EFFECTS OF BACTERIAL ENDOTOXINS ON METABOLISM

V. The Hyperreactivity of Mice Infected with Mycobacterium tuberculosis, Strain BCG*

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Suter, Ullman, and Hoffman (1) were the first to report that mice infected with an attenuated strain of *Mycobacterium tuberculosis* (BCG) are more susceptible than control animals to the lethal effects of bacterial endotoxins derived from Gram-negative organisms. This was confirmed by Howard *et al.* (2) who also showed that increased activity of the reticuloendothelial system (RES) exists at a time when the animals are most sensitive to endotoxin. This finding had not been anticipated since the RES is believed from other experiments to play an important role in an animal's response to endotoxin. "Blockade" of the RES by injections of colloidal agents sensitizes animals to endotoxin (3, 4) while induced tolerance to endotoxin is lost following similar treatment (5, 6). More recently, Suter and Kirsanow (7) elaborated the conditions necessary for the development of hyperreactivity toward endotoxin in BCG-infected animals. Infection with living mycobacteria is more effective than injection of an equivalent number of dead organisms. Maximum sensitivity develops after 7 to 9 days and persists for some time thereafter. The reaction is non-specific; *i.e.*, hyperreactivity occurs with endotoxins derived from different species of Gram-negative bacteria.

Our interest in this effect of BCG was prompted by the desire to increase the sensitivity of the urinary nitrogen excretion assay for endotoxin (8, 9). It soon became apparent, however, that BCG-infected mice respond to endotoxin in a manner quite distinct from those previously studied in our laboratory. This report summarizes the results on which this conclusion is based.

METHODS

BCG Infection.—Mice were injected intravenously with 4 mg (0.2 ml) of a BCG vaccine prepared by the Henry Phipps Institute, University of Pennsylvania. A shipment of vaccine

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was received at fortnightly intervals through the generous cooperation of Mr. H. J. Henderson, to whom we express our gratitude. From 80 to 120 mice were infected with a given shipment within 48 hours of its receipt. Until employed for experimental tests these animals were kept in a small darkroom laboratory maintained at 25 ± 2°C. An automatic electric switch gave 12 hours of illumination and 12 hours of darkness each 24 hour period.

Endotoxin.—Heat-killed *Salmonella typhimurium* suspended in isotonic solution of sodium chloride was prepared as previously described (10). The LD_{50} dose for this preparation was consistently found to be 2×10^9 cells when injected intraperitoneally into female mice weighing 20 ± 2 gm. This quantity of material weighs 1.5 mg. For the results to be described all injections of this crude material were given intraperitoneally. Purified lipopolysaccharide derived from *Escherichia coli* (Upjohn) was used in a few experiments.

Nitrogen Assays.—Techniques previously described were used to measure the urinary nitrogen excretion of mice during a 17 hour period of fasting (10) and to determine the total carcass non-protein nitrogen (NPN) (9) under relevant experimental conditions.

Carbohydrate Determinations.—Liver glycogen was evaluated by the method of Kemp and Kits van Heijningen (11). Muscle glycogen and total carbohydrate in mice were each determined by means of the same assay following tissue preparation as reported by Berry, Smythe, and Young (12).

ACTH Injections.—Mice were injected subcutaneously with 2 units of ACTH (Armour's acthar gel, 40 units per ml). Cortisone acetate (Nutritional Biochemicals, Cleveland) was given subcutaneously as a saline suspension stabilized with a drop of tween 80 at a dose level of 5 mg contained in 0.5 ml. Saccharated iron oxide (proferrin of Merck, Sharp and Dohme) was injected intravenously to "block" the RES of mice.

Inulin Clearance.—Kidney function of mice was evaluated by the inulin clearance test as described earlier (9). Blood inulin concentration was measured according to the method of Schreiner (13).

Adrenal Function Tests.—The *in vitro* secretion of adrenal corticoids in response to ACTH stimulation was measured according to the method of Elliot *et al.* (14) as modified by Saffron and Schally (15) and adapted to mouse glands by Berry and Smythe (9). Adrenal cholesterol levels were estimated by the method of Knobil *et al.* (16).

Adrenalectomies.—Mice were adrenalectomized under nembutal anesthesia (about 2 mg intraperitoneally per 20 gm mouse). The mice were maintained on 1 per cent saline and each animal was tested for the completeness of adrenal deprivement by the water excretion test of Beatty *et al.* (17). They were employed experimentally between 1 and 2 weeks postoperatively.

Miscellaneous.—CF 1 female mice (Carworth Farms, New York, New York) were used exclusively in these studies. Weekly shipments of animals weighing between 15 and 18 gm were received and they were held in the animal room for at least 1 week before being subjected to any experimental procedures. Only mice weighing 20 ± 2 gm were used. They were housed in small cages, 10 per cage with pine shavings as bedding. They were fed Dietrich and Gambrill specific pathogen-free mouse food *ad libitum* and water was available at all times.

