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Innate Immunity in Insects: The Role of Multiple, Endogenous Serum Lectins in the Recognition of Foreign Invaders in the Cockroach, *Blaberus discoidalis*.

Raymond Wilson, Changwei Chen, and Norman A. Ratcliffe

Unlike vertebrates, insects do not have an Ab-based nonself recognition system, and must rely totally on innate immunity to defend themselves from microbial invaders. The most likely candidates for recognizing foreign material in insects are the lectins, which have already been shown to be important in mammalian innate immunity. The hemolymph of the cockroach, *Blaberus discoidalis*, contains multiple lectins, designated BDL1, BDL2, BDL3, and GSL (β-1,3-glucan-specific lectin), two of which, namely BDL1 and GSL, have close similarities to acute phase reactants. These endogenous molecules, as well as Con A, wheat germ agglutinin, and *Helix pomatia* agglutinin, have been shown to induce an enhanced phagocytic response by *B. discoidalis* plasmatocytes. This effect is related to the carbohydrates presented on the surface of the microorganism and to the sugar specificities of the lectins. Thus, the mannose-specific lectins, BDL1 and Con A, both increase the phagocytosis of baker’s yeast and *Escherichia coli*, whereas the N-acetyl-D-glucosamine/N-acetyl-D-galactosamine-specific lectins, BDL2, wheat germ agglutinin, and *H. pomatia* agglutinin, induce the phagocytosis of *Bacillus cereus* and *E. coli*. GSL, specific for β-1,3-glucan, and the N-acetyl-D-galactosamine-specific BDL3, only enhance the phagocytosis of yeast and *B. cereus*, respectively. Phenylthiourea, an inhibitor of the prophenoloxidase system, caused either total, partial, or no inhibition of the lectin-induced increase in phagocytosis, indicating that this immune enhancement results, in some cases, from at least two closely linked mechanisms. These results show that the endogenous lectins in the cockroach hemolymph are capable of acting as nonself recognition molecules for a wide range of microorganisms, and thus obviate the necessity of Abs in these animals. *The Journal of Immunology*, 1999, 162: 1590–1596.

In vertebrates, the role of lectins as mediators of nonself recognition in the innate immune response has been well documented (1), with several mammalian lectins having an opsonic function and involved in the clearance of microbial agents (e.g., 2–5). The best studied of these proteins are the mannose-binding lectins, MBLs (6), which are an essential component of the vertebrate innate immune system, since MBL-deficient individuals are prone to recurrent infections during infancy (7). MBLs not only enhance the phagocytosis of virulent bacteria (2), but also activate the complement system through the classical pathway (8).

In invertebrates, including insects, due to the lack of Ab-based immunity, lectins probably play a major role in nonself recognition, with several reports of endogenous serum lectins having opsonic activity for invading pathogens (e.g., 9–14). If, however, lectins act in an analogous manner to Abs, then it is essential that multiple lectins are present in the circulation, each with different carbohydrate-binding specificities, and thus able to recognize a wide variety of invading organisms. To date, multiple lectins have been purified from only three species of insects, namely, the silkworm, *Bombyx mori* (15, 16); the American cockroach, *Periplaneta americana* (17–21); and the West Indian leaf cockroach, *Blaberus discoidalis* (22, 23). Two of the lectins from *P. americana*, i.e., the LPS-binding protein (11) and the *Periplaneta* lectin (14), have been reported to bind to, and increase the in vivo clearance of *Escherichia coli* from the hemolymph. In *B. discoidalis*, at least four lectins occur, namely, BDL1, BDL2, BDL3, and GSL, of which three, BDL1, BDL2 (22), and GSL (23), have been purified. Of these lectins, BDL1 has been shown to have properties similar to MBLs in terms of specificity, structure, and activation of complement (our unpublished data), and GSL has been shown to be similar to invertebrate C-reactive proteins.5 These endogenous lectins are also capable of enhancing the activation of the phenoloxidase system (24), a melanization cascade that is an important component of the insect immune defense system involved in numerous defense reactions, including encapsulation, nodulation, and phagocytosis (25).

