The antiserum was titrated against 4-week-old CBA/H and AKR thymocytes, showing a plateau of kill against CBA/H thymocytes out to a final dilution of 1/32 (mean kill on plateau = 87%). A final antibody dilution of 1/16 was used in all experiments. No activity was seen against AKR thymocytes.

Cytotoxicity testing

After 4 days in culture, experimental and control thymic explants were tested for the presence of Thy-1.2 positive cells, using the cytotoxic assay of Boyse *et al.* (1964) as modified in Schlesinger's two-step assay (1965) to minimize anticomplementary effects. Guinea-pig serum, absorbed with agar (Difco Noble Agar) to remove anti-thymocyte activity, was used as a source of complement (Cohen & Schlesinger, 1970). Results were corrected for non-specific background cell death:

$$\text{Corrected per cent kill} = \frac{\text{observed per cent kill} - \text{background per cent kill}}{100 - \text{background per cent kill}}.$$

RESULTS

Histology (7μm sections)

The development of small lymphocytes in corticosterone treated 13-day embryonic mouse thymus is summarized in Table 1. At the start of culture 13-day

Table 1. Effect of corticosterone on lymphocyte development in cultured embryonic mouse thymus

Corticosterone dose (μg/ml)	Presence of small lymphocytes after 6 days in culture
0	+++
1	+++
2	+++
5	+
10	+
50	+
250	0

Thirteen-day embryonic mouse thymic rudiments were cultured in medium containing corticosterone. After 6 days, cultures were scored for the presence or absence of small lymphocytes in tissue sections: highly lymphoid (+++), barely lymphoid (+) or alymphoid (0).

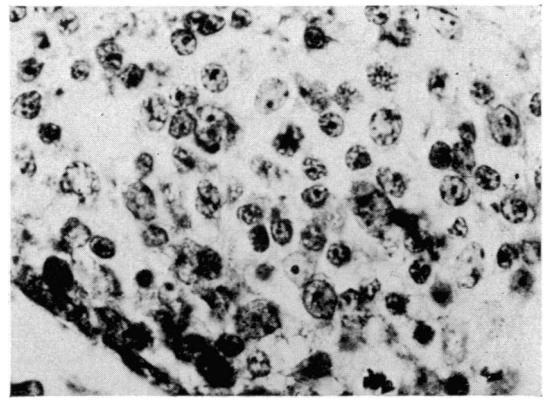

Figure 1. Thirteen-day embryonic mouse thymus cultured *in vitro* for 6 days in medium containing 0·5% ethanol (control for corticosterone cultures). In addition to some large and medium lymphocytes, many small lymphocytes can be seen. (Giemsa Stain × 1575.)

thymus was mainly epithelial with some large basophilic cells. After 4 days control cultures contained, in addition to epithelial cells, large, medium and some small lymphocytes. In some explants occasional basophilic cells could still be seen. After 6 days in culture many small lymphocytes were found (Fig. 1). The histological picture found in thymic rudiments cultured with 1 and 2 μg/ml corticosterone was similar to that found in control cultures.

Rudiments treated with 5, 10 and 50 μg/ml corticosterone possessed large and medium lymphocytes at both 4 and 6 days in culture. However, small lymphocytes were rare (Fig. 2).

After culture with 250 μg/ml only epithelial cells and cell debris were seen at either stage (Fig. 3).

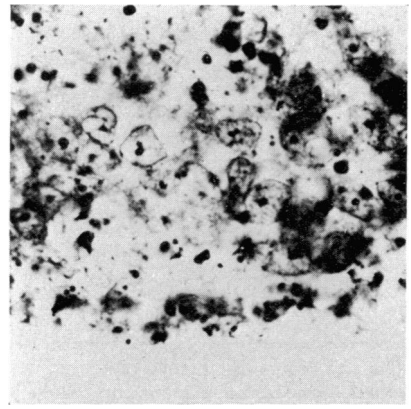

Figure 3. Thirteen-day embryonic mouse thymus cultured for 6 days *in vitro* in medium containing 250 μg/ml corticosterone. There are no lymphocytes. Only epthelial cells and cell debris are found. (Giemsa Stain × 1575.)

