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ADRENAL STEROID RELEASE BY VINBLASTINE SULFATE AND ITS CONTRIBUTION TO VINBLASTINE SULFATE EFFECTS ON RAT THYMUS

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Abstract—Measurement of plasma 11-hydroxycorticosteroid levels after vinblastine sulfate (VLB) injection (1 mg/kg) into nonadrenalectomized rats showed that corticosteroid levels were elevated by a factor of two over control level within 3 hr after injection, increasing to a factor of four within 12 hr. Since adrenal steroids were known to cause thymic involution associated with an inhibition of nucleic acid synthesis, VLB effects on thymus were compared in adrenalectomized and nonadrenalectomized rats. VLB (0·33–1·0 mg/kg) caused thymic weight loss in adrenalectomized rats. Incorporation of [^{14}C]thymidine and [^{3}H]uridine into cold-acid-insoluble material by thymocytes, obtained from adrenalectomized rats after they had been injected with similar doses of VLB, was also inhibited. The magnitude of each of these effects was similar to that seen in nonadrenalectomized rats. It was concluded that adrenal steroid release did not play a major role in the thymolytic effects of VLB. Adrenalectomized rats were shown to be more sensitive to the lethal effects of VLB than were nonadrenalectomized rats. Small doses of cortisol administered simultaneously with VLB could protect rats from the lethal effects of this agent.

VINBLASTINE sulfate (VLB) has been shown to have a wide spectrum of antitumor activity in laboratory animals[1,2] and to be an agent of therapeutic value in Hodgkin's disease, acute leukemia, and various lymphomas.[3–5] VLB has also been shown to cause the involution of normal lymphoid tissue.[6,7] The mechanism for VLB cytotoxicity is not known and may vary for different cell types.[1,7–9]

Only a limited number of studies on VLB action have been carried out using thymus tissue.[6,10,11] These studies suggested that VLB causes thymic involution and inhibits nucleic acid synthesis. All three reports dealt with studies on nonadrenalectomized rats, and it is possible that the observed effects might be due to or modified by adrenal steroid release, since cortisol has been shown to produce similar effects on lymphoid tissue.[12]

The present study deals with the effects of VLB on thymus tissue of adrenalectomized rats and compares these to effects seen in nonadrenalectomized animals. The possible influence of adrenal corticosteroids on the VLB response is also studied and discussed.

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MATERIALS AND METHODS

Animals. Male rats of the Sprague–Dawley strain (70–90 g) were obtained from Simonsen Laboratories, Gilroy, Calif., and maintained on standard laboratory Chow. Some rats were subjected to bilateral adrenalectomy under ether anaesthesia, and maintained in our animal quarters for 3–4 days before use. These animals received 0·9% NaCl in their drinking water after the operation; other animals, either unoperated or sham-operated, received similar care except that they received tap water.

Chemicals. [^{14}C]Thymidine (30 mc/m-mole) and [^{3}H]uridine (330 mc/m-mole) were purchased from New England Nuclear Corp., Boston, Mass.; vinblastine sulfate (Velban) was obtained from Eli Lilly & Company; cortisol (Cortef) was obtained from the Upjohn Company. Vinblastine was dissolved in 0·9% NaCl and administered intraperitoneally, while cortisol was suspended in No. 100 Vehicle, Upjohn Company, and injected intramuscularly. Minimum essential medium (Eagle) for suspension culture (Cat. No. F-14) and calf serum were obtained from Grand Island Biological Company.

Determination of plasma 11-hydroxycorticosteroids. Nonadrenalectomized male rats, weighing about 180 g before sacrifice, were kept in a light-controlled room with 12 hr of light and 12 hr of darkness per day for at least 5 days before use. Animals were disturbed as little as possible before and during the experiments. At intervals of 0·5, 1, 3 and 12 hr before sacrifice, separate groups of animals received injections of VLB (1 mg/kg), hydrocortisone (50 mg/kg) or an equal volume of saline as control. All the animals were decapitated at the same time and blood was collected in heparinized containers and immediately centrifuged. The supernatant plasma fraction was collected for 11-hydroxycorticosteroid assay by a fluorimetric method originally described by DeMoor *et al.*[13] and later modified by Mattingly.[14]

Preparation of thymocyte cell suspensions. Animals were killed at varying times after treatment and their thymus glands were excised and pooled in Petri dishes containing tissue culture medium. The glands were minced and transferred to a Dounce homogenizer with 20 vol. of tissue culture medium. This tissue mince was then dispersed by three very slow strokes with a loose-fitting pestle. The resulting cell suspensions were then filtered through three layers of cheesecloth and centrifuged at 200 *g* for 5 min. The supernatants were decanted and the cell pellets resuspended in 20 vol. of tissue culture medium. The cell suspensions were then placed in stoppered Erlenmeyer flasks under a 5% CO_2 and 95% air atmosphere and were incubated in a shaking water bath at 37° for 15 min. During this initial incubation period, some cell clumping occurred; clumps were removed by filtration through three layers of cheesecloth. The resulting cell suspensions were used for precursor incorporation studies. The above operations were carried out at room temperature except for the centrifugation step, which was at 4°.

