Interactions Between Endotoxic Lipopolysaccharides and the Complement System in the Sera of Lower Vertebrates[1] (34698)

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Endotoxic lipopolysaccharides (LPS) are ubiquitous biologically active products derived from the outer membrane of most, if not all, gram-negative bacterial species. In recent years it has become increasingly clear that the biological responses induced in the host by LPS are related in part to their potent ability to activate and generate biologically active peptides from the complement (C) system (1–3). The underlying mechanism(s) of these interactions is not yet understood. To date, studies of the C–endotoxin interaction have been performed only in the higher vertebrates. Further insights might be gained by investigations in the phylogenetic perspective. In the present work we studied the effect of LPS on C activity of lower animals. We found that LPS is able to consume C activity in very primitive as well as more highly developed vertebrates. These include the lower fishes, amphibians, reptiles, and birds.

Materials and Methods. The lower fishes were represented by the Agnathan *Petromyzon marinus* (sea lamprey), the Chondrichthyes *Ginglymostoma cirratus* (nurse shark) and *Heterodontus francisci* (horned shark), and the Osteichthyes *Polyodon spathula* (paddlefish) and *Cyprinus carpio* (carp). Two species of *Amphibia, Rana pipiens* (leopard frog) and *Rana catesbiana* (bull frog), were included. Species representative of the Reptilia were the *Chelydra* (snapping turtle) and *Naja haje* (Egyptian cobra). The chicken represented the *Aves.* For comparative purposes, sera of guinea pig and man were included in this study. All animals studied were mature, except in the investigations of nurse shark serum in which species two of the four animals examined were young.

The lamprey and sharks were bled by heart puncture under tricaine methanesulfonate (MS 222 anesthesia. The paddlefish were bled from the efferent branchial vein, while all other fish, amphibians, reptiles, and the guinea pig were bled by heart puncture. Blood from the chicken was collected from its brachial vein, while human blood was drawn from the antecubital vein. The samples were allowed to clot for 1 hr at room temperature, and the serum was separated after overnight retraction at 0°.

Endotoxic lipopolysaccharide was obtained from *Escherichia coli* strain 0127 by the trichloracetic acid extraction procedure of Boivin *et al.* (4), generously provided by Dr. W. W. Spink. Immune complexes of bovine serum albumin (BSA) and rabbit antibody were prepared at equivalence, and after five washes in normal saline, brought to a final concentration of 2 ng of protein/ml.

It was first necessary to establish detection systems suitable for the quantitation of hemolytic C in each species. The standard

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hemolytic indicator system which consists of sheep erythrocytes and rabbit antibody was suitable for assay of C in the sera of four poikilotherms (cobra, turtle, frog, and nurse shark) as well as in the guinea pig and man. However, this system was not suitable for assay of C in the sera of the other species because rabbit serum did not potentiate C-mediated lysis of sheep cells with their preabsorbed serums. When these respective sera were preabsorbed with intact sheep red cells or red cell stroma, an alternate system consisting of rabbit cells presensitized in EDTA with the heat-stable natural antibody of the test species was established for assay of C in the carp, paddlefish, horned shark, and chicken. Sensitization was performed in the absence of divalent cations in order to allow addition of antibody with minimal (if any) fixation of complement. In these cases, sensitization was considered optimal when sera preabsorbed with stroma induced maximal lysis of the indicator cells. We were satisfied that the lytic activity measured was analogous to the C system of higher vertebrates because it was heat labile, temperature dependent, potentiated by antibody, inhibited by EDTA, consumed by cobra venom factor (CVF) and, subsequently, found to be consumed by immune complexes (see below).

Indeed, presensitization in the manner just described seems to be an effective way to measure hemolytic C in the various lower vertebrates. An exception was seen only among the cyclostomes. In efforts to demonstrate a hemolytic complement system in the lamprey, antibody prepared in both the rabbit and the lamprey itself and indicator erythrocytes derived from the sheep, rabbit, frog, and lamprey were utilized in a variety of combinations. Nonetheless, as in previous studies (5, 6), classical hemolytic C activity still could not be demonstrated in this species (Table I).