The present study examines in detail, for the first time in an invertebrate, the role of multiple, endogenous lectins in immune recognition, in particular, the effects of the four endogenous serum lectins from *B. discoidalis*, and four exogenous lectins of comparable carbohydrate specificity, on the in vitro phagocytosis of representatives from three major groups of microorganisms, namely yeast and Gram-positive and Gram-negative bacteria. Each of these microorganisms has very different surface carbohydrate.

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3 Abbreviations used in this paper: MBL, mannose-binding lectin; BDL, *Blaberus discoidalis* lectin; GaINAc, N-acetyl-D-galactosamine; GIM, Grace’s Insect Medium; GlcNAc, N-acetyl-D-glucosamine; GSL, β-1,3-glucan-specific lectin; HPA, *Helix pomatia* agglutinin; proPO, prophenoloxidase; PTU, phenylthiourea; TPA, *Tetragnobulus purpureus* agglutinin; WGA, wheat germ agglutinin.

properties (26, 27), and we have shown that the activation of phagocytosis by each lectin is related both to the carbohydrates exposed on the surface of the phagocytosed particle and to the sugar specificity of the lectin.

Materials and Methods

Insects

West Indian leaf cockroaches (B. discoidalis) were kept in fiber-glass containers at 28°C, with a constant 12-h light/dark cycle. They were fed on dried cat food, banana skins, and apple cores, and given water ad libitum. Adult males and females were used for all of the experiments.

Bleeding of insects and preparation of monolayers

Cockroaches were cooled at −20°C for approximately 20 min and surface sterilized with 70% ethanol. Using a sterile 26G needle, 0.4 ml of anticoagulant (0.01 M EDTA (free acid), 0.1 M glucose, 0.062 M NaCl, 0.03 M trisodium citrate, 0.026 M citric acid (pH 4.6), 370 mM) was injected between the second and third abdominal sclerites. Insects were massaged gently to circulate anticoagulant before bleeding by piercing the arthrodial membrane of a posterior limb with a 21G needle, and 0.1 ml of hemolymph collected in a syringe containing 0.4 ml of anticoagulant. The contents of the syringe were mixed and ejected into a sterile 7-ml Bijou bottle containing 2 ml of Grace's Insect Medium (GIM; Sigma, Poole, Dorset, U.K.). In cases in which incubation in PTU (phenylthiourea) was required to inhibit the prophenoloxidase (proPO) system, the contents of the syringe were ejected into 2 ml of GIM containing a few crystals of PTU. Bleeding of the insects was performed in a 4°C constant temperature room to prevent hemolymph coagulation.

For hemocyte monolayer preparation, sterile 5-mm diameter glass coverslips were placed into the wells of a sterile, 96-well, flat-bottom microtiter plate (Nunc, Roskilde, Denmark), and 50 μl of diluted hemolymph was pipetted into each well. The plate was then incubated at 30°C for 20 min to allow the cells to attach to the glass coverslip. To remove any unattached cells, the monolayers were washed three times with 100 μl of GIM.

Lectin labeling of hemocytes

A number of FITC-labeled lectins were used to probe the surface carbohydrates of the B. discoidalis hemocytes, namely Con A, WGA, HPA, and TPA (Sigma). All lectins were dissolved in GIM at a concentration of 100 μg/ml, aliquoted, and stored at −20°C.

Monolayers were overlaid with 50 μl of FITC-labeled lectin and incubated at 29°C for 15 min in the dark. Unbound lectin was gently washed off three times with 100 μl of GIM. The cells were fixed with GIM-buffered 4% formaldehyde for at least 30 min at 29°C. The fixative was washed off with GIM, and the 5-mm coverslips, containing the monolayers, were removed from the microtiter plate wells, placed on microscope slides, then mounted using Kaiser’s glycerol gelatin (50% glycerin, 8% gelatin, 0.01% merthiolate). At least 250 cells per monolayer were then examined under UV light using a Zeiss photomicroscope II, and equipped with a barrier and excitation filter set number 487/009 for FITC epifluorescence.

