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**Adherence of *Salmonella typhimurium* to murine peritoneal
macrophages**

Al-Bahry, Saif Nasser, Ph.D.

University of New Hampshire, 1994

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**ADHERENCE OF *Salmonella typhimurium* TO
MURINE PERITONEAL MACROPHAGES**

BY

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DISSERTATION

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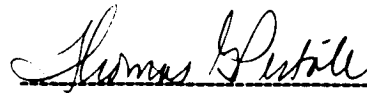
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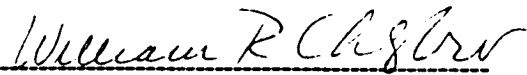
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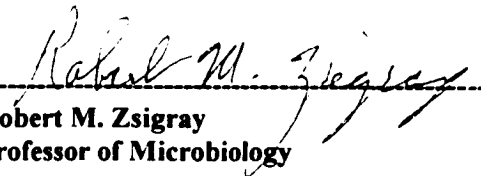
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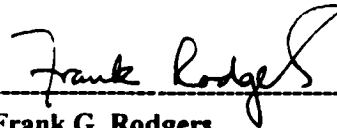
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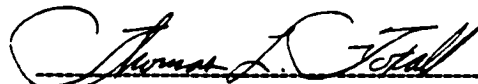
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GENERAL ABSTRACT

ADHERENCE OF *Salmonella typhimurium* TO MURINE PERITONEAL MACROPHAGES

by

Saif N. Al-Bahry

University of New Hampshire, May, 1994

Salmonella species are responsible for wide spread disease in both humans, and animals and macrophages (MØ) play a central role in host defenses against disease. Recent evidence has shown that MØ bear surface receptors involved in direct microbial recognition. Using the mouse model and a virulent strain of *S. typhimurium*, we have attempted to determine the means by which peritoneal MØ recognize this pathogen in the absence of serum factors. Bacterial adherence was monitored by direct microscopic visualization and by flow cytometry. Two strains of *S. typhimurium* strains 1826 and ATCC 14028 were used in this model. Adherence was much lower for strain 1826. Homologous wildtype lipopolysaccharide and, to lesser extent, Re glycolipid and the core sugar 2-keto-deoxyoctonate (KDO) effectively blocked bacterial binding. Two-deoxyglucose, known to inhibit phagocytosis via complement receptors (CR) or Fc receptors, and neutrophil elastase, which specifically cleaves CR1, each reduced binding. Monoclonal antibodies directed against the α and β chains of CR3 also reduced binding. Residual bacterial binding activity remained even when both CR1 and CR3 were blocked.

We suggest that the initial recognition is a multifactorial process involving ligands on the bacterial LPS and both CR1 and CR3 plus other as yet unidentified MØ structures. The role of other receptors on MØ was also examined. Antibody to C1q receptor enhanced binding of *Salmonella* to MØ, while antibodies to the Mac-2 and Mac-3 receptors had no effect. Using electrophoresis and Western blot techniques, we were able to isolate two MØ membrane-proteins each with molecular weight of 16 and 13 kilodaltons. The proteins were identified by labeled bacteria cells. Similarly, we have identified *S. typhimurium* outer membrane protein that binds to macrophage. The protein has a molecular mass of 44 kilodaltons and is expressed under anaerobic conditions.

GENERAL INTRODUCTION

I. *Salmonella* infection:

Salmonellosis continues to be one of the major catastrophic diseases around the globe (WHO News and Activities, 1991). About 12.5×10^6 cases of human salmonellosis are reported annually, excluding most of the self-limiting enterocolitis cases that go unreported (Groisman *et al.*, 1990). Forty thousand cases are reported in the United States alone, resulting in 500 fatalities and more than 50 billion dollars in health care expenses (Cohen *et al.*, 1986). About 25-30% of these cases in the United States are due to *Salmonella typhimurium* (Doyle *et al.*, 1990), which is also a prominent cause of salmonellosis in animals. In general, people with poor health are much more prone to even low doses of *Salmonella* infection. Animals are the main reservoir of *Salmonella* species (Goldstein *et al.*, 1986) with the exception of *S. typhi* for which humans are the only reservoir (Hardy, 1983). Salmonellosis occurs as enteric fever, gastroenteritis, empyema, bone and joint infections, or a combination of these complications (Hadfield *et al.*, 1985; Hardy 1983).

Most *Salmonella* infections arise from oral ingestion of contaminated water or food products of animal origin (Sherris, 1990). Internalized organisms proceed to the distal small bowel (Carter *et al.*, 1974). The majority of bacteria invade epithelial cells through their apical membrane (Groisman *et al.*, 1990) and associate with mouse ileal membranous (M) cells (Yokoyama *et al.*, 1987) where they become translocated to

Peyer's patches. Only strains that cause enteric fever penetrate past the basolateral membrane of the epithelial cell and travel to their principal target of infection, the macrophages underlying the lamina propria (Groisman *et al.*, 1990). These cells are professional phagocytic cells that form an important line of defense against invading microorganisms. The invading bacteria are subsequently transported within macrophages to regional lymph nodes, the spleen and the liver, where *Salmonella* may multiply further and become disseminated haematogenously (Finlay *et al.*, 1989_b).

The ability of salmonellae to cause severe infections is due to virulence determinants (Fig. 1). Some of the virulence factors and their mechanisms by which salmonellae initiate their infectivity are examined below.

A. Motility and chemotaxis

Salmonellae are highly motile, possessing flagella and chemotactic functions (Finlay *et al.* 1989_b). Motility plays an important role in invasion and establishment of colonization of some *Salmonella* species. *S. typhi* requires intact motility to adhere and to invade epithelial cells, and mutations that affect flagella, or chemotaxis render the microorganism unable to enter these cells. However, *S. typhimurium* motility is not required to invade epithelial cells. Flagella are also not crucial for growth and survival in the spleen and liver (Finlay *et al.*, 1988_b). Even though *S. typhimurium* flagella display positive chemotaxis and may play a role to initiate colonization, they are not required for invasion of epithelial cells.

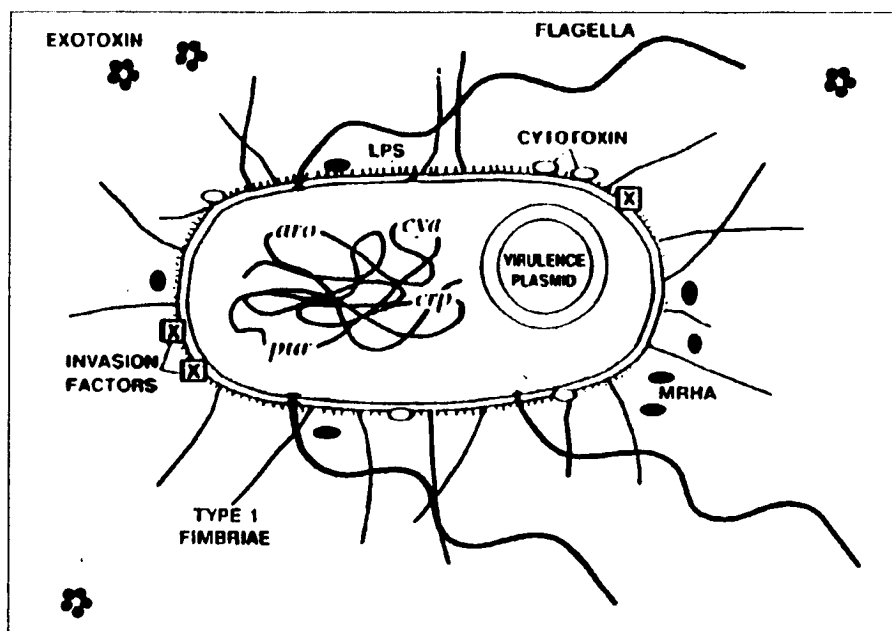


Fig. 1. Schematic drawing of proposed virulence factors of *Salmonella*. Finlay *et al.*, 1988.

B. Adherence

Binding of bacteria to host cells protects them from being swept from the ileum by peristalsis and thus enhances their ability to colonize the epithelia, multiply and invade the host (Sharon, N. 1987). *Salmonella* are capable of expressing various factors for adherence. One of these are type 1 pili (fimbriae) which are also produced by other members of enterobacteriaceae such as *E. coli* (Finlay *et al.*, 1988_b). These fimbriae are homogeneously dispersed on the bacterial cells (Sharon, 1987). Bacteria with type 1 fimbriae are genotypically capable of spontaneously shifting back and forth from a fimbriated to a non-fimbriated phase. This phase variation in *Salmonella* species contributes to their ability to evade host defense mechanisms (Lockman *et al.*, 1992). The distal tip of the fimbriae consists of a very small amount of an additional subunit that carries the carbohydrate-binding site (Sharon, 1987). This subunit is involved in mediating the adherence of bacteria to eukaryotic cells. Thus, type 1 pili provide bacterial cells with an advantage in colonizing epithelial cells in the intestinal tract (Lockman *et al.*, 1992).

C. Entry of *Salmonella* into the host

The precise area of entry of *Salmonella* species into their host is still unknown (Finlay *et al.*, 1989_b). There is a strong evidence that M cells within Peyer's patches (rather than columnar absorptive epithelial cells) preferentially ingest invasive organisms, including *S. typhimurium* and *S. typhi* (Yokoyama *et al.*, 1987). M cells exist in the follicle-associated epithelium of the Peyer's patches. Their function is to take up antigenic substances within the intestinal lumen and to be selectively transmitted to lymphocytes and macrophages and

antigen-presenting cells (Kamoi, 1991). However, association of salmonellae with M cells has not yet been correlated with systemic infection (Finlay *et al.*, 1989_b). It is likely that splenic infections originate from a source other than Peyer's patches, but not via the absorptive epithelial cells in the villi (Kamoi, 1991). M cells could also contribute to a determination of the host-specificity of various *Salmonella* species by providing a specialized epithelial cell with which to interact (Finlay *et al.*, 1989_b).

