THYMUS–MARROW IMMUNOCOMPETENCE

V. Hydrocortisone-Resistant Cells and Processes in the Hemolytic Antibody Response of Mice*

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Corticosteroids can suppress both humoral and cellular immune responses in experimental animals such as the mouse, rat, and rabbit. Until recently, it was thought that corticosteroids acted in these systems by means of lymphoid cell destruction. Several recent reports, however, indicate that some of the cells involved in the induction and expression of immune responses are corticosteroid-resistant. This was first shown in the graft-vs.-host (GVH)[1] system by Warner (1) who demonstrated that the few cells which remained in the chicken thymus after cortisone treatment had virtually all the thymic GVH potential. Blomgren and Andersson have recently confirmed this in the mouse (2). These findings indicate that a minority (about 5%) of the cells in the thymus are corticosteroid-resistant but are immunocompetent in the GVH system. The resistant cells appear to reside in the thymus medulla (3). The same pattern is true of the spleen; about 75% of the cells are corticosteroid-sensitive, while the resistant population contains all the spleen's GVH initiator potential (4).

The circulating antibody response of mice to sheep erythrocytes (SRBC) is markedly suppressed by corticosteroids given at about the same time as antigen (5). This response involves the interaction of at least two cells, one derived from the thymus and the other derived from bone marrow. Little is known about the effects of corticosteroids on the cooperating cells of this response. Compared with normal spleens, spleens from cortisone-treated donors have a decreased ability to transfer antibody-forming capacity to irradiated recipients (6). Since the spleen contains both marrow-derived and thymus-derived cells, such data do not indicate the corticosteroid-sensitive step. On the other hand, bone marrow cells (at least while in the marrow) are resistant to corticosteroids. This

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; GVH, graft-*versus*-host; MEM, minimal essential medium; PFC, plaque-forming cells; PHA-phytohemagglutinin; SRBC, sheep erythrocytes; TL system, thymus–leukemia system.

was learned from experiments where bone marrow cells from donors given large doses of cortisone were able to cooperate well with normal thymus cells when transferred to syngeneic irradiated recipients and stimulated with SRBC antigen (6). We undertook the present investigations to determine the hydrocortisone sensitivity of the thymus component in this system and the nature of the corticosteroid sensitivity of spleen.

Materials and Methods

Animals.—Male LAF_1 mice, aged 8–18 wk, were obtained from Jackson Laboratories, Bar Harbor, Maine. All mice in any one experiment were born on the same day and housed together in groups of six. Drinking water contained 0.02% sodium hypochlorite.

Hydrocortisone Treatment.—Some donors were given 2.5 mg of hydrocortisone acetate suspension (Cortril, Chas. Pfizer & Co., Inc., New York) intraperitoneally 2 days before their tissues were used in transfer. Control donors received a similar volume of the Cortril vehicle.

Irradiation.—Recipients received 1000 R in 10 min at a distance of 60 cm from an Atomic Energy of Canada Ltd. ^{60}Co source.

Cell Preparations and Transfer.—Single cell suspensions of spleen, femoral bone marrow, and thymus were prepared as described previously (4). Thymuses from hydrocortisone-treated donors were removed under 4 × loupe magnification to avoid taking adjacent lymph nodes. Suspensions in spinner-type minimal essential medium (MEM) were centrifuged at 400 *g* for 10 min and resuspended in MEM. Nucleated cells were counted, and portions were mixed according to the protocol so that each recipient received the appropriate number of cells and 5×10^8 SRBC intravenously in a volume of 0.5 ml. Booster injections of antigen consisting of 0.5 ml of 10% SRBC were given intraperitoneally on day 4. On the 8th day after transfer, the recipients were killed by cervical dislocation and the spleens removed.

Plaque-Forming Cell Determinations.—Plaque-forming cells (PFC) in the recipient spleens were determined by the direct method of Jerne, Nordin, and Henry (7), modified in that 60 mm Petri dishes were employed, and CO_2 was omitted during incubation.

RESULTS

Immunocompetence of Hydrocortisone-Resistant Cells in the Thymus.—2 days after administration of 2.5 mg of hydrocortisone acetate intraperitoneally, the thymuses of LAF_1 mice contained 2.0–6.3% of the number of nucleated cells of normal thymuses. Normal thymus cells, equivalent in number to one thymus, were given intravenously to 20 irradiated recipients; 20 others were given one thymus-equivalent of cells from hydrocortisone-treated donors. 10 mice in each group were also given 10^7 bone marrow cells, and all mice were given SRBC intravenously. A booster of SRBC was given on day 4, and PFC were determined on day 8. Neither normal nor hydrocortisone-treated thymus alone transferred a significant number of PFC, but they were equally effective in synergism with bone marrow (Table I). This experiment indicates that thymus "helper" cells are resistant to hydrocortisone.

