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Effects of Subchronic Steroid Therapy on Vitamin E-Deficient Rats (38480)

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Previous studies have shown that vitamin E and glucocorticoids, including triamcinolone, can stabilize isolated hepatic lysosomes, after administration of these agents to rats for 3 days (1, 2). Vitamin E and glucocorticoids can also stabilize erythrocyte membranes (3, 4) *in vitro*. Whether these agents impart their membrane-stabilizing properties by a common mechanism is unknown. It has been suggested that stabilization of cell membranes may be due to the interaction of such agents with certain proteins in the membranes (5). Indeed, glucocorticoids, vitamin E, and selenium can interact with plasma proteins and thereby induce a stabilizing effect, as manifested by decreased thermally induced aggregation of plasma proteins (6). If membrane labilization is important to the development of a vitamin E-deficient state, and vitamin E and glucocorticoids stabilize cellular or organelle membranes by a common mechanism, then glucocorticoids may be capable of attenuating vitamin E-deficiency or some of its characteristics.

The purpose of this study was, therefore, threefold: (a) to determine whether triamcinolone could supplant vitamin E in a vitamin E-deficiency state, by measuring its effect *in vivo* on the decreased body-weight gains and autohemolysis associated with this deficiency in rats; (b) to investigate the effects of subchronic steroid therapy *in vivo* on the hepatic lysosomes of normal and vitamin E-deficient rats; and (c) to determine whether a myopathy occurs during the deficiency state by measuring serum creatine phosphokinase.

Materials and Methods. Lysosomal enzymes. Male Holtzman rats (165–265 g) were fed a vitamin E-deficient diet (Nutritional Biochemicals Corp.) or a commercial stock diet[2] (Purina Laboratory Chow, 65 IU vitamin E/kg) for 43 days. Drugs or drug vehicle (0.9% saline) were administered once daily (i.p.). Food consumption (of vitamin E-deficient animals) and animal weights were measured every other day. A water soluble preparation of vitamin E (*d*-α-tocopherol polyethylene glycol 1000 succinate, 287 IU/g; Eastman Kodak Co.) was used to supplement deficient animals. Triamcinolone acetonide was obtained from E. R. Squibb.

Methods used for the enrichment of lysosomes have been previously described (1, 2, 7).

Enzyme assays. Acid phosphatase (AP, EC 3.1.3.2) and β-glucuronidase (BG, EC 3.2.1.31) activities were determined using methods previously described (1, 2, 7).

Erythrocyte (RBC) hemolysis tests. Hemolysis of RBC's was measured using modifications of methods previously described (8). Phosphate buffer was prepared by dissolving 14.2 g anhydrous dibasic sodium phosphate in distilled water, adding 20.1 ml 1.0 *N* HCl and diluting to 1.0 liter. Saline solutions contained 8.90 g NaCl made up to 1.0 liter with distilled water. Saline-phosphate buffer (9) consisted of equal parts of these solutions. Blood was obtained from rats on day 43 by retro-orbital eye puncture using heparinized capillary tubes. Four drops were added to each test tube containing 3.0 ml of saline-phosphate buffer. The resulting suspension was centrifuged (500 g, 4°, 10 min.) and the supernate discarded. The remaining RBC's were suspended in 4.0 ml of saline-phosphate buffer and incubated at 37° for 24 hr. After incubation, the RBC suspension was again centrifuged (25,000 *g*, 4°, 10 min.) and absorbance of the supernatant fluid (diluted 1:3) was measured at 540 nm (against a buffer blank) using a Bausch and Lomb Spectronic 20 colorimeter.

Serum creatine phosphokinase (CPK)

[1] Taken in part from a dissertation presented in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology.

[2] Content of vitamin E in stock diet (Purina Laboratory Chow) supplied by manufacturer. Manufacturer neither analyzes for selenium nor knows its content in stock diet.

TABLE I. CHANGES IN BODY WEIGHTS.

