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Endotoxin Induced Metabolic Alterations in BCG Infected (Hyperreactive) Mice (40286)

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Endotoxin given to laboratory animals causes a loss of glucose homeostasis which is followed by hypoglycemia and death (1, 2). The data accumulated to date suggest that the major factor responsible for hypoglycemia is a failure of gluconeogenesis rather than excessive glucose consumption, although the latter has been reported (3). Endotoxin induced hypoglycemia has been most extensively studied in mice by Berry and coworkers (4–7). These workers found that a key liver enzyme, phosphoenolpyruvate carboxykinase (PEPCK) which regulates gluconeogenesis is no longer susceptible to glucocorticoid induction after endotoxin poisoning (4, 6, 7). They have stressed the importance of this enzyme in endotoxin hypoglycemia presumably because this enzyme has a relatively short half life, i.e. 2 hr in rats while other gluconeogenic enzymes have longer half lives (6, 7). The published data indicate that three gluconeogenic enzymes, glucose-6-phosphatase, fructose-1,6-diphosphatase and PEPCK remain at normal or elevated activities in livers from endotoxin poisoned mice until about 12 hr. Thereafter, the activities fall (8, 9). The drop in enzyme activity corresponds with the development of hypoglycemia. A logical scenario for endotoxin induced hypoglycemia based on these observations is as follows: Mice given endotoxin fail to eat, and therefore, do not assimilate exogenous carbohydrates. Gluconeogenic enzymes are not induced by endogenous steroids elaborated in response to stress. As their level falls during normal turnover, particularly that of PEPCK, gluconeogenesis fails and hypoglycemia results.

Mice infected with *Mycobacterium bovis* BCG become hyperreactive to endotoxin and are killed approximately 1/1000th of the usual LD_{50} (10). These mice have a remarkably abbreviated clinical course with augmented clinical manifestations. One tenth of a microgram of endotoxin in BCG infected mice often causes profound hypoglycemia in 2 hr, and death with convulsions frequently occurs within 4 hr (11). This is unlike the response of normal mice which die after 17–24 hr and in which the hypoglycemia occurs later and is less severe. The response of the BCG mouse is, therefore, a caricature of that of the normal mouse.

The exaggerated responses and shortened time course of BCG mice provide a suitable model to study metabolic abnormalities caused by endotoxin. We studied the BCG mouse model previously and found that endotoxin induced hypoglycemia is largely due to defective gluconeogenesis (2). Where in the gluconeogenic pathway the defect lies is unknown. In addition, the rapidity with which profound hypoglycemia occurs in BCG mice given endotoxin (2 hr vs 17 hr for normal mice) suggests that failure of enzyme induction and normal enzyme turnover may not account for this abnormality.

In this paper we report experiments designed to determine if the hypoglycemia in endotoxin poisoned BCG mice is due to a selective defect in the gluconeogenic pathway or if there is a general perturbation of the pathway. The studies were performed between one and 2 hr after endotoxin (prior to profound hypoglycemia) to avoid the potential secondary effects of hypoglycemia and shock. Additional studies were performed to determine the effect of glucocorticoid and stimulation of gluconeogenesis by fasting on endotoxin hypoglycemia and mortality.

Materials and methods. Animals. Pathogen free, CD-1 female mice weighing 20–25 g were obtained from Charles River Breeding Laboratories, North Wilmington, Massachusetts. They were fed and watered *ad libitum* and housed in air conditioned quarters fully accredited by the American Association of Laboratory Animal Care. Unless otherwise indicated, all animals were fasted 18–24 hr prior to experimentation. The mice were ren-

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dered hyperreactive to endotoxin by a systemic infection with *Mycobacterium bovis* BCG given intravenously 13–16 days prior to use according to the method of Suter and Kirsanow (10). 0.2 ml of a 10–14 day culture of BCG in Dubos Liquid Broth (BBL) was injected via tail vein into unanesthetized, restrained mice.

Endotoxin. The endotoxin was prepared from a smooth strain of *Salmonella typhimurium.* The bacteria were grown in glucose minimal salts medium (M-9) supplemented with 0.1% Casamino Acids (Difco). At the stationary phase of growth they were killed with 0.2% formalin, harvested, and extracted by the phenol water procedure of Westphal *et al.* (12). Endotoxin challenge was by the intravenous route in 0.2 ml saline.

