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Effect of Hydrocortisone on the Reactivity of Thymus and Spleen Cells of Mice to *in vitro* Stimulation

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Summary. Treatment of mice with hydrocortisone reduced the number of thymus-cells to 6 per cent and the number of spleen-cells to 32 per cent of matched controls. Per spleen, an absolute decrease of cells carrying immunoglobulin receptors on the surface as determined by immunofluorescence was found and a smaller decrease in cells with theta-antigen. Equal numbers of thymus and spleen cells from hydrocortisone-treated mice and matched controls were cultured and stimulated with phytohaemagglutinin (PHA), pokeweed mitogen (PWM), allogeneic cells, and, following immunization, with keyhole limpet haemocyanin (KLH). Stimulation was assessed by incorporation of [^{3}H]thymidine into the acid precipitable fraction of the cultured cells. With thymus cells, hydrocortisone treatment increased the reaction to PHA and allogeneic cells. Thymus cells from untreated animals already gave a good response to PWM, further increased by treatment with hydrocortisone. With spleen cells, hydrocortisone treatment reduced the reaction to KLH, PWM, allogeneic cells and PHA in decreasing order. The results are discussed with reference to cells affected by hydrocortisone treatment and to the mechanism of *in vitro* lymphocyte stimulation.

INTRODUCTION

Lymphoid cells can be stimulated in culture by a variety of substances to transform into blast cells, to incorporate [^{3}H]thymidine and to divide (for review, see Naspitz and Richter, 1968). Lymphocyte stimulation is widely used in man for investigations concerning abnormalities of the immune system. During the last years, the concept of cooperation of different types of lymphocytes emerged. Experiments on mice and chickens indicate that at least two classes of lymphocytes are involved in the immune response, the thymus-dependent (T) and the bone-marrow-dependent (B) lymphocytes (for review, see Playfair, 1971). Therefore the systems of *in vitro* lymphocyte stimulation should be reevaluated in terms of the new concept.

Markers have been recognized in mice, specific for T-cells or for B-cells. The theta-antigen is specific for thymus and thymus-dependent peripheral cells in most strains of mice (Reif and Allen, 1966). Bone-marrow-dependent cells carry sufficient amounts of immunoglobulin incorporated in the surface to be recognized by immunofluorescence (Unanue, Grey, Rabellino, Campbell and Schmidtke, 1971).

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By treatment of mouse spleen cell suspensions with an antibody against the theta-antigen and complement, both the mixed lymphocyte reaction and the secondary reaction *in vitro* to soluble antigen are greatly reduced. However, the reaction to phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) is only moderately influenced (Vischer and Jaquet, 1972). Treatment of cells with anti-theta and complement may not eliminate all thymus dependent cells from lymphoid cell suspensions (Vischer, 1972). Hydrocortisone treatment of mice is supposed to select a population of mature T-cells within the thymus accompanied by the loss of less mature cortical lymphocytes (Warner, 1964; Blomgren and Anderson, 1969; Ishidate and Metcalf, 1963). Such a purified T-cell population might assist in further evaluation of the role of T-cells during the *in vitro* lymphocyte stimulation. Therefore, cells from thymus and spleen of hydrocortisone-treated mice were cultured and stimulated with PHA, PWM, allogeneic cells or specific antigen. The results indicate that hydrocortisone treatment has a much wider effect on lymphocyte stimulation than that to be expected by enrichment of mature T-cells only.

MATERIALS AND METHODS

Mice

Specific pathogen-free BALB/c and C57Bl/6 female mice (6–8 weeks initial age) were obtained from the Tierfarm AG, Sisseln, Switzerland. For each experiment, one batch of mice of the same age housed in the same cage were used. Some BALB/c were immunized with keyhole limpet haemocyanin (Calbiochem) (KLH) adsorded on bentonite (Gallily and Garvey, 1968). Mice were injected i.p. with 125 mg hydrocortisone acetate per kg body weight (Hydrocortone, Merck); control mice of the same lot were injected with 0·9 per cent saline. The animals were killed 2 days after injection and thymus and spleens removed. In experiments, where only thymus cells were used, the parathymic lymph nodes were marked by injection of a dilute solution in India ink 45 minutes before removal (Blau and Gaugas, 1968). Peritoneal exudates were induced by i.p. injection of 2 ml of 2·5 per cent gelatine 2 days before harvesting.

