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Adherence of *Salmonella typhimurium* to Murine Peritoneal Macrophages Is Mediated by Lipopolysaccharide and Complement Receptors

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Summary

Adherence of *Salmonella typhimurium* to mouse peritoneal macrophages (Mo) was monitored using a direct microscopic assay and flow cytometry. Competitive binding studies using wild-type lipopolysaccharide and derivatives confirmed a role for this moiety in bacterial adherence. Mo pretreated with 2-deoxy-D-glucose exhibited lower binding activity than did untreated controls, suggesting involvement of either Fc or complement receptors. Pre-exposing Mo to Fc fragments, however, failed to reduce bacterial binding, thus eliminating a role for Fc receptors in this process. Mo pretreated with neutrophil elastase exhibited a diminished ability to bind *S. typhimurium*, suggesting involvement of complement receptor 1. Monoclonal antibodies M1/70 and M18/2, specific for epitopes on the α and β chains, respectively, of complement receptor 3, also blocked this adherence. In each case we were unable to eliminate completely bacterial adhesion to Mo. Monoclonal antibodies to two additional Mo receptors, Mac-2 and Mac-3, did not block bacterial attachment. These data indicate that multiple mechanisms are involved in the initial adhesion of *S. typhimurium* to mouse Mo.

Introduction

Salmonellosis is a major infectious disease that occurs world-wide (27). About $1.25 \times 10^7$ cases of human salmonellosis are reported annually; many additional cases of self-limited enterocolitis remain unreported (9). Apart from the typhoid bacillus, animals are the main reservoir of *Salmonella* species and most human infections arise from oral ingestion of contaminated water or food products of animal origin.

Although the precise route of entry of *Salmonella* species into their host is still not completely known (4), they are ultimately transported across the intestinal mucosa to mononuclear phagocytic cells in the underlying lamina propria (9). *Salmonella* species are considered intracellular pathogens and invasion of eukaryotic cells is an essential step for virulence (5). Interaction of *S. typhimurium* and host Mo likely in-
volves receptor-ligand interactions between surface structures found on these respective cells. MØ are known to produce a wide variety of plasma membrane receptors (8).

A variety of microbial products cause activation of MØ. The premier example of this is lipopolysaccharide (LPS), which has multiple effects on these host cells. MØ activation results in enhanced expression of various receptors, including complement receptor (CR) 1 and CR3 (14) leading to increased phagocytosis (10, 13). Although these receptors have been implicated in opsonin-independent recognition and uptake of microorganisms (1, 21), other, lectin-like structures on the MØ have also been shown to mediate bacterial attachment through recognition of complementary sugar structures on the microbial surface (16).

The present study was developed to determine the role of LPS and key MØ receptors in the interaction of S. typhimurium and mouse MØ.

Materials and Methods

Chemicals. All reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise noted.

Bacteria. S. typhimurium ATCC strain 14028 was obtained from Difco, Inc. (Detroit, MI, USA). The cells were grown in LB broth (Difco) to late logarithmic growth phase at 37°C. The cell suspensions were centrifuged at 10000 × g for 1 h, washed twice in phosphate-buffered saline (PBS), pH 7.0, resuspended in PBS, and adjusted to a concentration of 2 × 10⁹ cells/ml in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA).

Macrophages. BALB/c mice, originally obtained from Charles River Laboratories (Wilmington, MA, USA), were bred on site. Male mice, 8–12 weeks of age, were injected intraperitoneally with 2 ml of aged Brewer thioglycollate broth (Difco). After 72 h the mice were sacrificed by CO₂ inhalation and the peritoneal exudate cells collected in 10 ml of cold Dulbecco's PBS (DPBS). The cell suspension obtained was centrifuged at 100 × g for 10 min to sediment the MØ; the cells were washed 3–4 times in 2 ml of cold RPMI medium and kept on ice until used. The cells were counted using standard hemocytometric techniques and the concentration adjusted for the appropriate assay.