D. *Salmonella* invasion of eukaryotic cells

Research on *S. typhimurium* pathogenesis with guinea-pig ileal cells suggests that bacteria interact with the apical epithelial surface and cause the microvilli and the terminal web of the brush border to degenerate (Finlay *et al.* 1989_b). It seems bacterial invasion occurs into two pathways. Some bacteria bind to the apical epithelial cell surface and invade those cells where they remain within a membrane-bound inclusion. *Salmonella* then proceed through the cell, and transcytose to the basal side of the epithelial cell. Other bacterial cells proceed between the zonular occludens of epithelial cells. These junctions become separated, then reseal after bacterial crossing. After bacteria have transcytosed, the brush border reassembles and the *Salmonella* proceed to Peyer's patches underlying lamina propria and to the reticuloendothelial system where they propagate within the mononuclear phagocytes in the liver, spleen, and lymph nodes resulting in systemic diseases (Groisman *et al.*, 1990).

Salmonella species enter eukaryotic cells and are considered intracellular pathogens (Finlay *et al.*, 1988_b). Invasion of eukaryotic cells is an essential step for virulence. It is

an intricate mechanism involving numerous factors. Bacterial protein and RNA synthesis is required (Finlay *et al.*, 1988_a). Epithelial cell surfaces cause the induction of several new bacterial surface proteins that are essential for invasion and virulence (Buchmeier *et al.*, 1990). These induced proteins are probably required for entry and possibly for intracellular survival. The invasion process requires viable *Salmonella*, as well as live host cells. It requires polymerization of cytoskeleton microfilaments. Host microtubules and intermediate filaments are not needed for *Salmonella* internalization (Finlay *et al.*, 1991; Shinji *et al.*, 1991).

1. Invasion loci

A genetic locus, *inv*, has been identified that affects the entry of *S. typhimurium* into epithelial cells, but it is not necessary for bacterial adherence (Finlay *et al.*, 1989_b). Several *Tn10* mutants in *S. typhimurium* have been identified that have low invasion capabilities. One of these mutants is avirulent in mice. It has decreased adherence and invasion capabilities for epithelial cells and macrophages in mice. This mutant is defective in its ability to synthesize proteins induced by epithelial cells, which are necessary for adherence and invasion.

E. Toxins

Salmonella species express at least three toxins: endotoxin (lipid A), enterotoxin (Baloda *et al.*, 1983) and cytotoxin (Finlay *et al.*, 1988_b). *Salmonella* gastrointestinal infections are usually accompanied by a cholera-like diarrhea. *Salmonella* enterotoxins are

closely related to cholera toxin. Both toxins elevate intracellular cyclic AMP levels by affecting adenylate cyclase.

Severe destruction occurs to the intestinal mucosal surface in *Salmonella* gastroenteritis. Villi are shortened by loss of their tip region, and other cytopathic changes result in tissue damage and bleeding. A non-lipopolysaccharide (LPS) compound of the *Salmonella* outer membrane has been shown to inhibit eukaryotic protein synthesis and to elongate CHO cells, both of which are signs of cytotoxic activity (Finlay *et al.* 1989_b).

The lipid A component of lipopolysaccharide (LPS) is a potent endotoxin, having many toxic effects. Most of these activities are due to interaction of endotoxin with macrophages and lymphocytes, causing activation of these cells (Langermans *et al.*, 1989). Activation of macrophages results in the release of several factors, which leads to a wide variety of biological effects such as pyrogenicity, leucocytosis, and hypotension (which can, in turn, lead to shock) (Finlay *et al.*, 1988_a).

F. Lipopolysaccharide

Lipopolysaccharide (LPS) is a complex molecule composed of three components: lipid A, core and O-side chain oligosaccharides (Finlay *et al.*, 1988_b) O-side chains are attached to the conserved core structure, which, in turn, is joined to the lipid A. Lipid A is central part of the bacterial outer membrane, and is a potent endotoxin. The O-side chains are composed of repeating oligosaccharides which vary among *Salmonella* species.

Mutations in the genes involved in core or O-side chain render *Salmonella* avirulent. LPS for *Salmonella* may also be involved in attachment to and invasion of intestinal epithelial

cells and resistance to serum-mediated killing. Even though LPS is a good activator of complement, it hinders the deposition of complement on the bacteria surface to various degrees, depending on its composition (Joiner, 1985). LPS may also contribute to intracellular survival within macrophages, perhaps by protecting the bacterium from oxygen radicals and other bactericidal substances (Finlay *et al.*, 1988_b)

II. Professional Phagocytes:

Our environment is filled with different pathogens, viruses, bacteria, fungi and parasites (Roitt *et al.*, 1989). These infectious agents can cause serious damage to their host. Most infections in normal individuals are of short duration and cause little damage due to the individual's immune system.

The immune system is divided into two functional classes: the adaptive immune system and the innate immune system. Innate immunity is the first line of defense against infectious agents and most pathogens are thwarted before they can initiate an infection. The adaptive immune system is activated if these first defenses are breached. This system produces a specific reaction to eradicate the invading agent.

The innate immune system includes various molecules and cells located throughout the body. The most critical cells are the leukocytes. The leukocytes are classified into two broad classes: phagocytes, including neutrophils polymorphonuclear monocytes and macrophages, which form part of the innate immune system, and lymphocytes, which mediate adaptive immunity. The former group is known as the professional phagocytic system.

A. Macrophage receptors

Because of the wide distribution of macrophages and variety of macrophage functions and potential ligands, macrophages are able to produce a wide variety of plasma membrane receptors (Gordon *et al.*, 1988). Macrophages express surface receptors of several superfamilies (Ig, integrin and a family of structurally related proteins that interact with C3b or C4b). In addition, there are various lectin-like receptors on macrophages, one of which, the mannosyl-fucosyl receptor (MFR), is related to a circulating acute phase mannose-binding plasma protein produced by the liver.

B. Activation of macrophages

Macrophages are activated by various products. Such activated leukocytes express their function in a non-specific way and are capable of showing enhanced microbicidal effects against different infective agents (Ögmundsdóttir, 1980).

A number of microbial products cause activation of macrophages (Roitt *et al.*, 1989). In the case of Gram-negative bacteria such as *Salmonella*, the lipid A portion of lipopolysaccharide (LPS) is a very potent activator of macrophages. It induces a variety of effects on macrophage function (Shinji *et al.*, 1991). Inflammatory molecules, including IL-1 (interleukin-1), TNF (tumor necrosis factor), and C3 (the third component of complement), are also generated. MØ activation results in the enhanced expression of various receptors, such as complement receptors CR1 and CR3 (Marra *et al.*, 1990), leading to enhanced phagocytosis (Ögmundsdóttir, 1980). LPS also induces the reorganization of microfilaments in macrophages, the breakdown of

phosphatidylinositides, the activation of protein kinase C, and the myristoylation and the phosphorylation of some proteins (Shinji *et al.*, 1991). Elevation of the intracellular calcium concentration has also been demonstrated.

C. Surface interactions between phagocytes and infectious targets:

Once phagocytes have arrived at a site of inflammation the phagocytes need to recognize the infectious agent. Attachment of the organism to the phagocyte surface is a critical step that precedes the uptake of the infective agent and subsequent killing of the organism. The binding is mediated by opsonin-independent and opsonin-dependent mechanisms.

1. Opsonin-independent mechanism

The opsonin-independent adherence occurs as a result of i) physical potentials, ii) lectins on the organism, such as the mannose-binding lectin on the fimbriae of *Salmonella* and *E. coli*, and iii) lectins on the phagocytes, such as complement receptor CR3 and related molecules (CR4 and LFA-1), which have multiple binding sites with different specificities and can bind to LPS of Gram negative bacteria.

a. Physical potentials influence cell-particle interactions

Classically, the association between particles and/or cells of different compositions and sizes is held to be a function of their electrostatic repulsion (living cells and other biological particles generally carry a negative net charge), and their van der Waals

attraction, also taking into account the interaction with and among the molecules of the liquid medium (Sharon, 1987). In the normal situation, negative surface potentials of both phagocytic and bacterial cells would prevent the establishment of contact between them. However, due to the small radius of curvature of bacteria and to the ability of phagocytes to elongate pseudopodia, that electrostatic repulsion usually can be eliminated. The tendency of phagocytes to extrude pseudopodia with extremities of small radii of curvature permits them to overcome the electrostatic repulsion and thus to initiate contact with most bacteria (Fig 2). As soon as an interparticle or intercellular distance of less than 8 Å is achieved, electrostatic repulsions become less important and van der Waals attractions become preponderant leading to adhesion. In addition, specific receptors may become sufficiently close to their ligands, e.g., Fc moieties, to attach to them via both electrostatic and van der Waals attractions.

b. The role of lectins on macrophages

Similar to other cells, phagocytes are coated with oligosaccharide units of membrane glycoproteins and glycolipids which are known as lectins (Sharon, 1984). Carbohydrate-lectin interactions facilitate cell-to-cell recognition in nonimmune phagocytosis.