Cultures

Cell suspensions were prepared and the cells cultured as described previously (Vischer and Jaquet, 1972). Equal numbers of viable spleen cells (5×10^{6}) or thymus cells (2×10^{6}) from hydrocortisone-treated mice and matched controls were cultured at 37° in 1 ml of RPMI 1640 (Microbiological Associates), buffered with HEPES (Calbiochem), and enriched with 5 per cent normal rabbit serum. 12×75-mm polystyrene tubes (Falcon Plastics) were used.

Stimulants were added in optimal concentration established after the plateau of the dose-response curve of normal spleen cells, in 5-μl amounts to cultures of BALB/c cells: PHA-P (Difco); PWM (Grand Island Biological Co.); KLH. In mixed lymphocyte cultures, equal numbers of BALB/c and C57Bl/6 cells were cultured together. All cells were cultured for 3 days, as preliminary experiments had indicated this time to be optimal. To measure the stimulation, 0·5 μCi of [^{3}H]thymidine (The Radiochemical Centre, Amersham, specific activity: 5·0 Ci/mmol) was added for the last 16 hours. At the end of the culture period, 10 per cent cold trichloracetic acid was added and the resulting precipitate washed and prepared for liquid scintillation counting as previously described. The results were expressed as the mean net cpm of triplicate cultures $\pm$ the standard deviation (SD).

Simultaneous cultures of cells obtained from individual animals from one batch of mice gave similar counts. However, the counts varied from one batch of mice to another. Therefore, in the tables only single experiments are reported, which have been repeated with similar results at least three times.

Fluorescent staining

The globulin fraction of an anti-serum against mouse immunoglobulins (anti-Ig) (from rabbits immunized with mouse serum fraction II (Mann Research Laboratories)), obtained by precipitation with ammonium sulphate at 33 per cent, was conjugated with fluorescein isothiocyanate (Baltimore Biological Co.) and evaluated by the method of Holborow and Johnson (1967). The conjugate had a fluorescein to protein ratio of 2·3 and contained 2·8 units of precipitating antibody. It reacted on immunoelectrophoresis with mouse immunoglobulins and slightly with transferrin. Anti-theta C3H was raised in AKR mice as described previously (Vischer and Jaquet, 1972).

Fresh spleen cells were stained in suspension in the following way: 0·1 ml cell suspension containing $2–8 \times 10^6$ cells were mixed with 0·1 anti-theta or normal AKR serum and incubated at 4° for 20 minutes. The cells were then washed three times with cold medium containing 3 per cent bovine serum albumin (Merz and Dade) and then mixed with 0·1 ml conjugate and 0·1 ml normal rabbit serum, incubated for a further 20 minutes at 4°, and washed as before. The cells were then placed on slides with a cytocentrifuge (Shandon) and examined with a Reichert Diapan microscope, fitted with an iodine quartz lamp, using a dark field condensor, a specific fluorescein interference filter and a GG 13 c barrier filter (Wild, Heerbrugg, Switzerland). The cells were evaluated under immersion at a final magnification of 480×. The staining patterns and the control for specificity are reported elsewhere (Vischer, 1972).

For statistical analysis, Student's *t*-test was employed.

RESULTS

THYMUS CELLS

As can be seen in Table 1, the cell number in the thymus of hydrocortisone-treated mice was reduced to 6·4 per cent of the number of matched controls. Stimulation of thymic cells of normal mice with all stimulants gave a much lower response than stimulation of cells obtained from hydrocortisone-treated mice. The normal high stimulation by PWM of thymic cells from untreated mice was further increased after hydrocortisone treatment (Table 2). Stimulation with allogeneic cells was also clearly higher after hydrocortisone treatment when compared with controls (Table 3). Thymic cells from both types of animals sensitized 4–8 weeks previously with KLH did not react to the antigen *in vitro*.

