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Function of the lipopolysaccharide-binding protein of *Periplaneta americana* as an opsonin

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Previously, we reported the purification of an LPS-binding protein from the hemolymph of the American cockroach that was specific for *E. coli* LPS. In this study we found that this protein participated in the clearance of *E. coli* cells injected into the abdominal cavity of the cockroach, and that hemocytes ingested *E. coli* cells treated with this LPS-binding protein in vitro. These findings suggest that this LPS-binding protein acts as an opsonin.

LPS-binding protein; Opsonin; Cockroach; *Periplaneta americana*

1. INTRODUCTION

Previously, we purified a lipopolysaccharide (LPS)-binding protein from the hemolymph of the American cockroach (*Periplaneta americana*) [1]. A unique feature of this protein is that, unlike many mammalian LPS-binding proteins [2–4], it does not bind to the lipid A region, but to the hexose core region of the LPS molecule. The alignment of the hexoses in this region seems to be important for the binding of this protein, because although it has strong affinity to *Escherichia coli* K12 LPS, it does not bind to *Salmonella minnesota* R60 LPS, which differs from *E. coli* LPS in the alignment of 4 hexoses at the proximal end of the carbohydrate chain [5–7].

We isolated a cDNA for this protein and determined its complete primary structure [8]. Results showed that there is a carbohydrate-recognition domain at the carboxyl terminal containing amino acid sequences that are conserved in various mammalian C-type lectins [9]. Northern blot analysis with this cDNA revealed that this LPS-binding protein is an acute phase responsive protein. Synthesis of mRNA for this protein was significantly enhanced by injection of foreign cells into the abdominal cavity of adult *Periplaneta* [8]. These results suggest that this LPS-binding protein participates in elimination of bacteria that have LPS with affinity to this protein.

Here we show that this LPS-binding protein does in fact act as an opsonin. Probably, this LPS binding protein is a defense molecule that facilitates ingestion of infecting bacteria by hemocytes.

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2. MATERIALS AND METHODS

2.1. *Animals and isolation of hemocytes*

A colony of the American cockroach (*P. americana*) was maintained in a plastic container at 27°C with dog biscuits and water [10]. Adult males and females were used throughout. For isolation of hemocytes, the insects were anesthetized on ice, a pair of mid and hind legs was cut off with fine scissors, and then 200 μl/insect of ice-cold modified Carlson's saline (NaCl 0.9%, NaH_2PO_4 0.02%, $NaHCO_3$ 0.012%, KCl 0.02%, $CaCl_2$ 0.02%, $MgCl_2$ 0.01%, glucose 0.003%) [11] was injected into the abdominal cavity. The hemolymph that exuded from the wound was collected, and the number of hemocytes in this hemolymph was counted. Hemolymph containing 5×10^5 hemocytes was inoculated into 24-well tissue culture plates and incubated for 1 h at 25°C. Adherent cells were washed 3-times with 500 μl of modified Carlson's saline and used for studies on uptake of *E. coli* cells.

2.2. *Assay of bacterial clearance*

Adult *Periplaneta* weighing about 1.5 g were anesthetized on ice and 10 μl of insect saline containing 1×10^6 *E. coli* K12 594 (*str*^{r}) cells was injected into the abdominal cavity through a joint of a hind leg. Then the mid and hind legs were cut off at various times, and 500 μl of ice-cold modified Carlson's saline was injected into the abdominal cavity. The hemolymph that exuded from the wound was collected and centrifuged at $110 \times g$ for 5 min to remove hemocytes, and numbers of viable *E. coli* cells in the supernatant were determined by plating.

2.3. *Uptake of* E. coli *by hemocytes in vitro*

E. coli K12 594 (*str*^{r}) cells were cultured in 5 ml of Antibiotic medium (Difco) containing 2 MBq [^{3}H]thymidine for 3 h to label DNA. Labeled cells were washed well and suspended in 500 μl of modified Carlson's saline. The specific activity of this cell suspension was usually $2–5 \times 10^5$ cpm/10^8 cells. For opsonization, 5×10^7 labeled *E. coli* cells were incubated with various concentrations of the LPS-binding protein for 1 h at 4°C in 25 μl of 10 mM Tris-HCl buffer, pH 7.9, containing 130 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$ and 0.1% bovine serum albumin. Then the cells were washed repeatedly with modified Carlson's saline with centrifugation at $10,000 \times g$ for 5 min, and finally suspended in 25 μl of the same saline. For examination of uptake of opsonized *E. coli* cells by hemocytes, 5×10^7 labeled cells were added to wells containing 5×10^5 substratum-adherent hemocytes. The volume of the medium (modified Carlson's saline) in the well was adjusted to 250 μl, and the culture plates were incubated for 30 min at 25°C. Then the medium was discarded, the hemocytes were

