LYSOSOMAL ACID HYDROLASES AND HYPERREACTIVITY TO ENDOTOXIN IN MICE INFECTED WITH BCG*

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During the course of some infections a state of diversely altered responsiveness develops not only to the causative pathogen but also to a variety of seemingly unrelated stimuli, possibly due to increased activity of the RES.[1] For example, animals infected with BCG develop a state of hyperreactivity to the lethal action of endotoxin prepared from Gram-negative bacteria (2, 3). This latter phenomenon is rather unexpected, since enhanced resistance or tolerance to endotoxin is generally associated with increased activity of the RES.

As one indication of enhanced activity of the RES induced by infection with BCG, increase of the content of lysosomal acid hydrolases in peritoneal MP[1] and liver was demonstrated (4). Furthermore, in a study of isolated subcellular fractions of liver homogenates the increase of the enzymes was proved to be mainly confined to the "large granular" fraction, which consisted of the bulk of mitochondria, lysosomes, and microsomes (4). The recent suggestion that lysosomes may represent a major target of endotoxin in tissue damage (5–8) is of great interest in this connection.

The present investigation was undertaken to detect a possible relationship between increased content of lysosomal enzymes and hyperreactivity to endotoxin in BCG-infected animals. Also the effect of endotoxin and PPD[1] on lysosomal enzymes in BCG-infected animals was compared. The results obtained indicate that BCG infection causes a lowered threshold of endotoxin tolerance of lysosomes, possibly resulting in the release of the enzymes into the blood upon administration of endotoxin. There appeared to be at least a difference between the action of endotoxin and PPD in hyperreactive animals.

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[1] The following abbreviations are used in this paper: RES, reticuloendothelial system; MP, macrophages, histiocytes, and monocytes; saline, physiologic sodium chloride solution; PPD, purified protein derivative from tuberculin.

Materials and Methods

The infection of mice with BCG, preparation of cell extracts for enzymatic determination, and enzymatic and chemical methodology were described in the preceding article (4). In brief, mice injected intravenously with BCG were used 10 to 14 days after the injection when hyperreactivity towards endotoxin was fully induced. Sonic oscillated MP, frozen and thawed liver homogenate, and citrated blood plasma were used for enzymatic determinations. *p*-Nitrophenylphosphate, phenolphthalein glucuronidate, and hemoglobin were used as substrates for acid phosphatase, β-glucuronidase, and cathepsin respectively.

Injection with Endotoxin.—An endotoxin preparation, Bacto lipopolysaccharide derived from *Escherichia coli* O55:B5 (Difco Laboratories, Detroit, Michigan), was used throughout the experiments. The endotoxin was suspended in saline[1] and 0.2 ml of appropriate dilutions of the suspensions was given intravenously.

Injection with PPD.—A purified tuberculin preparation prepared from *Mycobacterium tuberculosis*, strain RT23 was obtained from the State Serum Institute (Copenhagen, Denmark). PPD was suspended in saline with the aid of a small amount of NaOH solution, and after neutralization of the pH, 0.2 ml of the appropriate dilution of the suspension was administered intravenously.

Preparation of Blood Leucocytes for Enzymatic Assays.—Blood leucocytes were isolated by the method of Hammerstrom and Stoner (9), using polyvinylpyrrolidine. Blood withdrawn from the heart was mixed with an equal amount of 3.5 per cent polyvinylpyrrolidinone saline solution containing heparin and was allowed to stand undisturbed for 20 to 50 minutes. The supernatant, which was separated from packed red cells, was removed and centrifuged at 1000 RPM for 10 minutes. The blood leucocytes thus collected still containing some red blood cells were resuspended in saline and were then treated with sonic oscillation at 10 kc/second (Raytheon sonic oscillator) for 10 minutes.

RESULTS

Effect of Endotoxin on Acid Hydrolases in BCG-Infected Mice.—

Effect on plasma levels of acid hydrolases: In an attempt to elucidate the mechanism underlying the hyperreactive state to endotoxin of BCG-infected animals, the effect of administration of endotoxin on plasma levels of lysosomal acid hydrolases in such animals was examined. Mice infected with BCG and control normal mice were injected intravenously with endotoxin, and plasma levels of acid phosphatase and β-glucuronidase were determined before and after the injection. The results of a representative experiment, in which mice were injected with 10 μg of endotoxin, are summarized in Table I.