In the final assays, quantitative C titrations were performed with serum dilutions in 0.5 ml of buffer (Veronal-buffered glucose–saline at ionic strength 0.075 and containing 0.00015 M Ca^{2+}, 0.0005 M Mg^{2+}, and 0.1 mg/100 ml of gelatin) (7, 8). Washed

TABLE I. Detection of Hemolytic Complement in the Serum of Lower Vertebrates.[a]

					CH_{50}/ml		
Species	Indicator cell	Cell (no. × 10^7/ml)	Antibody species	Total vol (ml)	0° 18 hr	30° 1 hr	37° 1 hr
Man	Sheep	5.0	Rabbit	1.0	<10	160	180
Guinea pig	Sheep	5.0	Rabbit	1.0	15	1000	1840
Cobra	Sheep	5.0	Rabbit	1.0	110	230	23
Turtle	Sheep	5.0	Rabbit	1.0	1200	2400	130
Frog	Sheep	5.0	Rabbit	1.0	810	900	26
Nurse shark	Mature (25 cm)	5.0	Rabbit	1.0	1000	2100	29
	Young (12 cm)	5.0	Rabbit	1.0	—[b]	92	10
Chicken	Rabbit	2.5	Chicken	1.0	10	200	280
Carp	Rabbit	2.5	Carp	1.0	180	180	27
Paddlefish	Rabbit	2.5	Paddlefish	1.0	60	120	<10
Horned shark	Rabbit	2.5	Horned shark	1.0	40	40	<10
Lamprey	Sheep	5.0	Rabbit	1.0	<2	<2	<2
	Sheep	5.0	Lamprey	1.0	<2	<2	<2
	Rabbit	2.5	Lamprey	1.0	<2	<2	<2
	Frog	2.5	Lamprey	1.0	<2	<2	<2
	Lamprey	2.5	Lamprey	1.0	<2	<2	<2

[a] Reaction mixtures were incubated at 0° for 18 hr and at 30° and 37° for 60 min, 6.5 ml of normal saline was added; and after centrifugation, the hemoglobin released was determined spectrophotometrically. The CH_{50}/ml was determined by interpolation.

[b] Not done.

rabbit or sheep cells (2.5 and 5.0 × 10^7, respectively) were added in 0.5 ml and the mixture was incubated for 1 hr at 30 or 37°, or 18 hr at 0° (Table I). To determine the percentage of lysis, 6.5 ml of saline were added, the mixtures were centrifuged and the supernates were read spectrophotometrically at 412 mμ. The CH$_{50}$ was determined by interpolation in the usual way (7, 8). Hence a methodology for assay of hemolytic C activity in each of these species is provided. High titers were obtained in all animals studied except for the lamprey.

To test the interactions of LPS and preformed immune complexes with the hemolytic C system, 0.1 ml of serum sample and 0.1 ml of graded amounts of test reactant were preincubated for varying times and temperatures (Figs. 1 and 2). Buffer (0.8 ml) was added, serial dilutions were performed and the residual CH$_{50}$ was determined as described above.

Results. The results obtained when sera of the several species were reacted with endotoxic LPS are shown in Fig. 1. In each instance, whenever C was measureable, it was consumed by LPS. Hence, C-activation by this bacterial product seems to be a general phenomenon over a broad phylogenetic scale. For comparative purposes, these same sera were reacted with immune complexes prepared with bovine serum albumin and rabbit antibody. Again C consumption was noted in every instance. To our surprise, even though rabbit antibody had not enhanced the hemolytic C of four of the species for sheep red cells, immune complexes made with rabbit sera did consume their hemolytic C activity. The basis for this disparity is not yet clear. It would seem that immunoglobulins which cannot sensitize red cells to the 11-component function of hemolysis, nonetheless can initiate consumption of certain C components. Also, we have shown that even after removal of natural antibody to sheep cells from carp, paddlefish, and chicken sera, the rabbit antisera was ineffective as a hemolysin.

In the next group of experiments, quantitative aspects of the LPS–C interaction were

		Detectable C' Activity	C' Consumption by LPS	C' Consumption by 1000 µg LPS*	C' Consumption by 200 µg Protein Ag-Ab Complexes*
Mammalia	Man	+	+	90	55
	G Pig	+	+	80	85
Aves	Chicken	+	+	60	61
Reptilia	Cobra	+	+	85	95
	Turtle	+	+	65	75
Amphibia	Frog	+	+	90	40
Osteichthyes	Carp	+	+	80	50
	Paddle-fish	+	+	70	45
Chondrichthyes	Nurse Shark	+	+	60	50
Agnatha	Lamprey	0	—	—	—

FIG. 1. Complement (c') consumption by endotoxic LPS in sera of lower vertebrates. Endotoxic lipopolysaccharide (LPS) induced C consumption in all species in which hemolytic C activity was detected. The percentage of C consumed by 1000 μg of LPS preincubated with test sera at optimal temperatures is shown in Table I. C consumption by washed performed immune complexes (200 μg) of bovine serum albumin and rabbit antiserum is shown for comparison. The amount of C available in these various sera is shown in Table 1.

