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Endogenous and Exogenous Glucocorticoids Have Different Roles in Modulating Endotoxin Lethality in D-Galactosamine-Sensitized Mice†

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Endotoxin sensitivity and dexamethasone protection have been assessed in mice that were adrenalectomized and also treated with D-galactosamine at the time of endotoxin challenge. Our data establish that adrenalectomy did not detectably alter the magnitude of the increased sensitivity induced by D-galactosamine alone. Furthermore, protection provided by acute exogenous glucocorticoid treatment was still demonstrable in these mice and was not influenced by chronic experimentally induced glucocorticoid deficiency. Our data confirm that the adrenalectomized mouse model of endotoxin lethality is characterized by increased sensitivity to endotoxin and establish that the magnitude of this sensitizing effect is more than 100-fold. We also show for the first time that adrenalectomy causes an appreciable kinetic shift in the endotoxic crisis and that dexamethasone, given at the time of endotoxin challenge, will significantly reverse the increased sensitivity to lethality. Our results indicate that the protective effects of corticosteroids may involve important chronic as well as acute responses. In particular, we conclude that endogenous glucocorticoid need not always increase host resistance to endotoxin, nor does such a circumstance eliminate the possibility for exogenous glucocorticoid-mediated protective effects.

D-Galactosamine, given at the time of endotoxin challenge, can markedly sensitize mice, and other species, to the lethal effects of endotoxin. Decreases in the endotoxin 50% lethal dose (LD_{50}) of at least several orders of magnitude are routinely observed; most deaths occur 5 to 9 h following challenge (12). In mice otherwise equivalent but not treated with D-galactosamine, the first deaths following an endotoxin LD_{50} challenge usually occur at about 24 h (20).

Important to D-galactosamine sensitization is the ability of D-galactosamine to serve as a reversible hepatotoxin which functions by depletion of hepatocyte uridine nucleotides, with subsequent decreases in hepatic RNA and protein syntheses (7, 12). Cytokine gene expression in the Kupffer cell in response to endotoxin and other exogenous stimuli is unaffected by D-galactosamine treatment (6). Studies of animals challenged with lipopolysaccharide (LPS) and recombinant tumor necrosis factor alpha (TNF-α) indicate that TNF-α is an essential mediator of endotoxin lethality in normal (5, 23) and D-galactosamine-treated (9, 10, 14) mice.

A number of agents have been shown to protect mice against endotoxin lethality when tested in the D-galactosamine model. These include, among others, dexamethasone (10), antibody to TNF-α (10), hydrazine sulfate (21), antibody to LPS receptor (16), and pretreatment with low doses of either LPS or TNF-α (tolerance) (8, 11). Each of these agents also protects normal mice against the lethal effects of endotoxin, albeit in some cases with a lower efficacy. Thus, D-galactosamine-treated mice provide one means by which to examine protection against endotoxin and TNF-α under conditions of acute sensitivity.

Adrenalectomy is well recognized as one experimental method to increase the sensitivity of mice to the lethal effects of endotoxin (3, 17, 26), although the precise magnitude of the sensitization relative to other mechanisms, particularly D-galactosamine treatment, has not been quantitatively assessed. In an earlier report from this laboratory, hypophysectomy was shown to increase endotoxin lethality, with an approximately 1,000-fold decrease in the LD_{50}. A combination of pituitary removal and D-galactosamine treatment, however, increased endotoxin lethality only an additional fourfold beyond that seen among intact sham-operated, D-galactosamine-treated mice (21). We report the results of analogous experiments using adrenalectomized mice, with the objective of defining the relationship between adrenalectomy (with consequent elimination of endogenous glucocorticoid) plus dexamethasone treatment (introduction of exogenous glucocorticoid) and host defense against the lethal effects of endotoxin.

MATERIALS AND METHODS

Animals. CF1 female mice were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). They were of the same age and housed under the same conditions as in previous studies (6, 20, 21).