Binding assay to MØ monolayers. Adherence of S. typhimurium to MØ was determined by direct microscopic observation based on the procedure of Glass et al. (7) as modified in our laboratory (20). Each experimental system contained approximately 3 × 10⁵ MØ and 3 × 10⁸ bacteria. Mixtures were incubated at 37°C for 1 h in the presence of 5% CO₂.

Downmodulation assay. Microscope slides were pretreated with varying dilutions of the test substance in 0.1 m carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C (29). The slides were washed and MØ monolayers prepared as described above. Bacteria were added and the binding assay completed as noted above.

Lipopolysaccharide. Purified LPS, derived from S. typhimurium, and 2-keto-3-deoxyoctonate (KDO) were commercially obtained. Glycolipid, derived from Salmonella minnesota R595, was prepared in our laboratory by the method of Galanos et al. (6). LPS and KDO were each suspended in DPBS at a final concentration of 1 mg/ml. The R glycolipid was initially dissolved in acetone/ether (1:1 v/v), then DPBS was added to bring the contents to a final concentration of 0.1% acetone/ether and 0.5 mg/ml of the glycolipid.

Treatment of MØ with 2-deoxy-D-glucose. The basic procedure of Sung and Silverstein (24) was followed. In brief, MØ monolayers were incubated with varying concentrations of
this sugar for 1 hr at 37 °C, prior to the addition of bacteria. The binding assay was completed as described above.

Treatment of Mo with neutrophil elastase. Lyophilized human neutrophil elastase (110 U/mg) was dissolved in distilled water to a final concentration of 1 mg/ml, then further diluted in DPBS immediately prior to use. Appropriate concentrations were added to Mo monolayers, as described by Tosi et al. (26). The cells were incubated for 1 hr at 37 °C, then washed three times before the addition of bacteria. The binding assay was completed as indicated above.

Monoclonal antibodies. The following rat anti-mouse hybridomas were obtained from the American Type Culture Collection (Rockville, MD): M1/70.15.11.5 (ATCC TIB 128; anti-CD11b; ref. [22]), M18/2a.12.7 (ATCC TIB 218; anti-CD18; ref. [19]), M3/38.12.8.HL.2 (ATCC TIB 166; anti-Mac-2; ref. [11]), and M3/84.6.34 (ATCC TIB 168; anti-Mac-3; ref. [12]). For brevity these clones are referred to as M1170, M18/2, M3/38, and M3/84, respectively. M3/84 and M3/38 were grown in RPMI 1640 medium, M18/2 in RPMI 1640 medium plus 0.05 mM 2-mercaptoethanol, and M1/70 in Dulbecco's modified Eagle's medium with 4.5 g/L of glucose. In each case the medium was supplemented with 10% low-IgG fetal bovine serum (HyClone Laboratories, Logan, UT, USA), 2 mM L-glutamine, and penicillin-streptomycin. The supernatant fluid, containing antibody, was separated from the cells by centrifugation (100 x g). MAb were purified by 50% saturated (NH₄)₂SO₄ precipitation and affinity chromatography on a Protein G Superose® Fast Flow column (Pharmacia LKB Biotechnology, Piscataway, NJ, USA), equilibrated with 20 mM phosphate-buffered saline (PBS) containing 0.1% thimerosal. The IgG was eluted with 0.1 M glycine, pH 2.7, and collected as 1.5 ml fractions in tubes containing 45 μl of 1.0 M Tris·HCl, pH 9.0 to preserve the activity of the acid-labile IgG. Column size and flow rates were determined by rat IgG₂a and IgG₂b binding properties, as provided by the manufacturer.

Flow cytometry. Flow cytometry was used to examine the adherence of S. typhimurium to Mo in suspension. These assays were conducted using a FACScan model (Becton-Dickinson, FACS Systems, Sunnyvale, CA, USA) with computer-assisted evaluation of data (FACStarPLUS Lysys II software). Bacteria were labeled with lucifer yellow VS (23) by incubating S. typhimurium (10¹⁰ organisms) with 1 mg/ml of lucifer yellow in 0.1 M NaHCO₃, pH 9.5, for 2 h at room temperature, then washing the cells 5 x in DPBS (25). Washed peritoneal exudate cells, containing 95% Mo at 4 x 10⁶/ml, and labeled bacteria at 2 x 10⁹/ml were incubated for 1 h at 37 °C to allow for adherence. For blocking studies Mo were pretreated for 1 h at 37 °C with potential inhibitors prior to addition of the labeled bacteria. After 1 h the samples were fixed with an equal volume of 2% formaldehyde and subsequently examined by flow cytometry.