The phagocyte lectin-like receptors, including the β -glucan receptor and a 170-kD macrophage-specific mannose receptor, are expressed on the surface of tissue macrophages but not on circulating phagocytes (Kuhlman *et al.*, 1989). The mannose receptor interacts with mannose-rich particles like zymosan, resulting in engulfment of the

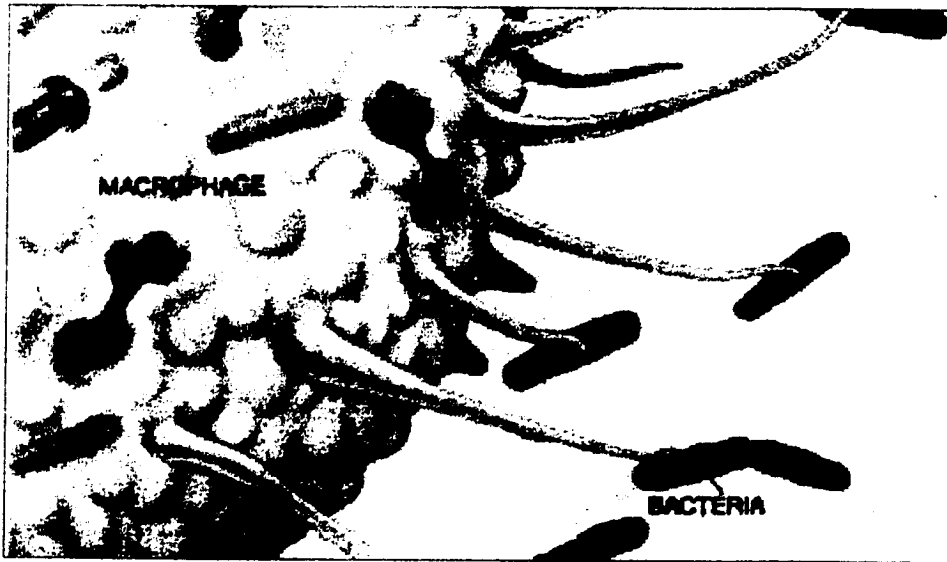


Fig. 2. Schematic diagram of macrophage attached to bacterial cells by its pseudopodia in the absence of opsonins. Some bacterial cells are being engulfed (Paul, W. 1994).

particles and the release of biologically active secretory products such as reactive oxygen intermediates, arachidonate metabolites, and neutral proteinases. This interaction, known as "lectinophagocytosis", most typically occurs between bacterial pili or fimbriae and their receptors on phagocytes (Cross *et al.*, 1990).

2. Opsonin-dependent mechanisms

Serum factors enhance the rate of uptake of microorganisms by phagocytes (Ofek *et al.*, 1988). These factors are called opsonins, and the process by which the phagocytes engulf microbial agents is known as opsonophagocytosis. Some of the well known opsonins are: i) complement deposited via the alternative or classical pathways. ii) antibody, which links bacteria to the Fc receptors on MØ. iii) other opsonins: mannose- and LPS-binding proteins, which link bacteria to MØ via specific receptors, such as mannose receptor.

The primary role of the opsonins is to provide a means of recognition between the phagocytes and their targets. This is especially important for those infectious agents that can escape lectinophagocytosis. The opsonins function by binding specifically in a lock-and-key manner to integral surface membrane molecules on the phagocytes on one side and to the bacteria on the other side, resulting in uptake of the opsonized particle.

Deposition of these opsonins on the surface of *Salmonella* species enhances their phagocytosis. However, there is growing evidence that, under conditions such as an opsonin-poor environment, *Salmonella* may be able to bind directly to host macrophages

in the absence of either antibody or complement. This direct association may be important for intracellular pathogens such as *Salmonella* to allow them to invade eukaryotic cells such as macrophages. Opsonin-poor environments may occur in immunodeficient hosts (e.g., immunosuppressed individuals, AIDS patients, or neonates) where the host is unable to synthesize opsonins (Baret, 1983) or in persons with defective opsonin receptors (Anderson *et al.*, 1987).

Persons with an inherited defect in their ability to synthesize the C3 complement component exhibit a depressed ability to opsonize bacteria and suffer repeated bacterial infections (Alper *et al.*, 1972). Also, rare individuals with a genetic defect known as leukocyte adhesion deficiency (LAD) are also more prone to repeated bacterial infections (Anderson *et al.*, 1987). These persons are unable to synthesize the α subunit of the LFA-1 superfamily of integrins, which includes complement receptor 3 (CR3). Other instances in which persons are more susceptible to *Salmonella* infections include patients with leukemia and lymphoma (Wolfe *et al.*, 1971). Also, people with diseased or injured tissue are highly susceptible to *S. typhimurium* infections (Uhari *et al.*, 1987). People with sickle cell anemia and malaria infections are also more susceptible to salmonella infections (Hornick, 1993). *Salmonella* infections which cause gastroenteritis in healthy individuals, *i.e.* *S. typhimurium*, cause more severe infections in immunocompromized individuals that usually leads to bacteremia (Goldberg *et al.*, 1993).

The precise initial adherence mechanism(s) used by *Salmonella* species and their interaction with the innate defense mechanism still remain largely undetermined. Therefore, the purpose of this study was to characterize the early events in *Salmonella*-

MØ interactions. Also, recent studies have suggested that newly synthesized proteins may play a role in binding of *Salmonella* to MØ membranes. Accordingly, we have studied these bacterial proteins and MØ receptors for their contribution to the initial binding of *Salmonella* to MØ membranes in the absence of opsonins.

Using the mouse model and a virulent strain of *S. typhimurium*, we have attempted to determine:

1. Which *Salmonella*-induced proteins are involved in the adherence to MØ.
2. The role of *Salmonella* LPS and its derivatives in the adherence process.
3. The role of complement receptors in their recognition of *Salmonella*.
4. What other MØ receptors may be involved in the adherence process.

CHAPTER 1

A COMPARATIVE ADHERENCE OF *Salmonella* *typhimurium* STRAIN 1826 AND ATCC STRAIN 14028

ABSTRACT

The adherence of *S. typhimurium* strain 1826 (originally isolated from human urinary tract and designated as a mouse-virulent strain with an LD₅₀ of ~10 organisms following intraperitoneal injection) was assessed using a visual binding assay. The strain bound poorly and required 9 hours of incubation to achieve maximum adherence. In contrast a second strain of *S. typhimurium*, ATCC 14028, (originally isolated from the liver of a cow dying of septicemia) exhibited significantly greater binding. Microbial adherence exceeded 80% at the third hour when incubated with MØ. Cytochalasin B at 50 µg/ml was used to prevent phagocytosis and uptake of microorganisms without effecting MØ viability.

INTRODUCTION

It is known that specific antibody directed at exposed structures on the microbial surface enhance phagocytosis and uptake of microorganisms via an Fc receptor by a process known as opsonization (Weir, 1984). Deposition of activated complement components on the surface of *Salmonella* species also enhances their phagocytosis (Joiner, 1985). There is growing evidence that, under some conditions, *Salmonella* may be able to bind directly to host macrophages in the absence of either antibody or complement (Wright *et al.*, 1989). This direct association may be an important requirement for intracellular pathogens such as *Salmonella* to allow them to adhere, invade and eventually survive intracellularly in eukaryotic cells such as macrophages. We used the mouse model to monitor adherence of *S. typhimurium* to MØ, to study the efficiency of this adherence in different media, and to determine the viability of MØ during the adherence assays.

MATERIALS AND METHODS

Preparation of bacterial stock culture.

Two strains were used for this study, *S. typhimurium* strain 1826 and *S. typhimurium* ATCC strain 14028. *S. typhimurium* 1826 was originally obtained from Professor K. Jann, Max Plank Institut für Immunbiologie, Freiberg and was designated as mouse-virulent strain (at the time of isolation) with an LD₅₀ of ~ 10 organisms following intraperitoneal injection. *S. typhimurium* 14028 was grown from a Preceptrol stock (Difco, Inc). It was originally isolated from a liver of a cow dying with septicemia and was designated as a virulent strain at the time of isolation.

S. typhimurium strains 1826 and ATCC 14028 were separately grown in 2 l of LB broth to late logarithmic phase at 37°C. The cell suspensions were transferred to 250-ml centrifuge bottles and centrifuged at 10,000 x g for 1 h. The cells were washed twice in phosphate-buffered saline (PBS), pH 7.0. The bacteria were resuspended in PBS containing 8% dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis) and counted using a Petroff-Hausser chamber (CA. Hausser and Son, Philadelphia, PA). The cell suspensions were stored at -70°C in 1 ml aliquots. Prior to use the thawed cells were washed three times and resuspended to a concentration of 2×10^9 cells/ml in RPMI 1640 medium (RPMI; Gibco Laboratories, Grand Island, NY).

Preparation of murine peritoneal MØ.

Balb/c mice, originally obtained from Charles River Laboratories (Wilmington, MA), were bred on site. Male mice, 8-12 weeks of age, were injected intraperitoneally with 2 ml of aged Brewer thioglycollate broth (Difco Laboratories, Detroit, MI) to elicit a MØ influx into the peritoneal cavity. After 48-72 hours the mice were sacrificed by CO₂ inhalation and pinned to a dissection board with the ventral side exposed. The abdomen was washed with 70% ethanol and an incision was made in the skin to expose the intact peritoneal membrane. Mice were injected with 10 ml of cold Dulbecco's phosphate buffered saline (DPBS; Hazelton, Inc.; 23-gauge, 1" needle). The carcass was gently shaken to dislodge MØ and the peritoneal fluid was withdrawn (18-gauge, 1-1/2" needle). This fluid was centrifuged at 100 x g for 10 min to sediment the MØ; the cells were washed 3-4 times, resuspended in 2 ml of cold RPMI 1640 medium, and kept on ice until used. The cells were counted using standard hemacytometric techniques and the suspension adjusted for the appropriate assay.