RESULTS

The Effect of BCG Infection on Urinary Nitrogen Excretion in Mice.—The quantity of urinary nitrogen excreted during a 17 hour period of fasting by mice at different times postinfection with BCG is indicated graphically in Fig. 1. During the first day or two there is a drop of 30 to 40 per cent below control levels followed by a progressive rise that continues for nearly a week. A maximum occurs at 9 days, an increase of almost 50 per cent above the control value. Another drop to about normal nitrogen output is followed by a steady

increase over the next 2 weeks that terminates with double the amount of nitrogen excreted by normal animals.

The precise basis for these changes is not clear. The initial drop in nitrogen output is associated with a rise in total carcass NPN equal to the decrease in urinary nitrogen output. Thus urinary nitrogen excretion at time zero is 13.6 mg per mouse per day and on the 3rd day postinfection with BCG is 9.4 mg, a difference of 4.2 mg. The total NPN in the carcass of normal mice is 14.6 mg, while 3 days after BCG the total carcass contains 19.2 mg, a difference of 4.6 mg. This finding suggests an impairment in kidney function but at no other time postinfection was this observed. Carcass NPN values are within norma

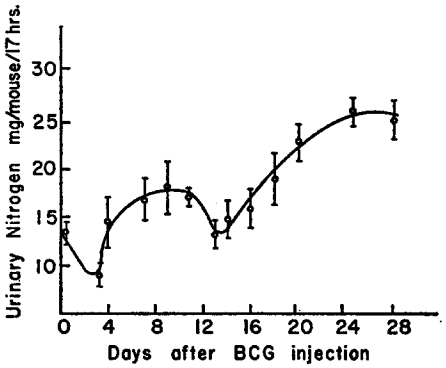

FIG. 1. Average urinary nitrogen excreted per mouse per 17 hours at different times postinfection with the attenuated BCG strain of *Mycobacterium tuberculosis*. Each point is the mean of at least eight separate determinations, each with urine from two mice, and the limits of the standard deviation are shown.

limits from 1 week to the end of the 4th week after the injection of the attenuated tubercle bacilli (see Table VIII below).

Timing of Hyperreactivity to Endotoxin in BCG-infected Mice.—The data of Table I summarize the results obtained when LD_{50} dosages of heat-killed *S. typhimurium* were determined in mice at the times specified after BCG infection. The final column gives the degree of sensitization as compared to the control value. Maximum sensitivity occurs at 2 weeks and corresponds to the time when urinary nitrogen excretion is at a minimum (Fig. 1). From this time on, hyperreactivity gradually decreases but is still apparent at 8 weeks.

An interesting relationship is made evident by the results presented in Table II. When a purified lipopolysaccharide derived from *E. coli* is given to mice 2 weeks postinfection with BCG, the LD_{50} dose is only 1/210th that required by normal controls. The mice are nearly three times more reactive to the lipopolysaccharide than to heat-killed cells, as the corresponding data of Table I show. This emphasizes the importance of the physical and, perhaps, chemical state of endotoxin in eliciting host response.

Effect of Reticuloendothelial Blockade on the Hyperreactivity of BCG-Infected Mice. Intravenous injection of colloidal particles sensitizes experimental animals to endotoxin (3, 4). The extent to which BCG-infected mice can be made more reactive to heat-killed *S. typhimurium* by such injections was determined with the results shown in Table III. The LD_{50} dose of endotoxin in normal mice injected with proferrin was less than 1/20 that for untreated con-

TABLE I

LD_{50} Dose in Number per Animal of Heat-Killed S. typhimurium Administered Intraperitoneally to Mice at Different Times Postinfection with the BCG Strain of Mycobacterium tuberculosis

Each value is calculated by the method of Reed and Muench (21).

Time postinfection with BCG	LD_{50} heat-killed *S. typhimurium*	Dose reduction compared to controls
Controls	2×10^9	
1 wk.	1.2×10^8	17 ×
2 wks.	2.7×10^7	74 ×
3 wks.	7.4×10^7	27 ×
4 wks.	2×10^8	10 ×
8 wks.	3.6×10^8	5.5 ×

TABLE II

LD_{50} Dose (Micrograms Per Mouse) of E. coli Lipopolysaccharide in Normal Mice and in Mice 2 Weeks Postinfection with BCG

Each value is calculated by the method of Reed and Muench (21).