Metabolic studies. Glucose determinations were performed using the "Glucostat" (Worthington Biochemicals) micromethod. A 20 μl sample of blood obtained from the retroorbital plexus was added to 1.0 ml distilled water and deproteinized with 0.5 ml 1.8% $Ba(OH)_2 \cdot 8H_2O$ and 0.5 ml 2.0% $ZnSO_4 \cdot 7H_2O$ solutions. One ml of the resulting supernatant fluid was added to one ml "Glucostat" reagent at room temperature. A standard curve was prepared for each series of reactions.

Glucose production *in vivo* was estimated by the net increase in blood glucose twenty minutes following an intraperitoneal injection of 100 μM glycerol or fructose. Endotoxin was given intravenously one hour before glycerol or fructose. In one experiment, the incorporation of ^{14}C into glucose from ^{14}C glycerol (3 μCi in 100 μM) was determined by measuring the cpm/mg glucose in blood obtained via cardiac puncture. The glucose was separated from 1.0 ml whole blood by passage through mixed bed resin columns as described by Corridor *et al.* (13). The effluent was qualitatively checked chromatographically to insure that the radiolabel resided with the glucose.

Substrate oxidation *in vivo* in mice was measured by methods previously described (2). The mice were adapted to a gas train in such a way that all expired air was bubbled through 5 ml NCS^{tm} (Nuclear Chicago Corp.) to collect CO_2. Aliquots (0.5 ml) were removed at 15-min intervals and counted in a Packard liquid scintillation spectrometer to determine the activity of $^{14}CO_2$. Tracer amounts of 1-[^{14}C]glycerol, 6-[^{14}C]glucose, 1-^{14}C glucose, or 1-[^{14}C]palmitate (New England Nuclear) were injected intravenously in control BCG infected mice and in BCG infected mice one hour after 1.0 μg endotoxin. The isotopes (specific activities 4.6–11.3 Ci per mM) were injected in 0.2 cc saline via tail vein.

The free fatty acid concentration in the sera of inividual mice was measured colorimetrically at 440 nm and compared with similarly treated standards of palmitic acid dissolved in chloroform. The free fatty acids were extracted from the sera by mixing 0.1 ml in 2.0 ml 0.2 M phosphate buffer (pH 6.5) and 6.0 ml chloroform. The mixture was shaken 2 min and after settling 15 min the upper layer was removed by aspiration. The chloroform layer was filtered into clean chloroform rinsed glass stoppered tubes to which 3.0 ml Cu-triethanolamine reagent was added and mixed. The color was developed by the addition of two drops of sodium diethyldithiocarbamate reagent before reading the OD at 440 nm (14).

Results. Previous experiments have shown that glucose production from pyruvate is decreased in BCG infected mice as early as one to 2 hr after endotoxin challenge. The gluconeogenic pathway from pyruvate to glucose involves all of the key gluconeogenic enzymes including phosphenolpyruvate carboxykinase. The pathways from fructose and glycerol do not. Therefore, if the endotoxin induced defect in gluconeogenesis is the result of the loss of a specific enzyme at the beginning of the pathway one would expect that glucose production from glycerol and fructose might be unaffected. The results presented in Tables I and II, however, indicate that the metabolic lesion is not limited to the loss of phosphoenolpyruvate carboxykinase, since glucose production *in vivo* was not sustained from glycerol or fructose. The injection of exogenous fructose and glycerol elevated blood glucose in the control mice, but did not prevent a decrease in blood glucose when given to BCG infected mice one hour after endotoxin. In a similar experiment, radiolabeled ^{14}C glycerol was used to insure that an actual decrease occurred in the incorporation

TABLE I. THE EFFECT OF EXOGENOUS FRUCTOSE ON BLOOD GLUCOSE CONCENTRATION IN BCG INFECTED MICE BEFORE AND AFTER ENDOTOXIN.

Treatment	Mean blood glucose, mg percent ± SE	
	Before fructose[a]	20 min after fructose
BCG infected controls (10)	91 ± 3	109 ± 2
BCG infected mice 1 hr after 1.0 μg endotoxin (10)	81 ± 9	65 ± 3

[a] 100 μ*M* fructose injected ip. ( ) Indicates the number of mice per group.

TABLE II. GLUCOSE PRODUCTION FROM GLYCEROL IN BCG INFECTED MICE BEFORE AND AFTER ENDOTOXIN.

