white nodules just above the aortic valve in a few rabbits in all 3 groups. Except for these lesions, microscopically visible as subintimal, fibrous calcified lesions, none of the animals showed severe medial alterations, but half of the d-thyroxine-injected and 2 of the saline-injected animals had scattered accumulations of metachromatic substance in the media between the elastic membranes. Considering the content of water, hexosamine, hydroxyproline and the hexosamine/hydroxyproline ratio no difference was seen between the 3 groups, whereas the uptake of ^{35}S sulfate in aorta showed a significant increase in the d-thyroxine group. The injected doses of d- and l-thyroxine were found to have a complete inhibitory effect on release of ^{131}I-labelled hormone from the thyroid gland. The effect was noted 24 hours after onset of injections.

Discussion. D-thyroxine showed an influence on the aortic wall similar to that of l-thyroxine(1,2). It enhanced the damaging effect of epinephrine and induced alterations in the mucopolysaccharides of the arterial wall. The alterations observed might accordingly be interpreted as a repair process elicited by injury to the arterial wall(2). However, even if injected in 5-fold higher doses, d-thyroxine alone in contrast to l-thyroxine (1), was unable to induce quantitative alterations in the hexosamine content. As the doses of d- and l-thyroxine exerted an equally inhibitory effect on release of thyrotrophic hormone, it seems reasonable to assume, that d-thyroxine as well as l-thyroxine exerts its influence on the arterial wall by a direct peripheral action and not *via* TSH.

Summary. D-thyroxine enhanced epinephrine-induced alterations in the aortic wall in rabbits, causing an increase in hexosamine content and uptake of ^{35}S sulfate. D-thyroxine alone increased uptake of ^{35}S sulfate. Compared to l-thyroxine used in previous experiments, d-thyroxine acted similarly but to a lesser extent. As the applied amounts of d- and l-thyroxine exerted an equally inhibitory effect on TSH, d-thyroxine as well as l-thyroxine probably exerts its damaging effect on the arterial wall by direct peripheral action.

1. Lorenzen, I., *Acta Endocrin.*, 1961, v37, 183.
2. ———, *ibid.*, 1961, v36, 197.
3. Elson, L. A., Morgan, W. T. J., *Biochem. J.*, 1933, v27, 1824.
4. Boas, N. F., *J. Biol. Chem.*, 1953, v204, 553.
5. Kirk, J. E., Dyrbye, M. O., *J. Geront.*, 1956, v11, 273.
6. Dyrbye, M. O., *ibid.*, 1959, v14, 32.
7. Neuman, R. E., Logan, M. A., *J. Biol. Chem.*, 1950, v184, 299.
8. Martin, C. J., Axelrod, A. E., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 461.
9. Moltke, E., *Acta Endocrin.*, 1957, v25, 179.
10. ———, *ibid.*, 1957, v24, 226.
11. Brown-Grant, K., v. Euler, C., Harris, G. W., Reichlin, S., *J. Physiol.*, 1954, v126, 1.
12. Lorenzen, I., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 440.

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Cytotoxicity of Adrenal Cortex Hormones on Normal and Malignant Lymphocytes of Man and Rat.* (26928)

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I have shown by the method of unstained cell counts(1) that cortisol and related hormones have an *in vitro* cytotoxic effect on cells derived from the rabbit thymus. The newly developed slide-chamber method(2) provided a means for extending this study to human lymphocytes. This method was used in this study to measure the toxic effect of cortisol on normal human and rat lymphocytes and also on human leukemic lymphocytes.

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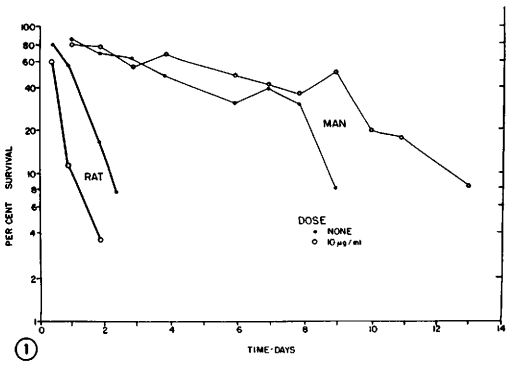
Methods. Three groups of individuals were used in these studies. First was a group of 7 normal men and women, employees of the Research and Laboratory services, to whom I am indebted. A second group consisted of 16 non-leukemic, ambulatory, male hospitalized patients with more or less normal hemograms. Specimens from these patients were obtained through courtesy of the Laboratory Service of Hines Hospital. A third group consisted of 12 men and 1 woman with chronic lymphocytic leukemia or lymphosarcoma in leukemic phase. Specimens from these patients were obtained from the Hematologic Sections of the Medical Service of Hines Hospital and of Hektoen Clinic of Cook County Hospital.