Visual binding assay.

Four types of diluents were tested to determine the optimal conditions for assessing the adherence of *S. typhimurium* to MØ. These were [1] DPBS, [2] RPMI 1640, [3] RPMI supplemented with essential amino acids and glutamine, and [4] RPMI supplemented with essential amino acids but without glutamine. MØ suspensions were diluted to 1×10^6 /ml in each medium (Glass *et al.* 1980). From each suspension 300 µl amounts were transferred to appropriate wells on chamber slides (Lab-Tek) and incubated for 1 h at 37

°C in a CO₂ incubator. Non-adherent cells were removed by gentle washing (2-3 times) with DPBS. After the last wash, 300 µl of suspension of *S. typhimurium*, adjusted to 1×10^9 bacteria/ml in the corresponding diluent, were added to the respective chambers containing the MØ monolayers. These mixtures were incubated for 1, 3, 6, and 9 h to allow bacteria to adhere to the MØ. Following this incubation, the MØ were washed 5 times with DPBS. The chamber systems were removed from the microscope slides and the cells were air dried. The slides were fixed in methanol and stained with Wright's stain [(Coligan *et al.* 1991); Leukostat, Fisher Scientific Co., Pittsburgh, PA]. The slides were washed with distilled water and air-dried. They were covered with a large coverslip, sealed, and examined at 1000 X magnification using an Olympus microscope, model BH2, Japan. MØ to which 5 or more bacteria were attached were considered positive. Five hundred MØ were examined for each system and each assay was performed in triplicate. The average of the three counts was used to determine the portion of MØ with adherent *Salmonella*.

Determination of cell viability:

Approximately 1×10^6 MØ/ml suspended in RPMI were incubated at 37°C in a CO₂ incubator. At time zero and every two hours up to 10 hours thereafter, 0.1 ml of the sample was removed and diluted 1:10 in trypan blue (Coligan *et al.*, 1991). The mixture was placed on a hemacytometer and examined under high dry magnification (440 x). This is a dye-exclusion assay; cells that stain blue are considered to be dead. The proportion of cells staining blue was used to determine cell viability.

Effect of cytochalasin B.

Three hundred microliters of a cell suspension containing 1×10^6 MØ/ml in RPMI were added to each slide chamber and the system incubated for 1 h. Varying concentrations (10, 50, 150, or 200 µg/ml) of cytochalasin B were added to each chamber and incubation was continued for 30 min. Approximately 300 µl of bacterial suspension containing 2×10^9 organisms/ml were added to each assay system and the reaction mixtures incubated for 3 h as above. Each chamber was washed five times with DPBS to remove non-adherent bacteria and the slides were stained and evaluated as previously described. Separate viability studies on the MØ were conducted in the presence of the test concentrations of cytochalasin B.

Statistical analysis.

Data were analyzed using factorial or repeated measures analysis of variance and, where appropriate, Scheffe's test of significance.

RESULTS

Adherence of S. typhimurium strain 1826 to murine peritoneal MØ:

Adherence of this bacterial strain to MØ was assessed in a visual binding assay in the presence of 4 different fluid systems: [1] DPBS, [2] RPMI without essential amino acids, [3] RPMI containing essential amino acids and glutamine, and [4] RPMI containing all essential amino acids but no glutamine. The mean adherence values and the standard error values were calculated; the data are shown in Fig. 1.1. Adherence of *S. typhimurium* 1826 to MØ was poor through the sixth hour for all four systems tested. Maximum adherence was seen at the ninth hour. At this time more than 70% of the MØ in RPMI without amino acids exhibited positive bacterial adherence, whereas the percentage of MØ with adherent bacteria in the other three systems ranged from 60% to 79%. Statistical analysis of the results indicated that adherence was significantly higher ($p \leq .0001$) when MØ were maintained in RPMI, compared to DPBS. Additionally no significant effect was seen for either supplemented RPMI medium compared to the basic RPMI system. However, the viability of MØ maintained in RPMI without amino acids dropped from 97% at time zero to 81% at 9 h [$p \leq .0001$; (Fig. 1.2)].

Adherence of S. typhimurium strain 14028 to murine peritoneal MØ:

Because of the poor adherence to strain 1826, we repeated these adherence studies with another strain known to bind to MØ. The results are shown in Fig. 1.3. After the

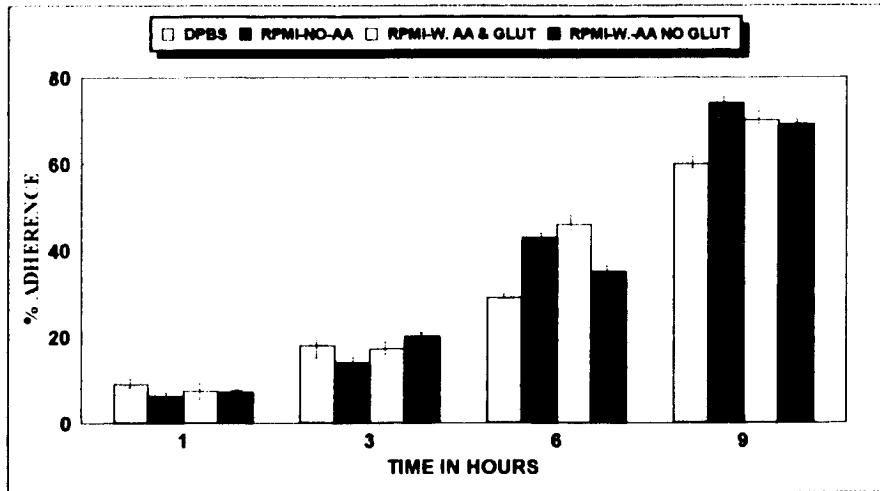


Fig. 1.1. Adherence of *S. typhimurium* strain 1826 to mouse peritoneal macrophage during the nine hour period.

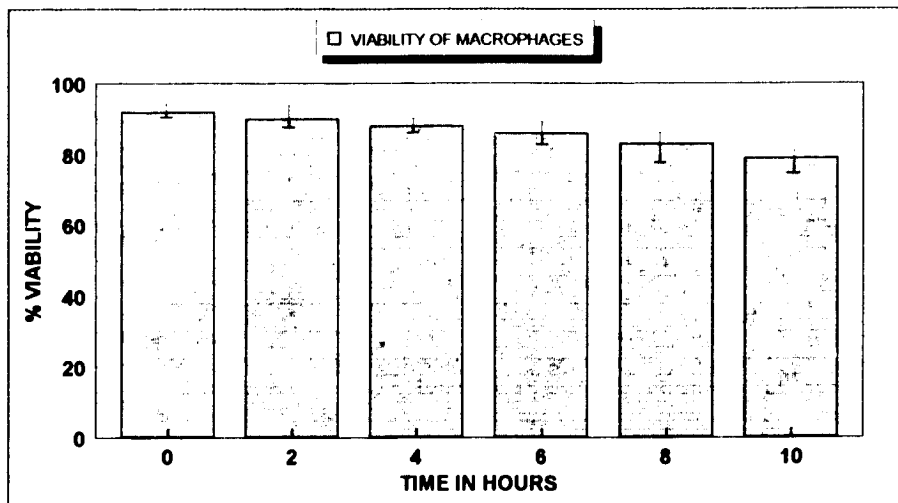


Fig. 1.2 Viability of macrophages grown in RPMI medium without essential amino acids, at time interval from 0 to 10 hours.

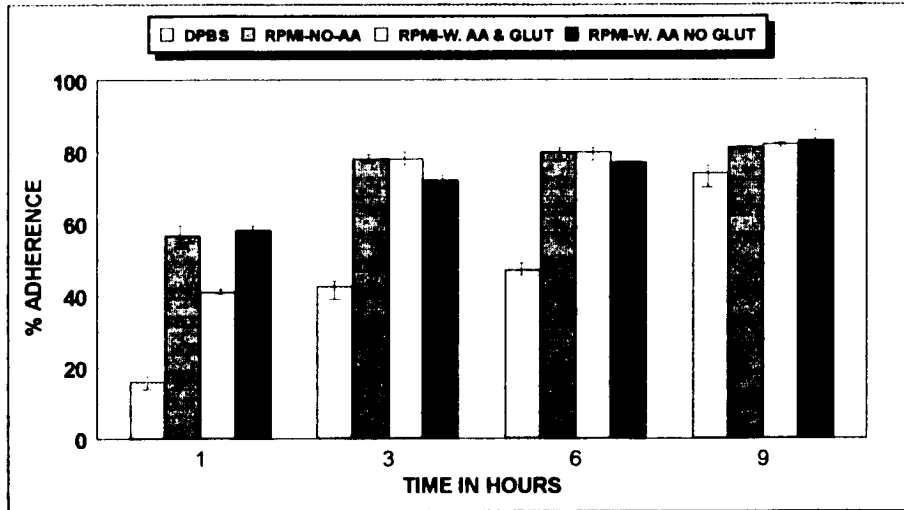


Fig. 1.3. Adherence of *S. typhimurium* ATCC strain 14028 to mouse peritoneal macrophages at various incubation times.

