

HETEROGENEITY IN THE DEVELOPMENT  
OF CYTOTOXIC T LYMPHOCYTES IN VITRO REVEALED  
BY SENSITIVITY TO HYDROCORTISONE

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When lymphoid cells are cultured in vitro with allogeneic cells, specifically cytotoxic lymphocytes develop (1). One in vitro system for the generation of cytotoxic lymphocytes which has been studied extensively is the mixed lymphocyte culture (MLC) (2) in which the allogeneic stimulus is provided by lymphocytes. Another system which was developed in our laboratory (3-5) involves sensitizing lymphoid cells against xenogeneic or allogeneic fibroblasts. Sensitization and generation of cytotoxicity in both the MLC and the antifibroblast reaction (AFR) systems were shown to be the function of T lymphocytes (2,5). However, it is not known whether the only difference between these two in vitro systems is related to the nature of the sensitizing cell (lymphocyte vs. fibroblast) or whether there are more fundamental differences which reflect heterogeneity of cytotoxic T lymphocyte (CTL) precursors and their differentiation mechanisms.

Another question involves the heterogeneity of T cells present in different lymphoid organs. For example, are mature lymphocytes within the thymus functionally equivalent to peripheral T cells?

Heterogenous populations of thymocytes and T cells can be distinguished on the basis of physical (6), surface (6,7), and functional (8-11) properties. Another property which has been used to distinguish between different types of thymocytes is their sensitivity to hydrocortisone (HC) in vivo (6,12).

In the present study we used hydrocortisone treatment in vivo as a probe to analyze and compare the generation of CTL by thymocytes and spleen cells in the MLC and AFR systems. We found that different pathways and/or precursors for the generation of CTL exist in the AFR and MLC systems, and, in addition, that the MLC reactivity of thymocytes and spleen cells are each affected in a different way by hydrocortisone treatment.

**Materials and Methods**

*Animals.* 6-wk old male C57BL/6J mice, obtained from The Weizmann Institute Animal Breeding Center, were used as donors of spleen and thymus cells.

*Hydrocortisone (HC) Administration.* 2 mg of hydrocortisone acetate (Frederiksberg Chemical Laboratories, Copenhagen) in a vol of 0.25 ml were injected intraperitoneally into mice. The mice

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were used as lymphocyte donors. 2 days later the spleens and thymus glands of these mice contained around 15% and 5%, respectively, of the original number of lymphoid cells.

**Antifibroblast Reaction.** Lymphoid cell suspensions were prepared and sensitized on allogeneic embryonic fibroblast monolayers as described previously (5,13,14). Briefly,  $30 \times 10^6$  lymphocytes, in 4 ml of Dulbecco's modified Eagle's medium (EM) supplemented with 15% inactivated fetal calf serum (FCS, Rehatuin N. F. S., Reheis Chemical Co., Chicago, Ill.) and  $5 \times 10^{-5}$ M 2-mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.), were seeded on irradiated C3H fibroblast monolayers in 60-mm plastic petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Medium was changed after 24 h. Sensitized lymphocytes were collected after 4 days by gentle pipetting and resuspended in fresh EM + 15% horse serum (Grand Island Biological Co., Grand Island, N. Y.) to a concentration of  $2 \times 10^6$ /ml. Aliquots of 0.5 ml were seeded in quadruplicate on  $^{51}\text{Cr}$ -labeled target fibroblast monolayers prepared 1-2 days earlier in 12 x 35 mm sterile glass vials (Kimble Products, Owens, Ill.). The ratio of effector to target cells was 10:1. Cytolysis was determined by measuring the amount of  $^{51}\text{Cr}$  released into the culture medium after subtracting the spontaneous release (usually 10-20%). Radioactivity was counted in a well-type sodium iodide crystal autogamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

**MLC Proliferative Phase (13,14).** To measure DNA synthesis,  $2 \times 10^6$  responder lymphocytes together with an equal number of 1,000 R irradiated syngeneic (control) or allogeneic C3H stimulator spleen cells in a total vol of 1 ml EM + 10% FCS, were cultured in triplicate in 17 x 100 mm Falcon plastic tubes for 4 days. 18 h before the end of culture, 2  $\mu\text{Ci}$  per tube of tritiated thymidine ( $^3\text{H}$ ]TdR, preparation TRA.120, Radiochemical Centre, Amersham, England) were added. Cells were collected on GF/C glass fiber filters washed twice each with cold saline, 5% TCA, and absolute ethanol. The filters were then dried and counted in 10 ml of toluene scintillation fluid, using a Packard Tri-carb scintillation counter.