Resistance of Thymus Cell Proliferation to Hydrocortisone.—The following experiment (Table II) was designed to determine whether thymus helper cells, shown in the first experiment to be hydrocortisone-resistant within the thymus,

TABLE I

*Helper Cell Activity of One Thymus-Equivalent from Normal or Hydrocortisone-Treated Donors in Adoptive Transfer of Humoral Immunity to Sheep Red Blood Cells**

Experiment	Mice/ group	Thymus donors	Thymus cells transferred	Marrow cells transferred	PFC/recipient spleen
1	10	Normal	70×10^6	—	10 (0–32)‡
(Donors 8	10	Normal	70×10^6	10^7	794 (468–1346)
wk old)	10	Hydrocortisone	1.4×10^6	—	12 (0–38)
	10	Hydrocortisone	1.4×10^6	10^7	613 (224–1680)
	6	—	—	10^7	16 (4–28)
2	10	Normal	24×10^6	—	51 (15–86)
(Donors 18	10	Normal	24×10^6	10^7	1020 (147–1893)
wk old)	10	Hydrocortisone	1.2×10^6	—	25 (14–37)
	10	Hydrocortisone	1.2×10^6	10^7	906 (213–1600)
	6	—	—	10^7	173 (50–280)

* The equivalent of one thymus from normal LAF_1 donors, or donors treated with 2.5 mg of hydrocortisone acetate 2 days before, was transferred with or without normal bone marrow to irradiated (1000 R) recipients on day 0, along with SRBC. SRBC were also given on day 4.

‡ Means and 95% confidence limits.

TABLE II

*Resistance to Hydrocortisone of Antigen-Induced Proliferation of Thymus Cells**

Group (number of mice)	Primary recipient		Secondary recipient	
	Transferred Day 0	Hydrocortisone treatment	Transferred Day 5	PFC/spleen Day 13
A (6)	Thymus	—	Primary spleen A + marrow + SRBC	150 (90–212)‡
B (6)	Thymus + SRBC	—	Primary spleen B + marrow + SRBC	840 (240–1440)
C (6)	Thymus + SRBC	−6 hr; +3 days	Primary spleen C + marrow + SRBC	650 (254–1046)
D (6)	Thymus + SRBC	+24 hr; +3 days	Primary spleen D + marrow + SRBC	639 (255–1012)

* Six irradiated (1000 R) primary recipients were given 25×10^6 normal thymus cells with or without SRBC on day 0. One group which received thymus + SRBC was given 2.5 mg of hydrocortisone acetate 6 hr before transfer and on day 3; another such group was given hydrocortisone acetate 24 hr after transfer and on day 3. On day 5, spleens were removed and one spleen-equivalent transferred, with 10^7 bone marrow cells and SRBC, to each of six irradiated secondary recipients. A booster of SRBC was given on day 9, and PFC determined on day 13.

‡ Means and 95% confidence limits.

become sensitive when stimulated by antigen to proliferate (8). Normal thymus cells were "educated" by transferring 25 × 10^6 cells (equivalent in this experiment to one-half normal thymus) with SRBC to irradiated syngeneic recipients. 5 days later, spleens were removed, single cell suspensions made, and 10^7 normal bone marrow cells were added to the equivalent of one such spleen.

TABLE III

*Reversal by Bone Marrow of the Suppressive Effect of Hydrocortisone on the Ability of Spleen to Transfer Adoptive Immunity to Sheep Red Blood Cells**

Group (number of mice)	Spleen cells transferred	Other cells transferred	PFC/recipient spleen	Calculated PFC/donor spleen
A (6)	Normal (1.5 × 10^6)	—	250 (120–530)‡	10,500 (4956–22,260)§
B (6)	Normal (1.5 × 10^6)	10^7 Bone marrow	971 (550–1720)	40,782 (22,932–72,576)
C (6)	Hydrocortisone-treated (1.5 × 10^6)	—	139 (80–230)	2,780 (1,660–4,680)
D (6)	Hydrocortisone-treated (1.5 × 10^6)	10^7 Bone marrow	1347 (800–2400)	27,480 (16,000–47,180)
E (6)	Hydrocortisone-treated (1.5 × 10^6)	3.5 × 10^7 Thymus	180 (86–376)	3,600 (1,720–7,520)

* 1.5 × 10^6 spleen cells from normal donors, or donors which had received 2.5 mg of hydrocortisone acetate 2 days previously, were transferred with SRBC to irradiated (1000 R) recipients. One group of recipients of normal spleen was also given 10^7 bone marrow cells. One group of hydrocortisone-spleen recipients was given 10^7 bone marrow cells, and another group 3.5 × 10^7 normal thymus cells. All groups received SRBC. An SRBC booster was given on day 4, and PFC were determined on day 8.