Normal mice	BCG-infected mice	Dose reduction compared to controls
175	0.83	210 ×

trols (line 1, Table III). One week after infection with BCG, proferrin sensitized the animals ninefold while at 2 and 3 weeks postinfection the LD_{50} dose was reduced by only one-half (lines 2, 3, 4, Table III). The reticuloendothelial system seems to play only a minor role, therefore, in the response of these animals to endotoxin.

Effect of ACTH on Urinary Nitrogen Excretion in BCG-Infected Mice.—It was previously reported (8, 10) that urinary nitrogen excreted in response to an injection of ACTH is at least double the control amount (line 1, Table IV). Changes in urinary nitrogen excreted by mice given ACTH at intervals after BCG infection are also reported in Table IV. After 1 week the increase in nitrogen due to ACTH is only about one-half (47 per cent) that of control mice and this drops to a minimum of about 20 per cent at both 2 and 3 weeks

TABLE III

Influence of Saccharated Iron Oxide (Proferrin) on LD_{50} Dose of Heat-Killed S. typhimurium in Normal Mice and in Mice at Different Times Postinfection with BCG

Each animal was injected intravenously with 0.1 ml of proferrin 2 hours prior to the intraperitoneal injection of heat-killed *S. typhimurium*. Each value is calculated by the method of Reed and Muench (21).

Time postinfection with BCG	LD_{50} of heat-killed *S. typhimurium* given to mice treated with		Ratio of doses
	Proferrin	Controls	
Controls	9.2×10^7	2×10^9	22
1 wk.	1.3×10^7	1.2×10^8	9
2 wks.	1.5×10^7	2.7×10^7	2
3 wks.	4×10^7	7.4×10^7	2

TABLE IV

Urinary Nitrogen Excretion in Mice at Different Times Postinfection with BCG with and without Subcutaneous Injections of 2 Units of ACTH

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Time postinfection with BCG	Urinary nitrogen excreted by		N due to ACTH	Per cent of normal
	Control mice	Mice given ACTH		
	mg/mouse/17 hrs.	*mg/mouse/17 hrs.*	*mg*	
Controls	13.6 ± 1.9 (9)	28.3 ± 3.8 (8)	14.7	100
1 wk.	17.1 ± 4.4 (46)	24.1 ± 5.0 (29)	7.0	47
2 wks.	14.7 ± 4.0 (28)	17.6 ± 4.0 (25)	2.9	20
3 wks.	22.8 ± 5.4 (20)	25.6 ± 4.0 (14)	2.8	19
4 wks.	25.2 ± 4.4 (7)	36.5 ± 5.8 (7)	11.3	77

postinfection. This increment is apparently independent of the control amount of nitrogen excreted by mice given no corticotrophin, *i.e.* the same amount of nitrogen (2.8 and 2.9 mg) is excreted following ACTH given at 2 weeks and 3 weeks, though control values are 14.7 mg and 22.8 mg, respectively. At 4 weeks, however, the response to ACTH is 77 per cent of normal.

There are several possible interpretations of these results. Among these are failure of the adrenals of BCG-infected mice to secrete normal amounts of glycocorticoids in response to ACTH and impairment in the ability of the

kidneys of these animals to clear nitrogen. Experiments described in the sections that follow were designed to discriminate between these possibilities.

Adrenal Cholesterol in Control and in BCG-Infected Mice after ACTH or Endotoxin.—Table V summarizes the data obtained on cholesterol content of adrenal glands (expressed as milligrams of cholesterol per 100 mg adrenal tissue) derived from normal mice and from mice 2 weeks after BCG infection. Values for mice fasted for 17 hours are given in the first line of Table V and are the same for the two groups. Four hours after the subcutaneous injection of

TABLE V

Cholesterol Content of Adrenal Glands of Normal Mice and Mice 2 Weeks Postinfection with BCG at 4 and 17 Hour Intervals after Subcutaneous Injection of Either 2 Units ACTH or an LD_{50} Dose of Heat-Killed S. typhimurium

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental treatment	Adrenal cholesterol in	
	Normal mice	BCG-infected mice
	mg/100 mg tissue	*mg/100 mg tissue*
Controls—17 hr. fasting	5.5 ± 0.9 (11)	5.4 ± 1.2 (10)
4 hrs. after 2 units ACTH	3.7 ± 0.9 (9)	5.4 ± 0.9 (10)
17 hrs. after 2 units ACTH	3.0 ± 0.4 (8)	3.7 ± 0.5 (11)
4 hrs. after LD_{50} dose of endotoxin	2.6 ± 0.5 (7)	4.8 ± 0.6 (8)
17 hrs. after LD_{50} dose of endotoxin	2.3 ± 0.7 (8)	3.6 ± 0.7 (10)

2 units of ACTH, cholesterol in glands of normal mice was significantly lowered (P = 0.1 per cent by rank test (18)) but was unchanged in glands of BCG-infected mice (line 2, Table V). Seventeen hours after the injection of ACTH, adrenal cholesterol in both normal and BCG-infected mice was significantly lower than in the corresponding fasted controls (line 3, Table V).

