Plasma β-Lysin and Lysozyme Following Endotoxin Administration and the Generalized Shwartzman Reaction[1] (35291)

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The bactericidal compounds β-lysin and lysozyme are present in high concentration in platelets and leukocytes, respectively. They have also been found free from these cellular elements in a number of normal body fluids including saliva and plasma. However, the quantity of extracellular β-lysin and lysozyme in these fluids is normally very small when compared with the quantity contained in leukocytes and platelets (1). Recent studies have elucidated several ways in which large quantities of β-lysin and lysozyme can be released from these cellular elements into the surrounding tissues and fluids within a few hours after stimulation (1, 2). The stimuli for such releases include: antigen–antibody reactions, inflammatory reactions, blood coagulation, and the intravenous injection of bacteria or other particulate material. The magnitude of these releases and their intimate association with such important immunological phenomena as antigen–antibody reactions, and inflammation leads to the speculation that these nonspecific bactericidal compounds may play a major role in the control of certain infections. The present results are another means by which β-lysin and lysozyme may be released from platelets and leukocytes into plasma.

Materials and Methods. The endotoxin used was an *Escherichia coli* lipopolysaccharide (Difco 0127:B8) prepared by the Boivin trichloroacetic acid procedure suspended in physiological saline solution (PSS). The generalized Shwartzman reaction (GSR) was induced by the iv injection of 100 μg of endotoxin/kg of body weight 24 hr after the iv injection of 10 μg of endotoxin/kg. Control rabbits were injected with PSS rather than endotoxin.

Blood samples were collected by cardiac puncture in sodium citrate (0.38%), and centrifuged for 5 min at 900*g* to obtain plasma. The leukocyte number was determined by the hemocytometer method utilizing Turk's acetic diluting fluid, and the platelet number was determined by the Brecher-Cronkite method. Techniques for the plasma β-lysin assay were previously described (3, 4). Lysozyme was assayed by Jollès' technique (5). Three times crystallized egg-white lysozyme (Reheis Chemical Company, Control No. C18306) was used as the enzyme standard.

A number of rabbits died during the *in vivo* experiments but each point in Fig. 1 and 2 is the average obtained from a minimum of eight rabbits. Student's *t* test was used in all statistical analyses. In accordance with standard statistical methods, a logarithmic transformation was employed in calculations involving β-lysin values.

Results. Figure 1 illustrates the alterations observed in platelet number and plasma β-lysin concentration following an endotoxin injection or the induction of a GSR. The β-lysin level was not significantly altered following a single iv injection of 10 or 100 g of endotoxin/kg even though the platelet number decreased by almost half. This is the first time we have observed a decrease in circulating platelets without a corresponding increase in plasma β-lysin. Apparently, the platelets are being removed from circulation without physically breaking up and releasing their β-lysin. Higher doses of endotoxin up to 1000 μg/kg were injected but in no case was a significant increase in plasma β-lysin ob-

[1] Supported in part by Research Grant AI-01171 and Predoctoral Fellowship GM-31764 from the U.S. Public Health Service.

