ELEVATION OF PROPERDIN LEVELS IN MICE FOLLOWING ADMINISTRATION OF BACTERIAL LIPOPOLYSACCHARIDES*

By MAURICE LANDY‡, Ph.D., and LOUIS PILLEMER, Ph.D.

(From the Walter Reed Army Institute of Research, Washington, D. C. and the Institute of Pathology, Western Reserve University, Cleveland)

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It is well established that certain bacterial products may alter non-specific defense mechanisms of the host (1, 2). Many examples have been described of a rapid and transient increase in resistance to experimental infection following the injection of killed bacteria (3–5) and bacterial products (5). However, the identity or character of the bacterial constituents responsible for this non-specific immunity has not been clearly defined, nor has the mechanism of this early resistance been elucidated. It is clear that it is not associated with detectable antibody formation.

The intravenous injection of cell walls of *Escherichia coli* and *Salmonella typhimurium* (6) evokes in mice this type of resistance to intraperitoneal challenge with *E. coli*. Zymosan (7), an insoluble carbohydrate derived from cell walls of yeast, produces a similar resistance against a variety of Gram-negative pathogens (6,8–10). Bacterial cell walls, and zymosan, also combine with properdin *in vitro* (11, 12) and alter serum properdin levels *in vivo* (9). Evidence now suggests that a relationship exists between the serum properdin levels of various animals and their resistance or susceptibility to certain experimental or natural infections. The properdin system (11), which consists of properdin, complement, and magnesium, kills or inactivates a variety of infectious agents *in vitro* and appears to be a factor in natural resistance.

It has been shown that a purified, protein-free lipopolysaccharide (endotoxin) derived from *S. typhosa* (13), combines with properdin *in vitro* (12). The injection in mice of a large amount (100 μg.) of this material causes a fall in their properdin titers during the first 12 to 24 hours; this drop is followed by an increase in titer which persists for 96 to 120 hours (12). Recently, it was reported (14) that a small amount (0.1 to 10 μg.) of a variety of bacterial lipopolysaccharides evokes in mice a rapidly developing, non-specific, transient, increase in resistance to infection with various Gram-negative pathogens.

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‡ Present address: Laboratory of Chemical Pharmacology, National Cancer Institute, Bethesda.

To resolve and integrate certain aspects of the aforementioned experimental work, it was felt that two issues had to be clarified further: first, the influence of such factors as time between injection and obtaining the blood sample, the character of the bacterial product, and the dose employed, on the nature, magnitude, and duration of the properdin response; and secondly, whether the elevated properdin titers reflect the ability of the host to resist Gram-negative infections.

The present work is concerned with the first of these two issues and shows that the dose of lipopolysaccharide and the interval from time of administration affect the degree to which these levels of properdin are increased. These alterations in properdin levels are also accompanied by the appearance in the serum of substances of endogenous origin; these are complex materials of high molecular weight.

The second issue, *viz.* the relationship of these findings to resistance to infection, is the subject of a forthcoming report (15) in which it is shown that the level of serum properdin during the infectious process appears to be of greater significance than the titer at the time of initiation of infection.

Materials and Methods

Bacterial Lipopolysaccharides[1].—The lipopolysaccharide derived from *Salmonella typhosa* 0901 by the isolation procedure of Webster *et al.* (13), containing 0.6 per cent nitrogen (of which 0.4 per cent is attributable to hexosamine), 2 per cent phosphorus, 33 per cent lipide, and 65 per cent polysaccharide was used in most of the experimental work. Incompletely characterized lipopolysaccharides prepared by Webster *et al.* from *Hemophilus pertussis*, *Shigella flexneri*, *Pseudomonas aeruginosa*, and *Serratia marcescens* by the aforementioned isolation procedure, with certain modifications, were also examined. Other lipopolysaccharide preparations generously provided by different investigators included the following: from *E. coli* 08 and 018, *S. typhosa* 0901, and rough variants of *Salmonella enteritidis* and *Salmonella schottmuelleri* (Dr. O. Westphal); from the rough variant of *S. typhosa* 0901 (Dr. M. Raynaud); from *Serr. marcescens* (Dr. M. Shear); from *Ps. aeruginosa* (Dr. M. Usdin); from *E. coli* 026, 055, and 0127, *Serr. marcescens* and *S. typhosa* 0901 (Difco Laboratories, Mr. H. W. Schoenlein).