Before the injection of endotoxin, plasma levels of both enzymes in BCG-infected mice were significantly higher than those in normal mice. After the intravenous injection with 10 μg of endotoxin, a substantial increase of acid hydrolase, especially of β-glucuronidase, was observed in BCG-infected mice. On the basis of results of individual experiments a 30- to 100-fold increase of β-glucuronidase and 3- to 8-fold increase of acid phosphatase were observed in BCG-infected mice 1 and 3 hours after the injection of endotoxin, while in control mice only a very moderate increase of β-glucuronidase and almost no change of acid phosphatase were noticed.

In further experiments of the same type carried out several times, varying

amounts of endotoxin were injected, *i.e.* 1 to 50 μg in BCG-infected mice, and 1 to 1250 μg in control mice. The results obtained were similar to those given in Table I. In some instances, plasma was collected from control mice 8 and 17 hours after injection of 1250 μg of endotoxin, since the course of the lethal shock induced by endotoxin in control mice appeared to be more delayed than in hyperreactive mice (2, 10). However, no significant change of acid hydrolases was observed under these conditions.

Mortality and plasma level of β-glucuronidase in response to endotoxin: Endotoxins derived from Gram-negative bacteria are known to exert their biologic

TABLE I

Effect of Administration of Endotoxin on Plasma Levels of Acid Hydrolases in Normal and BCG-Infected Mice

Hrs. after endotoxin (10 μg. i.v.)	Acid phosphatase*		β-Glucuronidase‡	
	Normal	BCG	Normal	BCG
Before	1.111 ± 0.551 (3)§	2.600 ± 0.943 (3)	0.7 ± 1.2 (3)	24.0 ± 12.5 (3)
1	1.278 (2)	9.000 (2)	3.0 (2)	602.5 (2)
3	0.933 ± 0.237 (3)	11.067 ± 1.467 (3)	2.3 ± 0.58 (3)	1157.8 ± 233.9 (3)

* Activity of acid phosphatase expressed as μmole of *p*-nitrophenol produced in 40 minutes per ml of plasma.

‡ Activity of β-glucuronidase expressed as μg of phenolphthalein produced in 1 hour per ml of plasma.

§ Number of determinations.

action in diverse manifestations. However, the hyperreactive state to endotoxin in BCG-infected animals has been demonstrable so far only in the lethal action of endotoxin (11, 12). The results described in the preceding section indicate clearly that BCG-infected mice responded to endotoxin quite differently from control mice by showing a rapid increase of acid hydrolases in the plasma. Thus lethal activity and effect on plasma level of β-glucuronidase of varying amounts of endotoxin was compared. Varying amounts of endotoxin, as shown in Table II, were injected intravenously into BCG-infected and control mice. The mice injected with each amount of endotoxin were divided into two groups. Mice of one group were observed for their mortality for 72 hours, and mice of the other group were used for determination of plasma level of β-glucuronidase 3 hours after the injection of endotoxin.

As can be seen in Table II, even 1250 μg of endotoxin caused only about a 3-fold increase of plasma level of β-glucuronidase in control mice, although this dose killed 6 out of 10 mice. By contrast, in BCG-infected mice a dose as small

as 0.4 μg of endotoxin not only killed 8 out of 10 mice but also caused about a 100-fold increase of plasma level of β-glucuronidase. Doses greater than 0.4 μg caused more than 90 per cent of mortality and about a 50- to 100-fold increase of plasma β-glucuronidase, while doses less than 0.4 μg caused almost no deaths and only a slight increase of plasma β-glucuronidase. Thus, the results in BCG-infected mice show a fairly good correlation between the extent of increase of plasma β-glucuronidase and the mortality in response to endotoxin.

TABLE II

Mortality and Plasma Level of β-Glucuronidase of Normal and BCG-Infected Mice in Response to Endotoxin

Endotoxin	Mortality*		β-Glucuronidase in plasma‡	
	Normal	BCG	Normal	BCG
(μg i.v.)				
1250	6/10	—	5.0	—
250	0/10	—	5.0	—
50	1/9	9/9	4.0	1305.0
10	0/9	9/9	4.0	470.0
2	—	8/9	—	680.0
0.4	—	8/10	—	1110.0
0.08	—	0/10	—	37.5
0.016	—	1/10	—	11.0
0	—	—	1.5	10.0

* Number deaths/number injected.

‡ Collected 3 hours after administration of endotoxin.

For further explanation, see Table I.