Incorporation system for thymocyte cell suspensions. One-ml aliquots of thymocyte cell suspensions were incubated in 15-ml Corex tubes at 37° with 5% CO_2 and 95% air as the gas phase for 5 min. One ml of tissue culture medium containing both [^{14}C]thymidine and [^{3}H]uridine was then added to each incubation vessel and incorporation was allowed to proceed for 30 min at 37° with gentle shaking. Samples were then transferred to ice and 8 ml of ice-cold tissue culture medium (without calf serum) was added to each sample. Cell suspensions were then centrifuged at 600 *g* at 4° for 5 min. The supernatants were aspirated and the pellets washed three times by resuspension in

5 ml of ice-cold tissue culture medium, followed by centrifugation as above. Five ml of 6% trichloro acetic acid (TCA) was then added to each cell pellet. The resulting precipitates were dispersed to a fine suspension with the aid of a Vibromixer and a glass rod. These suspensions were then centrifuged at 8000 *g* for 10 min and each of the precipitates was washed twice with 5 ml of 6%TCA (resuspension and centrifugation at 8000 *g*). The final pellets were designated as cold-acid-insoluble material and were used for assay of radioactivity.

Measurement of radioactivity. Cold-acid-insoluble material was retained on a Millipore filter and washed several times with cold 6% TCA. The final precipitate, along with the filter, was transferred to a scintillation counting vial and dried under an infrared lamp. Ten ml of toluene scintillation counting fluid [4 g 2,5-diphenyloxazole 0·1 g 1,4-bis-2-(5-phenyloxozylyl) benzene per liter of toluene] was then added and [^{3}H] and [^{14}C] activity determined in a Packard Tri-Carb scintillation spectrometer. Radioactivity was expressed as counts/min/μg of thymocyte DNA.

Determination of DNA. Cold-acid-insoluble material was hydrolyzed in 1 ml of 6% perchloric acid at 90° for 20 min. The hydrolyzed mixtures were chilled in ice and then centrifuged at 10,000 *g* at 4° for 10 min. Aliquots of the supernatant were used for deoxyribose determination by the method of Burton.[15]

RESULTS

The possibility of adrenal steroid release by VLB treatment *in vivo* was tested by measuring plasma 11-hydroxycorticosteroid levels in nonadrenalectomized rats at various times after injection of 1 mg VLB/kg (Fig. 1). Plasma levels in control saline-injected animals were relatively constant (8–10 μg/100 ml), except for the one-half hour time, where it was seen to be somewhat elevated (15 μg/100 ml); this increase was possibly the result of stress during manipulation of the animal. Steroid levels after VLB treatment were observed to rise from 8 to 20 μg/100 ml within 3 hr, and continued

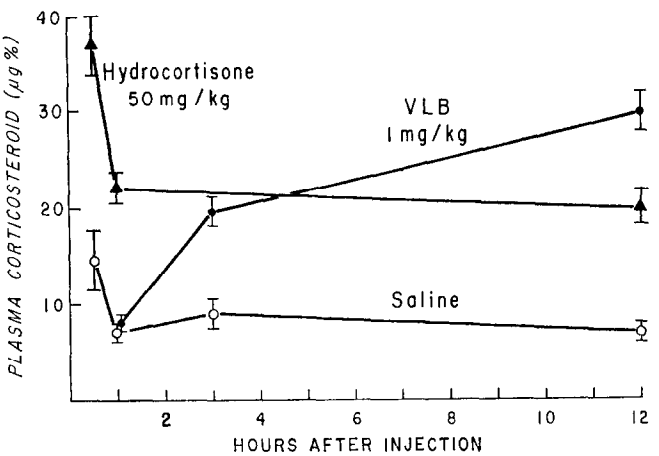

FIG. 1. Plasma corticosteroid level in μg per 100 ml at varying times after injection of saline, VLB (1 mg/kg) and cortisol (50 mg/kg). Blood was collected and plasma obtained as described in the Methods. Each point in saline- and VLB-treated groups represents the mean of separate assays for five rats ± standard error. Each point for cortisol-treated rats represents the mean of separate assays for three animals ± S.E.

to increase to 30 μg/100 ml by 12 hr. For comparative purposes, plasma corticosteroid levels were also determined after injection of cortisol (50 mg/kg). Plasma corticosteroid levels were markedly elevated (38 μg/100 ml) within one-half hour after cortisol injection, falling to about 20 μg/100 ml within 1 hr and remaining at this level for at least 12 hr after injection.