‡ Means and 95% confidence limits.

§ The number of PFC/recipient spleen was multiplied by the inverse of the fraction of a donor spleen represented by 1.5 × 10^6 cells; this was 20 in the case of hydrocortisone spleen, and 42 for normal spleen.

The mixture was transferred with SRBC to a second irradiated recipient; these mice were given boosters of SRBC on day 9 (4 days after transfer). PFC were determined 8 days after the second transfer (day 13). Proliferation of the thymus cells in response to antigen (in the first recipient) was shown by a significant PFC response in the second recipient (group B); when antigen was omitted in the first recipient few PFC were ultimately obtained (group A).

Groups of primary recipients of thymus cells and antigen were given 2.5 mg

of hydrocortisone acetate intraperitoneally either 6 hr before cell transfer (group C), or 24 hr afterwards (group D). Both groups were given 2.5 mg of hydrocortisone again 3 days after transfer, and their spleens were removed on day 5 for injection, with normal bone marrow and SRBC, into secondary recipients. PFC determined 8 days later were not significantly different from those in the control educated group (groups C and D vs. group B); both groups in which hydrocortisone was present during the phase of thymus cell proliferation showed education.

Locus of Hydrocortisone Effect on the Adoptive Response by Spleen.—This experiment was designed to locate the hydrocortisone-sensitive population of spleen cells by determining what cells could reconstitute a hydrocortisone-treated spleen. Normal spleen, and spleen from donors treated on day −2 with hydrocortisone acetate, were transferred to irradiated recipients with SRBC. The number of cells transferred in each case was 1.5×10^6, representing $\frac{1}{42}$ of a normal spleen or $\frac{1}{20}$ of a hydrocortisone spleen. PFC were determined, and to compare immunocompetence the number of PFC that theoretically would have been obtained if a whole donor spleen had been transferred was calculated (Table III). Equal spleen cell numbers were transferred rather than organ equivalents, and small numbers of spleen cells were used, so that spleen–marrow synergism could be more easily demonstrated (9).

As expected, spleen cells of mice treated with hydrocortisone were less able than normal spleen cells to produce PFC in irradiated recipients (group C vs. A). This immunosuppressive effect of hydrocortisone was in large measure reversed by transferring normal bone marrow with the hydrocortisone spleen (group D); addition of normal thymus was without effect (group E). The ability of hydrocortisone spleen to transfer PFC was enhanced tenfold by bone marrow, while the number of PFC obtained with normal spleen cells was enhanced only fourfold by bone marrow.

DISCUSSION

There is a small population (about 5%) of cells in the mouse thymus which survives treatment with 2.5 mg of hydrocortisone acetate (10). This population seems to contain all the immunocompetent cells in the thymus. In terms of cell-mediated immunity, the GVH initiator cells of normal thymus have been found in the hydrocortisone-resistant population (2, 4), indicating that, by this criterion, the 95% of thymus cells that are killed by hydrocortisone are not immunocompetent. The results presented above lead to a similar conclusion about thymus helper cells in the humoral immune response. One thymus from a hydrocortisone-treated donor induced as many antibody-forming cells (AFC) in normal bone marrow as did one normal thymus, although it contained only 5% as many cells. Thus, the active thymus helper cells are contained in the 5% or less of the thymus nucleated cells which are resistant to hydrocortisone. Andersson and Blomgren (11) have recently described similar results.

The response of thymic helper cells to antigen (education), which results in an expanded population of these cells (12), was also found to be resistant to hydrocortisone (Table II). Hydrocortisone was administered to one group of primary recipients before cell transfer, so that even the earliest events of lymphocyte–antigen interaction in the recipient would take place in the presence of the drug. Since corticosteroids might affect the circulation of transferred lymphoid cells, we included another group which received hydrocortisone 24 hr after cell transfer, by which time homing is virtually completed (13). Results in both groups were similar; hydrocortisone had little effect on thymus education.

The role of the great majority of thymus lymphocytes (those which are sensitive to hydrocortisone) is not established. It is probable that these cells, primarily situated in the cortex (3), are immunologically immature precursors of hydrocortisone-resistant medullary cells. Experiments using tritiated thymidine labeling have shown intense mitotic activity in the thymus cortex, and very much less in the medulla; as cortical cells divide, they appear to move into the medulla (14, 15). From these data, one might assume that hydrocortisone preferentially affects dividing cells, but our experiments with education for GVH (16) and helper activity in the antibody response (Table II) indicate that the situation is not that simple; in both these systems the proliferation of specific cells under antigenic stimulation (17) is hydrocortisone resistant. We suggest that lymphoid cells possess or lack, depending on their stage of maturation, a surface hydrocortisone receptor whose presence is essential for the cytotoxic effect of hydrocortisone to be manifest. There is experimental evidence for such a receptor (18).