SPLEEN CELLS

After hydrocortisone treatment the cell number in spleens decreased to 32 per cent of the controls (Table 1). In contrast to the thymic cells all stimulants used showed a markedly reduced reactivity, with the exception of PHA where the reaction was only moderately reduced (Tables 4 and 5). Whereas with thymic cells the baseline incorporation of unstimulated cultures increased after hydrocortisone treatment, there was a decrease with spleen cells.

TABLE 1

NUMBER OF NUCLEATED CELLS IN THYMUS AND SPLEEN OF BALB/C MICE AFTER TREATMENT WITH HYDROCORTISONE

	Matched normal mice number of nucleated cells $\times 10^6$ ($\pm$SD)	Hydrocortisone-treated mice	
		Number of nucleated cells $\times 10^6$ ($\pm$SD)	Per cent of controls ($\pm$SD)
Thymus (N = 10)	167·7 ($\pm$79·0)	11·0 ($\pm$6·0)	6·4 ($\pm$0·6)
Spleen (N = 10)	147·8 ($\pm$37·5)	47·9 ($\pm$17·3)	32·4 ($\pm$5·4)

TABLE 2

THYMUS CELLS: STIMULATION WITH PHA AND PWM*

	No stimulant cpm ($\pm$SD)†	PHA cpm ($\pm$SD)†	PWM cpm ($\pm$SD)†
Cells from control mice	345 ($\pm$86)	9501 ($\pm$174)	36716 ($\pm$4597)
Cells from hydrocortisone-treated mice	925 ($\pm$101)	103,315 ($\pm$100,00)	132,751 ($\pm$1276)

* 2×10^6 Cells cultured for 3 days in RPMI 1640 with 5 per cent rabbit serum.

† Mean net cpm of triplicate cultures $\pm$ standard deviation. 0·5 μCi [^{3}H]thymidine was added at 60 hours and radioactivity of the TCA precipitable fraction measured at 72 hours.

TABLE 3

THYMUS CELLS: STIMULATION WITH ALLOGENEIC CELLS*

	BALB/c cpm ($\pm$SD)†	C57Bl/6 cpm ($\pm$SD)†	Mean (cpm)	BALB/c and C57Bl/6 cpm ($\pm$SD)†
Cells from control mice	106 ($\pm$5)	109 ($\pm$6)	108	1548 ($\pm$63)
Cells from hydrocortisone-treated mice	243 ($\pm$25)	387 ($\pm$8)	315	8422 ($\pm$961)

* 2×10^6 Cells of each strain cultured alone or 1×10^6 cells of both strains cultured together for 3 days.

† See Table 2.

TABLE 4

SPLEEN CELLS: STIMULATION WITH PHA, PWM AND KLH

	No stimulant cpm ($\pm$SD)†	PHA cpm ($\pm$SD)†	PWM cpm ($\pm$SD)†	KLH cpm ($\pm$SD)†
Cells from control mice	1158 ($\pm$119)	90224 ($\pm$10068)	21426 ($\pm$2737)	34105 ($\pm$3418)
Cells from hydrocortisone-treated mice	719 ($\pm$31)	76665 ($\pm$5919)	3187 ($\pm$526)	1482 ($\pm$181)

* 5×10^6 Spleen cells from mice previously immunized with KLH, cultured for 3 days.

† See Table 2.

In further experiments, the composition of spleen cells from hydrocortisone-treated animals and the matched controls was investigated. Table 6 indicates that after hydrocortisone treatment a significant decrease of the proportion of Ig-receptor containing cells occurs, with a proportionate increase in theta-cells ($P<0{\cdot}001$). Both groups had a similar incidence of cells negative for both antigens. In absolute terms, when a whole spleen is considered and not a fixed amount of cells as used in cultures, both theta- and Ig-receptor cells decreased after hydrocortisone treatment.