Injection and Bleeding of Experimental Animals.—Female albino mice of the Bagg strain, weighing 14 to 16 gm. were employed. Since pilot experiments had indicated that the biological effects produced in mice by lipopolysaccharide injected intravenously or intraperitoneally were essentially identical, injections were made thereafter *via* the more convenient intraperitoneal route. The mice were exsanguinated by bleeding directly from the heart under chloroform anesthesia. Generally, 0.5 to 0.8 ml. of blood was obtained from each animal and blood from groups of 10 or more mice was pooled to yield a single serum speci-

[1] The term "lipopolysaccharide" is not intended to imply that such bacterial products are necessarily homogeneous or composed exclusively of polysaccharide and lipide. For the most part, these substances, as customarily prepared, also contain nitrogenous and other associated materials in varying amount. Until more is known about which features of chemical structure are essential for each or all of the many biological properties possessed by these complex materials, the terminology should be understood not to imply more than the foregoing.

men for properdin titration. In certain instances, as many as 100 mice were employed to obtain serum pools. Serum was frozen promptly and held at −55°C.

Properdin Titrations.—Serum samples were thawed at room temperature, aliquots were centrifuged at 35,000 *g* at 2°C. for 2 hours and the supernatants carefully decanted. The untreated serum and centrifuged sample were then tested for properdin by the zymosan assay (16); some samples were also tested in the bactericidal assay. All serum samples of any given experiment were titrated for properdin content on the same day and with the same reagents.

TABLE I

Properdin Levels of Mice Following Injection of Lipopolysaccharides

Effect of removal of high molecular weight substances

Lipopolysaccharide derived from	Amount injected i.p.	Treatment of serum pools prior to properdin assay	Hrs. elapsed between injection of lipopolysaccharide and exsanguination						
			3	6	12	24	48	72	120
			Units properdin per ml. of serum*						
	μg.								
Salmonella typhosa, 0901	10	None	24	9	12	15	24	24	24
		Centrifuged‡	12	18	30	36	36	36	24
Escherichia coli, 08	10	None	24	24	12	12	24	30	30
		Centrifuged‡	12	24	30	30	30	36	36
—	0.5 ml. of saline	None				12			
		Centrifuged‡				12			

* Groups of 100 mice were employed to obtain the serum pools representing each product-time interval.

‡ 35,000 *g* for 2 hours at 2°C.

RESULTS

In orientation experiments on the properdin titers of serum from experimental animals injected with 1 to 10 μg. of bacterial lipopolysaccharides, it was noted that the titers were erratic and failed to show any consistent trend or pattern. It had already been shown (12) that high molecular weight polysaccharides of bacterial and mammalian origin combine with properdin and inactivate C'3 *in vitro*. It was emphasized (12) that the presence of these substances in serum interferes both with properdin and C'3 assays and that high molecular weight polysaccharides first had to be removed by centrifugation at 35,000 *g* in order to obtain reliable titers.

A series of experiments was carried out to ascertain whether the removal of high molecular weight substances from serums obtained from animals receiving minute amounts of bacterial lipopolysaccharides would influence their properdin titers. Table I shows properdin titrations on mouse serums before and after centrifugation at 35,000 *g* for 2 hours. It will be noted that