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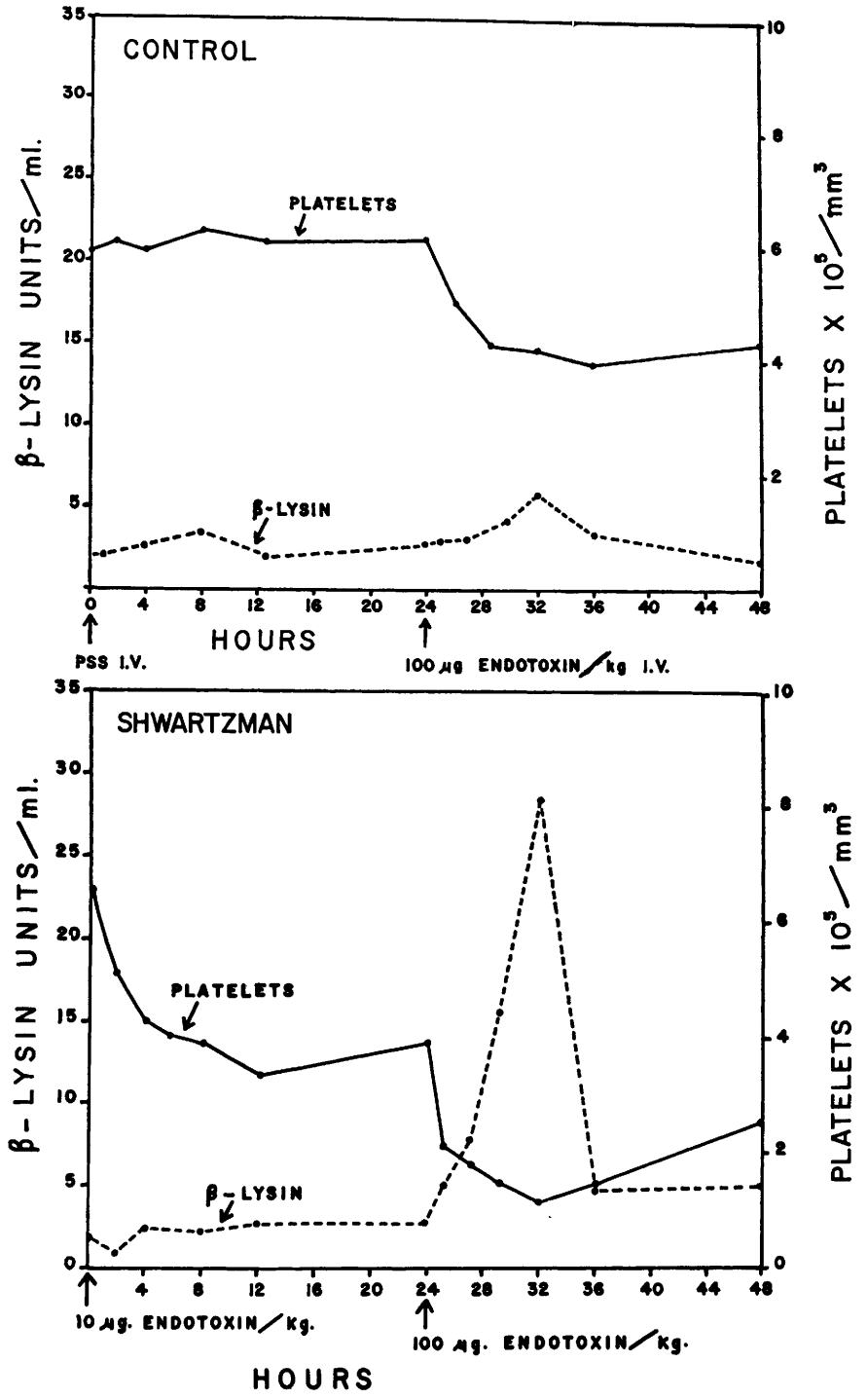

FIG. 1. Effect of endotoxin administration and the GSR on plasma levels of free β-lysin and circulating platelet.

served following a single injection.

The GSR induced with two injections of endotoxin resulted in a dramatic increase in the average β-lysin concentration and a substantial drop in circulating platelets (Fig. 1). A statistical analysis of this data indicated that the plasma β-lysin was significantly higher than that of animals receiving a single 100-μg endotoxin/kg injection in the period between 29 and 32 hr.