Lectins preincubated for 1 h with their respective inhibitory sugars were used as negative controls. The inhibitory sugars used for each lectin were as follows (the lectin that each sugar inhibits is included in the parentheses): N-Ac-D-mannose (0.2 M; Con A); N,N′,N″-triacetylchitotriose, a trimer of N-acetyl-d-glucosamine (4.5 mM; WGA); N-acetyl-d-galactosamine (1.5 mM; HPA); and α-D-(fucose (0.2 M; TPA).

To investigate whether Con A was binding to the same ligands as BDL1, monolayers were incubated with 50 μl of either BDL1 or GIM for 30 min at 29°C. The cells were then washed five times with 50 μl of GIM, before incubating the cells in 50 μl of FITC-labeled Con A for 15 min at 29°C. The monolayers were washed five times with GIM and fixed as described.

Culture and labeling of yeast and bacteria

Baker's yeast (Saccharomyces cereviiae; Westmill Foods, Maidenhead, Berks, U.K.) was grown up in YM broth (Difco, E. Molesley, Surrey, U.K.), overnight at 25°C, in a shaking water bath. The cultured cells were heat killed in a boiling water bath for 1 h, then washed three times in pH 7.4 TBS/CA++ (20 mM Trizma base, 77 mM NaCl, 10 mM CaCl2) by centrifugation at 650 × g for 10 min. The washed cells were then labeled with FITC by the method of Rohloff et al. (28) and resuspended at a concentration of 1 × 105 cells/ml in GIM.

E. coli K12 and Bacillus cereus PC2599 were treated in a similar manner to the yeast cells, except they were grown in nutrient broth at 37°C and 25°C, respectively. The resultant cultures were then processed in the same way as the yeast, except that they were centrifuged at 2000 × g.

Preparation of the B. discoidalis lectins

The endogenous lectins BDL1, BDL2, and BDL3 were purified from the serum of adult cockroaches by a combination of ammonium sulfate precipitation, DEAE-cellulose cation-exchange chromatography, and affinity chromatography, as described in Chen et al. (22). GSL was purified by a combination of gel filtration and blue-Sepharose chromatography, according to the method of Chen (23). The purified lectins were dialyzed against GIM for use in the phagocytosis assays. The BDL1 used for the dose-dependency curves was purified by the following immunoadfinity method, which yields much greater volumes of almost pure lectin than the method of Chen et al. (22), with only one major contaminating protein.

Mouse anti-BDL1 mAb 6B4 was purified from 500 ml of hybridoma supernatant using an immunoadfinity column, which yields much greater volumes of almost pure lectin than the method of Chen et al. (22), or, for GSL, the agglutination of yeast (23), and their concentrations were adjusted to 17,000 U/ml for BDL1, 5000 U/ml for BDL2, 10,000 U/ml for BDL3, and 760 U/ml for GSL, in which 1 U equals an agglutination titer of 1/1 mg of purified lectin.

Opsonization of yeast and bacteria

FITC-labeled bacteria and yeast suspensions (50 μl used per monolayer) were pelleted at 10,000 × g for 20 s. The supernatants were removed, and 50 μl of lectin were added with gentle agitation to resuspend the pellets, and incubated at 29°C for 1 h in the dark. The cells were pelleted by centrifugation, washed twice in 50 μl of GIM to remove any unbound lectin, and finally resuspended in GIM (50 μl). Inhibited lectin controls were prepared, as above, except the lectins were preincubated in their respective inhibitory sugars for 1 h at 29°C before addition to the bacteria or yeast. The inhibitory sugars used were: 0.2 M mannose for BDL1 and Con A; 0.2 M GlcNAc for BDL2; 1 mg/ml GalNAc for BDL3 and HPA; 1% laminarin for GSL; and 0.2 M fucose for TPA. BSA (100 μg/ml) was used as a proteinaceous nonlectin control.