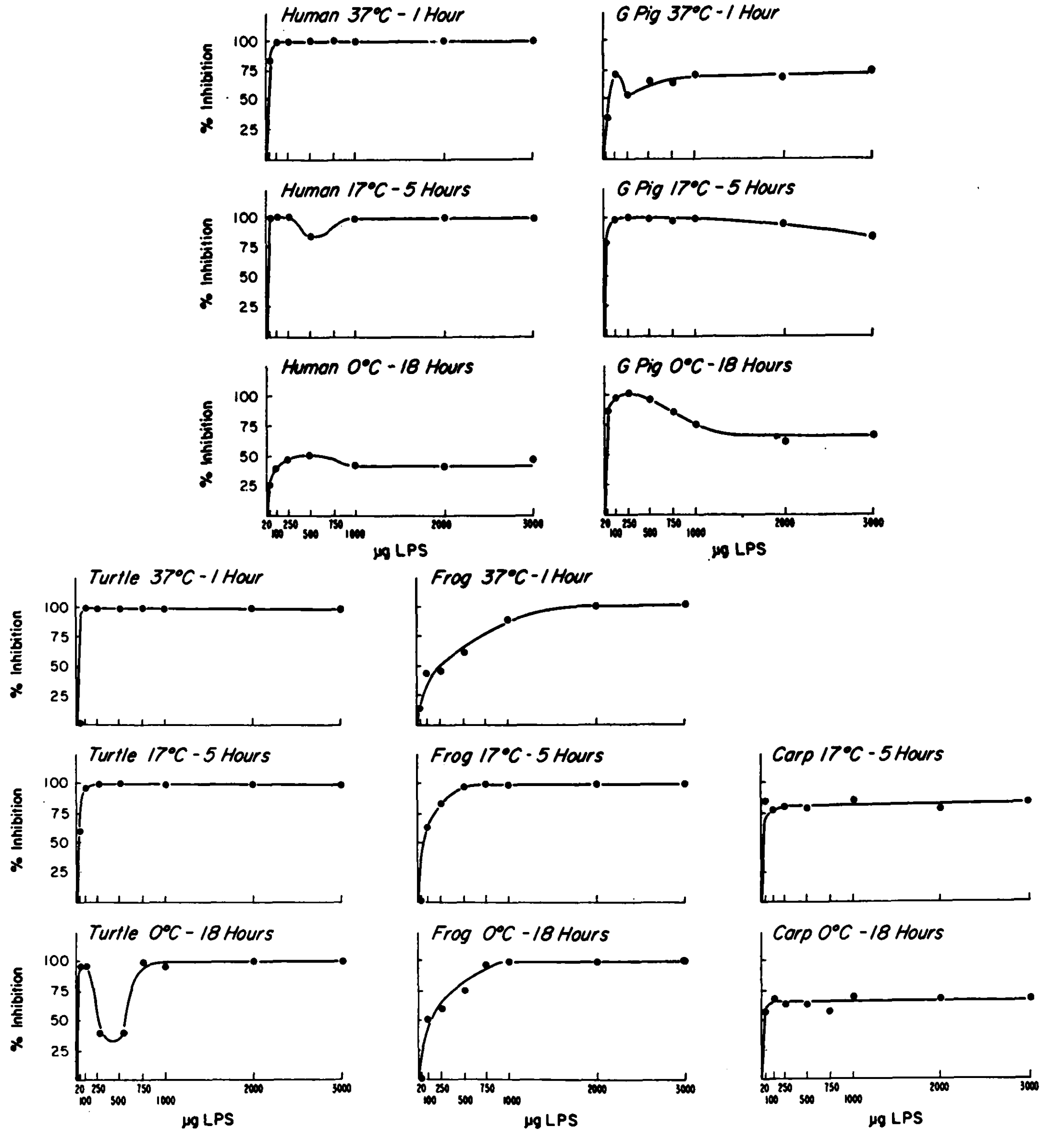

FIG. 2. Characteristic dose–response curves were seen when sera (0.1 ml) of man, guinea pig, turtle, frog, and carp, respectively, were preincubated with *E. coli* endotoxic lipopolysaccharide (0.1 ml) at the temperatures indicated, and the residual total hemolytic C titers were determined by assay during 1 hr at 30°. The C available is approximately as shown in Table I, with the exception cited below. Small amounts of LPS were sufficient to initiate C consumption in each species. Peaks of maximum C consumption were present at two distinct dose ranges in the turtle at 0° and to a lesser extent in guinea pig and man. Substantial (>75%) reduction of carp hemolytic activity was observed even in central specimens incubated with buffer in the absence of LPS; further, the carp had substantially lower detectable titers of C than did the other species. Therefore, it was difficult to evaluate the effects of LPS on carp C during incubation at 37°.