Adrenalectomy and recovery. Before surgery, mice were anesthetized with 350 mg of Avertin (Aldrich Chemicals, Milwaukee, Ws.) per kg. Adrenalectomy was performed by using the dorsolateral approach. The hair was removed from the dorsolateral area of the kidneys, and the skin was washed with povidone-iodine (Betadine) and 70% ethanol. A 0.5 to 0.7-mm incision was made in the skin with scissors, and the peritoneum was cut just posterior to the last rib on both sides. The right adrenal was grasped with a curved

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† This paper is dedicated to Professor Robert H. Abeles on the occasion of his 65th birthday.

forceps and removed with an iridectomy scissors; the left adrenal was removed in a similar fashion. Total adrenalectomy was verified visually; i.e., if two intact adrenal glands were not obtained, the animal was discarded from the study. The skin wounds were closed with 7-mm wound clips, and the animal was placed in a heated cage until fully recovered from the anesthesia. Following the operation, the mice were given 0.5% saline for drinking water. Sham-operated mice were handled in the same way except that the adrenals were not removed and saline was not used for drinking water. The time between surgery and endotoxin challenge was 9 days. Mice were allowed sufficient time to recover following surgery to eliminate the possibility of early endotoxin tolerance. Preliminary studies with sham-adrenalectomized mice showed little difference in lethal sensitivity to LPS between 3 and 10 days postsurgery.

Lethality studies. Mice were taken off feed at 10 p.m., except when noted, and then challenged with endotoxin intraperitoneally at 1 p.m. the next afternoon, at which time they were placed back on feed. D-Galactosamine (Sigma Chemical Co., St. Louis, Mo.) was added to phosphate-buffered saline (PBS) immediately before challenge and was given, along with sonicated LPS, intraperitoneally in a volume of 0.4 ml. The PBS was made immediately before use from sterile saline and the solid sodium phosphates. The D-galactosamine dose was 20 mg per mouse on the basis of 25-g body weight. Dexamethasone (American Regent Labs, Shirley, N.Y.) was diluted in sterile saline and was injected intraperitoneally at the time of challenge, i.e., without pretreatment, in a volume of 0.1 ml.

Chemicals. LPS was from *Salmonella enteritidis* and *Escherichia coli* O55 and was extracted by the aqueous phenol method (25).

Statistics. χ^2 contingency tables (1) and the Fisher exact probability test (19) were used to assess significance of results.

RESULTS

Kinetics of endotoxin lethality in adrenalectomized mice. The first set of experiments was designed to confirm the sensitizing effects of adrenalectomy on LPS lethality in mice and to establish quantitatively the extent of the sensitization relative to normal or sham-operated controls. Nine days following either adrenalectomy or sham operation, mice were challenged with graded doses of endotoxin, and the kinetics of lethality were subsequently assessed over the next 72 h. The results of that study, shown in Fig. 1 and 2, demonstrate that endotoxin-challenged, adrenalectomized mice manifest a significantly more rapid kinetics of lethality at doses of comparable lethality (Fig. 1). The mean time to death for adrenalectomized mice was on the order of about 12 h, whereas more than twice that time period was required in normal mice. Further, much lower doses of endotoxin were sufficient to cause lethality among adrenalectomized mice (Fig. 2). The endotoxin LD_{50} for the adrenalectomized mice was determined to be approximately 0.8 μg, compared with about 200 μg for normal mice. Of interest, the absolute time span wherein nearly all deaths were scored was similar in both groups.

Endotoxin lethality in adrenalectomized and D-galactosamine-treated mice. The effect of combined adrenalectomy and D-galactosamine was next determined by using experimental protocols similar to those described above. The collective results of three separate experiments are summarized in Table 1 and indicate that adrenalectomized