Phagocytosis experiments

The method described below is a modification of the technique described by Rohloff et al. (28). To each hemocyte monolayer, 50 μl of opsonized bacteria or yeast (1 × 105 cells/ml), or 50 μl bacteria or yeast treated with BSA or GIM alone, were added, and incubated at 29°C. After 1 h, the monolayer was washed five times with 100 μl of GIM, to eliminate any noninternalized particles. Monolayers were then overlaid with 50 μl of 0.2% trypan blue, and incubated in the dark for 5 min at 29°C. All traces of trypan blue were washed away with GIM, and the cells were fixed as above for the lectin-labeling experiments. At least 250 cells per monolayer were then examined under UV light for phagocytosed bacteria or yeast using a Zeiss photomicroscope II, as above.

Treatment of monolayers with laminarin

To demonstrate the effect of the activation of the proPO system on phagocytosis, monolayers were preincubated with laminarin, a known activator of proPO. Since laminarin, a β-1,3-glucan, is also an inhibitor of GSL receptor recognition, the proPO system was activated before the addition of the test particles. Adult cockroaches were bled, as described previously.
Table I. Staining of B. discoidalis hemocytes with FITC-labeled exogenous lectins

<table>
<thead>
<tr>
<th>FITC-Labeled Lectin</th>
<th>Inhibitory Sugar</th>
<th>Labeling of Plasmatocytes</th>
<th>Labeling of Granular Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>α-(+)-mannose (0.2 M)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>WGA</td>
<td>N,N′,N′-triacetylchitotriose (a trimer of GlcNAc; 1 mg/ml)</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>HPA</td>
<td>GalNAc (1 mg/ml)</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>TPA</td>
<td>α-D-(+)-fucose (0.2 M)</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Monolayers were incubated with FITC-labeled lectin solutions for 10 min in the dark at 29°C. At least eight monolayers were examined for each treatment. ++++, intense staining; ++, significantly brighter staining than controls; −, no significant staining compared with controls.

Results

Comparison of the purification methods of BDL1

The original method of purifying BDL1 by Chen et al. (22) yields approximately 100 μg of pure protein from 10 ml of serum. In comparison, the immunoaffinity method, described in this work, produces 100 μg of protein with a similar sp. act. to that purified by Chen et al. (22), but from only 1 ml of serum. The purified BDL1 is, however, not totally pure, as it is always copurified with a protein that has a mass of approximately 40 kDa under both reducing and nonreducing conditions in SDS-PAGE. Since the immunoaffinity-purified lectin and the pure lectin, when used at comparable sp. act., induced phagocytosis by the same amount, it was concluded that the contaminating protein was not contributing to the observed effects, and therefore the immunoaffinity-purified lectin was used in a limited number of experiments.

Lectin labeling of hemocytes

Con A, HPA, and WGA stained 100% of plasmatocytes from adult B. discoidalis, with Con A giving the most intense staining and WGA the weakest (Table I). Only Con A was observed to also bind to the granular cells. TPA did not bind to any of the cell types. Lectin binding was completely inhibited by incubating the lectins with their inhibitory sugars before their addition to the monolayers. There was no significant difference in the pattern of labeling between fixed and nonfixed hemocytes.

The monolayers incubated with BDL1 before the addition of Con A showed no observable difference in the binding of Con A to the hemocytes.

Phagocytosis of yeast

Nonopsonized yeast, incubated in GIM alone or in 100 μg/ml BSA, was phagocytosed by 6.56 ± 0.59% and 5.87 ± 0.56% of plasmatocytes, respectively (Fig. 1a). Granular cells did not exhibit any observable phagocytosis. This basal, nonspecific phagocytosis was increased to over 11% (Fig. 1a) when the monolayers were incubated with laminarin, a known activator of the proPO cascade, before the addition of yeast. This increase in phagocytosis due to laminarin was completely inhibitable by PTU (Fig. 1a), an inhibitor of phenoloxidase (30). Regarding phagocytosis of yeast in the presence of the endogenous lectins BDL1, BDL2, BDL3, and GSL, a fivefold increase in internalization, from 6.56 ± 0.56% to 31.54 ± 2.03%, was observed when yeast was opsonized with BDL1 (17,000 U/ml; Fig. 1a). This effect was completely inhibited by mannose (Fig. 1a), and was dose dependent (Fig. 2a), reaching a maximum level of phagocytosis at a concentration of 13,000 U/ml. Incubation of the yeast with GSL also induced a significant increase in phagocytosis from 6.22 ± 1.48% to 11.86 ± 1.02%, which was inhibited by preincubation of the lectin with laminarin (Fig. 1a). In contrast, pretreatment of yeast by either BDL2 or BDL3 had no effect on phagocytosis (Fig. 1a).