determined in five representative species (carp, frog, turtle, guinea pig, and man) at three different temperatures (Fig. 2). Mixtures of LPS (10, 20, 100, 250, 750, 1000, and 3000 μg) and test sera were preincubated at 0° for 18 hr, 17° for 5 hr, and 37° for 1 hr, respectively, and the residual CH_{50} was determined as described earlier. Two aspects of the dose–response relationships seemed of particular interest. Firstly, small amounts of LPS (10–50 μg) were sufficient for consumption of C at the ambient temperatures of the several species. Secondly, in certain instances (*e.g.*, 0° in turtle serum), maximal peaks of C consumption were seen at two distinct dose ranges, suggesting the participation of two separate factors initiating the endotoxin–complement interaction. This suggests that in the turtle and guinea pig at 0° one of these consumption reactions may be inhibited by excess LPS. Although there have been conflicting reports on the mechanism of initiation of the endotoxin–C interaction (1–3), there is substantial evidence that antibodies play a role in many endotoxin-induced reactivities. The observations here suggest that there are multiple factors which could consist of either different antibody classes or yet unknown nonantibody substances which are capable of achieving this activation. Thus, of the two zones of C consumption observed, only one was inhibited by excess antigens.

Discussion. Hemolytic C activity has been observed in many poikilothermic species (5, 6, 9–16). Indeed, there have been many similarities between the C systems of various poikilotherms and higher vertebrates. This led us to test the reactivity of the C system in representative vertebrates upon the addition of bacterial lipopolysaccharides, agents known to be potent activators of mammalian C. Again, basic similarities were found.

Indeed, wherever C was measurable, it was consumed by *E. coli* lipopolysaccharide. This consumption was featured by a time, temperature, and dose-dependence characteristic for each species. The pathway(s) initiating this interaction remains unclear. However, the ability of endotoxic LPS to activate and consume the amplifying biologically-active C system in all species tested speaks for a potential involvement of C in the mediation of the inflammatory reactions induced by bacterial products in vertebrates generally.

Summary. Endotoxic lipopolysaccharides (LPS) have potent ability to consume complement (C) components and generate biologically active peptides from the C system in normal mammalian serum. The present investigation shows that LPS derived from *Escherichia coli* has potent ability to consume hemolytic C in all vertebrate species in which C activity is detectable, including species as low phylogenetically as representative Chondrichthyes (horned shark, nurse shark), Osteichthyes (paddlefish, carp), Amphibia (bull frog, marine frog), and Reptilia (cobra, turtle). Hence, the consumption of C components in response to this ubiquitous bacterial product is a characteristic of vertebrate serum virtually throughout vertebrate evolution.

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1. Spink, W. W., and Vick, J., J. Exp. Med. **114**, 501 (1961).

2. Gewurz, H., Shin, H. S., Pickering, R. J., Snyderman, R., Lichtenstein, L., Good, R. A., and Mergenhagen, S. E., *in* "Cellular Recognition" (R. T. Smith and R. A. Good, eds.), p. 305. Appleton-Century-Crofts, New York (1969).

3. Mergenhagen, S. E., Snyderman, R., Gewurz, H., and Shin, H. S., Curr. Top. Microbiol. Immunol. **50**, 37 (1969).

4. Boivin, A., Mesrobeanu, J., and Mesrobeanu, L., C. R. Soc. Biol. **113**, 490 (1933).

5. Gewurz, H., Finstad, J., Muschel, L. H., and Good, R. A., *in* "Phylogenetic Approaches to Immunity" (R. T. Smith, R. A. Good, and P. A. Miescher, eds.), p. 105. Univ. Florida Press, Gainesville (1966).

6. Legler, D. W., and Evans, E. E., Proc. Soc. Exp. Biol. Med. **124**, 30 (1966).

7. Mayer, M. M., *in* "Experimental Immunochemistry" (E. A. Kabat and M. M. Mayer, eds.), p. 149. Thomas Springfield, Ill. (1961).

8. Nelson, R. A., Jr., *in* "The Inflammatory Process" (B. W. Zweifach, L. Grant, and R. T. McCluskey, eds.), p. 819. Academic Press, New York (1965).

9. Cushing, J. E., Jr., J. Immunol. **50**, 61 (1945).

10. Cushing, J. E., Jr., J. Immunol. **50**, 75 (1945).

11. Rice, C. E., Can. J. Comp. Med. **14**, 369 (1950).

12. Legler, D. W., and Evans, E. E., Proc. Soc. Exp. Biol. Med. **124**, 30 (1967).

13. Legler, D. W., and Evans, E. E., Trans. Amer. Fish. Soc. **96**, 237 (1967).

14. Feldman, H. A., and Miller, L. T., Science **131**, 35 (1960).

15. Mazzetti, L., Z. Immunitätsforsch. **18**, 132 (1913).

16. Nardi, F., Z. Immunitätsforsch. **94**, 505 (1938).

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