To avoid the complications of endogenous steroid release, VLB action on thymus tissue was studied in adrenalectomized animals. Preliminary VLB toxicity studies in adrenalectomized rats are shown in Table 1. Intraperitoneal injection of 1 mg VLB per kg body weight killed all adrenalectomized rats within 72 hr. No fatalities were

TABLE 1. REVERSAL OF LETHAL EFFECT OF VLB IN ADRENALECTOMIZED RATS BY THE ADMINISTRATION OF CORTISOL*

Treatment	Dose (mg/kg)	Fraction surviving at various times after injection			
		24 hr	48 hr	72 hr	96 hr
Vinblastine	1	3/6	1/6	0/6	—
Vinblastine + cortisol	1	6/6	6/6	5/6	5/6
Vinblastine	0·33	6/6	6/6	6/6	6/6

* Three groups of rats (6 animals/group) were injected intraperitoneally with VLB at doses of 1 and 0·33 mg/kg. One group receiving VLB at 1 mg/kg also received intramuscular injection of 1 mg cortisol/kg of body weight. Surviving animals were recorded at varying times after injection.

observed when normal (unoperated) and sham-operated animals were similarly treated. Simultaneous administration of cortisol (1 mg/kg) protected adrenalectomized rats from the lethal effects of VLB at the 1 mg/kg dose level. Smaller doses of VLB (0·33 mg/kg) permitted survival of adrenalectomized rats without a protective dose of cortisol.

Table 2 shows the effects of VLB on tissue weights of thymus, spleen, heart and liver for adrenalectomized, sham-operated and normal (unoperated) rats 24 hr after intraperitoneal injection of 1 mg VLB per kg body weight; similar experiments were also performed 12 hr after injection of VLB. Spleen weight was significantly decreased in unoperated, sham-operated and adrenalectomized animals by 12 hr after VLB injection, whereas thymus weight was significantly decreased only in adrenalectomized rats. By 24 hr, however, thymus weight was significantly decreased in both adrenalectomized and normal (unoperated) rats. Thymus weight in saline-treated, sham-operated rats was significantly less than thymus weight in normal (unoperated) or adrenalectomized rats (Table 2), probably as a result of adrenal steroid release in response to surgery. VLB did not cause any further decrease in thymus weight for sham-operated rats. The weights of heart and liver were also measured and found to be unaffected up to 24 hr after VLB treatment. The effect of VLB on water content of thymus, spleen, heart and liver was also studied 24 hr after drug injection. VLB did not significantly alter the water content of any of these tissues at that time.

Table 3 compares the effects of two dose levels of VLB on thymus weight for adrenalectomized and normal (unoperated) rats. The thymus of the adrenalectomized rats appears to be more sensitive to VLB at both the 0·33 and 1·0 mg/kg dose levels.

TABLE 2. THE 24-hr EFFECT OF VLB ON WET TISSUE WEIGHT*

Treatment	Thymus (mg/g body wt.)	P	Spleen (mg/g body wt.)	P
Normal (unoperated)				
Saline	3·47±0·26		4·10±0·15	
VLB (1 mg/kg)	2·85±0·12	< 0·05	2·42±0·17	< 0·001
Adrenalectomized				
Saline	4·30±0·27		4·13±0·42	
VLB (1 mg/kg)	3·03±0·25	< 0·01	3·45±0·16	< 0·05
Sham-operated				
Saline	2·61±0·15		3·89±0·22	
VLB (1 mg/kg)	2·53±0·31	> 0·10	2·80±0·42	< 0·05

* Normal, adrenalectomized and sham-operated rats were sacrificed 24 hr after treatment with saline or VLB. Their thymus glands and spleens were excised, blotted and weighed in well-sealed vials on a microbalance. Data represent the average of six separate determinations ± standard error of the mean (expressed as mg gland wet weight per g of pretreated rat body weight). P values were obtained from the Student's *t*-test (one-tail) between saline-treated and VLB-treated groups in normal, adrenalectomized and sham-operated animals.

Data are also provided showing that cortisol, in a dose (1 mg/kg) which protects against the lethal effects of 1 mg VLB/kg, does not significantly alter VLB effects on thymus weight.