Treatment	Daily dose (ip)	Body wt (g ± SEM)[a]			Body wt. (% increase ± SEM)[a]
		Day 0	Day 43	Increase	
Vitamin E-deficient diet					
+ Vehicle	2.0 ml/kg	225 ± 10	284 ± 8	59 ± 7	26 ± 2[b]
+ Triamcinolone acetonide	1.6 mg/kg[c]	—	—	—	—
	0.4 mg/kg	228 ± 12	176 ± 6	−52 ± 7	−23 ± 4[d]
	0.1 mg/kg	199 ± 8	191 ± 9	−9 ± 10	−5 ± 5[d]
+ Vitamin E	0.39 IU/kg	220 ± 12	266 ± 9	46 ± 7	21 ± 3[b]
+ Triamcinolone acetonide	0.4 mg/kg	173 ± 2	144 ± 4	−29 ± 3	−17 ± 2[d]
	0.1 mg/kg	199 ± 6	181 ± 5	−18 ± 7	− 9 ± 4[d]
Normal diet					
+ Triamcinolone acetonide	1.6 mg/kg[c]	—	—	—	—
	0.4 mg/kg	188 ± 7	205 ± 4	17 ± 8	9 ± 4[e]
	0.1 mg/kg	201 ± 4	276 ± 3	75 ± 2	36 ± 1[b]
+ Vehicle	2.0 ml/kg	203 ± 4	399 ± 9	196 ± 6	98 ± 2

[a] Standard error based on eight animals except for the group receiving the normal diet and triamcinolone acetonide 0.4 mg/kg; this group was represented by 12 animals.

[b] $P < 0.1$ compared to the normal controls.

[c] No animal survived past day 15.

[d] $P < .01$ compared to vitamin E-deficient group.

[e] $P < .05$ compared to vitamin E-deficient group.

assay. A portion of the blood obtained for the hemolysis test was centrifuged (25,000 *g*, 4°, 10 min.) and 10 μl of serum was used in the procedure for the colorimetric determination; outlined in Bulletin No. 520 published by Sigma Chemical Co. Statistical analysis of all data was carried out using the Student's *t* test.

Results. Although vitamin E-deficiency was not manifested by a loss in body weight (Table I), the percent increase in body weight in the vitamin E-deficient group was significantly smaller than for the normal controls (normal diet plus drug vehicle) on day 43. Vitamin E-deficient animals treated with either triamcinolone acetonide (0.1 or 0.4 mg/kg) alone, or concurrently with vitamin E (0.39 IU/kg), manifested a *decrease* in body weight. Animals maintained on a normal diet and treated with triamcinolone acetonide (0.1 mg/kg) manifested an increase in body weight, but this was significantly smaller than that observed with the normal controls. Animals maintained on a normal diet and treated with the steroid at a higher dose (0.4 mg/kg) showed a slight increase in body weight, but this was significantly smaller than both the vitamin E-deficient group and the normal controls. There were no differences in the body-weight gains in any of the groups maintained on the vitamin E-deficient diet and supplemented with vitamin E, compared to the vitamin E-deficient animals.

Autohemolysis of RBC's (Table II) was significantly elevated in the vitamin E-deficient animals (0.77 ± 0.06) compared to the normal controls (0.14 ± 0.01). Treatment of vitamin E-deficient animals with triamcinolone acetonide was ineffective in decreasing this elevated hemolysis. The higher dose level of vitamin E (0.39 IU/kg), however, prevented this phenomenon. Thus, by definition, these rats were vitamin E-deficient, that is, an abnormal condition observed in the absence of the required nutrient was corrected by its subsequent readministration. The amount of vitamin E administered, however, was nutritionally minimal, since supplemented rats gained less weight than those on a normal diet. Supplementation of vitamin E-deficient animals treated with triamcinolone acetonide with the higher dose level of vitamin E (0.39 IU/kg), which was previously shown to prevent autohemolysis, failed to affect the autohemolysis. Treatment of animals maintained on a normal diet with triamcinolone acetonide had no effect on

TABLE II. INHIBITION OF RBC AUTOHEMOLYSIS BY VITAMIN E.