Similar results were found when endotoxin was injected intraperitoneally. After 4 hours, adrenal cholesterol was less than one-half the control value in normal mice while in BCG-infected mice it was not changed significantly as judged by the rank test (18). These values are given in line 4, Table V. The last line of the table shows that adrenal cholesterol content is below control levels 17 hours after endotoxin in both groups but normal mice show the larger decrease.

The adrenals of BCG-infected mice are more refractory to ACTH and to

endotoxin than are the glands of normal mice as judged by decrease in cholesterol reserve. This observation may explain why mice infected with BCG show such a small increase in urinary nitrogen excretion after ACTH. Without release of glycocorticoid to promote protein catabolism, urinary nitrogen output cannot become augmented. Commensurate with this suggestion is the result that follows.

In Vitro Secretion of Adrenocorticoids by Glands from Normal and BCG-Infected Mice.—The *in vitro* secretion of corticoids by glands from normal mice and from mice 2 weeks after infection with BCG is summarized in Table VI. The adrenal cortex of BCG-infected mice is more refractory to stimulation with 0.01 unit ACTH than is that of normal animals ($P = 0.05$ by rank test (18)). The quantity of corticoids released under these conditions represents

TABLE VI

In Vitro Corticoid Synthesis in Response to 0.01 Unit ACTH by Pooled Glands Taken from Mice 2 Weeks Postinfection with BCG and from Control Mice

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Corticoid synthesis in response to 0.01 unit ACTH by glands from	
Normal mice	BCG-infected mice
mg/100 mg tissue 1 hour	*mg/100 mg tissue 1 hour*
24.7 ± 3.0 (6)	20.1 ± 3.2 (7)

the conversion of a minute fraction of the cholesterol present. Adrenals contain 5.5 mg cholesterol per 100 mg of whole glands (Table V) and secrete as total corticoid only 20 to 35 μg per 100 mg. Thus, the ability to secrete corticoids *in vitro* may be quite different from *in vivo* activity as judged by cholesterol depletion (Table V).

Renal Clearance of Inulin in Endotoxin-Poisoned BCG-Infected Mice.—In a previous report impairment of renal function was demonstrated in mice 17 hours after an LD_{50} dose of endotoxin (9). One of the tests that made this evident was plasma clearance of inulin. Thirty minutes after intravenous injection of inulin (20 mg per mouse) normal animals contained 0.20 mg inulin per ml of blood while those poisoned with endotoxin retained 1.49 mg per ml (line 1, Table VII). The same test, carried out with mice 1, 2, 3, and 4 weeks postinfection, yields results summarized in Table VII. BCG infection alone has no influence on inulin clearance. Endotoxin poisoning (LD_{50} dose) altered renal function at the end of the 2nd and 3rd weeks of BCG infection in such a way that all mice fell into two distinct groups. At 2 weeks (line 3, Table VII), half the mice clear inulin normally and the remaining half behave as poisoned nor-

mal mice. Three weeks after BCG infection, a similar result is obtained with a different proportion of animals. By the 4th week postinfection, all mice given endotoxin had impaired inulin clearance. Normal mice injected with a dose of heat-killed cells that is LD_{50} for mice 3 weeks after BCG infection show no retention of inulin (last line, Table VII).

TABLE VII

Inulin Remaining in Blood of Normal Mice and of Mice 2 Weeks Postinfection with BCG 30 Minutes after an Intravenous Injection of 20 Mg Inulin per Mouse

All determinations were made on mice 17 hours after an LD_{50} dose of heat-killed *S. typhimurium* or on mice merely fasted for 17 hours. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Time postinfection with BCG	Inulin remaining 30 min. after injection into mice given	
	No endotoxin	Endotoxin (LD_{50} dose)
	mg/ml blood	*mg/ml blood*
Control mice	0.20 ± 0.06 (10)	1.49 ± 1.0 (9)
1 wk.	0.21 ± 0.07 (8)	1.63 ± 0.8 (8)
2 wks.	0.22 ± 0.09 (10)	0.24 ± 0.06 (10)
		1.31 ± 0.7 (10)
3 wks.	0.21 ± 0.04 (10)	0.29 ± 0.05 (5)
		1.86 ± 0.8 (12)
4 wks.	0.19 ± 0.04 (8)	2.21 ± 0.16 (7)
Control mice given "BCG" LD_{50}		0.24 ± 0.08 (10)

Certain difficulties arise in comparing endotoxin-poisoned normal mice with endotoxin-poisoned BCG-infected mice. An LD_{50} dose of endotoxin rarely kills normal mice before 18 hours or after 36 hours while BCG-infected mice die as early as 4 hours and rarely after 12 hours. For the data of Table VII, therefore, all animals in the normal group were available for testing while only about 50 per cent of the BCG-infected animals remained. Whether the surviving mice that cleared inulin normally were typical or not of the group that succumbed was partially answered by the next experiment. An LD_{75} dose of endotoxin was given to mice 2 weeks postinfection with BCG. The survivors

at 17 hours were then used to test inulin clearance. After 30 minutes, 2.35 mg inulin per ml of blood was found. Apparently those mice originally capable of excreting inulin normally were killed by the endotoxin. The uncertainty associated with these findings, however, dictated the experiments now to be described.