Smears

The size distribution of lymphoid cells in control and experimental (10 μg/ml hormone) cultures was compared (Table 2). In the absence of corticosterone, 31% of all lymphoid cells were small lymphocytes. The remaining 69% were medium and large cells. In contrast, in cultures containing 10 μg/ml corticosterone only 2% were small, while 98% were medium and large lymphocytes.

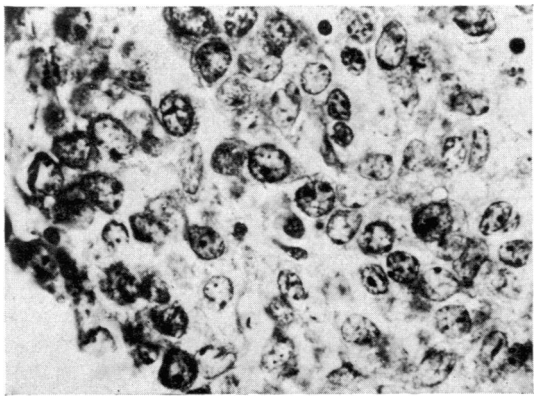

Figure 2. Thirteen-day embryonic mouse thymus after 6 days *in vitro* in medium containing 10 μg/ml corticosterone. The explant contains both large and medium lymphocytes can be seen. Giemsa Stain × 1575.)

Cytotoxicity testing

The results of cytotoxicity tests on thymic rudiments after culture for 4 days with various doses of corticosterone are given in Table 3. In control cultures 37% cells were Thy-1.2 positive. After corticosterone doses of 0·2 and 1 μg/ml the percentage of Thy-1.2 cells was elevated (51% and 43% respectively). In contrast, higher concentrations of hormone led to a progressive reduction in the percentage of Thy-1.2 positive cells (2 μg/ml: 27%; 5 μg/ml: 4%; 10 μg/ml: 0%). In each

Table 2. Effect of corticosterone on size distribution of lymphocytes within embryonic mouse thymus

Corticosterone dose (μg/ml)	Small lymphocytes	Medium/large lymphocytes	Total lymphocytes	Per cent small lymphocytes
0	93	207	300	31
10	5	295	300	2

Thirteen-day mouse thymic rudiments were cultured in medium containing either 0 or 10 μg/ml corticosterone. After 4 days in culture the size distribution of lymphocytes was scored in smear preparations.

Table 3. Effect of corticosterone on per cent of Thy-1.2 positive cells in cultured embryonic mouse thymus

Corticosterone dose (μg/ml)	Per cent Thy-1.2 positive cells
0	37 (36,37)
0·2	51 (50,52)
1	43 (40,45)
2	27 (26,28)
5	4 (4, 5)
10	0

Thirteen-day mouse thymic rudiments were cultured in medium containing corticosterone. After 4 days the per cent Thy-1.2 positive cells was assessed in cytotoxicity tests. Each value of per cent Thy-1.2 positive cells is the mean of values (given in brackets) obtained in two independent experiments, each from a minimum of thirty-six pooled thymic lobes (to provide sufficient cells for a cytotoxicity test). Unfortunately the pooled nature of the data make them unsuitable for statistical analysis.

experiment those cells that were Thy-1.2 negative (unstained by Trypan blue) appeared large and round under phase contrast microscopy.

DISCUSSION

In the adult mouse the thymus is composed of an epithelial matrix containing lymphoid cells. The lymphocytes of the outer cortex are mainly large and medium, while those nearer the medulla are small. Medullary lymphocytes are less densely packed and range in size from large to small, although the majority are small. Small lymphocytes in the cortex are characterized by: cortisone sensitivity, high Thy-1, low H-2, TL positive and absence of immunological competence. Those of the medulla are slightly larger, cortisone resistant, low Thy-1, high H-2, lack TL and are immunocompetent (reviewed in Cantor & Weissman, 1976).