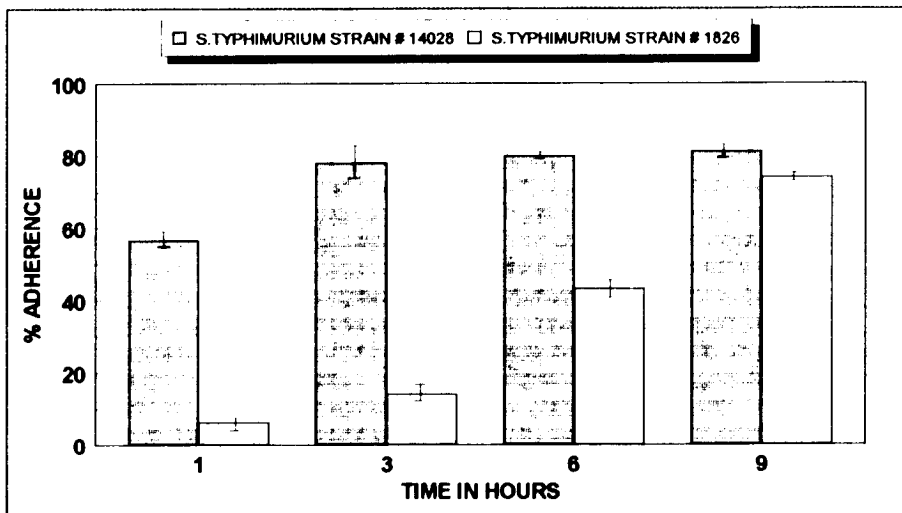


Fig. 1.4. Comparative adherence of two strains of *S. typhimurium* to mouse peritoneal macrophages at various incubation times.

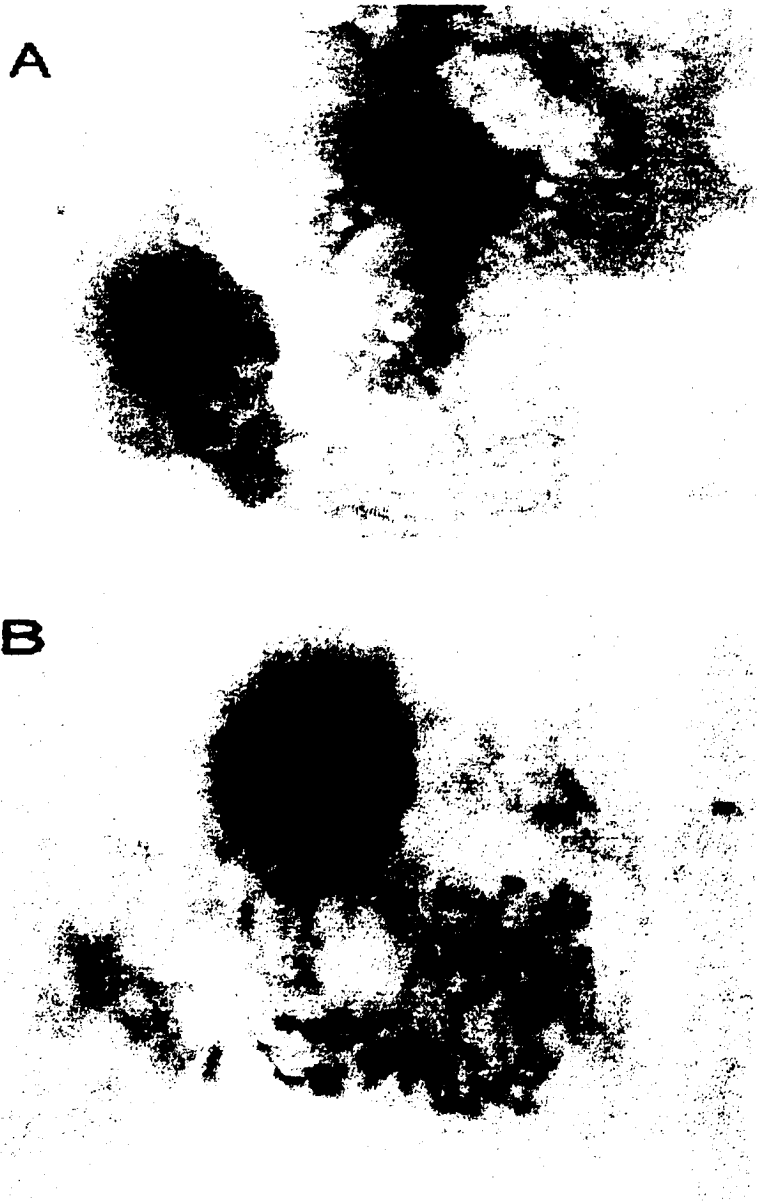


Fig. 1.5 A micrograph showing adherence of *S. typhimurium* to MØ at the third hour of the assay. (A) Adherence of *S. typhimurium* strain 1826 and (B) adherence of *S. typhimurium* ATCC strain 14028

first hour of incubation in DPBS less than 20% of the MØ were scored positive for bacterial adherence, whereas at 9 h the value was 83%. In contrast, the bacterial adherence seen in the various RPMI formulations each began markedly higher at 1 h, approached 80% at 3 h, and remained at this level for the rest of the experiment. A comparison of the adherence of these two strains of *S. typhimurium* to MØ in basic RPMI is shown in Fig. 1.4. Figures 1.5-A and -B are micrographs taken at the third hour of the experiment for the strain 1826 and 14028, respectively. Note the number of bacteria between the two strains bound to each macrophages.

Based on these findings, strain 14028 was used for the remainder of my studies. Also, since RPMI without added amino acids yielded higher adherence values and showed no obvious advantage over the supplemented forms, it was used in subsequent assays. Finally, because high adherence values were obtained at the third hour of the assay, this incubation time was used in subsequent studies.

Effect of cytochalasin B on adherence of S. typhimurium to MØ:

Because of the difficulty of enumerating phagocytosed bacteria, cytochalasin B was used to block the uptake of adherent salmonellae by MØ. These studies were conducted to determine whether this compound had any effect on the actual binding step. Overall, the effect of this chemical on binding was highly significant ($p \leq .0001$). In the absence of cytochalasin B, approximately 70% of the MØ had 5 or more bacteria adhered to them (Fig 1.6). This percentage increased as the concentration of the drug increased, to a maximum of 93% adherence in the presence of 50 µg/ml of cytochalasin B. At higher

doses the binding values decreased in an inverse pattern to where, at the highest concentration tested (200 $\mu\text{g/ml}$), the percentage of MØ with adherence bacteria was comparable to the control with no drug present.

Viability studies of the MØ in the presence of cytochalasin B were also performed, using the dye exclusion assay (Fig. 1.7). Viability remained constant at approximately 94% at drug concentrations up to 50 $\mu\text{g/ml}$. Higher doses of this material yielded a decrease in viability to a level of approximately 85% at 100 $\mu\text{g/ml}$ and 83% at the 200 $\mu\text{g/ml}$, each significantly different ($p = .0009$ and $p = .0001$, respectively) from the control. Based on the information derived from the effect of cytochalasin B on adherence and viability, we routinely pretreated MØ with 50 $\mu\text{g/ml}$ of cytochalasin B in experiments involving visual assessment of binding.

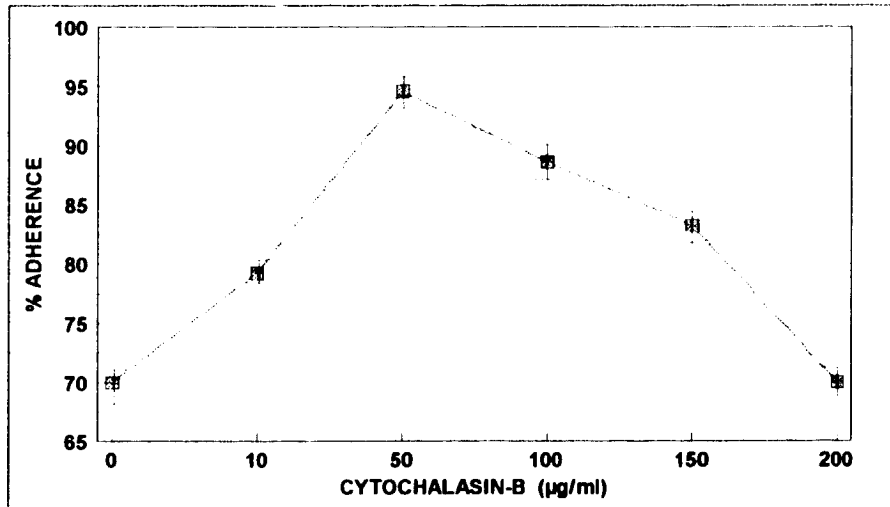


Fig. 1.6. Effect of cytochalasin B on the adherence of *S. typhimurium* strain 14028 to mouse peritoneal macrophages

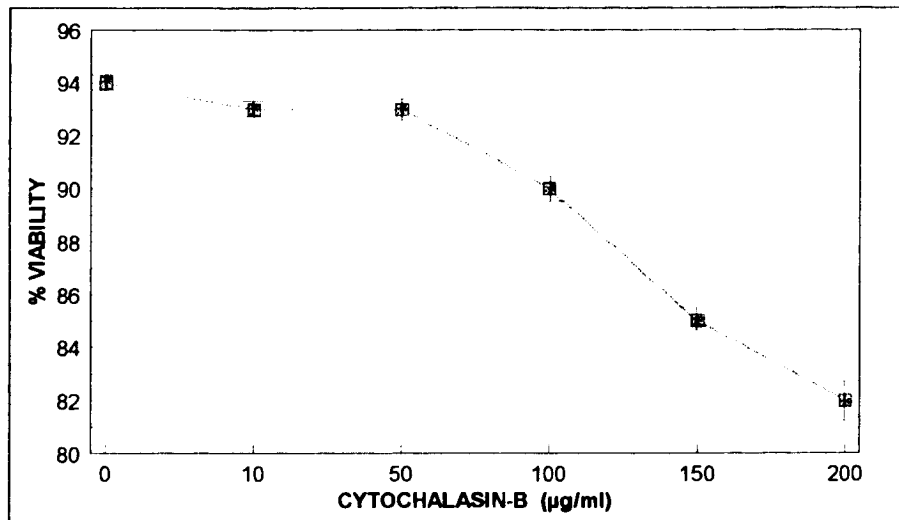


Fig. 1.7. Viability of macrophages at different concentration of cytochalasin B.