Blood and Carcass NPN in Normal and BCG-Infected Mice after Endotoxin.— An impairment of renal function in endotoxin poisoning results in uremia and a statistically significant accumulation of NPN in the carcass of mice (9). Measurements of this type in BCG-infected mice were made with the results presented in Table VIII. BCG infection alone at either 2 or 4 weeks does not

TABLE VIII

Blood and Total Carcass Non-Protein Nitrogen (NPN) in Normal Mice, in Mice 2 and 4 Weeks Postinfection with BCG, and in Adrenalectomized Mice 17 Hours after an LD_{50} Dose of Heat-Killed S. typhimurium and at Death

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental treatment	Non-protein nitrogen in	
	Blood	Carcass
	mg per cent	*mg/mouse*
Normal mice		
(1) Controls	37.3 ± 3.8 (10)	14.6 ± 1.3 (19)
(2) 17 hrs. after endotoxin	86.9 ± 15.2 (15)	18.1 ± 2.6 (16)
(3) At death		33.9 ± 5.3 (9)
2 wks. post BCG		
(1) Controls	40.9 ± 3.8 (8)	14.9 ± 1.2 (11)
(2) 17 hrs. after endotoxin	46.7 ± 9.4 (12)	15.9 ± 3.1 (7)
(3) At death		16.5 ± 1.5 (9)
4 wks. post BCG		
(1) Controls	40.8 ± 3.6 (9)	15.0 ± 1.3 (10)
(2) 17 hrs. after endotoxin	141 ± 34.0 (7)	21.4 ± 3.2 (7)
Adrenalectomized mice		
(1) Controls	48.9 ± 5.7 (6)	15.6 ± 0.8 (7)
(2) 17 hrs. after endotoxin	109 ± 23 (6)	22.1 ± 4.9 (12)

alter blood or carcass NPN. Both normal mice and mice 4 weeks postinfection show, respectively, more than a doubling and tripling of blood NPN and statistically significant increases in carcass NPN. However, mice given endotoxin 2 weeks after BCG infection show no significant changes in either blood or carcass NPN.

These same animals assayed at the moment of death from endotoxin poisoning have essentially normal values for carcass NPN while in control mice at death it more than doubles. These data clearly indicate, therefore, that changes in renal physiology seen typically in endotoxin-poisoned animals are not present in the BCG-infected animals at the time when they are most hyperreactive.

The NPN content of blood and carcass of adrenalectomized mice is also presented in Table VIII. The increase in each of these values 17 hours after endotoxin is similar to that in normal mice. Impaired adrenal function will not alone account, therefore, for the unique metabolic behavior of the BCG-vaccinated animals.

Carbohydrate Levels in Mice at Different Times Post BCG Infection.—The short survival time of BCG-infected mice poisoned with endotoxin (compared with that of control mice) and the convulsive nature of their death prompted determination of carbohydrate levels in mice after BCG inoculation. The results are given in Table IX. Both liver and muscle glycogen decrease to a minimum level at 2 weeks and then increase at 3 and 4 weeks postinfection. These changes are reflected in total carbohydrate expressed both as per gram of carcass and per mouse. When these mice are most sensitive to endotoxin they also contain minimum carbohydrate reserve.

Effect of Endotoxin on Carbohydrate Levels of Control and BCG-Infected Mice. The same percentage decrease in carbohydrate reserve in normal mice and in mice 2 weeks after infection with BCG is found 5 hours after endotoxin. This can be seen in the data presented in Table X. The BCG-infected mice start with lower initial carbohydrate and end lower than control animals even though the absolute change is smaller.

The 17 mg of carbohydrate remaining in BCG-infected mice 5 hours after endotoxin approaches the 10 mg found in mice of each group at death, as the last line of Table X shows. These findings suggest that the short survival time of BCG-infected mice given endotoxin may be causally related to the exhaustion of carbohydrate.