The thymus of the 13-day mouse embryo is composed of epithelial and large basophilic cells. The latter are thought to be the lymphoid precursors since their presence in the thymus is essential for thymocyte development (Owen & Ritter, 1969). After 4 days in culture (no hormone) most basophils have disappeared, being replaced by large, medium and a few small lymphocytes. After 6 days in culture many small lymphocytes are found. At this stage in similar experiments (14-day thymus + 4–6 days in culture) the majority of thymocytes carry the Thy-1 antigen, and although the thymus is not divided into cortex and medulla as it is in the adult, both 'medullary' (TL+) and 'cortical' (G-v-H active) small lymphocytes have been identified (Owen & Raff, 1970; Ritter, 1971). The system is therefore highly suitable for a study of the differential corticosteroid sensitivities of the various thymus lymphocyte subpopulations as they develop.

Results show that low doses of corticosterone (1 μg/ml, 2 μg/ml) had little effect on the histological picture of cultured 13-day embryonic thymus. Intermediate doses (5, 10 and 50 μg/ml) had no visible effect on the large and medium lymphocytes, but reduced small lymphocytes from 31–2% of the lymphocyte population (Table 2). The highest hormone dose (250 μg/ml) destroyed all lymphoid cells, leaving only epithelial cells intact. Thus, in the embryonic thymus cortisone sensitivity is inversely related to lymphoid cell maturity. This fits well with earlier work in young adult mice (Dougherty *et al.*, 1962; Ishidate & Metcalf, 1963). Only small lymphocytes (with exception of those in medulla) were sensitive to low corticosteroid doses, while higher levels killed the large and medium cortical cells too. Weissman (1973) has used this high dose sensitivity (2·5 mg/mouse equivalent to 700 μg/ml) in combination with transcapsular ^{3}H-thymidine labelling to show that the large cortisone sensitive lymphocytes of the outer cortex are the progenitors of at least some medullary cortisone resistant small lymphocytes.

A second approach to the embryonic situation was to analyse the effects of corticosterone in terms of Thy-1.2 development. After 4 days in normal culture medium (0·5% ethanol and no hormone) 37% of thymus cells carried Thy-1.2 antigen. The presence of 5 μg/ml corticosterone abolished this development, although a few small lymphocytes could be identified histologically after as much as 50 μg/ml (Tables 1 and 2). It is possible that these could represent a 'medullary' small lymphocyte subpopulation, which bearing less Thy-1.2 would be harder to detect. Alternatively a very small positive result could be obscured by the 'background' in the cytotoxicity tests.

The most interesting effect, however, was seen at low hormone levels (0·2 and 1·0 μg/ml) where the percentage of Thy-1.2 positive cells were greater than

in control cultures. Thus corticosterone at a low concentration appears to have a potentiating effect upon embryonic thymus lymphopoiesis, probably via stimulation of both cell division and maturation. Other observations on low dose corticosteroid enhancement have been reported, showing increased mitotic activity for adult rat thymocytes in suspension *in vitro* (Whitfield *et al.*, 1970) and elevated immunoglobulin production by antigen stimulated rabbit and unstimulated human peripheral lymphocytes *in vitro* (Ambrose, 1970; Smith *et al.*, 1972). It is unlikely that the elevated Thy-1.2 kill seen in cytotoxicity tests of 4-day cultures is due to hormone induced cytolysis, since lymphocyte death occurs within 8 h of first contact with corticosteroids, and since the half life of these hormones is approximately only 120 min (Burton *et al.*, 1967; Claman, 1972). It is also unlikely to result from developmental variability in the 13-day explants at the start of culture, as each per cent Thy-1.2 value is derived from seventy-two to ninety-six thymic lobes (six to eight litters of mice).

The mechanism of action and biological function of corticosteroid influence on lymphoid tissue are unclear. Potentiating effects may be mediated at the cell surface via increased intracellular cyclic AMP (Whitfield *et al.*, 1970; Singh & Owen, 1975), while lymphocyte death may result from different mechanisms involving entry of the hormone into the cell, where binding in cytoplasm and nucleus leads to cellular disruption and death (Burton *et al.*, 1967; Baxter, Harris, Tomkin & Cohn 1971). Work presented in this paper demonstrates an increase in embryonic thymus lymphopoiesis after culture in a low concentration of corticosterone (maximal effect at 0·2 μg/ml). In view of the physiological level found in rodent plasma (0·12–0·29 μg/ml corticosterone, van der Vies, 1961) it is proposed that under normal conditions *in vivo* one function of corticosterone is to enhance the production of thymus small lymphocytes.

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