Treatment	Mean blood glucose, mg percent ± SE		cpm/mg glucose $\times 10^{-4}$
	Before glycerol	20 min after glycerol	
BCG infected control mice	Exp 1		
	115 ± 6 (10)[a]	142 ± 5 (10)	
	Exp 2		
	111 ± 9 (10)	158 ± 9	9.6 ± 1 (10)
BCG infected mice 1 hr after 1 μg endotoxin	Exp 1		
	76 ± 10	56 ± 5	
	Exp 2		
	91 ± 12 (10)	66 ± 18 (10)	3.6 ± 1 (10)

[a] ( ) Indicates the number of mice per group.

of ^{14}C label into blood glucose. The results shown in Table II indicate that the incorporation of ^{14}C into blood glucose in the experimental group was only about one-third the incorporation which occurred in the control mice not given endotoxin.

Because an increased oxidation of glycerol might account for its decreased incorporation into glucose, the *in vivo* oxidation of glycerol was measured between one and 2 hr after endotoxin by collecting expired $^{14}CO_2$ after ^{14}C glycerol injection. Figure 1 shows the cumulative counts per minute of $^{14}CO_2$ collected from a group of BCG infected mice and a group of BCG mice given endotoxin. The results show decreased glycerol oxidation after endotoxin.

Since an increased oxidation of glucose could result in an apparent decrease in incorporation of ^{14}C into blood glucose by its loss as expired $^{14}CO_2$, the *in vivo* oxidation of 1-[^{14}C]glucose and 6-[^{14}C]glucose was measured in mice after endotoxin. The results presented in Fig. 2 show that endotoxin caused decreased oxidation of both 1-[^{14}C]glucose or 6-[^{14}C]glucose. Oxidation of the 6-[^{14}C]glucose was depressed more than that of 1-[^{14}C]glucose.

Endotoxin LD_{50}'s were determined in fasted and fed BCG infected mice to determine if the fasting state, which enhances gluconeogenesis via endogenous steroids, influenced survival after endotoxin challenge. Fasted mice would have a stimulated gluconeogenic pathway and little stored carbohydrate while fed mice would have less active gluconeogenesis and much stored carbohydrate. The results presented in Table III show no difference in the responses of fasted and fed mice.

The effect of treatment of mice pre- and postchallenge with pharmacologic doses of

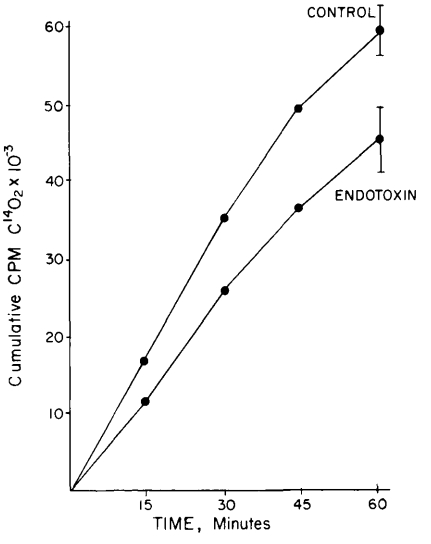

FIG. 1. Glycerol oxidation *in vivo* in BCG infected mice before and after endotoxin challenge. The endotoxin treated mice were given one μg endotoxin iv one hour prior to the injection of [^{14}C]glycerol. Each point represents the mean cumulative counts per minute of expired $^{14}CO_2$ from five individual mice. Vertical bars indicate ± SD.

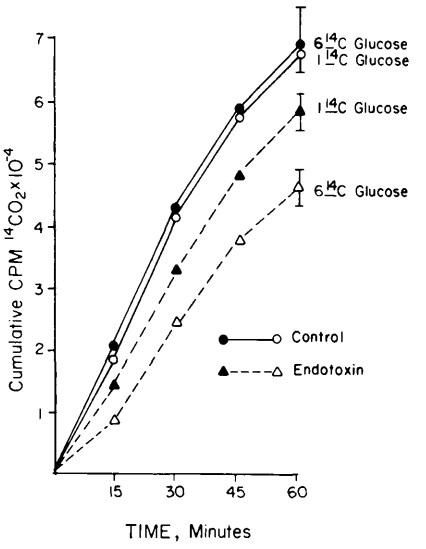

FIG. 2. Glucose oxidation *in vivo* in BCG infected mice before and after endotoxin challenge. The endotoxin treated mice were given one μg endotoxin iv 1 hr prior to the injection of [^{14}C]glucose. Each point represents the mean cumulative counts per minute of expired $^{14}CO_2$ from five individual mice. The vertical bars indicate ± SD.