within 3 hours after administration of lipopolysaccharide the uncentrifuged serum had a properdin titer of 24 units/ml., while the same serum centrifuged and the serums of control mice had a titer of 12 units/ml. The results of previous studies on the *in vitro* interaction of polysaccharides with the properdin system (12) suggest that a properdin-polysaccharide complex may be formed at this time that would inactivate C'3 and thereby give erroneous high titers in the zymosan assay for properdin. It is apparent that between 6 hours and approximately 3 days following administration of lipopolysaccharides the untreated serums of these mice show lower, and at times, erratic properdin values. On the other hand, after centrifugation the same serums present a consistent pattern of early rise in properdin levels reaching a maximum of approximately 3 times normal, which is maintained for several days. Thus a series of events follows the injection of 10 μg. of lipopolysaccharide into mice, *viz.*, during the first 3 hours part of the serum properdin appears to form a complex with a new substance in serum which inactivates C'3. This is followed after 6 to 12 hours by the appearance in the blood of high molecular weight substances which interfere with the inactivation of C'3 by zymosan. These high molecular weight substances persist for 48 to 72 hours after which time the properdin levels are not altered by centrifugation. The appearance of these high molecular weight substances in the circulation shortly after injection of lipopolysaccharide suggests that they are elaborated or released by the host following stimulus or injury by lipopolysaccharide. While lipopolysaccharide previously has been shown to combine with properdin *in vitro* the quantities required are of a completely different order of magnitude inasmuch as the *in vitro* interactions require 100 to 200 times the amount necessary for the described *in vivo* effects.

The nature of these host products is now being investigated. Preliminary evidence suggests that they are protein–lipide–polysaccharide complexes. In this connection it is particularly significant that Shear and Perrault recently have succeeded in isolating polysaccharide complexes from a variety of mammalian tissues (17) and that these also interact both *in vitro* and *in vivo* in diverse ways with the properdin system (18). These results, therefore, suggest that following the injection of lipopolysaccharide, substances are elaborated into the blood which interfere with the assay of properdin. Serums containing these substances require high speed centrifugation, for their removal, before assays are conducted.

Effect of Lipopolysaccharides of Varied Generic Origin.—Lipopolysaccharides derived from smooth and rough strains of *Escherichia*, *Hemophilus*, *Pseudomonas*, *Salmonella*, *Serratia*, and *Shigella* were tested for their effect on properdin levels of mice. These products were of varied generic origin and it is also noteworthy that they were prepared in six different laboratories employing different methods of fractionation and purification. A total of 16 products was examined and 3 selected preparations were studied thoroughly. Blood speci-

mens drawn 24 hours after intraperitoneal injection of 1 or 10 μg. of each of the different lipopolysaccharides disclosed that, in most instances, 10 μg. elevated properdin levels and in a few instances even 1 μg. caused a significant rise. A definitive study may well reveal quantitative and perhaps even qualitative differences between these products. Thus the factor or factors responsible for increasing properdin levels can be isolated by varied procedures from most Gram-negative bacterial species of both smooth and rough colonial types. It is again pointed out that these products may not be single chemical entities and probably consist of various polysaccharides, proteins, and lipides, differing qualitatively and quantitatively in their constitution and structure.

TABLE II

Properdin Levels of Mice Following Injection of Lipopolysaccharide

Time-dose relationships

Amount of *S. typhosa* lipopolysaccharide injected i.p.	Hrs. elapsed between injection of lipopolysaccharide and exsanguination						
	1	6	12	24	48	72	120
	Units properdin per ml. of serum*						
μg.							
100	18	18	9	12	36	36	36
10	18	24	30	36	36	30	24
1	18	24	24	30	24	24	24
0.1	18	18	18	18	18	18	18
0.5 ml. saline	18	18	24	18	18	18	18

* The properdin values given were obtained on serum pools from groups of ten mice at each dose-time combination. All sera were centrifuged for 2 hours at 35,000 *g* prior to properdin assay.

Time and Dose Relationships.—In the foregoing sections, it was shown that changes in properdin levels occurred within a few hours after injection of lipopolysaccharide. However, the effect of the nature of the product, the amount injected and time elapsed between injection and bleeding upon the character and magnitude of the response remained to be determined. The results of a typical experiment as given in Table II show: (*a*) the injection of 100 μg. caused a fall in properdin titer between 12 and 24 hours which was succeeded by a marked rise which persisted for at least 5 days; the results with this high dosage are essentially identical with those reported (12) earlier; (*b*) the injection of 10 μg. produced an increase as early as 6 hours which then rose to a maximum of 36 units/ml. at 24 hours and persisted up to 72 hours. Attention should be called to the fact that this elevation occurred without the prior fall in properdin titers observed when large doses of zymosan are employed. This effect may, therefore, be due to a direct stimulation of the synthesis