DISCUSSION

Salmonella infections continue to be a major disease throughout the world (WHO news and activities, 1991). Individuals at high risk include infants, adults over 60 years old, and immunocompromised persons. Despite the fact that this microorganism is susceptible to a variety of antibiotics and that it elicits a significant antibody response, the incidence of this disease continues to increase. The present study was conducted to evaluate the role of innate cellular defenses in coping with this bacterial pathogen.

S. typhimurium strain 1826 was originally obtained from Professor K. Jann, Max Plank Institut für Immunbiologie, Freiberg, Germany. The strain was isolated from human urinary tract infection and was earlier designated as a mouse-virulent strain with an LD₅₀ of ~10 organisms following intraperitoneal injection. Following experiments that were conducted in our laboratory to study the adherence of group B streptococci to mouse peritoneal MØ which were conducted in DPBS medium (Sloan and Pistole, 1992), adherence of strain 1826 to MØ was initially tested in DPBS. However, poor adherence was seen during the first 3 hours of the assay. Significant improvement was seen during the 6th to 9th hour of the assay (Fig. 1.1). As a result of this poor adherence, three forms of RPMI 1640 were studied. Unsupplemented RPMI 1640 medium, RPMI 1640 supplemented with essential amino acids except for glutamine, and RPMI 1640 containing essential amino acids including glutamine. Glutamine is usually supplemented separately as it is unstable and has a short half-life (Freshney, 1983). Substitution of DPBS with

RPMI 1640 medium, even when supplemented with amino acids, did not enhance bacterial adherence. During the incubation time, MØ viability dropped from approximately 95% to near 80%. Because of these findings, we sought another relevant strain of this pathogen.

ATCC strain 14028 of *S. typhimurium* has been used for the adherence studies by many other laboratories (Alpuche, 1992; Buchmeier *et al.*, 1991; Fields *et al.*, 1986; Groisman, 1989; and Langermans *et al.*, 1989). Similar adherence studies conducted using the *S. typhimurium* strain 14028 yielded much improved results compared to those obtained when using the strain 1826. Microbial adherence reached 60% by one hour when the assay was carried out in RPMI (Fig 1.3). By the third hour adherence reached or exceeded 80% and maintained this level through completion of the study (9 hours). As with strain 1826, adherence of *S. typhimurium* 14028 to MØ in DPBS was relatively poor. We concluded the following: [1] *S. typhimurium* 1826 is a poor binder in this assay system when compared to the ATCC strain 14028; [2] assays conducted using RPMI either alone or supplemented with amino acids yield higher binding levels than those using DPBS; [3] strain 14028 achieved maximal adherence by 3 h. Consequently, all subsequent studies used *S. typhimurium* 14028, unsupplemented RPMI, and a 3-h incubation time.

Another complication of this adherence assay is the possibility that bacteria that have adhered to the MØ surface may be engulfed and not observed in the visual assay, leading to deceptively low adherence values. To prevent phagocytic uptake, we pretreated MØ with cytochalasin B. Cytochalasins are metabolite components derived from several species of molds (Carter, 1967) and have been shown to prevent phagocytic uptake

(Greenberg *et al.*, 1990). *In vitro* studies showed that *S. typhimurium* invasion to epithelial cells is inhibited by cytochalasins B (Francis *et al.*, 1992) probably by blocking the assembly of F-actin (Greenberg *et al.*, 1990). Our studies indicated that 50 µg/ml of this chemical was the optimal concentration. Higher concentrations resulted in loss of viability for the MØ. Although cytochalasin B inhibits phagocytic uptake (Sveum *et al.*, 1985), it did not affect bacterial adherence in our studies.

CHAPTER 2

THE ROLE OF LIPOPOLYSACCHARIDE AND COMPLEMENT RECEPTORS IN THE ADHERENCE OF *Salmonella typhimurium* TO MURINE PERITONEAL MACROPHAGES

ABSTRACT

Adherence of *S. typhimurium* strain 14028 to mouse MØ was monitored using a direct microscopic visual assay and flow cytometry. Competitive binding studies using wildtype lipopolysaccharide and derivatives confirmed a role for this moiety in this adherence. MØ pretreated with neutrophil elastase exhibited a diminished ability to bind to *S. typhimurium*, suggesting involvement of complement receptor 1 (CR1). Monoclonal antibodies M1/70 and M18/2, specific for epitopes on the α and β chains, respectively, of β_2 integrin CR3, also blocked this adherence. In each case, we were unable to completely eliminate this bacterial adherence, suggesting that multiple mechanisms are likely involved in recognition and uptake of this pathogen by MØ. We investigated the role of other receptors by using specific antibodies against these receptors. One of the antibodies that recognize C1q receptor caused enhancement of bacterial binding to macrophages. Antibodies to other receptors, including Mac-2 and Mac-3, and C1q itself did not block bacterial adherence to MØ, suggesting that these components are not involved in the binding process.

INTRODUCTION

A major structure on the outer surface of *Salmonella* species and many other Gram-negative bacteria is lipopolysaccharide (LPS). This macromolecule consists of a lipid core known as Lipid A that is intimately associated with the outer membrane, an adjacent core sugar region, and terminal sugar side chains extending distally from the cell envelope (Fields *et al.*, 1988). LPS is known to activate host MØ (McCleish *et al.*, 1989; Marra *et al.*, 1990). It causes the activated macrophage to secrete various inflammatory molecules, including interleukin-1, tumor necrosis factor and the third component of complement (C3) (Shinji *et al.*, 1991). LPS also causes rearrangement of microfilaments in macrophages (Finlay *et al.*, 1991), generation of protein kinase C, degradation of phosphatidylinositides (Shinji *et al.*, 1991), and elevation of the intracellular calcium concentration (Marra *et al.*, 1990). The myristoylation and the phosphorylation of some proteins has also been demonstrated (Shinji *et al.*, 1991). *Salmonella* species are known to escape opsonization by rapid alteration of their lipopolysaccharide (LPS) phenotype and other surface antigens (Kwahara *et al.*, 1989).

LPS also prevents activation and deposition of complement on the bacterial cell surface. In the case of facultative intracellular pathogens LPS has been shown to enhance phagocytosis by professional phagocytes (Cooper *et al.*, 1984) and to inhibit intracellular killing after engulfment (Fields *et al.*, 1988). One of the important consequences of LPS stimulation of MØ is a dramatic augmentation in the cell surface expression of the

complement receptors for C3b (CRI; McCleish *et al.*, 1989) and for iC3b (CR3; Kauffmann, 1989; Paccaud *et al.*, 1990). In fact, Patarroyo (1989), claimed that some MØ receptors are able to recognize *Escherichia coli* directly without participation of complement components. On the other hand, Wright, *et al.* (1986) showed in down-modulation assays that adherence of *E. coli* to human MØ was completely blocked when the MØ were initially cultured on a surface coated with LFA-1, a member of the β_2 integrin family (Fig 2.1).

In general, increased surface expression of CRI (Fig 2.2) and CR3 facilitates attachment of pathogens to MØ. Unlike other known MØ receptors, such as the Fc receptor (Kauffmann, 1989) and the LPS-binding protein receptor (Wright *et al.*, 1990), that require initial opsonization of the pathogen (*i.e.*, they mediate opsonin-dependent phagocytosis), CRI and CR3 can bind to parasites in the absence of opsonins (*i.e.*, they mediate opsonin-independent phagocytosis; Kauffmann, 1989). Interestingly, attachment of pathogens to CRI or CR3 causes ingestion of pathogens without stimulation of the intracellular oxidative response. As a result, intracellular pathogenic microorganisms can abuse these receptors to gain a safe entry into the intracellular compartment of MØ. Sometimes binding of intracellular parasites to certain receptors on a MØ surface does not initiate a microbicidal response, while in other cases binding to a MØ receptor leads to microbial killing (Russell *et al.*, 1988). It seems that the choice of receptor used during phagocytosis could be one of the possible means by which an intracellular parasite enhances its successful invasion into the MØ and determines the outcome of the potential

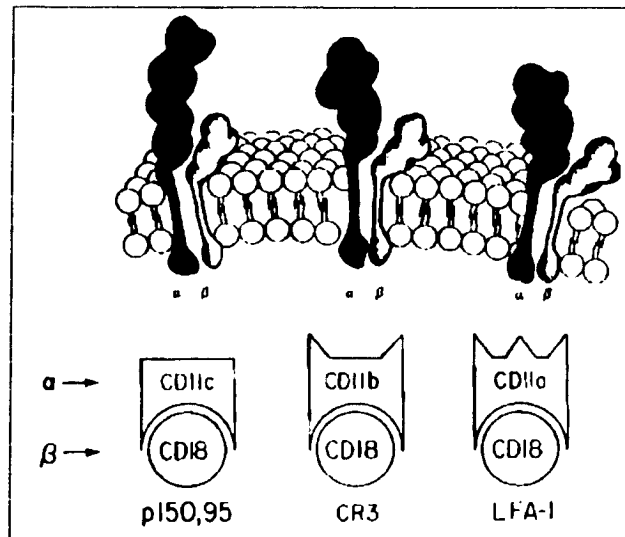


Fig. 2.1. Schematic representation of β_2 family of white blood cell integrins. The integrins are composed of two subunits: the identical β -chain associated with unique α -chains that are different in each protein. (Wright *et al.* 1988; and Todd *et al.* 1988).