Effect of desensitization on mortality and plasma β-glucuronidase in hyperreactive mice: Hyperreactivity of BCG-infected animals towards lethal action of endotoxin has been shown to be decreased by desensitization with endotoxin (11, 12). Since results in the preceding experiments demonstrated close correlation between increase in plasma β-glucuronidase and the hyperreactive state to lethal action of endotoxin, the effect of desensitization on the plasma level of β-glucuronidase in hyperreactive mice was examined.

Mice infected with BCG were divided into 2 groups; the mice of one group were desensitized with endotoxin and the mice of the other group were kept without the treatment. The following schedule was adopted for desensitization. On the 10th day after BCG infection a series of intraperitoneal injections with endotoxin for 7 consecutive days was begun injecting 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 μg, respectively. On the day following the last desensitizing injection, varying amounts of endotoxin were injected intravenously into the

desensitized and the untreated mice, and the mortality and plasma level of β-glucuronidase were examined as in the preceding experiment. The results are shown in Table III.

As expected, hyperreactivity of BCG-infected mice to the lethal effect of endotoxin was markedly decreased by the desensitization. In the desensitized group 3 out of 9 mice were killed by 250 μg of endotoxin, while in the untreated control group 4 out of 9 mice were killed by 0.4 μg of endotoxin. In concordance with this decrease of hyperreactivity, desensitized mice responded to endotoxin

TABLE III

Effect of Desensitization with Endotoxin on Mortality and Plasma Level of β-Glucuronidase in Hyperreactive Mice in Response to Endotoxin

Endotoxin	Mortality		β-Glucuronidase in plasma	
	Control (BCG)	Desensitized	Control (BCG)	Desensitized
μg i.v.				
1250	—	9/9	—	255.0
250	—	3/9	—	95.0
50	9/9	2/9	1230.0	80.0
10	9/9	0/9	1255.0	28.5
2	8/9	0/9	1345.0	27.0
0.4	4/9	0/9	295.0	22.5
0.08	0/9	—	15.5	—
0.016	0/8	—	24.0	—
0	—	—	17.5	9.0

For explanations see Tables I and II.

with a much smaller increase of β-glucuronidase in the plasma. Desensitized mice responded to the injection of 250 μg of endotoxin with about a 10-fold increase of plasma level of β-glucuronidase, while in the control BCG-infected mice 2 μg of endotoxin caused an 80-fold increase. The results also indicate that even desensitized mice exhibit a fairly good correlation between the degree of increase of plasma β-glucuronidase and mortality in response to endotoxin.

Effect of endotoxin on acid hydrolases in MP, liver, and blood leucocytes: To detect the source of the acid hydrolases released into the plasma of BCG-infected mice in response to endotoxin, these enzymes were determined in MP, liver, and blood leucocytes before and after the injection of endotoxin. No significant depletion of acid hydrolases was found in these cells after administration of endotoxin in normal or BCG-infected mice.

In BCG-infected mice intravenous injection of 10 μg of endotoxin appeared to cause decrease, though statistically not significant, of acid phosphatase and

β-glucuronidase in all three types of cells 3 hours after the injection, while in the normal mice endotoxin caused almost no change or, in some cases, rather an increase of the enzymes. These results do not appear to give us a clearcut answer in regard to the origin of acid hydrolases released into the plasma of BCG-infected mice in response to endotoxin. However, when the protein content in a whole liver (about 250 mg in BCG-infected and 140 mg in normal mice) and the ubiquitous distribution of phagocytic cells in a whole animal are considered, a slight decrease in acid hydrolases per mg of cellular or tissue protein may represent the release of a significant amount of these enzymes into the circulation. Furthermore, a significant decrease of the number of MP in the peritoneal cavity was observed invariably in BCG-infected mice after injection of endotoxin.

Effect of PPD on Acid Hydrolases in BCG-Infected Mice.—The data obtained in the preceding sections indicate that hyperreactive mice respond to endotoxin by the release of a large amount of acid hydrolases, especially β-glucuronidase, into the blood. Since previous experiments (11–13) had suggested that delayed hypersensitivity played little if any role in hyperreactivity to endotoxin, the effect of PPD on acid hydrolases in the plasma was determined in normal and BCG-infected mice.

TABLE IV

Effect of Administration of PPD on Plasma Levels of Acid Hydrolases in Normal and BCG-Infected Mice

Hrs. after PPD*	Acid phosphatase (3)		β-Glucuronidase (4)	
	Normal	BCG	Normal	BCG
Before	0.787 ± 0.121	1.573 ± 0.266	2.0 ± 2.3	20.8 ± 8.1
2	0.630 ± 0.221	2.347 ± 0.277	2.1 ± 1.7	260.4 ± 41.3

* 2500 μg and 1000 μg intravenously in normal and BCG mice respectively.
For further explanations see Table I.