Of the exogenous lectins tested (Fig. 3a), incubation of yeast with Con A caused a dose-dependent (Fig. 4a), threefold increase in phagocytosis from 6.56 ± 0.59% to 19.13 ± 0.97%, which was inhibitable by 0.2 M mannose. WGA also induced a significant enhancement in phagocytosis from 6.56 ± 0.59% to 11.86 ± 1.02%, which was approximately the same level as that observed with laminarin-inubcated monolayers (Fig. 1a). HPA and TPA had no effect on the phagocytosis of yeast. The effects of both the endogenous and exogenous lectins on the phagocytosis of yeast are summarized in Table II.

Phagocytosis of E. coli

The basal level of phagocytosis of E. coli in GIM (5.36 ± 0.78%) and BSA (6.65 ± 1.86%), and the activation due to laminarin (10.10 ± 2.25%; Fig. 1b) were similar to those observed with...
yeast. The endogenous lectins BDL1, BDL2, and BDL3 all induced significant increases in the phagocytosis of *E. coli* from 4.80 ± 0.96% to 26.05 ± 2.39%, 15.02 ± 2.96%, and 35.04 ± 9.01%, respectively. All of the increases were inhibitable by their respective sugar ligands, namely mannose (BDL1), GlcNAc (BDL2), and GalNAc (BDL3). The enhancement of phagocytosis of *E. coli* by BDL1 was dose dependent (Fig. 2a), reaching a maximum level of phagocytosis at approximately 9000 U/ml. BDL2 and BDL3 were not tested for dose dependency due to inadequate supplies of purified lectin. In contrast to the other endogenous lectins, preincubation of *E. coli* with GSL (Fig. 1b) resulted in no increase in the internalization of *E. coli*. When BDL1 (4500 U/ml) and BDL2 (5000 U/ml) were incubated together, the observed level of phagocytosis (14.91 ± 5.88%) was not significantly different from that seen with each lectin individually at comparable concentrations (17.09 ± 3.73% and 15.02 ± 2.96%, respectively).

With the exogenous lectins (Fig. 3b), those with specificity for mannose, GlcNAc, or GalNAc, i.e., Con A, WGA, and HPA, all augmented the uptake of *E. coli* to a similar extent from 4.80 ± 0.96% to 26.05 ± 2.39%, 31.49 ± 6.79%, and 26.82 ± 6.81%, respectively. The effects produced by the exogenous lectins were all dose dependent and reached a maximum at approximately 80 μg/ml (Fig. 4b). In the case of WGA, there also appeared to be a minimum activation concentration (20 μg/ml) below which no significant increase occurred (Fig. 4b).

**Phagocytosis of *B. cereus***

The basal level of phagocytosis of *B. cereus* in GIM alone (6.74 ± 1.72%), the level of phagocytosis in the presence of BSA (8.10 ± 3.97%), and the level of phagocytosis due to the activation of the proPO cascade with laminarin (9.60 ± 1.57%) were similar to those observed with yeast and *E. coli* (Fig. 1c). In this case, preincubation of bacteria with BDL1, BDL3, and GSL caused no activation of phagocytosis, whereas BDL2 induced a very high level at 49.25 ± 6.91%, which was almost completely inhibitable by 1 mg/ml GlcNAc (Fig. 1c). The dose dependency of BDL2 was sigmoidal in character (Fig. 2b), with a minimum activation concentration of approximately 900 U/ml and a phagocytic maximum at 5000 U/ml.