Treatment	Daily Dose (ip)	Absorbance (±SEM)[a]
Vitamin E-deficient diet		
+ Vehicle	2.0 ml/kg	0.77 ± 0.06[b]
+ Triamcinolone acetonide	1.6 mg/kg[c]	—
	0.4 mg/kg	0.78 ± 0.06[b]
	0.1 mg/kg	0.85 ± 0.08[b]
+ Vitamin E	0.39 IU/kg	0.24 ± 0.06[d]
+ Triamcinolone acetonide	0.4 mg/kg	0.65 ± 0.03[b]
	0.1 mg/kg	0.65 ± 0.08[b]
Normal diet		
+ Triamcinolone acetonide	1.6 mg/kg[c]	—
	0.4 mg/kg	0.36 ± 0.13[e]
	0.1 mg/kg	0.25 ± 0.11[d]
+ Vehicle	2.0 ml/kg	0.14 ± 0.01

[a] Standard error based on eight animals except for the group receiving the normal diet and triamcinolone acetonide 0.4 mg/kg; this group was represented by 12 animals.
[b] $P < 0.01$ compared to normal controls.
[c] No animal survived past day 15.
[d] $P < 0.01$ compared to vitamin E-deficient controls.
[e] $P < 0.05$ compared to vitamin E-deficient controls.

autohemolysis compared to the normal controls.

There was no significant decrease in food consumption in any of the groups maintained on the vitamin E-deficient diet and supplemented with vitamin E (Table III), compared to the vitamin E-deficient controls. Animals maintained on the vitamin E-deficient diet and given triamcinolone (0.4 mg/kg) alone, or supplemented with vitamin E (0.39 IU/kg), consumed significantly less food (compared to vitamin E-deficient controls) in the latter stages of the study. Rats on a normal diet consume about 20–25 g of food per day (1).

Initial experiments were conducted to compare absorbance values (directly proportional to the amount of creatine formed) with Sigma Units of CPK/ml of serum. This relationship was found to be linear. The serum enzyme concentrations obtained for this assay were all well within the defined linear limits of the assay.

TABLE III. WEEKLY FOOD CONSUMPTION.

Treatment	Daily Dose (ip)	Food consumption (g ± SEM)[a] Week 1	2	3	4	5	6
Vitamin E-deficient diet							
+ Vehicle	2.0 ml/kg	172 ± 13	214 ± 16	249 ± 15	272 ± 13	286 ± 22	244 ± 21
+ Triamcinolone acetonide	1.6 mg/kg[b]	—	—	—	—	—	—
	0.4 mg/kg	158 ± 16	198 ± 16	256 ± 19	212 ± 18[c]	209 ± 9[d]	237 ± 8
	0.1 mg/kg	209 ± 9	235 ± 8	246 ± 4	239 ± 18	243 ± 9	255 ± 5
+ Vitamin E	0.39 IU/kg	152 ± 12	179 ± 11	259 ± 37	268 ± 26	268 ± 8	331 ± 1[d]
+ Triamcinolone acetonide	0.4 mg/kg	139 ± 4	205 ± 9	205 ± 6[e]	180 ± 6[d]	165 ± 9[d]	188 ± 17
	0.1 mg/kg	208 ± 7	221 ± 13	238 ± 11	226 ± 6[d]	221 ± 19[e]	241 ± 7

[a] Standard error based on eight animals per group, two animals per cage. [d] $P < 0.01$ compared to vitamin E-deficient controls.
[b] No animal survived past day 15. [e] $P < 0.05$ compared to vitamin E-deficient controls.
[c] $P < 0.02$ compared to vitamin E-deficient controls.

TABLE IV. SERUM CREATINE PHOSPHOKINASE LEVELS OBTAINED ON DAY 43.

Treatment	Daily dose (ip)	Units of activity (±SEM)[a]
Vitamin E-deficient diet		
+ Vehicle	2.0 ml/kg	33 ± 7 (5)
+ Triamcinolone acetonide	1.6 mg/kg[b]	—
	0.4 mg/kg	33 ± 4 (6)
	0.1 mg/kg	50 ± 7 (8)
+ Vitamin E	0.39 IU/kg	29 ± 6 (6)
+ Triamcinolone acetonide	0.4 mg/kg	69 ± 6[c] (6)
	0.1 mg/kg	60 ± 9[d] (8)
Normal diet		
+ Triamcinolone acetonide	1.6 mg/kg[b]	—
	0.4 mg/kg	48 ± 6 (12)
	0.1 mg/kg	44 ± 5 (8)
+ Vehicle	2.0 ml/kg	42 ± 5 (8)

[a] Standard error based on the number of animals shown in parentheses.

[b] No animal survived past day 15.

[c] $P < 0.01$ compared to vitamin E-deficient controls.