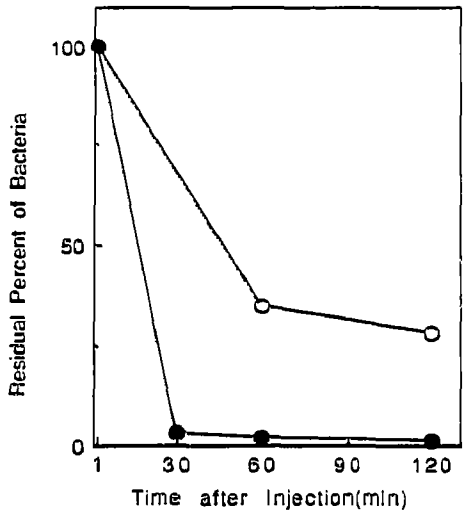

Fig. 1. Clearance of *E. coli* from the hemolymph of adult *Periplaneta*. After injection of 1×10^6 *E. coli* cells at time 0, adult *Periplaneta* were kept at 27°C (●) or 4°C (○). Hemolymph was collected from 4 insects each at the indicated times, and the numbers of viable *E. coli* cells in pooled hemolymph samples were measured.

washed extensively, and trichloroacetic acid-precipitable radioactivity was measured.

3. RESULTS AND DISCUSSION

3.1. *Participation of the LPS-binding protein in clearance of* E. coli

To determine whether the LPS-binding protein is a defense molecule, we examined whether it participates in the clearance of *E. coli* introduced into the abdominal cavity of adult *Periplaneta*. For this, we first investigated the fate of injected *E. coli* cells by injecting 1×10^6 streptomycin-resistant *E. coli* K12 cells into adult *Periplaneta*, and collecting their hemolymph at various times after the injection. Hemocytes were removed by centrifugation, and the number of viable bacteria in the hemolymph was determined by plating diluted hemolymp samples on agar plates containing 100 μg/ml of streptomycin. Change in the number of bacteria in the hemolymph as a percentage of the number in the hemolymph 1 min after injection of *E. coli* was plotted against time. As shown in Fig. 1, injected bacteria disappeared rapidly from the hemolymph when the insects were kept at 27°C, but the clearance of *E. coli* was delayed significantly at 4°C. As *Periplaneta* hemolymph does not contain appreciable bactericidal activity (unpublished result), this clearance of *E. coli* was supposed to be due to ingestion of bacteria by the hemocytes.

Then we tested if the LPS-binding protein participates in this process. For this, *E. coli* cells were injected simultaneously with various amounts of LPS prepared from *E. coli* or *S. minnesota* [12], the hemolymph was collected 1 h later and the number of

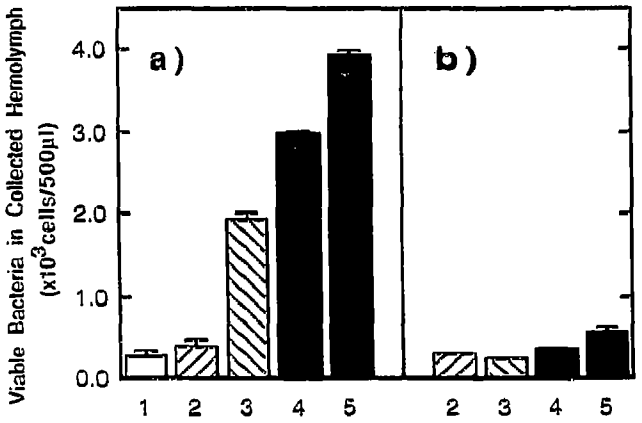

Fig. 2. Inhibition of clearance of *E. coli* by LPS. Adult *Periplaneta* were treated with 1×10^6 *E. coli* cells with various amounts of LPS prepared from *E. coli* (a) or *S. minnesota* (b), and kept for 1 h at 27°C. Hemolymph was collected and its content of viable *E. coli* cells was measured. The amounts of LPS injected were: (1) none (saline alone); (2) 200 μg; (3) 300 μg; (4) 400 μg; (5) 500 μg. Means for 4 insects are shown with SD.