TABLE 5

SPLEEN CELLS: STIMULATION WITH ALLOGENEIC CELLS*

	BALB/c cpm (±SD)†	C57Bl/6 cpm (±SD)†	Mean (cpm)	BALB/c and C57Bl/6 cpm (±SD)†
Cells from control mice	1158 (±119)	3731 (±152)	2444	18645 (±2296)
Cells from hydrocortisone-treated mice	719 (±31)	2464 (±444)	1591	4176 (±380)

* 5×10^6 cells of each strain cultured alone or $2{\cdot}5\times10^6$ cells of both strains cultured together for 3 days.
† See Table 2.

TABLE 6

SPLEEN CELLS: CELLS WITH IG-RECEPTORS OR THETA-ANTIGEN

	Per cent (±SD) cells with Ig-receptors	Per cent (±SD) cells with theta-antigen	Per cent (±SD) unstained cells
Cells from normal mice ($n = 8$)	35·4 (±4·8)	49·9 (±4·3)	14·8 (±2·6)
Cells from hydrocortisone-treated mice ($n = 8$)	20·4 (±6·2)*	64·1 (±5·8)*	15·5 (±3·8)

* Significantly different from the normal controls ($P<0{\cdot}001$).

Hydrocortisone treatment impairs macrophage function when used in high dosage (Fauve and Pierce-Chase, 1967). Therefore, in two experiments peritoneal exudate cells from normal mice were added to sensitized spleen cell suspensions (3 or 6×10^5 macrophages per culture of 5×10^6 cells) and stimulated with KLH. No restoration of the reaction could be observed.

DISCUSSION

Corticosteroids can suppress both humoral and cellular immune responses in experimental animals, probably by means of lymphoid cell destruction. Some of the cells involved in the induction and expression of immune response are however corticosteroid resistant. Esteban (1968) established that the short-lived lymphocytes of lymphoid organs are more susceptible to the destructive effect of hydrocortisone than are the long lived lymphocytes. A minority of about 5 per cent of the cells of the thymus are corticosteroid resistant and are immunocompetent in a graft-versus-host assay (Warner, 1964; Blomgren and Anderson, 1969). The resistant cells appear to reside in the thymus medulla (Ishidate

and Metcalf, 1963) and to consist of immunologically competent, mature thymus cells. Similar competent cells have been demonstrated within the thymus by other means (Raff, 1971; Leckband and Boyse, 1971). Similarly, the cells in the spleen capable of initiating a graft-versus-host reaction are hydrocortisone resistant (Cohen, Fishbach and Claman, 1970). The helper cell effect of thymus-dependent cells is also hydrocortisone resistant, whereas the bone-marrow-derived precursors of antibody formation in the spleen, but not in the bone-marrow, are damaged by hydrocortisone (Cohen and Claman, 1971).

Two difficulties arise in the interpretation of the experiments presented in this paper. First, hydrocortisone treatment of mice might not only influence the numbers of lymphoid cells and their distribution in tissues, but might affect the surviving cells in other ways, changing their capacity for reactions *in vitro*. Second, quantification of *in vitro* lymphocyte stimulation is difficult. None of the currently used methods is really satisfactory and, therefore, the reactivity of cell populations to stimulants can only be expressed in relative terms, comparing grossly the counts within one experiment.

The results concerning the improved reactivity of hydrocortisone resistant thymus cells in the *in vitro* response to PHA and allogeneic cells are well in line with the concept of selective survival of mature T-cells after hydrocortisone treatment. They indicate that these reactions depend primarily on thymus-dependent cells as indicated by other experiments (Takiguchi, Adler and Smith, 1971), and confirm the results from Blomgren and Svedmyr (1971). More surprising is the finding that PWM stimulates thymus cell suspensions from both hydrocortisone-treated and normal animals, with a relatively smaller enhancement of the reaction after hydrocortisone treatment, when compared to PHA. PWM also can stimulate spleen cells from thymectomized, irradiated and bone-marrow reconstituted mice, and from nude mice, which have a congenital absence of the thymus (Stockman, Gallagher, Heim, South and Trentin, 1971; Janossy and Greaves, 1971). PWM seems to stimulate various types of cells, including B- and T-cells.