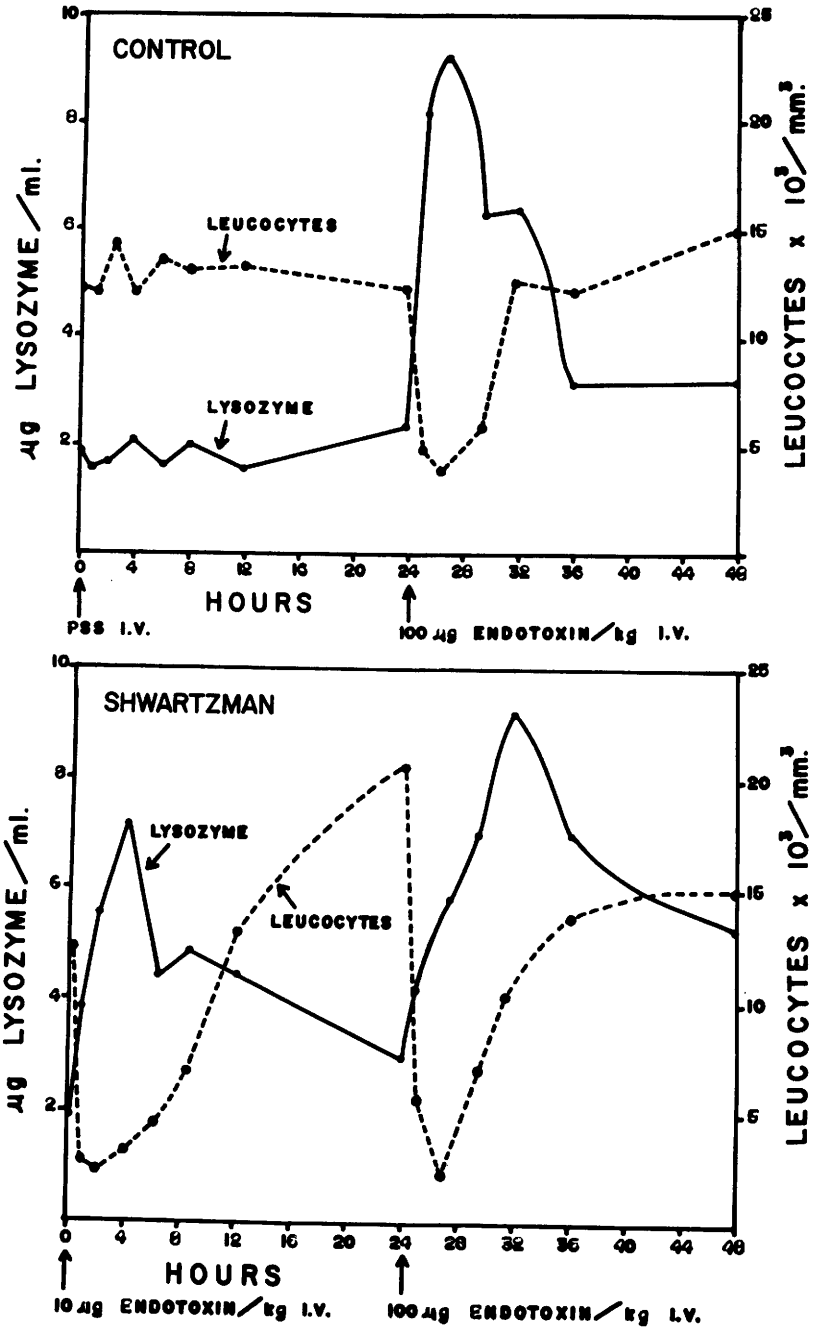

FIG. 2. Effect of endotoxin administration and the GSR on plasma levels of free lysozyme and circulating leukocytes.

TABLE I. Extracellular Lysozyme Levels Following the Addition of Endotoxin to Whole Blood *in vitro*.

Sample	Lysozyme (μg/ml); additions to blood samples: PSS	2 μg of endotoxin/ml	*p* value
Blood from normal rabbits	1.1 ± 0.24[a]	1.1 ± 0.24	>0.9
Blood from rabbits previously injected with endotoxin	1.2 ± 0.09	2.8 ± 0.31	<0.001

[a] Mean ± standard error.

A similar statistical comparison of platelet concentrations revealed that platelet levels in the GSR were significantly lower at all times except at 48 hr. At this time the platelets in animals recovering from the Shwartzman reaction were returning toward normal. This release of β-lysin could be attributed to *in vivo* blood coagulation which is known to occur during the GSR.

The changes in leukocyte number and plasma lysozyme concentrations following the iv injection of endotoxin and the GSR are illustrated in Fig. 2. In all cases, lysozyme increases were accompanied by leukocyte decreases and lysozyme decreases were accompanied by leukocyte increases. As with β-lysin and platelets, a statistical analysis of the data revealed that the bleeding procedures did not significantly alter either lysozyme or leukocytes. In contrast to the negative β-lysin results, a single injection of endotoxin resulted in a dramatic increase in lysozyme and a corresponding decrease in leukocytes. These results are in accord with those reported by Ribble (6) who observed plasma lysozyme increases following single injections of typhoid vaccine. These alterations in leukocytes and lysozyme were significant through the 30th hour for the leukocytes and 32nd hour for the lysozyme. After these times the leukocyte and lysozyme values were almost back to normal levels.

A comparison of the data obtained following the iv injection of 100 μg of endotoxin/kg with the data obtained following the induction of a GSR, revealed only two significant differences. The starting leukocyte number was higher in the Shwartzman reaction where the animals had been treated 24 hr earlier with endotoxin, and the lysozyme level was slightly lower in the GSR animals 1 hr post-100 μg injection. Because of positive results following a single endotoxin injection, it was not possible to attribute these changes in leukocytes and lysozyme to the GSR.

Little kidney damage was evident in these reactions elicited using *E. coli* endotoxin 0127:B8. Similar doses of *E. coli* endotoxin 055:B5 caused multiple kidney lesions in 9 of 11 rabbits. Samples from these rabbits showed the typical GSR changes in platelets, leukocytes, β-lysin, and lysozyme. However, the releases of β-lysin and lysozyme did not correlate with the production of kidney lesions.