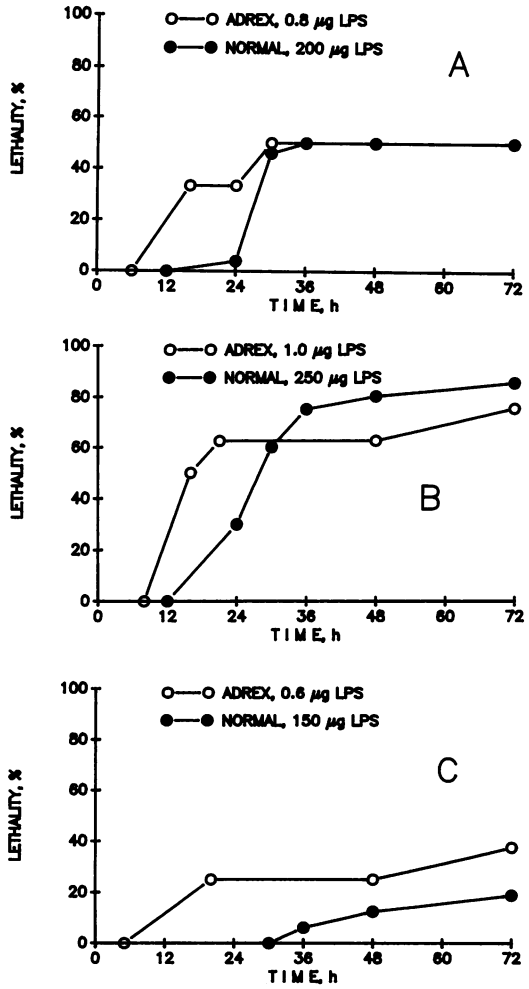

FIG. 1. Kinetics of endotoxin lethality for adrenalectomized (adrex) and normal mice. Endotoxin doses, relative to LD_{50}: (A) LD_{50}; (B) $>LD_{50}$; (C) $<LD_{50}$. Endotoxin doses, magnitude, are as shown. Numbers of mice: adrenalectomized, 8 mice per dose; normal, 150 and 250 μg of LPS, 16 mice per dose; 200 μg of LPS, 24 mice per dose.

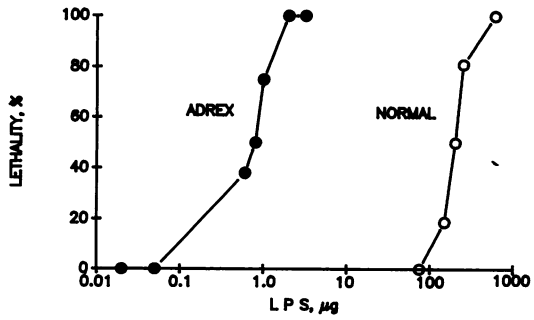

FIG. 2. LPS dose response, at 72 h, of endotoxin lethality for adrenalectomized (adrex) and normal mice. Number of animals per dose: adrex, 0.02, 0.05, and 3.2 μg of LPS, 5 mice each; 0.6, 0.8, 1.0, and 2.0 μg of LPS, 8 mice each; normal, 75 and 600 μg of LPS, 8 mice each; 150 and 250 μg of LPS, 16 mice each; 200 μg of LPS, 24 mice (20). Adrex: LPS, *E. coli*; normal: LPS, *S. enteritidis*.

TABLE 1. Sensitivity to endotoxin lethality in D-galactosamine-treated, adrenalectomized or sham-operated mice[a]

LPS(μg)	Lethality (no. of deaths/total)	
	Sham + galN	Adrex + galN
0.002	ND	0/4
0.004	1/4	1/14
0.008	2/4	1/4
0.010	2/5	2/5
0.016	8/8	4/4
0.040	8/8	14/14

[a] Data are cumulative from three experiments. Lethality, 72 h. LPS, *S. enteritidis*. Sham + galN, sham operated plus D-galactosamine; adrex + galN, adrenalectomy plus D-galactosamine; ND, not done.

and sham-operated mice manifest similar lethality profiles in response to D-galactosamine and endotoxin. These results demonstrate that, under conditions in which adrenalectomy increases lethal sensitivity to endotoxin more than 100-fold (see, e.g., Fig. 2), there is no overall increase in sensitivity in D-galactosamine-treated mice, which are already at a high level of sensitivity.