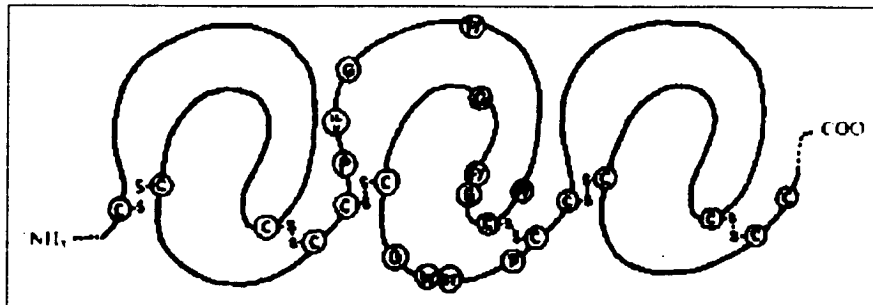


Fig. 2.2. Schematic of proposed structure of CR1. (Law, 1988).

pathogen (Connor *et al.*, 1991).

Because *Salmonella* species are considered to be facultative intracellular pathogens, the interaction of microorganism and MØ is a potentially complex one. Although the MØ has the potential to destroy this organism, it is also a protected site in which the bacteria can reside (Langermans *et al.*, 1990). Indeed, because of their mobility, MØ may play an important role in dissemination of *Salmonella* throughout the body. It is believed that MØ activated by, *e.g.*, primed T cells, may develop the ability to kill these intracellular pathogens (Roitt *et al.*, 1990). However, activation of MØ does not necessarily lead to destruction of internalized *Salmonella* (Langermans *et al.*, 1990).

The early events in the innate defenses against *Salmonella* remain incompletely clear. The goal of this research is to determine the role of LPS in the binding of *Salmonella typhimurium* to MØ and to identify and characterize the MØ receptors involved in this recognition in the absence of exogenous opsonins. This information will no doubt prove valuable in understanding how these microorganisms initiate disease and aid us in developing useful strategies to prevent such infections.

MATERIALS AND METHODS

Visual assay for detecting microbial attachment

The procedure of Glass *et al.* (1981) was used with modification in our laboratory (Sloan and Pistole 1992). MØ monolayers were prepared as described earlier (Chapter 1), then test reagents were added and the systems incubated for an additional 3 h at 37°C under 5% CO₂ tension. The monolayers were washed and overlaid with RPMI containing the corresponding test substance, then bacteria were added and the assays completed as described in Chapter 1. For studies testing the effects of added substance on bacterial adherence, the data were normalized with controls set at 100%.

Flow cytometry:

A fluorescence-activated cell sorter (FACS) was used to examine the adherence of *S. typhimurium* to MØ in suspension. One advantage of this technique over the visual binding assay is it can be used to quantify adherent bacteria without washing (Bjerknes *et al.* 1989). The assay was conducted using a FACScan model [Becton-Dickinson, FACS Systems, Sunnyvale, CA]. Data analysis was facilitated using the accompanying LysisII software. Bacteria were labeled with Lucifer yellow fluorochrome dye (Sigma; Sveum *et al.*, 1985). This dye binds covalently to amino acid and sulfurhydryl groups on the bacterial surface. Labeling was accomplished by incubating 2×10^9 *S. typhimurium* and 1 mg of lucifer yellow dissolved in 1 ml of 0.1 M NaHCO₃, pH 9.5, for 2 h at room

temperature. The cells were washed 4-5 times in DPBS. Peritoneal MØ at a concentration of 4×10^6 cells/ml were used as is or pretreated with various test substances (Table 1) and incubated for 1 h at 37°C. Labeled bacteria at a concentration of 2×10^9 organisms/ml were added to the treated MØ and incubation was continued for an additional 3 h at the same temperature. The cells were then fixed with formaldehyde (to a final volume of 1% formaldehyde). Samples were stored at 4°C prior to analysis.

The emission of labeled bacteria was recorded at 530 nm after excitation by the argon laser at 488 nm. Forward scatter and side scatter were recorded in the linear mode and fluorescence at 530 nm was recorded in the logarithmic mode. The forward scatter threshold was adjusted to exclude unbound bacteria, platelets, and erythrocytes and a live gate set around the reference system containing MØ and labeled bacteria. The data were represented as histograms plotting the logarithmic value of the fluorescence versus the number of MØ.

Effect of MØ viability to mediate bacterial adherence:

MØ monolayers prepared as earlier described (Chapter 1) were treated with 1% formaldehyde for 5, 15, and 30 min, then washed three times before the addition of the bacterial suspension. The binding assay was completed as usual. Suspension of MØ treated with 1% formalin for 30 min, then washed three, times, were used to conduct adherence assays, which were analyzed by flow cytometry.

Lipopolysaccharides and derivatives:

Purified LPS (derived from *S. typhimurium*), and 2-keto-3-deoxyoctonate (KDO) were obtained from Sigma. Glycolipid derived from *Salmonella minnesota* Re 595 (Fig 2.3), previously prepared in our laboratory by the method of Galanos *et al.* (1969), was also used. With the exception of Re-glycolipid, each test material was suspended in DPBS, pH 7.2, to a final concentration of 1 mg/ml. The Re-glycolipid was initially dissolved in acetone/ether (vol. 1:1). Then DPBS was added to bring the contents to a final concentration of 0.1% acetone/ether and 0.5 mg/ml Re-glycolipid.

Down-modulation assay:

Each chamber slide was pretreated with a test substance in bicarbonate buffer, pH 9.7, containing the test substance, and allowed to incubate overnight at 4°C. The chambers were washed and MØ monolayers were prepared as described above. The binding assay was completed with the addition of bacteria and subsequent processing as previously detailed.

Treatment of MØ with 2-deoxy-D-glucose (2-DG):

The basic procedure of Sung-Sang *et al.* (1985) was followed. In brief, MØ monolayers were incubated with varying concentrations of 2-DG for 1 h at 37°C prior to the addition of the bacterial suspension. The binding assay was completed as described above.

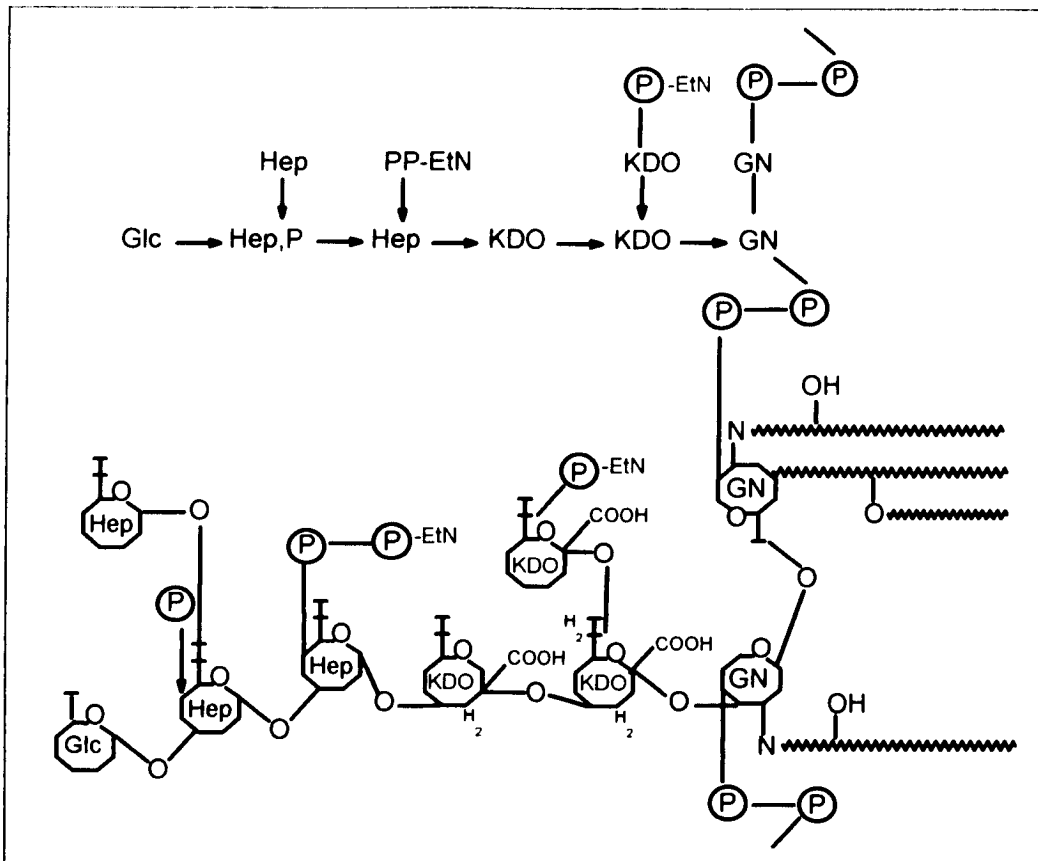


Fig. 2.3. Representation of the inner core and Lipid A glycolipid structure of *S. mimesota* mutant strain Re 595. (Lüderitz *et al.*, 1973).