[d] $P < 0.05$ compared to vitamin E-deficient controls.

Data on CPK (Table IV) suggest that a myopathy occurred in the vitamin E-deficient rats treated with triamcinolone (0.1 and 0.4 mg/kg) and supplemented with vitamin E (0.39 IU/kg). These two groups of animals manifested a significantly higher serum level of this enzyme (60 ± 9 and 69 ± 6 Sigma Units/ml of serum, respectively) than any other group tested. There were, however, no differences in the serum enzyme levels of the other groups compared to the normal controls (42 ± 5 Sigma Units/ml of serum).

Experiments conducted previously in this laboratory (1, 2) to determine whether substrate concentrations used in the lysosomal enzyme assays were sufficient for the amount of enzyme released thermally or hypotonically indicated that absorbance values (used to express enzyme activity) were directly proportional to the volume (amount) of free lysosomal enzymes added. The volumes of enzyme solutions used for testing acid phosphatase and β-glucuronidase (0.05 ml and 0.15 ml, respectively) were located within the linear portions of these assays.

There were no significant differences in the thermally-induced release of acid phosphatase or β-glucuronidase from hepatic lysosomes of rats maintained on the vitamin E-deficient diet, or those on the vitamin E-deficient diet supplemented with vitamin E compared to the normal controls, Table V. Increased release of acid phosphatase (51 ± 1) was observed in the group receiving the normal diet and treated with triamcinolone (0.1 mg/kg) compared to both the normal controls (38 ± 4), and vitamin E-deficient controls (36 ± 1). Increased release of β-glucuronidase (55 ± 8) occurred in rats on the vitamin E-deficient diet and treatment with triamcinolone (0.4 mg/kg) compared to the normal controls (40 ± 2).

Discussion. Results of the present study demonstrate that glucocorticoids cannot be substituted for vitamin E in vitamin E-deficient rats as assessed by changes in body weight or autohemolysis of RBC. Body-weight gain was minimal in animals receiving minimal amounts of vitamin E (vitamin E-deficient diet) and maximal in those animals maximally supplemented with vitamin E (normal diet). However, for the groups treated with triamcinolone (whether on a vitamin E-deficient diet, a vitamin E-deficient diet supplemented with vitamin E, or maintained on a normal diet), body-weight gain was inversely proportional to the daily dosage. Administration of triamcinolone (0.4 mg/kg) to rats on a normal diet resulted in less body-weight gain than normal controls, and a greater *loss* in body-weight in vitamin E-deficient animals than in the animals receiving triamcinolone and a minimal amount of vitamin E (0.39 IU/kg).

Vitamin E-deficiency was also manifested by an elevated RBC autohemolysis, Table II. This assay has been used as a rather sensitive index of vitamin E-deficiency (8, 9). Plasma and RBC levels of α-tocopherol diminish very early in the development of vitamin E-deficiency (9–11). Daily administration of vitamin E (0.39 IU/kg) prevented this hemolysis. A dosage of vitamin E (0.39 IU/kg) sufficient to reverse autohemolysis observed in vitamin E-defi-

TABLE V. THERMALLY-INDUCED (45°) RELEASE OF LYSOSOMAL ENZYMES FROM HEPATIC LYSOSOMES BY CHRONIC ADMINISTRATION OF TRIAMCINOLONE ACETONIDE IN NORMAL ANIMALS AND IN VARIOUS STATES OF VITAMIN E-DEFICIENCY.

Treatment	Daily dose (ip)	Enzyme release (% ± SEM)[a]	
		AP[b]	BG[b]
Vitamin E-deficient diet			
+ Vehicle	2.0 ml/kg	36 ± 1	35 ± 4
+ Triamcinolone acetonide	1.6 mg/kg[c]	—	—
	0.4 mg/kg	46 ± 4	55 ± 8[d]
	0.1 mg/kg	37 ± 3	42 ± 1
+ Vitamin E	0.39 IU/kg	30 ± 2	42 ± 4
+ Triamcinolone acetonide	0.4 mg/kg	42 ± 3	41 ± 3
	0.1 mg/kg	30 ± 3	36 ± 3
Normal Diet			
+ Triamcinolone acetonide	1.6 mg/kg[c]	—	—
	0.4 mg/kg	43 ± 2	33 ± 2
	0.1 mg/kg	51 ± 1[d, e]	43 ± 1
+ Vehicle	2.0 ml/kg	38 ± 4	40 ± 2

[a] Standard error based on four assays except for the group receiving the normal diet and triamcinolone acetonide 0.4 mg/kg; this group was represented by six assays.