Hydrocortisone treatment *in vivo* seems to have a more complex effect on spleen cells than on thymus cells. In the spleen, the number of nucleated cells is reduced to 32 per cent of the normal controls. Differentiation shows a marked decrease in the number of Ig-receptor, bone-marrow-derived cells and a smaller decrease in the number of theta-cells. For the cultures however, fixed numbers of cells were used, thus giving an increase in theta-cells and a decrease in Ig-receptor cells in the spleen cultures from hydrocortisone treated animals. The reduction of the number of theta-cells in the spleen could indicate that some cortisone sensitive cells leave the thymus and make up at least part of the theta-positive, thymus-dependent spleen cell population in normal mice.

All stimulants gave a reduced effect on the spleen cells from hydrocortisone-treated animals. The results with allogeneic cells are in contrast with the findings of Blomgren and Svedmyr (1971) who did not find a decrease in the mixed lymphocyte reaction using spleen cells from hydrocortisone-treated animals. These authors apparently used mitomycin-treated cells from normal mice for stimulation in a one-way system. These cells could have contributed in an unknown way to render the cells from hydrocortisone-treated animals more efficient to react with allogeneic cells (Janis and Bach, 1970). From our experiments, it seems that for a strong reaction in mixed lymphocyte cultures mature T-cells alone are not sufficient. Results with staining of the transformed cells for Ig-receptors and theta-antigen, after stimulation with allogeneic cells or PHA, indicate that a certain number of Ig-receptor cells are stimulated during these reactions (Vischer, 1972). As the

reactivity to PHA was only little decreased, it is likely that stimulation with this substance is different from stimulation with allogeneic cells. This is substantiated by the observation of Colley, Shih Wu and Waksman (1970), who demonstrated by fractionation of thymic cells that different cell types are stimulated by allogeneic cells and PHA. The diminished response with PWM can be explained by its wide reactivity. At least two cell types with which it can react are diminished after hydrocortisone treatment in the spleen: the bone-marrow dependent Ig-receptor cells and some of the perhaps immature theta-cells.

The secondary response to KLH *in vitro* also was markedly reduced. Both theta-bearing and Ig-receptor cells are necessary for initiation of the response (Vischer and Jaquet, 1972) and during culture both Ig-receptor cells and theta-bearing cells transform to blast-like cells and incorporate [^{3}H]thymidine (Vischer, 1972). The reduction of Ig-receptor cells might account for the diminished response.

Macrophage function in mice is impaired after treatment with a high dose of hydrocortisone (10 mg) (Fauve and Pierce-Chase, 1971). Macrophages seem to be necessary for mixed lymphocyte reaction (Gordon, 1968) and the secondary reaction against antigens (Seeger and Oppenheim, 1970), but not for the reaction of the non-specific stimulants PHA (Oppenheim, Leventhal and Hersh, 1968) and PWM (Gajl-Peczalska, Meuwissen and Good, 1969). That the hydrocortisone treatment used in the present experiment ($\sim$2·5 mg) does not affect the macrophages sufficiently to account for the decreased reactivity can be inferred from two findings: thymus cells from treated animals give an improved reaction against allogeneic cells, and addition of normal peritoneal macrophages to the cultures of spleen cells obtained from hydrocortisone treated, KLH-sensitized mice did not restore the reaction.

From the experiments reported it can be concluded that hydrocortisone treatment of mice has a deleterious effect on both immature thymus cells and part of the bone-marrow-dependent cells. Which of the B-cells are affected is not known, but it seems that cells affected by hydrocortisone are important for the potentiation of the mixed lymphocyte reaction, the reaction to PWM, and for the secondary stimulation by KLH. The PHA response seems to be less dependent on these cells. In addition, a population of thymus-dependent cells with theta-antigen must reside in spleens, which are affected by hydrocortisone.

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