viable bacteria in the hemolymph was determined. As shown in Fig. 2, the number of viable bacteria in the hemolymph increased significantly with an increase in the amount of LPS from *E. coli* LPS, but did not change appreciably on injection of *S. minnesota* LPS. These results suggested that the LPS-binding protein in the hemolymph is needed for the clearance of *E. coli*: injection of *E. coli* LPS would neutralize this protein with consequent increase in the number of viable cells, whereas *S. minnesota* LPS would not neutralize this protein.

Then we tested the effect of antibody against the LPS-binding protein [1] on clearance of *E. coli*. Increasing amounts of antibody were injected with 1×10^6 *E. coli*

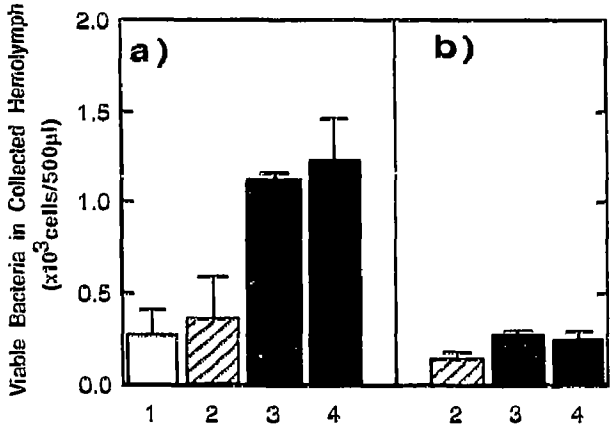

Fig. 3. Inhibition of clearance of *E. coli* by antibody against the LPS-binding protein. After injection of 1×10^6 *E. coli* cells with various amounts of antiserum against the LPS-binding protein (a) or preimmune serum (b), adult *Periplaneta* were kept for 1 h at 27°C. Then the hemolymph was collected and its content of viable *E. coli* cells was measured. The amounts of serum injected were: (1) none (30 μl saline alone); (2) 10 μl; (3) 20 μl; (4) 30 μl. Means for 4 insects are shown with SD.

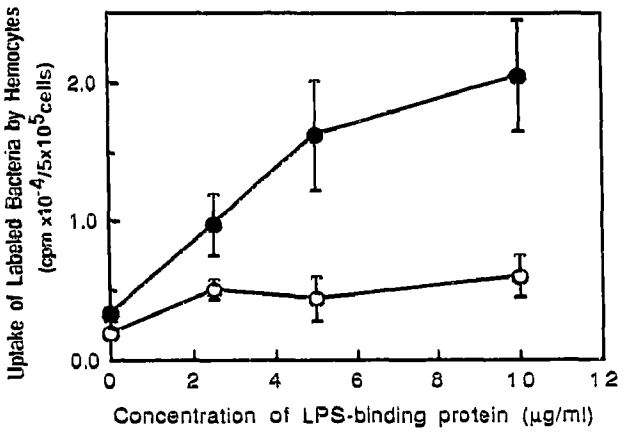

Fig. 4. Uptake of LPS-binding, protein-treated *E. coli* cells by hemocytes in vitro. Labeled *E. coli* cells (5×10^7) were treated with indicated concentrations of the LPS-binding protein, added to monolayers of hemocytes (5×10^5), and incubated at 25°C (●) or 4°C (○) for 30 min. Then the hemocytes were washed well and acid-precipitable radioactivity associated with them was measured. Means for 4 insects are shown with SD.

cells, the hemolymph was obtained 1 h later, and the number of viable bacteria was determined. As shown in Fig. 3, the number of viable bacteria in the hemolymph increased with an increase in the amount of antibody, but injection of pre-immune serum instead of the antiserum had no appreciable effect. These results suggest