Statistical analyses

Data from blocking and down-modulation studies on Mo monolayers were analyzed using factorial analysis of variance and, where appropriate, Scheffe's test of significance (StatView 4.01, Abacus Concepts, Berkeley, CA, USA).

Results

Role of LPS and its derivatives on bacterial adherence to Mo

The ability of purified LPS from S. typhimurium to inhibit attachment of whole S. typhimurium to Mo monolayers was assessed in bacterial binding assays. The results, presented in Fig. 1A, indicate a direct correlation between the concentration of LPS and the degree of inhibition. The concentration of LPS needed to achieve 50% inhibition of bacterial adherence was ~280 μg/ml. To determine which part of the
Fig. 1. Effect of pretreating Mø with LPS or LPS fractions on the adherence of S. typhimurium. A. LPS components were added to Mø monolayers at various concentrations and the systems were incubated for 1 h. Excess LPS material was removed by washing and the bacteria were added. Adherence was determined by microscopic examination. Data are expressed as means ± 95% confidence limits. Adherence refers to the number of Mø out of 500 examined with 5 or more bacteria attached. B. LPS components (400 µg/ml) were added to suspensions of Mø and the mixtures incubated for 1 h. Bacteria were added to the mixtures with an additional 1 h incubation. Samples were fixed in formaldehyde and adherence was determined by flow cytometry. A shift to the left in these binding histograms indicates diminished adherence. Key: A = LPS treated, B = KDO treated, C = Lipid A treated, D = control.

LPS molecule is involved in adherence of S. typhimurium, we examined the effects of two derivatives, glycolipid derived from the R595 mutant of S. minnesota and purified KDO, in bacterial binding assays. As shown in Fig. 1A, each derivative exhibited inhibitory activity; however, Mø pretreated with wild-type LPS exhibited a greater dose-dependent inhibitory effect (p < 0.014 compared to lipid A; p < 0.004 compared to KDO). Fluorescence histograms, derived from flow cytometry studies and shown in Fig. 1B, indicate that each preparation reduced the mean fluorescence intensity compared to the untreated control, thus supporting results from the visual binding assays.

Effect of 2-deoxy-D-glucose on bacterial adherence

To determine whether Fc or complement receptors were involved in the adherence of S. typhimurium, we treated Mø with 2-deoxy-D-glucose, then performed binding assays. When treated Mø monolayers were tested, binding of these bacteria was reduced in a dose-dependent manner over the range of 100 to 500 µg/ml, with ~ 400 µg/ml being the concentration that effected a 50% inhibition of bacterial adherence (Fig. 2A; p < 0.0001 compared to untreated control). A comparable study using suspensions of Mø and monitored by flow cytometry yielded similar results (Fig. 2B).
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Fig. 2. Effect of 2-deoxy-D-glucose on the adherence of *S. typhimurium* to *Mø*. A. Monolayers of *Mø* were pretreated with various concentrations of this agent for 1 h, then washed. B. Suspensions of *Mø* were incubated with 2-deoxy-D-glucose (500 μg/ml) for 1 h. See legend to Fig. 1 for additional experimental information. Key: A = 2-deoxy-D-glucose treated; B = control.

**Effect of neutrophil elastase on bacterial adherence**

Neutrophil elastase has been shown to specifically cleave CR1 (CD35; ref 26). *Mø* pretreated with this enzyme were tested for their ability to mediate adherence of *S. typhimurium*. As seen in Fig. 3A, a dose-dependent reduction in bacterial attachment to *Mø* monolayers was observed over the concentration range tested (p < 0.0001 compared to untreated control). The concentration of elastase that yielded a 50% reduction in bacterial adherence was ~18 μg/ml. The inhibitory effect of this enzyme was confirmed in flow cytometry studies (Fig. 3B), suggesting that CR1 is involved in this adherence.