The leukocytes from human or rodent blood were concentrated, washed, and resuspended in equal parts of normal homologous serum and tissue culture medium 199 (Difco). Cell suspensions were also prepared from a normal human spleen and a lymph node. The cortisol succinate was the commercial preparation Solu-Cortef (Upjohn). The cell suspension plus hormone was put up in slide-chambers(2) composed of 2 large cover slips separated by a metal disk 0.9 mm in thickness. The slide-chambers were incubated at 37°C.

The numbers of viable lymphocytes were counted one or more times a day in an area 0.04 × 10 mm by means of an inverted phase microscope with special accessories. The criteria for viable lymphocytes have been described(2). The counts enabled the construction of survival curves for treated and untreated lymphocytes. Survival curves were summarized by the index, the 10% survival time; *i.e.*, time in days during which 90% of the lymphocytes died or were killed and 10% survived.

The effect of a reagent was calculated by the arbitrary formula: the logarithm of 10% survival time of untreated lymphocytes minus that of 10% survival time of treated lymphocytes. An index of 0 means no effect of the reagent at a specific dosage. The same index was used as a measure of sensitivity of the lymphocytes to the reagent at a given dose.

Results. Rat blood lymphocytes. Suspensions of rat blood lymphocytes were incubated with and without cortisol succinate (10 μg/ml). Fig. 1 presents survival curves of rat blood lymphocytes in a typical experiment. It is seen that the viable lymphocytes in the control suspension decreased slowly and that 10% of rat lymphocytes survived 2.14 days. In contrast, the 10% survival time was only 0.90 days for lymphocytes treated with 10 μg/ml.



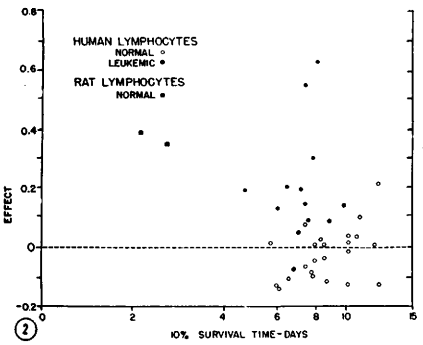


FIG. 1. Survival curves of rat and human lymphocytes incubated with and without cortisol succinate, 10 μg/ml. Abscissa represents days of incubation; ordinates, logarithms of percentage of lymphocytes surviving incubation.

FIG. 2. Effect of 10 μg/ml of cortisol succinate on lymphocytes from the blood of 23 non-leukemic individuals (○), of 13 leukemic patients (●), and of 2 rats (■). Abscissa represents 10% survival time of untreated suspension; ordinates, the effect as measured by the formula given under *Methods.*

The effect of varying doses of cortisol succinate on rat lymphocytes is shown in Table I. It is seen that 0.1 μg/ml produced a moderately toxic effect. Increasing the dosage to 100 μg/ml produced little, if any, increase in toxicity. A dosage of 1000 μg/ml was, however, highly toxic.

Human blood lymphocytes. The effect of

TABLE I. Effect of Adrenal Cortical Hormones on Normal Lymphocytes from Human and Rat Blood.

Blood lymphocytes from	Reagent	Effect* Dose (μg/ml) 1000	100	10	1	.1	.01
Rat	Cortisol succinate	.75	.45	.38	.54	.39	.16
	Cortisol		.35	.23	.22	.24	.13
	Corticosterone		.58	.30	.26	.16	.05
Man	Cortisol succinate	.55	.03	−.06	−.09		
	Cortisol		.20	.14	.02		
	Corticosterone		.14	.04	.01		

* Effect = log (10% survival time of untreated lymphocytes) minus log (10% survival time of treated lymphocytes).

cortisol succinate on blood lymphocytes of a patient is shown in Fig. 1. The patient had a normal hemogram. The control untreated lymphocytes in 50% human serum had a 10% survival time of 8.7 days, much longer than that observed for rat blood lymphocytes in 50% rat serum. The long 10% survival time of human lymphocytes as compared to that of rat lymphocytes has been observed previously(3). The lymphocytes treated with 10 μg/ml of hormone had a 10% survival time of 12.3 days, 34% greater than the control. Cortisol succinate caused, if anything, longer survival of the cells. According to these experiments, 10 μg/ml had a toxic effect on rat lymphocytes but not on human lymphocytes. The sensitivity of rat and human lymphocytes is also compared in Table I. A dose as small as 0.01 μg/ml was toxic to rat blood lymphocytes but doses of 10 and 100 μg/ml were not appreciably toxic to human cells.