In vitro *studies*. These studies were undertaken to determine whether the β-lysin and lysozyme results obtained *in vivo* could be duplicated in a simple *in vitro* system. Blood samples for these studies were obtained from both normal rabbits and rabbits injected 24 hr earlier with 10 μg of endotoxin/kg. The citrated blood from each animal was divided into two 10-ml aliquots. One aliquot was treated with sufficient endotoxin to make a final concentration of 2 μg/ml and the other received a similar quantity of PSS. Following incubation for 2 hr at 37°, the leukocyte number was determined and the plasma was collected and assayed for β-lysin and lysozyme. As shown in Table I, endotoxin did not induce lysozyme release in normal blood, but endotoxin stimulated a lysozyme release in blood obtained from rabbits injected previously with endotoxin. Although this release was not as great as the release observed *in vivo*, it was significant and took place in all blood samples tested. The lysozyme increase in this *in vitro* imitation of a Shwartzman reaction did not appear to be

due to leukocyte autolysis, since the leukocyte counts in endotoxin-treated samples and samples treated with PSS were comparable. The release of lysozyme from phagocytes following a single injection of endotoxin *in vivo* appeared to be indirect, since the addition of endotoxin to whole blood *in vitro* did not cause a release of lysozyme or a decrease in leukocytes, even in sample treated with 50 μg of endotoxin/ml. The β-lysin results in this experiment were inconclusive since only 10 of 18 blood samples from rabbits previously injected with endotoxin showed increases in β-lysin.

Discussion. A number of interesting findings have been reported from experiments designed to determine the effect of bacterial endotoxin on rabbit platelets (7–11). An *in vivo* study of the distribution and clearance of circulating endotoxin revealed that most if not all the endotoxin present in blood elements was associated with platelets (9). Des Prez *et al.* (7, 8) reported that *in vitro* exposure to endotoxin caused platelets to aggregate and to release numerous substances including: 5-hydroxytryptamine, platelet factor 3, platelet phospholipid, and a heat-stable bactericidin active against *Bacillus subtilis* (β-lysin). In view of these findings, it was anticipated that an iv injection of endotoxin would release β-lysin from platelets into plasma. However, such a release was not observed in this study. These results may be explained by the differences in endotoxin dosage and in endotoxin preparation. In these *in vivo* experiments, the highest endotoxin dosage used was 100 μg of endotoxin/kg of body weight (which was near the lethal level). Assuming that there is approximately 70 ml of blood/kg body weight in the rabbit, the final endotoxin concentration in the blood would have been only 1.4 μg/ml. In contrast, Des Prez *et al.* used 100 μg of endotoxin/ml in their *in vitro* experiments. The other factor which could have been responsible for the β-lysin release reported by Des Prez *et al.* related to the particulate nature of their endotoxin preparation. In his 1967 publication, Des Prez pointed out that ". . . endotoxin-platelet interaction requires rather large particles of endotoxin. Supernatant fluids from endotoxin suspension centrifuged at high speeds are completely inactive" (11). Mustard *et al.* have reported the release of a number of biologically active substances following interaction between platelets and many types of particles (12). Furthermore, a recent report from this laboratory describes the release of β-lysin from platelets following exposure to bacteria (2). In order to specifically ascribe the release of β-lysin from the platelet to endotoxin, it would be necessary to first eliminate the effect of platelet particle interaction.

The lysozyme results obtained following endotoxin injection and the GSR were as expected in the light of earlier work (2, 6, 18). However, the addition of endotoxin to citrated whole blood *in vitro* did not result in a release of lysozyme. A lysozyme release was anticipated since Kerby had reported an *in vitro* endotoxin-induced lysozyme release from a purified suspension of human leukocytes (14). The reason for this difference is not clear, but it may also relate to the endotoxin preparation. An earlier report from this laboratoy demonstrated that leukocytes would release *in vitro* in the presence of particulate matter (2).

Even though the lysozyme levels of animals treated with endotoxin and animals given the GRS were comparable, it is apparent from the *in vitro* studies that there were differences. Leukocytes present in the blood of animals primed from the Shwartzman reaction released lysozyme following endotoxin exposure, whereas leukocytes in the blood of normal animals did not. Horn and Collins (13) have shown that large numbers of granulocytes from the bone marrow enter the circulation following the first endotoxin injection. Many of these granulocytes may be immature and therefore more susceptible to endotoxin or perhaps they become sensitized by the initial endotoxin injection. In any case, these leukocytes responded to endotoxin by releasing lysozyme, whereas normal leukocytes were refractive.

Summary. A single iv injection of from 10 to 1000 μg of endotoxin/kg into rabbits did not alter the plasma β-lysin levels, but did cause a marked decrease in platelet number. The same endotoxin injections resulted in a 4- to 5-fold increase in plasma lysozyme and

a dramatic drop in circulating leukocytes. Both β-lysin and lysozyme were released during generalized Shwartzman reaction. These increases in β-lysin and lysozyme were accompanied by corresponding decreases in platelets and leukocytes, respectively. *In vitro* studies revealed that leukocytes in citrated whole blood, obtained from animals primed for the Shwartzman reaction, released lysozyme following the addition of endotoxin; whereas leukocytes in blood taken from normal animals did not.

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Received Sept. 1, 1970. P.S.E.B.M., 1971, Vol. 136.