Injections of glucose, on the other hand, are incapable of protecting BCG-infected mice against the lethal effects of endotoxin. Animals at the time of maximum hyperreactivity (2 weeks after BCG infection) were given intravenous injections of isotonic glucose, 10.8 mg per mouse, 1 hour before an LD_{50} dose of endotoxin and 1, 3, and 5 hours after endotoxin. This is a total of 43.2 mg of glucose within 6 hours but only 11 of 20 mice survived, while nine of 20 survived when saline alone was injected instead of the glucose. Either the glucose

TABLE IX

Carbohydrate Levels in Mice at Different Times Postinfection with BCG

All values are for fed animals. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Time post BCG Infection	Liver glycogen	Muscle glycogen	Carcass weight	Total carbohydrate	Total carbohydrate
days	*per cent by weight*	*per cent by weight*	*gm*	*mg/gm*	*mg/mouse*
0	5.5 ± 0.6 (27)	0.33 ± 0.06 (15)	11 ± 1.0 (15)	6.5 ± 1.2 (15)	72 (15)
7	4.0 ± 1.2 (12)	0.30 ± 0.06 (12)	12.8 ± 1.0 (12)	7.3 ± 1.9 (12)	94 (12)
14	1.3 ± 0.6 (8)	0.18 ± 0.05 (8)	13.8 ± 1.5 (8)	3.0 ± 0.9 (8)	42.5 (8)
21	2.0 ± 0.7 (7)	0.27 ± 0.02 (7)	13.2 ± 1.2 (7)	5.0 ± 1.2 (7)	66 (7)
28	2.7 ± 0.6 (8)	0.25 ± 0.05 (6)	14.6 ± 2.3 (8)	4.8 ± 1.1 (8)	70 (8)

TABLE X

Changes in Carbohydrate Reserves of Normal Mice and Mice 2 Weeks Postinfection with BCG Induced by an LD_{50} Dose of Heat-Killed S. typhimurium

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental treatment	Liver glycogen for		Muscle glycogen for		Total carbohydrate for	
	Controls	BCG-infected	Controls	BCG-infected	Controls	BCG-infected
	per cent by weight	*per cent by weight*	*per cent by weight*	*per cent by weight*	*mg/mouse*	*mg/mouse*
Fed	5.5 ± 0.6 (27)	1.3 ± 0.6 (8)	0.33 ± 0.06 (15)	0.18 ± 0.05 (8)	88 (15)	43 (8)
5 hrs. after LD_{50} endotoxin	1.4 ± 0.4 (6)	0.1 ± 0.06 (10)	0.16 ± 0.05 (7)	0.14 ± 0.06 (5)	41 (7)	17 (10)
At death from LD_{50} endotoxin	0.1 ± 0.05 (8)	0.1 ± 0.04 (9)	0.06 ± 0.02 (8)	0.08 ± 0.02 (4)	10 (5)	10 (4)

is not actually involved in the outcome of endointoxication or else the BCG-infected mouse cannot properly metabolize glucose. Preliminary findings tend to support the latter view (see below).

Protein-Carbohydrate "Balance" Following Cortisone Injection in BCG-Infected Mice.—Table XI summarizes data which relate amount of protein catabolized in response to cortisone administration with carbohydrate anabolized under the same conditions. Total protein is calculated from the increase in

urinary nitrogen excreted after the hormone is injected while the change in carbohydrate is measured directly in the homogenized carcass from which the digestive tract has been removed prior to homogenization. Determinations were carried out under fasting conditions in normal mice, adrenalectomized mice, and in mice 2 and 4 weeks postinfection with BCG. Control animals show 92 per cent as much carbohydrate anabolized as protein catabolized. The corresponding value for adrenalectomized mice is 21 per cent and for BCG-inoculated animals it is 47 per cent at 2 weeks and 88 per cent at 4 weeks. The smaller

TABLE XI

Carbohydrate Anabolized and Protein Catabolized in Response to 5 Mg Cortisone Acetate in Normal Mice, Adrenalectomized Mice, and in Mice 2 and 4 Weeks Postinfection with BCG

Total carbohydrate values are the product of the mean values for total carbohydrate per gram of carcass and mean carcass weight. Total protein is the mean urinary nitrogen excreted multiplied by 6.25. The number of separate determinations on which the calculations are based is shown in parentheses.

Experimental treatment	Total carbohydrate			Total protein			Carbohydrate / Protein
	Without cortisone	With cortisone	Due to cortisone	Without cortisone	With cortisone	Due to cortisone	
	mg/mouse	*mg/mouse*	*mg/mouse*	*mg/mouse*	*mg/mouse*	*mg/mouse*	*per cent*
Controls	22 (8)	70 (8)	48	83 (6)	135 (6)	52	92
Adrenalectomized	20 (11)	45 (10)	25	97 (11)	214 (10)	117	21
BCG 2 wks.	34 (12)	58 (14)	24	82 (7)	133 (8)	51	47
BCG 4 wks.	29 (10)	115 (10)	86	161 (10)	259 (10)	98	88

the ratio of carbohydrate to protein, the greater the susceptibility to endotoxin. Thus the adrenalectomized mice had an LD_{50} 1/250th that of controls while 2 weeks after BCG infection it was 1/74th and at 4 weeks it was 1/10. Similar findings have been reported elsewhere (19) for mice exposed to simulated altitude.