There are also considerable experimental data which support the view that surface features change as thymic lymphocytes mature. The cells of the thymus medulla, but not those of the cortex, are stimulated by phytohemagglutinin (PHA) (19), which probably involves binding of the mitogen to a surface receptor (20). Thymus-derived cells in peripheral lymphoid tissues are also PHA stimulable (21); thus, it appears that the ability to be stimulated by PHA is a marker for mature thymus cells. In a study of the thymus-leukemia (TL) system of thymus antigens, a small number of TL-negative cells were identified in the thymuses of TL-positive strains of mice (22). This minority contained most of the GVH initiators; thus, the cells expressing TL antigen were not immunocompetent. Schlesinger and Golakai have demonstrated that the TL-positive thymus cells are also hydrocortisone sensitive (23). Whether the TL-negative cells tend to be located in the medulla has not been established. Taking all these data into account, we suggest that most thymic cortical lymphocytes are: rapidly dividing (14), corticosteroid sensitive (3), immunoincompetent (4, 16), TL positive in those mice possessing the TL antigen (23), unresponsive to PHA (19), and relatively poor in the expression of H-2 antigens (23). Thymic medullary lymphocytes represent the converse in each case.

Spleen cells from mice treated with hydrocortisone were poor at transferring AFC to irradiated mice. This immunosuppressive effect of hydrocortisone could be on any of the three cell types known to be involved in humoral immune responses, and found in spleen: the macrophage (the in vivo counterpart of the adherent cell), the thymus-derived helper cell, or the AFC precursor. We consider the macrophage to be an unlikely candidate, since the irradiated recipient probably supplies an excess of these cells (24); more directly, cortisone treatment has been found not to affect the production of adherent cells active in in vitro antibody synthesis systems[2] and peritoneal macrophages are relatively unaffected by cortisone (25).

Our data suggest that, in the spleen, the marrow-derived AFC precursor and not the helper cell is the cell sensitive to hydrocortisone, since the immunocompetence of hydrocortisone–spleen can be restored by bone marrow and not by thymus, i.e., hydrocortisone-spleen is deficient in AFC precursors relative to normal spleen. Several other pieces of evidence are in keeping with this conclusion; another thymus-derived cell in the spleen, the GVH initiator, is hydrocortisone resistant (4), and the mature GVH (2, 4) and helper cells (11, and above) of the thymus itself are resistant. In a recent report, Andersson and Blomgren (11) were unable to show an effect of cortisone on the adoptive transfer of humoral immunity by spleen, but they used a dose of cortisone which, in their mice, was not immunosuppressive.

The maturation of the AFC with respect to corticosteroid sensitivity appears to be complex. The bone marrow serves as a reservoir of AFC precursors, and probably populates the spleen and lymph nodes with these cells as required (26). Levine and Claman have shown that, within the bone marrow itself, the AFC precursor is resistant to high doses of cortisone (6). By the time these cells have populated the spleen, they have become relatively hydrocortisone sensitive (see above). The mature AFC, however, is hydrocortisone resistant.[3] A considerable amount of further evidence is required to relate the changes in corticosteroid sensitivity of the AFC precursor to immunocompetence. This cell is much more difficult than the thymus cell to study because of the relative paucity of surface features as yet identified, and because functional tests that do not depend on the antigen-induced production of antibody, similar for example to PHA stimulation, are not available.

SUMMARY

Corticosteroids suppress the humoral antibody response of mice to sheep erythrocytes. This response depends on interactions between thymus-derived helper cells and bone marrow-derived antibody-forming cell precursors (AFC precursors). Previous experiments had shown that spleen cells (a mixture of thymus-derived and marrow-derived cells) were sensitive to corticosteroids

[2] Talmage, D. W., and J. Radovich. Unpublished results.

[3] Levine, M. A. Unpublished results.

while AFC precursors in the bone marrow were resistant. The present experiments showed that the thymus of a mouse given 2.5 mg of hydrocortisone acetate, although containing only about 5% of the number of cells of a normal thymus, was as effective as a normal thymus in cooperating with bone marrow when transferred to irradiated syngeneic mice and stimulated with SRBC. The proliferative response of thymus helper cells to SRBC was also resistant to hydrocortisone.

In this context, the majority of thymic cells are in the cortex, are rapidly dividing, are sensitive to corticosteroids and are not immunocompetent. A small number of thymic cells, probably located in the medulla, are resistant to corticosteroids, but are immunocompetent since they can serve as helper cells.

The hydrocortisone-sensitive phase of the splenic response to SRBC was found to be the bone marrow-derived AFC precursor since spleens from hydrocortisone-treated donors had immunocompetence restored by normal bone marrow but not by normal thymus cells.

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