**MLC Effector Phase (Cell-Mediated Lympholysis, CML) (14).**  $30 \times 10^6$  responder lymphocytes plus  $15 \times 10^6$  irradiated stimulator spleen cells in a total vol of 6 ml of EM + 15% FCS were cultured in 60-mm plastic petri dishes for 5 days. After 3 days, 3 ml of culture medium were replaced with fresh medium. The sensitized cells were collected from the cultures and the CML assay was carried out as described previously (14). Briefly, 0.5-ml aliquots containing  $1 \times 10^6$  sensitized cells plus  $1 \times 10^4$   $^{51}\text{Cr}$ -labeled target blast cells induced by concanavalin A (Miles-Yeda, Rehovot, Israel) were cultured for 4 h in 12 x 75 plastic tubes. The reaction was terminated by adding 1.5 ml cold phosphate-buffered saline and centrifuging the tubes. The extent of CML was determined by measuring the amount of  $^{51}\text{Cr}$  released into the medium. Spontaneous release, determined in the presence of fresh, unsensitized, lymphocytes, usually around 20%, was subtracted from the values of experimental release, to give the net CML.

## Results

*The Effect of HC Treatment on the AFR and MLC Reactions of Thymocytes.* Thymocytes from HC-treated and from normal mice were sensitized in both the AFR and the MLC systems, and the extent of cytolysis of target cells

TABLE I  
*The Effect of Hydrocortisone Treatment In Vivo on the MLC and AFR Reactivity of C57BL/6J Thymocytes\**

HC treatment	AFR % cytolysis	MLC proliferation				CML % cytolysis
		cpm (syngeneic)	cpm (allogeneic)	$\Delta$ cpm	SI $\ddagger$	
-	-0.9 $\pm$ 1.8	795 $\pm$ 65	5,381 $\pm$ 563	4,586	6.8	-1.4 $\pm$ 1.1
+	18.8 $\pm$ 1.5	1,709 $\pm$ 167	32,047 $\pm$ 6904	30,338	18.7	15.6 $\pm$ 1.3

\* Thymocytes from normal, untreated C57BL/6J mice, and from mice injected 2 days previously i.p. with 2 mg HC were sensitized against irradiated C3H fibroblasts (AFR) or lymphocytes (MLC).

$\ddagger$  SI, stimulation index.

was determined. In addition we measured DNA synthesis in the MLC. It can be seen (Table I) that thymocytes from normal, untreated, mice did not become sensitized in the AFR system, this being a consistent finding in 10 experiments. In addition, they did not generate CTL in the MLC, although in several other experiments low but significant cytolysis could be produced by normal thymocytes stimulated in a MLC. The same thymocytes underwent significant MLC activation which, however, was low compared to that of normal spleen cells (see Table II). After HC treatment *in vivo*, each of the three functions measured was markedly increased. CTL were now readily demonstrable after sensitization of HC-resistant thymocytes in both AFR and MLC reactions.

*The Effect of HC Treatment on the AFR and MLC Reactions of Spleen Cells.* A completely different picture was obtained when we tested the effect of HC treatment on the reactivity of spleen lymphocytes. We observed a clear dissociation between AFR reactivity on one hand, and the two phases of the MLC on the other (Table II).

TABLE II  
*The Effect of Hydrocortisone Treatment In Vivo on the MLC and AFR Reactivity of C57BL/6J Spleen Cells\**

HC treatment	AFR % cytolysis	MLC proliferation				CML % cytolysis
		cpm (syngeneic)	cpm (allogeneic)	$\Delta$ cpm	SI	
-	8.7 $\pm$ 0.3	6,149 $\pm$ 564	39,129 $\pm$ 6314	32,980	6.4	40.9 $\pm$ 3.2
+	33.8 $\pm$ 0.7	1,408 $\pm$ 81	2,790 $\pm$ 373	1,382	2.0	9.8 $\pm$ 1.3

\* Spleen cells from normal, untreated C57BL/6J mice and from mice injected 2 days previously *i. p.* with 2 mg HC were sensitized against irradiated C3H fibroblasts (AFR) or lymphocytes (MLC).

Thus, lymphocytes in the spleen of mice that were treated with hydrocortisone showed a greater capacity to become sensitized against allogeneic fibroblasts *in vitro*. The AFR reactivity of these spleen lymphocytes was increased to levels characteristic of normal lymph node lymphocytes.<sup>1</sup> In contrast to the augmentation of the AFR response, the MLC reactivities of the spleen lymphocytes were markedly depressed. Both DNA synthesis and the cytolytic responses of spleen cells to allogeneic MLC stimulation were inhibited after HC treatment. These findings were confirmed in six consecutive experiments using BALB/c as well as C57BL mice.