or release of properdin rather than to a mechanism which represents overcompensation "rebound" on the part of the host. (*c*) The effect of 1 μg. was qualitatively similar to that obtained with 10 μg. except that the increase in properdin titer was somewhat less, (*d*) essentially no alteration in properdin levels occurred following injection of either 0.1 μg. of lipopolysaccharide or of 0.5 ml. of saline. Indeed the constancy of the values in control animals is noteworthy. Thus, in the mouse, the quantity of lipopolysaccharide employed determines not only the rate at which properdin levels rise, but also the magnitude and duration of this elevation.

In a subsequent publication (15) it will be shown that in mice treated identically with those sacrificed for properdin assay the degree of susceptibility or resistance to infection was on the whole related to the properdin titers as here described. Thus, the injection of 10 μg. of lipopolysaccharide resulted in increased resistance as early as 6 hours and this resistance to

TABLE III

Properdin Levels in Mice Following Challenge with Salmonella typhosa

Treatment 24 hrs. prior to challenge	Challenge	Handling of serum pools prior to properdin assay	Pre-challenge	Hrs. elapsed between challenge and exsanguination				
				3	6	12	18	24
			Units properdin per ml. of serum					
10 μg. *E. coli* lipopolysaccharide	50 million *S. typhosa* Ty_2 in saline injected i.p.	None	18	15	18	18	24	30
		Centrifuged*	30	18	18	24	42	42
0.5 ml. saline		None	18	18	18	12	6	6
		Centrifuged	18	18	18	12	6	6

* 2 hours at 35,000 *g*.

challenge (with *S. typhosa* or *E. coli*) was maintained for 24 hours or more. As shown above, the properdin titers were elevated during this period.

Influence of Infection on Properdin Response.—In the foregoing experiments, properdin titers were determined in normal mice. Other experiments were undertaken to determine titers during infection. During the course of this work, (15), it became apparent that the properdin levels of the animal prior to infection did not indicate the ability of the host to maintain this titer during infection. It also became clear that following the initiation of an experimental infection, host-parasite interactions become extremely complex and the properdin levels during this period are altered from those of the prechallenge pattern.

It has been observed (19) that properdin titers fall in mice following injection of lethal doses of viable *E. coli*. However, studies have not been reported on animals previously treated with lipopolysaccharide and then experimentally infected. The results of one such experiment (Table III)

shows this properdin response by the host. The data reveal that the properdin values for the control mice are quite unaffected by centrifugation while the properdin levels in lipopolysaccharide-treated animals exhibit marked differences following centrifugation of serum. This shows that the production of endogenous high molecular weight substances occurs in response to the lipopolysaccharide stimulus or injury. Fig. 1 graphically portrays the relationship of the bacteriological sequence of events to the serum properdin alterations which occur following initiation of infection. In control mice, properdin titers

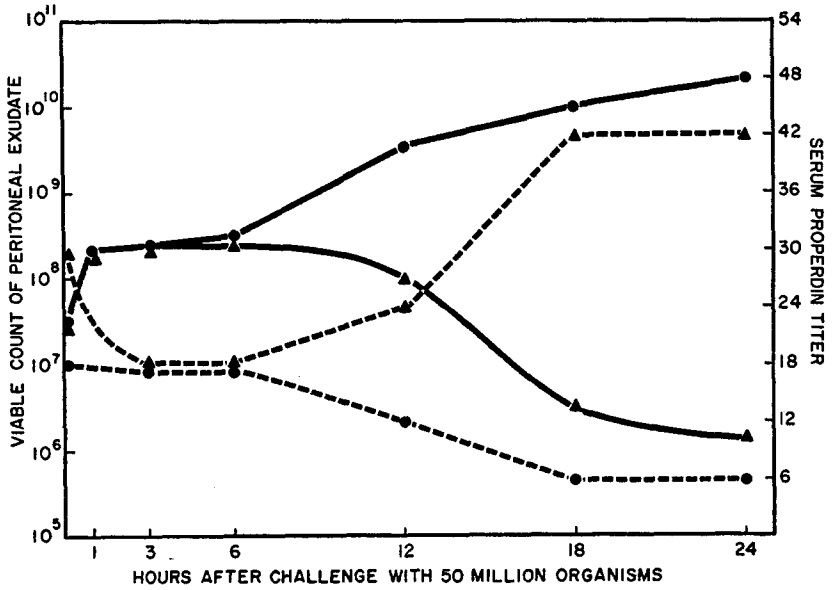

FIG. 1. Non-specific resistance to infection with *S. typhosa* developed in mice following administration of a bacterial lipopolysaccharide.