Nucleic acid synthesis was studied in thymocyte cell suspensions obtained from adrenalectomized rats which had been injected with saline or VLB. Two doses of VLB (0·33 and 1·0 mg/kg) given alone or simultaneously with a protective dose of cortisol

TABLE 3. EFFECT OF VLB ON RAT THYMUS WEIGHT*

Treatment	Normalized thymus weight: Nonadrenalectomized (mg thymus/g body wt.)	% of Control	P	Adrenalectomized (mg thymus/g body wt.)	% of Control	P
Saline	4·07±0·16			3·97±0·15		
VLB (0·33 mg/kg)	4·51±0·49	110	> 0·10	3·22±0·25	81	< 0·05
Saline	3·47±0·26			4·30±0·27		
VLB (1·0 mg/kg)	2·85±0·12	82	< 0·05	3·03±0·25	70	< 0·005
Saline	—			3·97±0·15		
VLB (1·0 mg/kg + cortisol (1·0 mg/kg)	—	—	—	2·87±0·13	72	< 0·001

* Adrenalectomized and normal (unoperated) rats were sacrificed 24 hr after treatment and thymus tissue was prepared as described in the legend of Table 2. Results presented are the averages of six determinations ± standard error of the mean. Thymus weights from VLB-treated rats are also expressed as per cent of control (saline-treated). Level of significance was determined using the Student's *t*-test (one-tail).

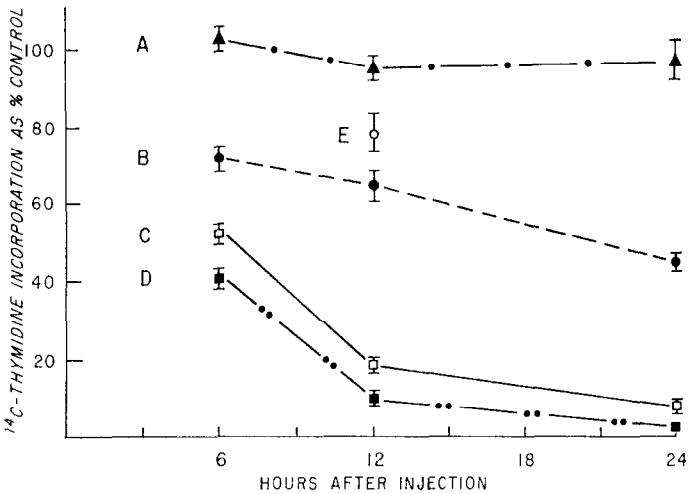

FIG. 2. Effect of VLB on incorporation of [^{14}C]thymidine into cold-acid-insoluble material of thymocytes. Adrenalectomized rats (from four to six per group) were treated as follows: (a) cortisol, 1 mg/kg; (b) VLB, 0·33 mg/kg; (c) VLB, 1 mg/kg plus cortisol, 1 mg/kg; (d) VLB, 1 mg/kg; (e) VLB, 0·33 mg/kg plus cortisol, 1 mg/kg. Adrenalectomized rats treated with an equivalent volume of saline were used as controls. Rats were sacrificed by decapitation at varying times after treatment, and incorporation of [^{14}C]thymidine and [^{3}H]uridine into cold-acid-insoluble material was assayed as described in the Methods. The amount of DNA per incubation tube was 695 μg, and incorporation was calculated in terms of counts/min/μg of DNA. Control incorporation for [^{14}C]thymidine and [^{3}H]uridine was 24·1 $\pm$ 1·3 and 11·4 $\pm$ 0·8 counts/min/μg DNA respectively. Data were normalized to per cent of control incorporation. Each point represents the mean of four to twelve determinations $\pm$ S.E.

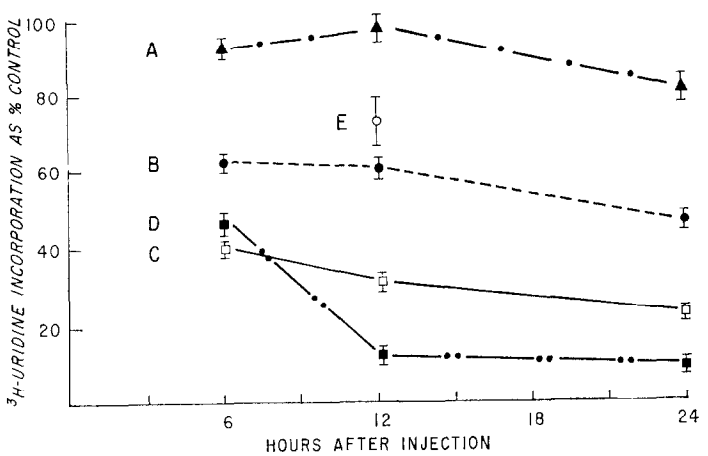

FIG. 3. Effect of VLB on incorporation of [^{3}H]uridine into cold-acid-insoluble material of thymocytes. Data were derived from the experiment described in the legend for Fig. 2.