### Discussion

In the present study we compared and analyzed the effects of HC treatment *in vivo* on the reactivity of thymocytes and spleen lymphocytes in two *in vitro* systems which generate CTL—the AFR and MLC systems. Two main findings emerged from these experiments: first, the treatment of mice with HC led to a marked increase in the generation of CTL by spleen cells stimulated in the AFR, and to a marked decrease in both DNA synthesis and CTL stimulated in the MLC system. This dissociation could not be ascribed to the difference between the target cells used in the two systems (fibroblasts vs. concanavalin A-induced

<sup>1</sup> Altman, A. Unpublished results.

blasts) since we have found that both types of cells can be lysed by lymphocytes sensitized in either AFR or MLC systems.<sup>2</sup> It is conceivable that splenic precursors of CTL in the AFR and MLC systems are different from each other with respect to their sensitivity to HC. Another possibility is that the precursors of CTL in both systems are the same and that HC acts on other cells which modulate the generation of CTL. For example, it was postulated that cells reacting to MLR gene products by proliferation amplify the generation of killer cells in the MLC (2,9,15). We found that MLC stimulus, in the form of allogeneic stimulator lymphocytes or MLC supernates, can augment sensitization and generation of CTL in the AFR system (13,14). Thus, the activity of HC-resistant effector cells or their precursors may be dependent on amplifying cells which are sensitive to HC. Indeed, HC treatment severely depressed proliferation of spleen cells in the MLC.

Treatment with HC, on the other hand, could augment the generation of CTL by the AFR possibly by inhibiting HC-sensitive precursors of splenic cells which can suppress sensitization and generation of CTL. In preliminary experiments we found that the addition of one-fourth the amount of normal spleen cells to cultures of HC-treated spleen cells suppressed the augmented generation of CTL in the AFR. According to these assumptions such modulating processes (amplification and suppression) act differentially in the AFR and MLC systems.

Contradictory findings about the effect of HC on the proliferation of spleen cells in the MLC have been reported. Vischer (16) found a marked depression of reactivity in a two-way MLC by HC treatment, while Blomgren and Svedmyr found no such effects in a one-way MLC (17). It should be mentioned that in our hands HC treatment left only about 15% of the original number of nucleated cells in the spleens, in contrast to a significantly higher proportion (30–50%) which was reported previously (16,17). However, we found in five consecutive experiments that the same treatment, when given to 3-mo old mice (compared to 6-wk old mice used in the present study), left about 40% of the cells in the spleen, without depressing the MLC reactivity. Thus, the effects of HC on certain lymphocyte populations in mice may be different at different ages.

Our second finding concerns the differential effect of HC on the reactivity of thymocytes and spleen cells. Populations of lymphocytes remaining in the thymus after treatment with HC showed increased reactivity in both the AFR and MLC, while those remaining in the spleen showed increased AFR and decreased MLC reactivity. The effects of HC on the thymus are compatible with the results of other studies which reported increased cell-mediated reactivity of thymocytes (16,17). This effect has been explained by the selective removal from the thymus of immature cells, and the resulting enrichment in mature immunocompetent thymocytes (18,19). Thus, the MLC-reactive cells in the thymus appear to be represented by the minor population of medullary, HC-resistant lymphocytes (17). On the other hand, in the spleen the bulk of MLC-reactive cells may be found in the pool of short-lived, HC-sensitive, T<sub>1</sub> lymphocytes (6,20). In addition, several reports point to the fact that the mature, HC-resistant thymocytes are not functionally equivalent to peripheral T lymphocytes (21,22).

<sup>2</sup> Altman, A. Unpublished results.

### Summary

In the present study we used hydrocortisone (HC) treatment *in vivo* as a probe to analyze two different *in vitro* systems for the generation of cytotoxic T lymphocyte (CTL), namely the antifibroblast reaction (AFR) and the mixed lymphocyte culture (MLC) system. We found that cells remaining in the thymus after HC treatment had increased reactivity in these two systems. However, the same treatment in the spleen severely depressed the MLC reactivity in both the proliferative and the cytolytic phases, while markedly increasing the AFR reactivity. These findings demonstrate heterogeneity of CTL precursors and/or their pathways of differentiation into effector cells. In addition, MLC-reactive cells in the thymus appear to be distinct from such cells in the spleen, as judged from their differential sensitivity to HC.

The authors are grateful to Mrs. R. Karakash for technical assistance and to Dr. M. Feldman for his support.

*Received for publication 16 June 1975.*

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