It is particularly interesting to compare the amount of protein catabolized in the different groups of mice. It was the same in control mice and in mice 2 weeks after BCG infection. It was double this amount in adrenalectomized mice and in mice 4 weeks postinfection with BCG. Since each group shows a different ratio of carbohydrate to protein and different sensitivity to endotoxin, perhaps protein metabolism is less important to survival than the handling of carbohydrate. The biochemical basis for these findings is not understood, despite a sizable literature on the subject (8–10, 12, 19).

DISCUSSION

The hyperreactivity of BCG-infected mice appears to be due to more than an intensification of the normal response to endotoxin. The animals die sooner, they show few of the symptoms commonly associated with endotoxin poisoning such as conjunctivitis, diarrhea, ruffled fur, and the general loss of activity. The LD_{50} dose differs in absolute amount in normal and in BCC-infected mice but the dose for adrenalectomized mice is even smaller. Nevertheless, adrenalectomized mice behave symptomatologically and metabolically more like normal mice than do BCG-infected animals. The times of death from an LD_{50} dose of endotoxin are approximately the same for adrenalectomized and normal mice. They show conjunctivitis and diarrhea and, in contrast to the BCG-treated animals, accumulate non-protein nitrogen in blood and carcass. This implies an oliguria, an impaired plasma clearance of inulin by kidneys of these mice and, inferentially, a decrease in glomerular filtration pressure probably associated with a drop in blood pressure and a general shock syndrome.

Such obvious differences in the behavior of adrenalectomized and BCG-treated mice speak eloquently enough against any suggestion that the hyperreactivity of the latter is to be explained on the basis of adrenocortical insufficiency induced by the infection. However, intracutaneous infection of rabbits with a virulent bovine strain of tubercle bacillus is known to result in adrenocortical hemorrhage (20). This may occur in our mice since the data of Tables V and VI are indicative of a diminished responsiveness of the adrenal cortex. Perhaps one must seek beyond the imbalance of this one hormone for changes in other endocrines before real understanding of these interactions is possible. These studies point unmistakably to errors that may arise in assuming that all animals react uniformly to endotoxin. The BCG-infected mouse is exceptional in its response to endotoxins but it is the only one studied in sufficient detail to make its unusual metabolic behavior evident. There will probably be others if a search is made. But one exception is enough to establish that endotoxins kill some experimental animals without the usual changes associated with endotoxic death.

SUMMARY

The greater susceptibility to the lethal effects of bacterial endotoxin (heat-killed *Salmonella typhimurium* or *Escherichia coli* lipopolysaccharide) in mice infected with an attenuated strain of *Mycobacterium tuberculosis* (BCG) was confirmed. It reached a maximum at 2 weeks postinfection and gradually diminished for an additional 6 weeks.

At the time of maximum susceptibility several metabolic and physiological differences became apparent. BCG-infected mice die sooner (4 to 12 hours) and without the diarrhea, conjunctivitis, and general symptomatology associated with endotoxin deaths of normal animals. Reticuloendothelial blockade results

in only a small change in reactivity to endotoxin, in contrast to normal mice. Subcutaneous injection of 2 units of ACTH is followed by no significant increase in urinary nitrogen excretion while in control animals it more than doubles. Plasma clearance of intravenously administered inulin is approximately normal in BCG-infected mice 17 hours after an LD_{50} dose of endotoxin but control mice similarly treated show renal impairment. In line with this result is the absence of elevated carcass non-protein nitrogen (NPN) following endotoxin poisoning or at the moment of death from endotoxemia in the hyperreactive animals in contrast to the two- to threefold increase in carcass NPN in normal mice under similar conditions. Body carbohydrate is at a minimum and becomes depleted to a level approximating that found at death more rapidly in BCG-infected mice given endotoxin than in controls. There is also a lower ratio of carbohydrate anabolized to protein catabolized following cortisone administration to BCG-infected mice than in control mice. This is found in adrenalectomized mice and in stressed animals and is reported elsewhere.

Some of the differences just described can be attributed to a refractory adrenal cortex. There is less depletion of adrenal cholesterol *in vivo* and lower corticoid synthesis *in vitro* than in normal mice yet this is not fundamentally responsible for the greater susceptibility of BCG-infected animals to endotoxin since adrenalectomized mice, which are even more susceptible, are metabolically and physiologically more comparable to normal mice than to BCG-infected mice. One can conclude, therefore, that the hyperreactivity of BCG-infected mice is more than an intensification of the normal response to endotoxin.