Dexamethasone protection of endotoxin-challenged mice. One of the more likely possibilities for the increased sensitivity of mice to endotoxin following adrenalectomy would be a consequence of endogenous glucocorticoid loss. We therefore assessed the capacity of an exogenously administered glucocorticoid, dexamethasone, to protect against endotoxin lethality in sham-adrenalectomized or adrenalectomized groups of mice under conditions in which the animals were or were not treated with D-galactosamine at the time of endotoxin challenge. The dose of dexamethasone was varied between 0.4 and 400 μg per mouse. As shown in Table 2, dexamethasone doses of either 40 or 400 μg provided significant protection to adrenalectomized mice when mice were challenged with 0.03 μg of LPS in the presence of D-galactosamine. A similar degree of protection was observed with the sham-operated mice also treated with LPS and D-galactosamine. The LPS dose, in each instance, was approximately threefold greater than the LD_{50}. Perhaps the most important finding from these experiments is that prior adrenalectomy appeared not to have any significant impact on the extent of dexamethasone reversal of endotoxin lethality. This finding would be consistent with the fact that adrenalectomy does not increase sensitivity to endotoxin lethality in these D-galactosamine-sensitized mice (Table 1).

In the absence of sensitization by D-galactosamine, however, adrenalectomized mice were significantly more responsive to the protective effects of dexamethasone than were sham-operated mice (Table 2). This rather significant difference is consistent with the previously noted increased sensitivity to endotoxin lethality among these adrenalectomized mice (Fig. 2). Notable protection of adrenalectomized mice is realized at doses of dexamethasone as low as 4 μg; in contrast, protection of sham-operated mice was apparent only at the 400-μg dose of dexamethasone and then only at LPS doses no more than two- or three-fold above the LD_{50} (Table 2). As with the data shown in Fig. 1, the majority of the deaths among adrenalectomized, endotoxin-challenged mice occurred within the first 24 h of challenge (15 of the 16 cumulative deaths) compared with only 4 of the 15 corresponding deaths of sham-operated mice occurring during the same period.

Dexamethasone counteracts adrenalectomy-derived endotoxin sensitivity. The results summarized above appear paradoxical in that adrenalectomy did not increase lethality in D-galactosamine-treated mice (Table 1), yet glucocorticoid administration nevertheless provided rather significant protection. These results suggest the unanticipated possibility that sensitivity to, and dexamethasone protection against, endotoxin lethality might not be intimately related phenomena. As shown in Table 3, relatively high doses (400 μg) of dexamethasone, similar to those required to protect sham-operated mice (Table 2), protected adrenalectomized mice against even relatively large doses (20 μg) of endotoxin. It also conferred resistance, albeit transitory, to 200 μg of LPS (an approximate dose 250 times the LD_{50}; Fig. 2). Thus, dexamethasone, in massive doses and given at the time of challenge, can still overcome, to a major extent, the increased endotoxin lethality associated with removal of the adrenals.

DISCUSSION

Exogenous glucocorticoids, such as dexamethasone, have been shown previously to protect mice against the lethal

TABLE 2. Effect of dexamethasone against endotoxin lethality in adrenalectomized, D-galactosamine-treated mice[a]

Mouse group[b]	LPS (μg)	Lethality (no. of deaths/total)[c]				
		Saline	Dexamethasone (μg)			
			0.4	4.0	40	400
Adrex + galN	0.030	5/5	ND	5/5	2/5**	1/5*
Sham + galN	0.030	5/5	ND	5/5	1/5*	1/5*
Adrex	0.4	3/5	1/5	1/5	ND	ND
	0.8	4/5	3/5	0/5*	ND	ND
	1.0	3/3	3/3	0/3*	0/3*	0/3*
	2.0	3/3	3/3	3/3	0/3*	0/3*
	4.0	3/3	2/2	1/2	0/2*	0/2*
Sham	150	0/5	ND	ND	0/5	0/5
	200	3/3	2/3	2/3	2/3	0/3*
	300	4/5	ND	ND	2/5	1/5**
	400	4/4	3/3	3/3	2/3	2/3
	600	4/4	3/3	3/3	3/3	3/3

[a] Lethality, 72 h. LPS, *S. enteritidis*. Dexamethasone was given at the time of challenge.

[b] Adrex + galN, adrenalectomy plus D-galactosamine; sham + galN, sham operated plus D-galactosamine.

[c] *$P < 0.05$, χ^2 contingency tables and Fisher exact probability test; **$P < 0.05$, χ^2 contingency tables, and $P < 0.10$, Fisher exact probability test. ND, not done.