TABLE III. ENDOTOXIN LD_{50}'S IN FED AND FASTED BCG INFECTED MICE.

Dose Endotoxin (μg)	Fed dead/total	Fed mean time to death (hours)	Fasted dead/total	Fasted mean time to death (hours)
3.2	5/5	5.5	4/5	5.6
0.8	5/5	4.8	4/5	4.9
0.2	4/5	5.3	4/5	7.0
0.05	1/5	—	1/5	—
LD_{50}[a]	0.126 μg		0.163 μg	

[a] LD_{50}'s were obtained by the method of Reed and Muench (15).

hydrocortisone was studied to evaluate protection against endotoxin in BCG mice. The results presented in Fig. 3 show blood glucose concentrations and mortality after 1.0 μg endotoxin when 3 mg hydrocortisone (Solu Cortef, Upjohn) was administered either before or after endotoxin. Cortisone reduced mortality significantly only in the group given cortisone prior to endotoxin. However, the rate at which blood glucose fell was diminished quickly i.e. within 2 hr, in all groups receiving steroids. In a similar experiment, the mice were challenged with less endotoxin (0.1 μg). The results were similar except that mortality was also reduced in the group given cortisone 30 min after endotoxin. These experiments show that cortisone given before or after endotoxin challenge rapidly lessens the rate at which blood glucose falls and, depending on the timing, prolongs survival or prevents death.

Fatty acid oxidation is important in providing energy and reducing equivalents to drive the gluconeogenic pathway. Therefore, palmitate oxidation was measured in BCG infected mice and in similar mice between 1 and 2 hr after endotoxin challenge. The results shown in Fig. 4 show that the *in vivo* oxidation of palmitate was reduced about 50% in the endotoxin poisoned mice. This apparent reduction in palmitate oxidation, however may be due to an *in vivo* pool size difference. Table IV, showing the serum free fatty acid levels in BCG infected mice before and after endotoxin, indicates that endotoxin caused a 77% increase in circulating free fatty acids. The effective specific activity of the injected isotope would therefore be decreased in mice given endotoxin, and this could account for decreased $^{14}CO_2$ evolution even though the rate of fatty acid oxidation is

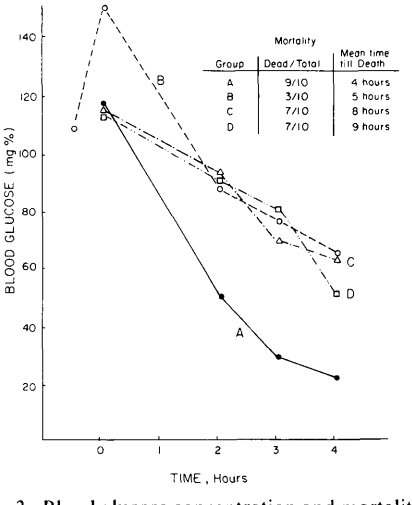

FIG. 3. Blood glucose concentration and mortality in BCG infected mice given 1.0 μg endotoxin (A) and 3.0 mg hydrocortisone 30 min before endotoxin (B), and 30 min and 1 hr after endotoxin (C and D). Each point represents the mean blood glucose of 10 mice.

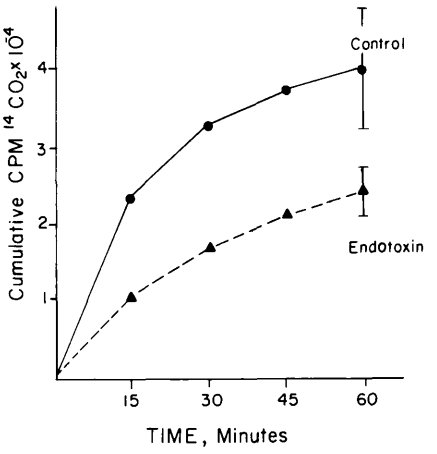

FIG. 4. Palmitate oxidation *in vivo* in BCG infected mice before and after endotoxin challenge. The endotoxin treated mice were given one μg endotoxin iv one hour prior to the injection of 1-[^{14}C]palmitate. Each point represents the mean cumulative counts per minute of expired $^{14}CO_2$ from five individual mice. The vertical bars indicate ± SD.

unchanged. On the other hand, a similar explanation for the reduction in glucose oxidation is untenable since the concentration of blood glucose is less in the endotoxin poisoned BCG infected mice than in control mice not given endotoxin.