Fig. 2 and Table II summarize the effect of 10 μg/ml on blood lymphocytes of 16 non-leukemic patients and 7 normal individuals. The average effects were −0.04 and −0.02 respectively, which are not significant (Table II). In only one test, a positive effect of 0.20 was observed (Fig. 2). However, a repeat test on this individual failed to substantiate significant sensitivity of the lymphocytes to the hormone.

It may be concluded that a dosage of 10 μg/ml of cortisol succinate had no perceptible cytotoxic effect on human blood lymphocytes of non-leukemic individuals. In contrast, rat blood lymphocytes were sensitive to dosages as low as 0.01 μg/ml.

Human leukemic lymphocytes. Thirteen patients with chronic lymphocytic leukemia or with leukemic lymphosarcoma were tested one or more times for the sensitivity of their blood lymphocytes to cortisol succinate (Table II and Fig. 2). The table shows that 10 μg/ml of cortisol succinate was slightly but significantly toxic to the lymphocytes of 13 leukemic patients but was not toxic to lymphocytes of 23 non-leukemic individuals. Fig. 2 shows that the results varied from no sensitivity for leukemic lymphocytes of 4 patients to moderate sensitivity (0.53 and 0.60) for the cells of 2 patients. Six repeat tests were done on 5 leukemic patients. The effect found on a second and third test deviated from the original test by 0.010 to 0.101. Average deviation was 0.044. In other words, the leukemic lymphocytes remained as sensitive to cortisol succinate on a repeat test as on the first test.

Other tissues and other corticosteroids. A normal human spleen and a lymph node were available for testing. The spleen was removed incidentally during a partial gastrectomy for a gastric ulcer. A suspension prepared from the spleen consisted largely of normal lymphocytes. Lymphocytes of the spleen and lymph node were as resistant to cortisol succinate as blood lymphocytes (Table II).

The toxic effects of other corticosteroids on rat and human blood lymphocytes were studied. The free steroids, cortisol and corticosterone, were dissolved in methyl alcohol. 0.1 cc of the required dilution of these steroids was placed in the center of a large cover slip. The alcohol was allowed to evaporate

TABLE II. Effect of 10 μg/ml of Cortisol Succinate on Human and Rat Lymphocytes in 50% Homologous Serum.

Source of lymphocytes	No. of individuals or animals	Avg 10% survival time (days) Untreated lymphocytes	Lymphocytes + reagent	Effect* (avg)	Stand. error
Rat blood	6	2.43	1.43	.282	.045
Human blood					
Normal individuals	7	9.31	9.86	−.042	.049
Non-leukemic patients	16	8.78	9.36	−.024	.017
Leukemic patients†	13	7.73	5.14	.194	.053
Human					
Lymph node—normal	1	5.75	5.56	.015	
Spleen— "	1	8.78	8.90	−.006	

* See footnote, Table I.
† Diagnoses: Chronic lymphocytic leukemia and lymphosarcoma in leukemic phase.

leaving a fine deposit. The cell suspension was then added to the precipitated steroid on the cover slip and the slide-chamber was completed and sealed. Both cortisol and corticosterone were toxic to rat lymphocytes (Table I). On the other hand, survival of human lymphocytes was not appreciably affected by moderate doses of cortisol or by corticosterone.

Discussion. Dougherty and White(4) demonstrated that injections of adrenal cortical hormones and adrenocorticotropin into mice and rats resulted in lymphopenia and atrophy of lymphoid tissue. The findings have been amply confirmed by others, as indicated by Valentine *et al.*(5). The *in vivo* findings stimulated many *in vitro* studies. Corticosteroids were found to have an *in vitro* cytotoxic effect on rat and rabbit lymphocytes according to the unstained cell count method(1), tissue culture(6) and, in the present study, the slide-chamber method. The *in vitro* and *in vivo* findings agree that rat lymphocytes are sensitive to glucocorticoids.

Results of studies by different workers on *in vivo* effects of glucocorticoids on lymphocytes in man have been variable(5,7,8). According to a review by Valentine *et al.*(5), it has not been conclusively demonstrated that adrenal cortical hormones produce lymphopenia in man. The authors, therefore, raised the question whether hematologic experiments with corticosteroids on mice and rats can be extrapolated to man. Similarly Long (9) concluded that there are cortisone-sensitive and cortisone-resistant species. Long's conclusion was based on reports of *in vivo* effects of cortisone on body weight, gamma globulin synthesis, and antibody production. According to these *in vivo* criteria, the rat and rabbit are cortisone-sensitive and man is cortisone-resistant.