(1 mg/kg) were investigated. Thymocyte suspensions obtained from these rats were exposed simultaneously to [^{14}C]thymidine and [^{3}H]uridine for 30 min, and incorporation of radioactivity into cold-acid-insoluble material was determined. The results for [^{14}C]thymidine and [^{3}H]uridine are shown in Figs. 2 and 3 respectively. VLB inhibited incorporation of both precursors, and in each case the magnitude of inhibi-

tion increased with dose and time after injection. Cortisol slightly reduced VLB inhibition of incorporation (5–15 per cent) for both precursors. The protective dose of cortisol alone (no VLB) was without significant effect on [^{14}C]thymidine incorporation and had only marginal effects on [^{3}H]uridine incorporation.

DISCUSSION

Preliminary observations in our laboratory showed that VLB (1 mg/kg) injected into rats caused decreased food consumption, weight loss, mild diarrhoea and piloerection within 12 hr. Measurement of blood corticosteroid levels in rats at varying times after VLB administration (Fig. 1) showed marked increases up to four times control values within 12 hr. For comparative purposes, blood corticosteroid levels were also determined after an intramuscular injection of 50 mg cortisol per kg of body weight, a dose known to cause thymic involution and to inhibit protein and nucleic acid synthesis in lympoid tissue.[12] Although the time course for the rise in the blood corticosteroid level differs after administration of VLB or cortisol, similar blood steroid levels were attained. These results confirmed our initial suspicions about steroid release by VLB, and raised a question as to the contribution of these steroids to the observed thymic involution.

To avoid complications arising from endogenously released adrenal steroids, VLB action on thymus tissue was studied in adrenalectomized animals. Preliminary studies on adrenalectomized rats (Table 1) revealed that an intraperitoneal dose of 1 mg VLB/kg would kill 50 per cent of these animals within 24 hr, whereas this dose was without lethal effect on normal (nonadrenalectomized) rats; single doses of 0·33 mg VLB/kg were without lethal effect in adrenalectomized rats. The lethal effect of VLB in adrenalectomized rats was markedly reduced by simultaneous injection of cortisol at a dose of 1 mg/kg. This dose of cortisol was without effect on thymic weight or nucleic acid synthesis. No exploration was made regarding the minimum dose or time–action curve for the protective effect. A similar protective effect by cortisol has been reported by Denckla and Dewey[16] for tryptamine toxicity seen in adrenalectomized rats. The underlying mechanism(s) for this protection, however, is not clear.

Data on tissue weight (Table 2) show that VLB is able to cause thymic involution in adrenalectomized and normal (unoperated) rats. The degree of involution appeared to be greater in adrenalectomized rats as compared to nonadrenalectomized rats (Table 3), suggesting that cortisol may in some way ameliorate VLB effects on thymus gland, as well as protect against the lethal effects. Cortisol (1 mg/kg), however, did not alter the degree of VLB-induced involution in adrenalectomized rats. The fact that heart and liver weights are not altered also indicates that the effects of VLB on tissue weight are specific for certain tissues (thymus and spleen). Since there is no significant change in water content in any of these tissues, the involution of thymus and spleen must represent a true loss of tissue mass.

The data in Figs. 2 and 3 show that thymocytes obtained from rats pretreated with VLB have a decreased ability to incorporate labeled-thymidine and uridine into cold-acid-insoluble material. The magnitudes of these effects were shown to be dependent on dose and time after drug treatment. It is noteworthy that significant inhibition of nucleic acid synthesis had occurred within 6 hr after VLB administration, whereas no detectable changes in thymus weight were observed at this time. Further, although

significant thymic involution was observed in adrenalectomized rats 12 hr after VLB administration, the percentage inhibition of nucleic acid synthesis was disproportionately greater than thymic weight reduction. These results suggest that the inhibitory effects of VLB on nucleic acid synthesis precede thymic weight loss.

Cortisol (1 mg/kg) administered concurrently with VLB did not potentiate VLB-induced inhibition of either DNA or RNA synthesis. Instead, a slight protective effect due to cortisol was observed. Further, the magnitude of VLB-induced inhibition of nucleic acid synthesis reported here for adrenalectomized rats is close to that reported by Richards *et al.*[10] for nonadrenalectomized rats. These results would suggest that the endogenously released adrenal steroids do not play a significant role in VLB-induced inhibition of thymus nucleic acid synthesis.

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