▲, mice injected with 10 μg. *coli* lipopolysaccharide 24 hours prior to challenge; ●, control mice; —, viable count; ----, properdin titer.

remain constant during the period of 3 to 6 hours post challenge. However, by 12 hours, when deaths first begin to occur, there is a significant drop in properdin which in time becomes even more pronounced as an increasing proportion of the animals become moribund. These changes are correlated with increasing bacterial populations in these animals. On the other hand, mice treated with 10 μg. of *coli* lipopolysaccharide 24 hours prior to challenge exhibit a completely contrasting pattern of bacteriologic and properdin responses. Indeed at 12 to 18 hours post challenge, at the very time when bacterial populations are rapidly rising to lethal levels and the properdin reserves are being depleted in the controls, the lipopolysaccharide-treated mice are not only maintaining their original properdin levels but these are, in fact, increased within 12 hours to a value of 42 units, which is extremely high for

this species. At the very same time bacterial multiplication is rapidly brought under control and the number of organisms decline to well below lethal levels. Thus at the time the control mice are dying rapidly, the properdin titer of the lipopolysaccharide-treated animals is 7 times higher than the controls.

DISCUSSION

The present work shows that the administration of a single small dose of bacterial lipopolysaccharide produces alterations of major proportions in the properdin level of mice. What lends special significance to this observation is the fact that, heretofore, such elevated properdin titers had been observed after the administration of a variety of high molecular weight polysaccharides only when it had been preceded by an initial depression of properdin titer. The elevation of properdin, as here reported, is not dependent upon the prior

TABLE IV

The Activities of Certain Products of Microbial Origin on Properdin

Product	Amount required for	
	Removal or inactivation of 8 units of properdin *in vitro*	Elevation of mouse properdin levels *in vivo*
	mg.	*mg.*
Bacterial cell walls	0.5–5.0	0.1–1.0
Bacterial lipopolysaccharides	0.5–3.0	0.001–0.01
Dextrans	1–5	0.2–6
Zymosan	0.5–5.0	0.1–1.0

removal of properdin by the agent injected; and perhaps may be quite unrelated to this removal. The data obtained in time-dose studies, such as depicted in Table II, suggest that rather high doses of lipopolysaccharide (100 μg.) are required to depress properdin titers. If this fall in properdin were the result of the combination of properdin and bacterial lipopolysaccharide, it would be expected that it would take place shortly after injection of the lipopolysaccharide. However, the data shown in Table II clearly indicate that during the first 6 hours after injection of 100 μg., properdin titers remain unaltered and it is not until 12 hours after injection that a pronounced fall in properdin concentration is observed. This suggests that in mice even with a high dose of lipopolysaccharide the fall in properdin is not caused by formation of complexes with the injected bacterial lipopolysaccharide.

The large amounts of cell walls, zymosan, levans, and dextrans required to produce the alterations in properdin titer suggest that these effects may be qualitatively different from the bacterial lipopolysaccharides. The activities of these products in combining with properdin *in vitro* and in raising the level of properdin *in vivo* are summarized in Table IV. It will be seen that these substances have comparable activity in their interaction *in vitro* with proper-

din. However, in their stimulation of elevated properdin levels in mice it is evident that, on a weight basis, the lipopolysaccharides possess activity of an entirely different magnitude. Inasmuch as zymosan, dextran, and cell walls are all of microbial origin, the possibility remains that they may contain small amounts of materials resembling bacterial lipopolysaccharide which are actually responsible for the elevated properdin levels reported for these materials.