The present *in vitro* work showed that glucocorticoids in small doses were cytotoxic to blood lymphocytes of the rat but had no perceptible effect on blood lymphocytes of 23 men. Both *in vitro* and *in vivo* work indicates that rat lymphocytes are sensitive to glucocorticoids but human lymphocytes are resistant.

It seems of particular interest that cortisol succinate was, on the average, more cytotoxic to leukemic than to normal lymphocytes. In contrast, several other reagents, including x-rays and nitrogen mustard were, on the average, equally toxic to normal and leukemic lymphocytes(10). However, the sensitivity of the leukemic lymphocytes to the steroid was usually only slight. Only 2 of 13 patients had leukemic lymphocytes that were moderately sensitive.

Shaw *et al.*(11) administered prednisone to 18 patients with chronic lymphocytic leukemia. The therapy usually resulted in an increase in lymphocyte count of the blood, suggesting that the lymphocytes of the blood of the leukemic patients were not sensitive to prednisone therapy.

Shaw *et al.*(11) also found that 2 of 18 patients showed a decrease in blood lympho-

cytes as a result of prednisone therapy. In the present study, 2 of 13 patients had lymphocytes which were moderately sensitive to cortisol succinate. The parallel *in vivo* and *in vitro* findings suggest further studies to determine whether there is a correlation between *in vitro* sensitivity of leukemic lymphocytes to cortisol and *in vivo* response of the leukemic patient.

Summary. Cortisol succinate, cortisol, and corticosterone in small doses were toxic to lymphocytes from rat blood and rabbit thymus. The toxicity was indicated by a reduction in *in vitro* survival time of the lymphocytes, according to a slide-chamber method. Minimal toxic effects were produced by 0.01 μg/ml of cortisol succinate and cortisol and by 0.1 μg/ml of corticosterone. In contrast, lymphocytes from the blood of normal individuals and from non-leukemic patients were resistant to 10 μg/ml of cortisol succinate. These experiments suggest a species difference in the sensitivity of lymphocytes from man as compared to those from the rat and rabbit.

Similar tests were done on the lymphocytes from the blood of 13 patients with chronic lymphocytic leukemia or with lymphosarcoma in leukemic phase. In general, the leukemic lymphocytes were more sensitive to cortisol succinate (10 μg/ml) than normal lymphocytes. Two of the 13 patients had lymphocytes which were very sensitive to the corticosteroid.

1. Schrek, R., *Endocrinology,* 1949, v45, 317.
2. ———, *A. M. A. Arch. Path.,* 1958, v66, 569.
3. ———, *Am. J. Clin. Path.,* 1959, v31, 112.
4. Dougherty, T. F., White, A., *Am. J. Anat.,* 1945, v77, 81.
5. Valentine, W. N., Craddock, C. G., Jr., Lawrence, J. S., *Blood,* 1948, v3, 729.
6. Trowell, O. A., *J. Physiol.,* 1953, v119, 274.
7. Ingle, D. J., *J. Clin. Endocrinol.,* 1950, v10, 1312.
8. Copeman, W. S. C., editor, *Cortisone and ACTH in Clinical Practice,* London; Butterworth and Co., Ltd., 1953,
9. Long, D. A., *Int. Arch. Allergy,* 1957, v10, 5.
10. Schrek, R., Friedman, I. A., Leithold, S. L., *J. Nat. Cancer Inst.,* 1958, v20, 1037.
11. Shaw, R. K., Boggs, D. R., Silberman, H. R., Frei, E., III, *Blood,* 1961, v17, 182.

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Experimental Phenylketonuria in Rats.* (26929)

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Auerbach, Waisman and Wyckoff(1) found that rats fed a diet containing 5% DL-phenylalanine or 2.5% each of DL-phenylalanine and L-tyrosine showed a 2-fold increase in plasma phenylalanine levels and an increased excretion of urinary phenylpyruvic acid. Similar observations were reported recently by Huang *et al.*(2). However, Neill and Langford(3) failed to demonstrate any phenylketonuria in rats fed a synthetic diet containing as high as 3.75% each of L-phenylalanine and L-tyrosine. The slightly elevated plasma phenylalanine levels obtained by previous investigators(1,2) were much lower than those found in phenylketonuric patients or in experimental phenylketonuric monkeys(4,5). The increased excretion of phenylpyruvic acid by rats fed DL-phenylalanine(1,2) is in all probability due to presence of the D-isomer, since D-phenylalanine can be deaminated by D-amino acid oxidase in kidney to form phenylpyruvic acid(6). Therefore, it seemed appropriate to obtain additional information in rats fed excessive quantities of the L-isomer of phenylalanine before the observations made in rats, monkeys and man could be compared biochemically.

Methods and materials. Eighteen-day-old

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