**Effect of anti-Mø MAb on bacterial adherence**

Three MAb, with specificity for epitopes on the β2 integrins, were tested for their ability to block attachment of *S. typhimurium* to *Mø*. In downmodulation studies *Mø* monolayers developed on surfaces coated with either MAb M1/70 or M18/2 exhibited significantly lower bacterial binding levels than those developed on MAb 5C6-coated surfaces (p = 0.025 and p = 0.0024, respectively; Fig. 4A). The concentration of MAb needed to achieve a 50% reduction in bacterial adherence was ~80 μg/ml for M1/70 and ~85 μg/ml for M18/2. There was no significant difference in the inhibitory effect of M1/70 and M18/2 (p = .65). *Mø* suspensions exposed to either M1/70 or M18/2 prior to addition of labeled bacteria exhibited reduced mean fluorescence in flow cytometry studies, compared to untreated controls (Fig. 4B), confirming the results obtained in down-modulation studies. MAb 5C6 was not used in the flow cytometry studies.
Fig. 3. Effect of neutrophil elastase on the adherence of *S. typhimurium* to Mø. A. Monolayers of Mø were pretreated with varying concentrations of elastase for 1 h, then washed. B. Suspensions of Mø were incubated with elastase (30 μg/ml) for 1 h. See legend to Fig. 1 for additional experimental information. Key: A = elastase, B = control.

Fig. 4. Effect of MAb to β2 integrins on the adherence of *S. typhimurium* to macrophages. A. Macrophage monolayers were developed on surfaces coated with various concentrations of these MAb. Bacteria were added and the systems incubated for 1 h. B. Suspensions of Mø were pretreated with MAb (100 μg/ml) for 1 h. See legend to Fig. 1 for additional experimental information. Key: A = M18/2 treated, B = M1/70 treated, C = control.
Fig. 5. Effect of combined treatments on bacteria adherence to macrophages. Monolayers of Mø were pretreated with two anti-β2 MAbs (M1/70 and M18/2, 50 or 100 μg/ml each) with and without treatment with neutrophil elastase (30 μg/ml), then washed. See legend to Fig. 1 for additional experimental information.

Because inactivation of CR1 and blocking of CR3 each produced partial inhibition of bacterial binding, we tested combination treatments to determine whether complete blockage of bacterial adherence could be obtained. Combinations of MAb M1/70 and M18/2 (50 and 100 μg/ml each) and of these two MAb plus neutrophil elastase (20 μg/ml) to inactivate CR1 failed to achieve complete inhibition when tested on Mø monolayers (Fig. 5; p = .28 for combined treatment vs. treatment with two MAb alone), suggesting a role for other, as yet unidentified Mø receptors.

Discussion

Salmonella spp. are considered intracellular pathogens (5) and invasion of eukaryotic cells is an essential step in the infectious process. Although factors such as specific antibody and complement can enhance the uptake of microorganisms by phagocytes, recognition mechanisms independent of these opsonins have also been described (16). The present study was initiated to identify possible structures on the surface of both the microbial pathogen and the host defense cell involved in non-opsonin-mediated recognition.

Our basic assay system was one using Mø that had been allowed to adhere to glass slides (20). Because of the possibility of non-specific blocking or steric hindrance, in some studies we included a second approach in which the putative inhibitor was bound to glass slides; Mø monolayers were subsequently developed on these pretreated slides. The rationale here is that receptors that are free to move within the plane of the membrane and that recognize the bound substance will become depleted on the apical surface as they form complexes with the glass-bound reagent. Such receptors...
are said to be "downmodulated" (29). Finally, because of the numerous washing steps required in each of these studies, loosely associated bacteria may be removed, thereby yielding inappropriately low binding values. To circumvent this problem, we developed an adherence assay using cell suspensions in which no washing steps were used. In this system fluorescently labeled bacteria attached to Mø were detected by flow cytometry.