[b] AP = acid phosphatase; BG = β-glucuronidase.

[c] No animal survived past day 15.

[d] $P < 0.02$ compared to normal controls.

[e] $P < 0.01$ compared to vitamin E-deficient controls.

cient rats, was *ineffective* when given concurrently with triamcinolone. The latter plus the absence of increased autohemolysis in animals receiving triamcinolone and maintained on a normal diet, suggest vitamin E-deficiency may increase the toxic effects of subchronic triamcinolone therapy. The lowest daily dosage of vitamin E (0.39 IU/kg) found to be effective in preventing the autohemolysis of RBC in these studies was only 70% of a previously reported minimal requirement (0.55 IU/kg) necessary for this effect (12).

Vitamin E-deficiency had no effect on the fragility of hepatic lysosomes, Table V. Since labilization did not occur, we can only speculate that the duration of vitamin E-deficiency was not sufficient for such to occur. In acute studies, triamcinolone, after administration for three days, stabilized hepatic lysosomal membranes of rats with respect to release of acid phosphatase or β-glucuronidase, and this effect is dose related (1). The present studies indicate that the subchronic effects of steroids in normal animals are not the same as those reported in acute studies (1). This is based on the observation that subchronic administration (43 days) of triamcinolone to normal rats failed to stabilize hepatic lysosomes compared to normal controls.

That triamcinolone could not substitute for vitamin E as a membrane stabilizer (in vitamin E-deficiency), was shown by its ineffectiveness in preventing increased RBC autohemolysis, Table II, and by increased CPK levels in rats, Table IV. These data indicate that glucocorticoids and vitamin E produce their membrane-stabilizing effects (1–4) by different mechanisms. Since a readily reproducible labilization of RBC's was manifested by all animals on the vitamin E-deficient diet and labilization of lysosomes was not apparent, one may conclude that autohemolysis of RBC is a more sensitive index of vitamin E-deficiency. Whether a longer period of vitamin E-deficiency would have resulted in labilization of hepatic lysosomes is speculative. However, degeneration and atrophy of their testes or muscle apparently requires 20–25 wk of deficiency in rats (10). By the end of 6 wk, vitamin E levels in liver are only about 10–20% of initial values, using mature or weanling rats (10). The pos-

sibility of depleting the remainder of the vitamin E seems unlikely, since after the first 2 or 3 wk of depletion, asymptotic values for liver and plasma were being obtained (10).

In conclusion, it was shown that steroids and vitamin E probably act by different mechanisms in stabilizing membranes, and steroids cannot supplant vitamin E in deficiency states. Indeed, they are more toxic in the absence of vitamin E.

Summary. Experiments were carried out to determine the effectiveness of steroid therapy in vitamin E-deficiency, as measured by autohemolysis of isolated RBC's, body-weight gain, serum creatine phosphokinase activity, and stabilization or labilization of isolated hepatic lysosomes. Results of such experiments would indicate whether triamcinolone acetonide could supplant vitamin E in vitamin E-deficiency states via its ability to stabilize various membranes. Autohemolysis induced by vitamin E-deficiency could not be prevented by daily administration of triamcinolone. Daily dosages of 0.1 and 0.4 mg/kg (ip) triamcinolone given concomitantly with replacement vitamin E (at sufficient dosages to reverse the autohemolysis) resulted in an increased autohemolysis. No changes in lysosomal membrane fragility were noted when hepatic lysosomes were obtained from vitamin E-deficient rats. Treatment of vitamin E-deficient rats with triamcinolone resulted in a greater attenuation of body-weight gain. Creatine phosphokinase levels were not augmented in vitamin E-deficient rats. Vitamin E-deficient rats supplemented with vitamin E and treated with triamcinolone, manifested an increase in creatine phosphokinase. It was therefore concluded that although triamcinolone and vitamin E possess a common ability to stabilize membranes and proteins, their mechanisms must be different since triamcinolone could not substitute for vitamin E in a deficiency state. Indeed, triamcinolone was found to be more toxic in the absence of vitamin E.

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