Effect of PPD on Plasma Levels of Acid Hydrolases.—Mice infected with BCG 10 to 14 days previously and control mice were injected intravenously with 1000 and 2500 μg of PPD respectively. Plasma levels of acid phosphatase and β-glucuronidase were determined 2 hours after the infection. These doses of PPD were chosen because 1000 μg of PPD was close to an LD_{50} in BCG animals, and furthermore some difficulty was experienced in making a homogeneous suspension containing more than 2500 μg of PPD in 0.2 ml.

Table IV shows that 1000 μg of PPD caused about 1.5- and 12.5-fold increases of acid phosphatase and β-glucuronidase, respectively, in plasma of BCG-infected mice. In control mice 2500 μg of PPD caused almost no change in plasma levels of both enzymes. The results of several experiments in which

amounts less than 1000 μg of PPD were given to BCG-infected mice, showed no change of plasma level of the enzymes in such animals.

The small increase of plasma β-glucuronidase in BCG-infected mice elicited by PPD could either be attributed to specific delayed-type hypersensitivity or to the effect of a small amount of endotoxin contaminating PPD. To examine this possibility, the following experiment was carried out.

Effect of Desensitization with Endotoxin on Increase of Plasma Acid Hydrolases Caused by PPD.—Mice infected with BCG were divided into 2 groups; the mice of one group were desensitized with endotoxin according to the same

TABLE V

Effect of Desensitization with Endotoxin on Mortality and Plasma Levels of Acid Hydrolases in BCG-Infected Mice in Response to PPD

PPD	Mortality		Acid phosphatase in plasma		β-Glucuronidase in plasma	
	Control (BCG)	Desensitized	Control (BCG)	Desensitized	Control (BCG)	Desensitized
μg i.v.						
2500	9/9	6/8	7.200	1.100	450.0	13.0
1000	9/9	3/8	6.160	1.260	393.8	39.0
0	—	—	1.500	1.160	11.0	6.0

For further explanations see Tables I and II.

schedule as in the previous experiment, and the mice of the other group were kept without the treatment. On the day following the last injection with endotoxin, 1000 and 2500 μg of PPD were injected intravenously into the desensitized and the untreated mice, and the mortality and plasma levels of acid phosphatase and β-glucuronidase were examined as in the preceding experiment. The results are shown in Table V.

Desensitization with endotoxin decreased not only the mortality but also the extent of elevation of plasma acid hydrolases of BCG-infected mice in response to PPD. Thus, in the desensitized group, 3 out of 9 mice survived the injection of 2500 μg of PPD, while in the BCG-infected control group 1000 μ of PPD killed all of 9 mice. Parallel with the decrease of the mortality in the desensitized group, PPD caused almost no change of plasma level of acid phosphatase and only about 2- to 6-fold increase of β-glucuronidase, while in BCG-infected control mice PPD caused about 4- and 40-fold increases of acid phosphatase and β-glucuronidase, respectively. These results are similar to those obtained in the desensitized mice in response to endotoxin (see Table III), and may be suggestive for contamination of the PPD preparation with endotoxin.

DISCUSSION

The results presented in this paper deal with the effect of endotoxin on the levels of acid hydrolases in the hyperreactive state induced by infection with BCG. Studies of hyperreactivity to endotoxin induced by BCG or other infections suggested that it represented a phenomenon in its own right, in as much as the state of heightened sensitivity applies only to the lethal action of endotoxin and to none of the other manifestations of its biological activities. Furthermore, there is little, if any, indication that this reaction can be attributed to immediate or delayed hypersensitivity or that it is related to the Shwartzman phenomenon (11–14). The results reported here demonstrated that hyperreactive mice respond to endotoxin by the release of acid hydrolases, especially of β-glucuronidase, into the blood. The magnitude of increase of these enzymes in the plasma showed fairly good correlation with the degree of sensitivity of the mice to the lethal effect of endotoxin. In addition, desensitization of hyperreactive mice with endotoxin reduced in a parallel fashion both activities of endotoxin. It cannot be decided yet whether this release of hydrolases represents an essential link in the development of endotoxin shock or is merely a symptom of it.