WGA and HPA both gave similar highly significant enhancements of phagocytosis (28.09 ± 7.36% and 28.42 ± 10.08%, respectively (Fig. 3c)), which were significantly inhibited by their specific sugar ligands, while Con A and TPA had no effect on the internalization of *B. cereus*. Both the enhancements due to WGA and HPA were dose dependent and exhibited their maximum augmentation of phagocytosis at approximately 80 μg/ml, with HPA also showing a minimum activation concentration of 40 μg/ml (Fig. 4c). The effects of both endogenous and exogenous lectins on the phagocytosis of *B. cereus* are summarized in Table II.

The presence of laminarin in monolayers treated with lectin-opsonized test particles had no effect on the lectin-induced enhancement of phagocytosis for any of the microorganisms tested.

**Effect of PTU on the lectin-mediated enhancement of phagocytosis**

PTU is a potent inhibitor of the proPO system, a melanization cascade that is involved in numerous immunologic reactions in...
invertebrates, including nonself recognition processes (31). In the present study, since it has been shown previously that lectins and the proPO system interact (24), PTU was used to determine whether proPO was involved in the lectin-mediated enhancement of phagocytosis.

The presence of PTU produced a total inhibition of the BDL1 and GSL-induced phagocytosis of yeast (Fig. 5a), to levels (7.71 ± 2.53% and 5.47 ± 1.32%, respectively) that were not significantly different from the basal level of phagocytosis in GIM alone. In contrast, phagocytosis was completely unaffected by PTU when BDL1, or BDL2, was incubated with E. coli, with the presence of PTU causing a 40% reduction in phagocytosis, from 48.11 ± 6.99% to 28.93 ± 3.54% (Fig. 5a). This PTU-inhibited phagocytosis was still significantly (p < 0.0001) higher than the nonstimulated level due to GIM alone.

With the exogenous lectins, PTU had no significant effect on the phagocytosis of WGA-incubated yeast or Con A-incubated E. coli (Fig. 5b). Only partial inhibition of the lectin-enhanced phagocytosis was observed with all the other lectin/test particle combinations that induced an increase in phagocytosis. All of these partially inhibited levels of phagocytosis were still significantly higher (p < 0.005) than the respective basal levels of phagocytosis.

Table II. Summary of the effects of endogenous and exogenous lectins on the phagocytic rate by the B. discoidalis plasmatocytes.

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Specificity</th>
<th>Yeast</th>
<th>E. coli</th>
<th>B. cereus</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDL1</td>
<td>Man &gt; GalNAc &gt; Glc, Gal</td>
<td>+++</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>BDL2</td>
<td>GalNAc &gt; Gal &gt; GlcNAc</td>
<td>–</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>BDL3</td>
<td>GalNAc &gt; Gal &gt; Fuc</td>
<td>–</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>GSL</td>
<td>β-1,3-glucan</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Con A</td>
<td>Man &gt; Glc</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>WGA</td>
<td>Chitotriose &gt; GlcNAc</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>HPA</td>
<td>GalNAc &gt; GlcNAc</td>
<td>–</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>TPA</td>
<td>Fuc</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Symbols are as follows: ++++, enhanced phagocytic rate to over 30%; ++, enhanced phagocytic rate to between 20% and 30%; +, enhanced phagocytic rate significantly but to a level below 20%; and −, no enhancement of the phagocytic rate.
Discussion

Since insects lack the Ab-based, nonself recognition system that is so important in the vertebrate immune response, they have to rely entirely upon innate immune defense mechanisms. Like mammals, in which the MBLs activate the complement system and enhance phagocytosis, lectins have also been shown to play an important role as mediators of innate immunity in insects. For example, the multiple lectins in *B. discoidalis* hemolymph are capable of enhancing nonself recognition and ingestion of foreign invaders by activating the proPO system (24), which, like mammalian complement, consists of a complex cascade of immune proteins.