TABLE 3. Ability of dexamethasone to reverse adrenalectomy-mediated endotoxin sensitivity[a]

Control or dexamethasone (μg)	LPS (μg)	Lethality (no. of deaths/total)[b] at time after challenge		
		24 h	48 h	72 h
Control	20	3/3		
4	20	2/4	2/4	2/4
40	20	0/4*	3/4	3/4
400	20	0/4*	0/4*	0/4*
Dexamethasone				
4	200	4/4		
40	200	2/4	3/4	3/4
400	200	0/5*	2/5	2/5

[a] Lethality, 72 h. LPS, *S. enteritidis*. Dexamethasone or the control (saline vehicle) was given at the time of challenge.
[b] *$P < 0.05$, χ^2 contingency tables and Fisher exact probability test.

effects of endotoxin, with a period of pretreatment suggested to be important to the observed protection (2). The available evidence would suggest further that dexamethasone protection occurs at the level of the macrophage (10) by down-regulation of the TNF-α response to endotoxin (4). This may well be an important mechanism by which the protective effects are manifested. However, other studies suggest additional potentially relevant targets for beneficial effects of glucocorticoids. These include, for example, increased expression of phosphoenolpyruvate carboxykinase in the liver (13, 15) and regulation of arachidonic acid metabolism (22, 24). Adrenalectomy and hypophysectomy, which serve to deplete endogenous sources of glucocorticoids, have effects opposite those of glucocorticoids, including increased sensitivity to the lethal effects of endotoxin (3, 17, 21, 26), lowering of hepatic phosphoenolpyruvate carboxykinase (15), and increased serum TNF-α levels in endotoxin-challenged animals (17, 26).

In the present study, we have established that, while D-galactosamine and adrenalectomy both significantly sensitize mice to the lethal effects of endotoxin, adrenalectomy did not quantitatively increase the degree of endotoxin lethality established in D-galactosamine-treated mice. Since these agents increase sensitivity to endotoxin by (presumably) totally different mechanisms, there would appear to be no a priori reason for the sensitization effects not to be cumulative. Nevertheless, acute replacement of adrenal glucocorticoids by dexamethasone administration still provided a degree of protection against endotoxin lethality in such mice. The experiments to examine dexamethasone protection in adrenalectomized versus sham-adrenalectomized mice, with or without D-galactosamine treatment, allow direct comparison of protective efficacy. In this respect, whereas the sham-adrenalectomized mice that were not given D-galactosamine were only marginally responsive to the protective effects of dexamethasone given at the time of challenge (as would be anticipated from earlier studies [2]), such was not the case with the adrenalectomized and/or the D-galactosamine-treated mice. Indeed, in both of the last instances, significant ($P < 0.01$) levels of protection were observed even with only modest doses of dexamethasone. Further, in preliminary experiments, 400 μg of dexamethasone given as late as 2 h after endotoxin challenge still provided protection to normal mice challenged with 150 to 300 μg of LPS (1 of 20 versus 9 of 20 deaths [19a]). Effective dexamethasone protection without pretreatment has also been demonstrated previously in D-galactosamine-sensitized mice (10).

The failure of hypophysectomy in our earlier published studies (21) or adrenalectomy (the present study) to substantially increase sensitivity to endotoxin in D-galactosamine-treated mice serves to emphasize the point that the importance of endogenous glucocorticoids to host defense against endotoxin (or even bacterial infection) may depend critically on the particular circumstances of the animal model. In addition, it would seem that the lack of an endogenous glucocorticoid contribution to endotoxin resistance need not preclude potential protective efficacy by exogenous glucocorticoid under the same experimental conditions. Conversely, the ability of exogenous glucocorticoids to protect adrenalectomized mice need not preclude the possibility that the increased sensitivity to endotoxin otherwise seen in these mice may, in part, arise from factors not specifically related to glucocorticoid depletion. Of relevance to the last point, it has been reported that adrenaline has the capacity to down-regulate LPS-stimulated TNF-α production (18), which would have an impact on endotoxin lethality totally unrelated to glucocorticoid production per se. In any case, however, the inability of adrenalectomy to sensitize animals to endotoxin lethality under a given set of experimental conditions may provide a useful model in which to assess the role of cytokines and other factors in mediating and protecting against endotoxin shock, specifically as it relates to adrenal function.

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