There is evidence that endotoxin may act as a releaser of lysosomal enzymes. Thus, Martini (5) showed that administration of endotoxin to rats facilitated the release of cathepsin from skeletal muscle and liver, and Weissmann and Thomas (6) found that the injection of endotoxin into rabbits caused subsequently the release *in vitro* of acid hydrolases from lysosomes prepared from the liver. This reaction could not be elicited entirely *in vitro*. In addition, Janoff *et al.* (15) observed about a 2-fold increase of plasma levels of acid phosphatase and β-glucuronidase after administration of one LD_{50} of endotoxin into normal rats. The magnitudes of increase of plasma levels of acid hydrolases in hyperreactive mice upon injection of endotoxin were shown to be much greater, amounting to a 3- to 8-fold increase of acid phosphatase and a 30- to 100-fold increase of β-glucuronidase. The reaction in control mice was about the same as that observed by Janoff *et al.* (15).

The rise of plasma levels of hydrolases in response to endotoxin in hyperreactive animals may be ascribed to an altered state of sensitivity of the lysosomal membrane to endotoxin with resulting release of its enzyme content. Alternatively, it may be due to an impairment of degradation or elimination of enzymes in the blood. Little information is available in this respect. The discrepancy of response of acid phosphatase and β-glucuronidase may indicate some rather significant difference between the various enzymes contained in lysosomes. As suggested by Sawant *et al.* (16) the degree of binding of an enzyme to the lysosomal membrane could determine its release. On the other hand, a difference in the rate of decay of various enzymes has been made responsible for differences in their blood level of tolerant animals during trau-

matic shock (15). The reported effect of desensitization with endotoxin on the release of enzymes by shock supports the former interpretation. The lack of release of β-glucuronidase after desensitization is not due to the depletion of lysosomes of this enzyme, since levels of the enzyme in the liver remained the same after the procedure. One has to assume that endotoxin exerts its action on the lysosomal membrane, and desensitization renders the latter more refractory (6, 7, 15).

The specific activities of acid hydrolases in liver, MP, and blood leucocytes of hyperreactive mice were found to decrease after administration of endotoxin. Although these results are statistically not significant, the loss of an almost undetectable amount of enzyme from the RES as a whole may suffice for the enzyme found in the plasma. The finding that MP disappear from the peritoneal cavity from mice hyperreactive to endotoxin after intraperitoneal injection of endotoxin supports this concept. Guinea pigs with delayed hypersensitivity to tuberculin show a similar phenomenon upon injection of the antigen (17).

The similarity of effects of large amounts of PPD and small amounts of endotoxin on acid hydrolases of hyperreactive mice suggests several explanations. At the surface it appears to support the view of Stetson (18) that most manifestations of endotoxin are mediated through reactions similar, if not identical, to delayed hypersensitivity. However, the fact that desensitization of hyperreactive mice with repeated injections of endotoxin decreased not only the effect of endotoxin but also that of PPD suggests a contamination of PPD with endotoxin as originally proposed by Stetson (18). There remains the possibility that delayed type hypersensitivity also causes release of acid hydrolases from lysosomes.

The findings reported and discussed in this paper, as well as those by Weissmann and Thomas (6) and Thomas (8) demonstrating the rupture of leucocyte granules with release of particle-bound enzymes in Shwartzman and Arthus reactions, suggest that further studies of these enzymes may help in the elucidation of endotoxin action and especially of hyperreactivity to it.

SUMMARY

Experiments are reported dealing with the correlation between activities of lysosomal acid hydrolases and hyperreactivity to endotoxin induced by BCG infection. Acid hydrolases were determined quantitatively in peritoneal MP, liver homogenate, and plasma of normal and hyperreactive mice. Mice infected with BCG not only exhibited a hyperreactive state to lethal effect of endotoxin, but also responded to endotoxin by rapid increase of acid hydrolases, especially of β-glucuronidase, in the plasma; whereas control mice responded to endotoxin by almost no change in plasma acid hydrolases. The extent of increase of β-glucuronidase in plasma of hyperreactive mice was shown to correlate fairly well with the degree of hyperreactivity to the lethal effect of endotoxin.

Desensitization of such animals with endotoxin was found to cause a decreased response of plasma β-glucuronidase parallel with decreased mortality. A large amount of PPD exerted the similar effect to that of endotoxin in hyperreactive mice. Furthermore, the effect of PPD was decreased by desensitization of such animals with endotoxin, a fact which suggests contamination of PPD with endotoxin.

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