Discussion. The data in this paper show that endotoxin causes a general derangement of the gluconeognic pathway in BCG-mice. The poisoned animals were unable to make glucose efficiently from glycerol or fructose. Previously we showed that glucose production from pyruvate was also impaired (2). Since these substrates enter the gluconeogenic pathway at different levels, a single lesion is unlikely to be responsible for the abnormality. In addition, the data also show that the apparent decreased incorporation of the substrates into glucose could not be caused by accelerated catabolism. The rates of oxidation of both glucose and glycerol were diminished.

Corticosteroids in pharmacological doses protected BCG-mice from the lethal effect of endotoxin when preadministered. Even when given as long as 30 min after endotoxin, steroids exerted a rapid sparing effect on blood glucose. The rapidity with which this sparing effect occurred i.e. within 2 hr, raises a question as to whether the effect was by glucocorticoid induced production of gluconeogenic enzymes. Increased enzyme production in response to glucocorticoids is a relatively slow process. The rise in enzyme is slow and usually preceeded by a lag of 2–3 hr (16, 17). It seems more likely in this setting that the steroid was preventing some of the toxic effects of endotoxin and thereby lessening hypoglycemia or, alternatively, was activating gluconeogenic enzymes. This argument is also supported by the observation that protection against endotoxin requires pharmacological doses of corticosteroids while only physiological doses are sufficient for enzyme induction.

The study of fasted and fed mice given endotoxin also raises questions about the failure of enzyme induction by corticosteroids as a cause of hypoglycemia in BCG mice. Fasted animals have an active gluconeogenic pathway with elevated levels of gluconeogenic enzymes (7), while fed animals have high stores of carbohydrate but low gluconeogenic activity. When challenged with endotoxin fed animals rapidly deplete their glycogen stores and then have to depend on their low gluconeogenic activity. One might think that the fasted animal with high gluconeogenic activity might have the advantage in survival. However, in spite of this increase in gluconeogenesis, the outcome is the same. Stimulation of gluconeogenesis by endogenous physiological amounts of glucocorticoid, therefore, offers no protection.

The data also suggest that abnormal substrate oxidation may also be partially responsible for endotoxin induced hypoglycemia. The oxidation of fatty acids are required for the production of energy and reducing equivalents to drive the gluconeogenic pathway. Palmitate oxidation was diminished in BCG-mice given endotoxin. However, because of the increase in free fatty acids in the blood of

TABLE IV. THE EFFECT OF ENDOTOXIN ON SERUM FREE FATTY ACID LEVEL IN BCG INFECTED MICE.

Treatment	Free Fatty Acids (μeq/ml serum) ±SE
BCG infected control mice	.97 ± .03 (14)[a]
BCG infected mice 2 hr after endotoxin	1.72 ± .03 (15)

[a] ( ) Indicates the number of mice.

BCG-mice after endotoxin a correction has to be made for fatty acid pool size. Fatty acids increased about 77%. Palmitate oxidation decreased by about the same amount. The conclusion is that there was no real change in fatty acid oxidation. However, during normal homeostasis a profound fall in blood glucose should result in an increase in fatty acid oxidation. The failure of fatty acid oxidation to increase suggests that loss of homeostatic regulation after endotoxin includes lipid as well as carbohydrate metabolism.

Summary. The cause of hypoglycemia induced by endotoxin in BCG infected mice was investigated. The major abnormality, known to be defective gluconeogenesis, was studied to determine whether a specific point in the gluconeogenic pathway is involved or whether the derangement is more general. The inability of endotoxin poisoned mice to synthesize glucose from glycerol and fructose in addition to pyruvate indicated that the entire pathway was in disarray. The *in vivo* oxidation of glucose, glycerol and palmitate to CO_2 was reduced, indicating that enhanced aerobic oxidation was not responsible for the hypoglycemia. This decrease in substrate oxidation also suggests that impaired gluconeogenesis may be due to decreased energy available to maintain the gluconeogenic pathway. Pharmacologic doses of glucocorticoids were protective in endotoxin poisoned BCG infected mice. The rate of development of hypoglycemia was rapidly lessened, and mortality reduced. The data suggest that steroids confer protection by preventing or interfering with some of the toxic effects of endotoxin or perhaps by activating glyconeogenic enzymes. It is unlikely that glucocorticoid mediated enzyme induction plays an anti-endotoxin role in this model.

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