Previously, reports of opsonic activities of insect lectins have demonstrated an enhanced phagocytic rate against Gram-negative bacteria (11, 14), fungal blastospores (9, 13), or erythrocytes (10). In the cockroach, *B. discoidalis*, four lectins, each exhibiting different carbohydrate specificities, have been isolated (22, 23). Two of these lectins, BDL1 and GSL, have been shown to share functional and structural similarities to known acute phase reactants. The present study demonstrates for the first time in an insect that multiple endogenous serum lectins are capable of potentiating the phagocytosis of a variety of very different microorganisms. The observed effects are inhibited significantly by the sugar ligands of the lectins and are, therefore, dependent on the carbohydrate recognition domain of the lectins. Thus, the lectins are probably acting as bridging molecules, by binding to the external polysaccharides of the bacteria and yeast and then to receptors on the surface of the plasmatocytes. This is substantiated by the use of the exogenous lectins, of similar sugar specificities to the *B. discoidalis* endogenous lectins, which produce analogous opsonic effects to those observed with the endogenous lectins, and which were also shown to be capable of binding to the surface of the plasmatocytes. The binding of the endogenous lectins to the hemocyte receptors is probably independent of the carbohydrate recognition domain, at least in the case of BDL1, since no observable reduction in the intensity of binding to the plasmatocytes by Con A was observed in the presence of BDL1.

To examine the role of the *B. discoidalis* lectins in the immune system of *B. discoidalis*, it is essential to first have a knowledge of the polysaccharides exposed on the surface of each microorganism tested. For this reason, well-studied bacteria and yeast were used. First, baker’s yeast, *S. cerevisiae*, is known to have an abundant supply of polymannans on its cell surface (32), which would provide numerous binding sites for the mannose-binding lectins, BDL1 and Con A. Both of these lectins induced a high level of phagocytosis of yeast. Another group of polysaccharides, abundant in yeast cell walls, are the β-1,3-glucans (27). GSL, as its name suggests, binds exclusively to β-1,3-glucans (23), and induced phagocytosis to a level comparable with laminarin-activated cells. This level is surprisingly small, considering the high agglutinating activity of GSL toward yeast, and may suggest that the physiologic role of GSL is more orientated toward the activation of the proPO system, or some other unknown function, than directly toward nonself recognition. Chitin, a polysaccharide composed of repeating units of GlcNAc and N-acetylmuramic acid, is also present, in small amounts, on the yeast cell surface (33). The presence of the GlcNAc residues should provide binding opportunities for BDL2 and WGA. HPA should also bind to the GlcNAc residues, since, although its highest specificity is for GalNAc, it also has an affinity for GlcNAc (Table II). However, neither BDL2 nor HPA had any effect on phagocytosis of yeast, and WGA, which has a very high specificity for chitin, only induced an increase comparable with that obtained by activation of the proPO cascade by laminarin. These results seem to suggest that the chitin in yeast is not present in sufficient quantities to induce a large phagocytic response by *B. discoidalis* plasmatocytes.

*E. coli* is a Gram-negative bacterium, and, therefore, the major constituent of its outer cell membrane is LPS (26). LPS is basically composed of a membrane-bound lipid A moiety bound to a polysaccharide chain of variable length and composition dependent on the strain of bacterium. For these experiments, the laboratory strain K12 was used, which has a relatively simple carbohydrate structure (26, 34) since it lacks the highly variable O-antigenic portion of the polysaccharide chain. Of the lectins tested, only GSL and TPA failed to significantly increase the phagocytosis of *E. coli*, which, on examination of the structure of the K12 LPS, is fairly predictable since neither β-1,3-glucan nor fucose is present. There are, however, numerous binding sites for BDL1 and Con A, since both have activity toward glucose (Table II), while BDL2 and BDL3 can bind to the galactose residues, and BDL2, WGA, and HPA can interact with the terminal GlcNAc residue of *E. coli* K12 LPS (Table II). However, the structure of LPS alone does not explain the high levels of phagocytosis obtained with either WGA or HPA, since the terminal GlcNAc residue is not always present (26). The levels of phagocytosis observed with WGA and HPA are, therefore, probably due to binding of the enterobacterial common O antigen that is found on the cell surface of all members of the *Enterobacteriaceae*. This is a 7-kDa molecule composed of repeating units of GlcNAc and N-acetyl-d-mannosaminuronic acid cross-linked to palmitic acid residues. BDL2 is probably also capable of binding to the GlcNAc residues in this molecule.