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BIBLIOGRAPHY

1. Suter, E., Ullman, G. E., and Hoffman, R. G., Sensitivity of mice to endotoxin after vaccination with BCG (Bacillus-Calmette-Guerin), *Proc. Soc. Exp. Biol. and Med.*, 1958, **99**, 167.
2. Howard, J. G., Biozzi, G., Halpern, B. N., Stiffel, C., and Moutan, D., The effect of *Mycobacterium tuberculosis* (BCG) infection on the resistance of mice to bacterial endotoxin and *Salmonella enteritidis* infection, *Brit. J. Exp. Path.*, 1959, **40**, 281.
3. Good, R. A., and Thomas, L., Studies on the generalized Shwartzman reaction. II. The production of bilateral cortical necrosis of the kidneys by a single injection of bacterial toxins in rabbits previously treated with thorotrast or trypan blue, *J. Exp. Med.*, 1952, **96**, 625.
4. Bennett, I. L., Jr., Further investigation of effect of colloidal materials upon the Shwartzman phenomenon, *Proc. Soc. Exp. Biol. and Med.*, 1952, **81**, 248.
5. Beeson, P. B., Tolerance to bacterial pyrogens; role of the reticuloendothelial system, *J. Exp. Med.*, 1947, **86**, 39.
6. Beeson, P. B., Effect of reticulo-endothelial blockade on immunity to the Shwartzman phenomenon, *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 146.

7. Suter, E., and Kirsanow, E. M., Hyperreactivity to endotoxin in mice infected with Mycobacteria. Induction and elicitation of the reaction, *Immunology*, 1961, **4**, 354.
8. Berry, L. J., and Smythe, D. S., Effects of bacterial endotoxins on metabolism. III. Nitrogen excretion after ACTH as an assay for endotoxin, *J. Exp. Med.*, 1961, **113**, 83.
9. Berry, L. J., and Smythe, D. S., Effects of bacterial endotoxins on metabolism. IV. Renal function and adrenocortical activity as factors in the nitrogen excretion assay for endotoxin. *J. Exp. Med.*, 1961, **114**, 761.
10. Berry, L. J., and Smythe, D. S., Effects of bacterial endotoxins on metabolism. II. Protein-carbohydrate balance following cortisone. Inhibition of intestinal absorption and adrenal response to ACTH, *J. Exp. Med.*, 1959, **110**, 407.
11. Kemp, A., and Kits van Heijningen, A. J. M., A colorimetric micro-method for the determination of glycogen in tissues, *Biochem. J.*, 1954, **56**, 646.
12. Berry, L. J., Smythe, D. S., and Young, L. G., Effects of bacterial endotoxins on metabolism. I. Carbohydrate depletion and the protective role of cortisone, *J. Exp. Med.*, 1959, **110**, 389.
13. Schreiner, G. E., Determination of inulin by means of resorcinol, *Proc. Soc. Exp. Biol. and Med.*, 1950, **74**, 117.
14. Elliot, F. H., Birmingham, M. D., Schally, A. V., and Schombaum, E., Micro-estimation of steroids produced by rat adrenals *in vitro*, *Endocrinology*, 1954, **55**, 721.
15. Saffron, M., and Schally, A. V., *In vitro* bioassay of corticotropin: modification and statistical treatment, *Endocrinology*, 1955, **56**, 523.
16. Knobil, E., Hagney, M. G., Wilder, E. J., and Briggs, F. N., Simplified method for determination of total adrenal cholesterol, *Proc. Soc. Exp. Biol. and Med.*, 1954, **87**, 48.
17. Beatty, C. H., Bocek, R. M., and Peterson, R. D., Water tolerance test for functioning adrenal cortical tissue in the rat, *Am. J. Physiol.*, 1954, **117**, 287.
18. White, C., The use of ranks in a test for significance for comparing two treatments, *Biometrics*, 1952, **8**, 33.
19. Berry, L. J., and Smythe, D. S., Effect of pure oxygen at reduced pressures on metabolic changes in mice, *Am. J. Physiol.*, 1962, **203**, 155.
20. Lurie, M. B., Abramson, S., Allison, M. J., Harris, T. N., and Heppleston, A. G., Constitutional factors in resistance to infection. III. On the mode of action of estrogen and gonadotropin on the progress of tuberculosis, *Am. Rev. Tuberc.*, 1949, **59**, 198.
21. Reed, L. J., and Muench, H., A simple method of estimating fifty percent endpoints, *Am. J. Hyg.*, 1938, **27**, 493.