Since LPS is a predominant structure on the surface of Gram-negative bacteria such as S. typhimurium, it is a likely candidate for mediating bacterial adherence to host cells. Our data support a role for this outer membrane component in bacterial attachment to murine Mø, since free LPS inhibited this process. Wright and Jong (28) proposed that LPS mediates attachment of Escherichia coli to human Mø, possibly via the glucosamine disaccharide backbone of the lipid A molecule. Our data are consistent with theirs; however, the ability of free KDO to partially block bacterial attachment suggests that more than one site on the LPS molecule may be involved in the S. typhimurium system. Our finding that wild-type LPS is a more effective inhibitor of bacterial attachment than the O-side chain-deficient glycolipid from the Salmonella R mutant is in agreement with those of Mroczenski-Wildey et al. (15) and Finlay and Falkow (4), who showed that Salmonella species with disrupted O-side chains or core structures adhered poorly to epithelial cells.

Non-opsonin-mediated binding of Mycobacterium avium (17) and E. coli (18) has been found to occur via receptors for the Fc portion of immunoglobulin molecules and for complement. Sung and Silverstein (24) demonstrated the inhibitory effect of 2-deoxy-D-glucose on the uptake of bacteria via Fc receptors as well as CR1 and CR3. Similarly, Sloan and Pistole (21) showed that this chemical blocked adherence of group B streptococci to murine peritoneal Mø. In the present study 2-deoxy-D-glucose was found to block adherence of S. typhimurium, suggesting that one or more of these Mø receptors is involved in this binding.

Saturating Fc receptors on these Mø with either Fc fragments derived from mouse polyclonal antibodies or an irrelevant mouse MAb failed to reduce bacterial adherence to those cells (Al-Bahry and Pistole, unpublished data), suggesting that Fc receptors are not involved in the direct recognition of S. typhimurium. The involvement of CR1 in the adherence process was confirmed using Mø treated with neutrophil elastase, an enzyme that specifically cleaves CR1 (26). MAb M1/70 and M18/2 each blocked bacterial adherence, confirming a role for both the α and β subunits of CR3 in attachment of S. typhimurium. These results are similar to those of Wright and Jong (28) in which they demonstrated a role for the β subunit in the recognition of E. coli LPS and confirmed that adherence of this organism occurred at an epitope distinct from the C3bi binding site (30). In our studies, however, bacterial adherence was also reduced by blocking with MAb to the α subunit, suggesting that CD11b is also an important determinant on Mø involved in recognizing S. typhimurium. The MAb 5C6, which recognizes a distinct epitope on CD11b (3), exhibited no blocking activity, confirming the specificity of our findings.

Both Mac-2 (11) and Mac-3 (12) are highly expressed on thioglycollate-elicited Mø. Although they seemed likely candidates for bacterial receptors, we were unable to demonstrate any significant reduction in bacterial adherence when Mø were pretreated with MAb to either membrane component (Al-Bahry and Pistole, unpublished data).

In no instance could we completely inhibit bacterial adherence, even by blocking multiple receptors simultaneously, suggesting the presence of additional mechanisms
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involved in this recognition process. Gordon et al. (8) reported that receptors for complement and other components on phagocytic cells can synergize in the binding and ingestion of microorganisms. Strong support of this concept was provided by Bermudez et al. (2), who confirmed the role of three MØ receptors – CR3, the fibronectin receptor, and the mannose-fucose receptor – in the uptake of Mycobacterium avium by human MØ. Other receptors, including CR1, Fc receptor, transferrin receptor, mannose receptor, and possibly a separate β-glucan receptor were also found to be involved in nonopsonic uptake of M. avium by both blood monocytes and alveolar MØ (17).

It is also possible that these phagocytic cells either synthesize new receptors or recycle existing ones, leading to expression or re-expression of the same structures that had been originally blocked. Additional studies are required to determine whether such possibilities contribute to the results obtained in the present study.

In conclusion, adherence of S. typhimurium to murine MØ can occur in the absence of exogenous opsonins. Results from inhibition and down-modulation studies suggest that multiple mechanisms are involved. Ongoing studies in our laboratory are directed at identifying these additional, as yet unidentified, MØ receptors.

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