Finally, *B. cereus*, like all Gram-positive bacteria, has a thick peptidoglycan layer covering the entire surface of the bacterium (26). Peptidoglycan is comprised of repeating units of GlcNAc and N-acetylglucuronic acid linked by short polypeptide chains, and from this it is obvious why only those lectins with a specificity toward GlcNAc (BDL2, WGA, and HPA; Table II) were able to induce a significant increase in the phagocytosis of *B. cereus*.

It has previously been shown that both endogenous *B. discoidalis* lectins and exogenous lectins are capable of activating the proPO system (24), a melanization cascade important in many invertebrate immune reactions. Also, phagocytosis of bacteria, by insect hemocytes, is enhanced by the activation of the proPO system by laminarin, a β-1,3-glucan, as demonstrated both in the present study and in previous work (35). Therefore, to determine whether the lectin-induced enhancement of phagocytosis was mediated through the activation of the proPO system, or was operating through an independent mechanism, the proPO system was specifically inhibited by PTU.

Three different effects were observed with the various combinations of lectins and microorganisms when the proPO system was inhibited with PTU. First, as was the case with BDL1- and GSL-induced phagocytosis, the presence of PTU completely inhibited the lectin-induced increase in phagocytosis of yeast. Since both of these lectins have been demonstrated to cause an increase in the activation of the proPO system (24), it is probable that BDL1 and GSL are inducing the phagocytosis of yeast via the activation of the proPO system. Second, the opposite effect was observed, with PTU having no effect on the enhanced phagocytosis of *E. coli* in the presence of BDL1, BDL2, or Con A, or the WGA-induced phagocytosis of yeast, indicating that a proPO-independent activation pathway is involved. It may be that the lectin-mediated binding of *E. coli* to the surface of the hemocytes causes direct activation of the phagocytic machinery, possibly through a secondary messenger cascade, such as the binding of small GTPases, which are known to be involved in mammalian phagocytic mechanisms (36). Finally, an intermediate effect was observed with BDL2-opsonized *B. cereus*, and with the majority of the treatments...
opsonized with the exogenous lectins. In all of these cases, the level of enhancement of phagocytosis induced by the lectins was reduced by 40–50%, but remained at a level significantly higher than the basal level, indicating that both the proPO-dependent and proPO-independent mechanisms are involved in producing the lectin-induced level of phagocytosis.

The results presented in this work demonstrate that the multiple serum lectins found in *B. discoidalis*, and also exogenous lectins, are capable of acting as nonself recognition molecules for a variety of potential pathogens, with each lectin potentiating a response with a different set of microbes (summarized in Table II). Since insects lack the Ab-based recognition system found in the vertebrates, the ability of a panel of lectins to potentiate the uptake of a variety of microorganisms is especially important in terms of invertebrate nonself recognition. This study represents the first evidence in an invertebrate that multiple, endogenous serum lectins, of different carbohydrate specificities, are capable of acting as opsonins toward a range of very different microorganisms. Such multiple lectins in cockroaches and related insects not only obviate the need for an Ab-based immune system, but also the presence of antibacterial peptides, which, although present in many other insect species, are apparently absent in cockroaches (unpublished observation). Once an invading microorganism has been recognized and phagocytosed by the numerous circulating hemocytes in *B. discoidalis*, it can be dealt with by intracellular killing mechanisms as those based on superoxide generation (37).

In conclusion, this present study emphasizes the importance of innate immunity based on multiple lectins and activation of a complex, complement-like proPO cascade that is sufficient in cockroaches to obviate the need for circulating antibacterial peptides. The efficacy of the system is clearly illustrated by the huge success of these insects that apparently thrive in extremely inhospitable, microbial-rich environments.

References