It is also noteworthy that the purified *typhosa* lipopolysaccharide prepared in this laboratory interacts with properdin but does not inactivate C'3 (12). However, incompletely characterized products obtained in this laboratory by the same basic isolation procedure were shown to inactivate C'3 in addition to reacting with properdin. Since zymosan and certain dextrans, levans, and cell wall preparations inactivate C'3, similar inactivation by certain preparations of lipopolysaccharides may indicate contamination with cellular material. The ability of bacterial lipopolysaccharides to inactivate C'3 may, therefore, prove to be a useful test for evaluating the purity of lipopolysaccharides, or at least their freedom from certain cellular materials.

The appearance of high molecular weight substances in serum following injection of small amounts of endotoxin is a highly significant, but unexpected, addition to an already complex system. Indeed, it will be reported subsequently that lipopolysaccharides evoke similar substances in man but produce high molecular weight agents in rabbits which behave quite differently (20). The discovery that different species elaborate endogenous substances which react differently with properdin may yet lead to a better understanding of antibacterial defense mechanisms, as well as the full effects of lipopolysaccharides in individual species.

Even the limited tests thus far completed make it clear that while most lipopolysaccharides cause a rise in properdin levels, their activity varies considerably with respect to the quantity required and the magnitude and duration of the effect they produce. It remains to be determined whether these differences are related to the bacterial species from which the lipopolysaccharide is derived, to the cultural conditions, or to the isolation procedure employed.

Variations in the general endotoxic properties of bacterial lipopolysaccharides have also been observed by other workers and this is, in effect, a further indication that these materials are heterogeneous and extremely difficult to characterize. Furthermore, as pointed out above, additional information on the relationship of chemical composition to biological activity is necessary to evaluate these substances. On the basis of the results here obtained, it may be concluded that lipopolysaccharides, derived from a number of bacterial genera, from numerous serotypes, from both smooth and rough strains, even though prepared by diverse isolation procedures have in common the ability to elevate properdin titers.

The mouse generally is considered to be refractory to the endotoxic action of bacterial lipopolysaccharides in that it exhibits none of the characteristic syndrome elicited in certain susceptible species. This would suggest that a high degree of susceptibility to the lethal action of bacterial lipopolysaccharides is not necessary for the observed changes in properdin concentration in the host. Indeed, it is noteworthy that, in mice, the increase in properdin levels following injection of lipopolysaccharide, appeared quite unrelated to the acute toxicity of the preparations employed. Thus, the lipopolysaccharide derived from *S. typhosa* 0901 is extremely toxic (LD_{50} = 200 μg.) while the product from *E. coli* 0127 is, for all practical purposes, non-lethal (LD_{50} > 5000 μg.). Nevertheless, the administration of 10 μg. in each case increased the properdin levels from an initial value of 12 units to 36 units per ml.

The effect of challenge with *S. typhosa* on properdin levels in mice, previously treated with lipopolysaccharide, is of special interest. The profound contrast in the postchallenge sequence of events occurring in lipopolysaccharide-treated and control animals suggests that the properdin levels of animals prior to infection may give an incomplete picture of the host's reaction to the infective agent. Indeed, evidence presented in this report, and others to follow, indicates that the extent to which the host has been "conditioned" is of prime importance. The rapidity and extent with which properdin is subsequently mobilized in response to the stimulus of infection may prove to be important in determining the fate of the host.

SUMMARY

The administration of a single small dose of bacterial lipopolysaccharide produces in mice a considerable rise in properdin levels. This is accompanied by an early, transient, non-specific increase in resistance to certain bacterial infections. Bacterial lipopolysaccharides were shown to possess far greater activity than other substances previously studied in bringing about an elevation of properdin levels.

After the injection of bacterial lipopolysaccharides, high molecular weight substances appear in the circulation, which interfere with the combination of properdin with zymosan and thus affect the assay of properdin.

The administration of small amounts of bacterial lipopolysaccharides to mice at appropriate times before experimental infection "conditions" the mice so that they maintain normal or elevated properdin titers during the infectious process in contrast to control mice which show a progressive decline in properdin to low levels and death.

The significance of this observation and its relationship to natural resistance to Gram-negative pathogens are considered.

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