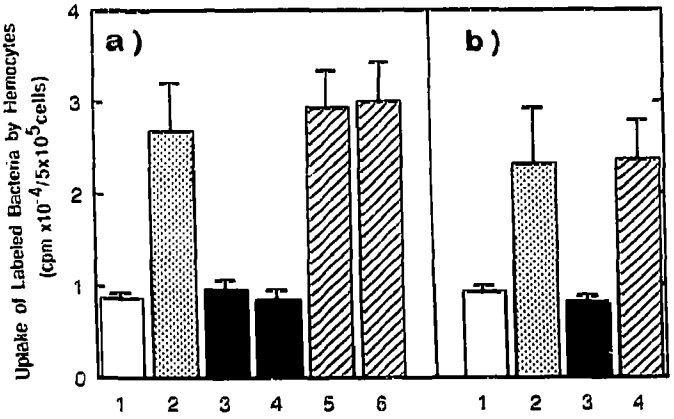

Fig. 5. Effects of LPS and antibody on the LPS-binding protein-dependent uptake of *E. coli* by the hemocytes. (a) The LPS-binding protein (10 μg) was incubated with increasing amounts of LPS for 30 min at 25°C. Then labeled *E. coli* cells were opsonized with this LPS-treated, LPS-binding protein and their uptake by the hemocytes was examined. The amounts of LPS used were (3) 1 mg/ml of *E. coli* LPS; (4) 100 μg/ml of *E. coli* LPS; (5) 1 mg/ml of *S. minnesota* LPS; (6) 100 μg/ml of *S. minnesota* LPS. Columns 1 and 2 show uptakes of *E. coli* opsonized with 0 and 10 μg of the LPS-binding protein, respectively. (b) The LPS-binding protein (10 μg) was treated with 12.5 μl of saline or antiserum against the LPS-binding protein or pre-immune serum. Then *E. coli* cells were opsonized with the pretreated LPS-binding protein, and their uptake by hemocytes was examined. LPS-binding protein was treated with: (2) saline; (3) antiserum; (4) pre-immune serum. Column 1 shows uptake without opsonization (control). Means for duplicate measurements are shown with SD.

that the antibody neutralized the LPS-binding protein in situ, and resulted in an increase in the viable cell number in the hemolymph. From these 2 experiments, we concluded that this LPS-binding protein participates in the clearance of injected *E. coli* cells.

3.2. *Participation of hemocytes in bacterial clearance mediated by the LPS-binding protein*

As the LPS-binding protein has no bactericidal activity, clearance of injected *E. coli* cells in adult *Periplaneta* is supposed to be due to their ingestion by hemocytes. Therefore, we tested whether *Periplaneta* hemocytes ingest *E. coli* cells in vitro. Substratum-adherent hemocytes were prepared, and a fixed number of [^{3}H]thymidine-labeled *E. coli* cells was incubated with the hemocytes for 30 min at 25°C. Then the radioactivity associated with the hemocytes was measured. As shown in Fig. 4, the radioactivity associated with the hemocytes was low after addition of labeled bacteria alone to the hemocytes. However, pre-incubation of the *E. coli* cells with the LPS-binding protein before their addition to the hemocytes resulted in a significant increase in the radioactivity associated with the hemocytes, with an increase in the amount of the LPS-binding protein used. These results suggest that the LPS-binding protein facilitates ingestion of the bacteria by the hemocytes. At present it is unknown whether the bacteria are ingested or simply attached to the hemocytes in the presence of the LPS-binding protein. But as the radioactivity associated with the hemocytes did not increase appreciably, even in the presence of the LPS-binding protein, when the hemocytes and bacteria were incubated at 4°C, we assume that most of the radioactivity detected was that of ingested bacteria. *E. coli* LPS or antibody against the LPS-binding protein inhibited the opsonin activity of the LPS-binding protein, but *S. minnesota* LPS or pre-immune serum had no effect, as shown in Fig. 5. These results may mimic the situation in vivo. Probably, LPS-binding protein opsonizes injected *E. coli* cells, making them readily ingestable by hemocytes. We assume that there are various LPS-binding proteins with different LPS specificities in the hemolymph of adult *Periplaneta* that act as opsonins when bacterial infection occurs.

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