Treatment of MØ with neutrophil elastase:

Lyophilized, human neutrophil elastase (110 U/mg; Sigma) was dissolved in distilled water to a final concentration of 1 mg/ml, divided into single use aliquots, and stored at -70°C, thawed immediately prior to use. Working concentrations (10, 20, and 50 µg/ml) of this enzyme were prepared in DPBS and added to MØ monolayers, as described by Tosi *et al.* (1990). The cells were incubated for 1 h at 37°C, then washed three times before the addition of bacteria. Combination treatments of neutrophil elastase and monoclonal antibody (MAb) to β_2 were also studied. The binding assay was completed as described above.

Blocking assays with monoclonal antibodies:

Three MAb, recognizing different epitopes on members of the leukocyte integrin family, were used (Fig 2.1). Their properties are summarized in Table 1. Hybridomas secreting M1/70 and M18/2 were obtained from American Type Culture Collection (Washington, D.C.). Purified MAb 5C6 was obtained from Serotec (Kidlington, Oxford). Two additional MAb that recognize epitopes on the non-integrin receptors, Mac-2 and Mac-3, were also used (American Type Culture Collection, Washington, D.C.). Rabbit polyclonal antibody to C1q receptors were generously provided by B. Ghebrehiwet. Varying concentrations of each MAb were added to MØ monolayers and the mixtures incubated at 37°C for 1 h. Polyclonal rat antibody at a concentration of 100 µg/ml was used to block the Fc receptors prior to the addition of MAb. Bacteria were added and the binding assay was completed as previously described.

Treatment of MØ with cytochalasin B

MØ were suspended in 300 µl and treated with 50 µg/ml of cytochalasin B as described previously in Materials and Methods (Chapter 1). Lucifer yellow-labeled bacteria were added and the assay was completed as previously described. The results were obtained using flow cytometry.

Statistical analysis

Data from visual binding and down-modulation assays were analyzed using factorial analysis of variance and, where appropriate, Scheffe's test of significance.

Table 1: Antibodies against macrophage receptors.

MAB	Short Name	Type	Epitope	Source	Isotype	Reference
M1/70.15.115 ^a	M1/70	Monoclonal	CD11b, C3bi binding site	Rat	IgG2b	Springer, T. 1979
M18/2a.12.7 ^a	M18/2	Monoclonal	CD18 of LFA-Family	Rat	IgG2a	Sanchez, F. 1983
5C6	5C6	Monoclonal	CD11b Mg ²⁺ -dependent binding site	Rat	IgG2b	Rosen, H. 1987
M3/38.12.8.HL.2	α MAC2	Monoclonal	-	Rat	IgG _{2a,k}	Ho, M. <i>et al.</i> , 1982
M3/84.6.34	α MAC3	Monoclonal	-	Rat	IgG _{1,k}	Springer, T. <i>et al.</i> , 1981
α C1q Receptor (R112)	α C1q R (R112)	Polyclonal	-	Rabbit	-	Ghebrehewit
α C1q Receptor (R235)	α C1q R (R235)	Polyclonal	-	Rabbit	-	Ghebrehewit
α Whole C1q	α C1q whole	Polyclonal	-	Rabbit	-	Ghebrehewit
α C1q globular head	α C1q Head	Polyclonal	-	Rabbit	-	Ghebrehewit

RESULTS

Three assays were used to determine adherence of *S. typhimurium* to mouse peritoneal MØ: a visual binding assay on MØ monolayers, down-modulation studies and flow cytometry studies. A photomicrograph of the visual binding assay has been previously presented (See Fig 1.5). As seen in Fig 2.4, suspension of MØ and *S. typhimurium* exhibited lower levels of bacterial adherence when the mixtures were centrifuged and suspended in buffer, compared to untreated controls. Whether the cells were fixed with formaldehyde, then washed or before fixation made no difference.

Because the visual binding assay detects only extracellularly associated bacteria, cytochalasin B was added to prevent phagocytic uptake by the attached MØ. Flow cytometry studies were conducted to determine whether treatment with cytochalasin B affected the level of bacterial adherence. As shown in Fig. 2.5., at the concentration used in visual binding assays (50 µg/ml), there was no detectable effect of this chemical. Since lucifer yellow, the fluorescent label used in the flow cytometry studies, remains detectable even inside phagocytic cells, cytochalasin B was not used in these studies.

Effect of MØ viability on adherence to mediate bacterial adherence

To determine whether bacterial adherence requires viable MØ, these cells were treated with 1% formaldehyde for 5-30 min prior to the addition of bacteria. The results are depicted in Fig. 2.6. Treated MØ exhibited a diminished ability to bind bacteria

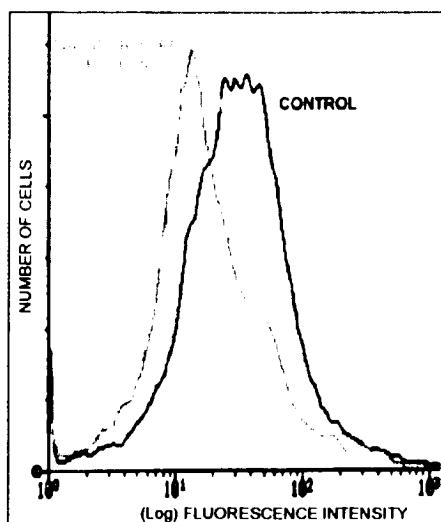


Fig 2.4. Effect of washing on the level of bacterial adherence to MØ in suspension as determined by flow cytometry. MØ-bacterial mixtures washed either before or after fixing with formaldehyde were compared to control system for which no washing step was included.

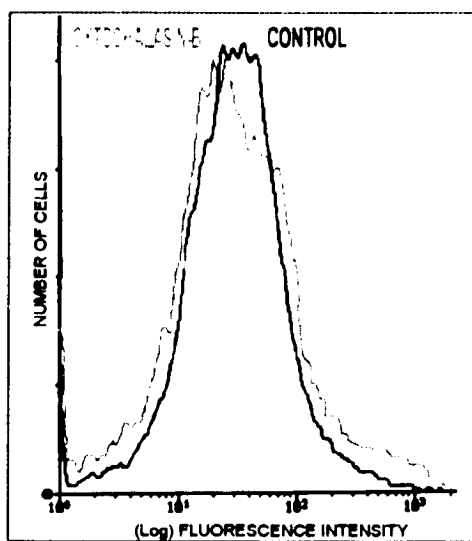


Fig. 2.5. The effect of cytochalasin B on the adherence of *S. typhimurium* to MØ, as determined by flow cytometry.

compared to untreated controls as detected by both visual binding assay and the FACS.

Role of LPS and its derivatives in bacterial adherence to MØ

Since LPS is the 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. Accordingly, blocking studies were conducted in which MØ were preincubated with wild-type LPS. Because native LPS tends to form micelles in aqueous fluids (P. Cohen, personal communication), this approach may lead to nonspecific blocking of bacterial adherence. Thus, we also used down-modulation studies to assess the effect of LPS on this adherence. The results of these two studies are shown in Fig. 2.7. Two findings are noteworthy. First, there is a direct correlation between the amount of LPS incorporated into these assays and the degree of inhibition. At the lowest concentration tested, 100 µg/ml, the level of inhibition was approximately 50% whereas at 400 µg/ml, the inhibition increased to nearly 80%. The second finding is that results from the two assays were similar.

To determine which part of the LPS molecule is involved in bacterial adherence to the MØ, two derivatives were examined. The glycolipid from the *S. minnesota* Re595 is composed of the lipid A core and the KDO portion of the saccharide core (see Fig. 2.3). KDO is an acidic sugar that connects the lipid and saccharide core regions. The results, shown in Fig. 2.8, indicate a modest but comparable inhibitory effect for KDO and Re glycolipid over the concentration range tested. In contrast, MØ pretreated with wild-type LPS exhibited a greater dose-dependent inhibition effect over this same range ($p < .014$

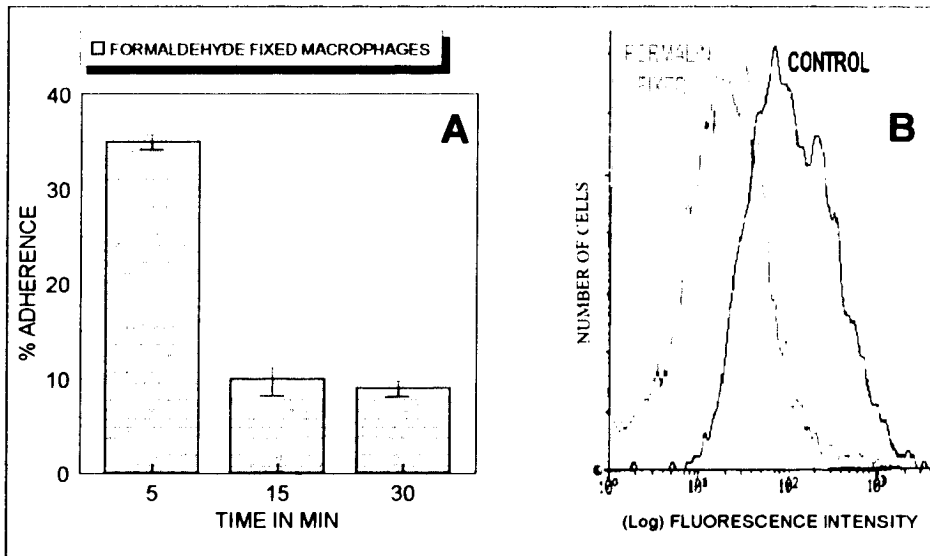


Fig. 2.6. Bacterial adherence to MØ treated with 1% formaldehyde prior to the addition of bacteria. (A) Visual binding assay, (B) FACS assay.

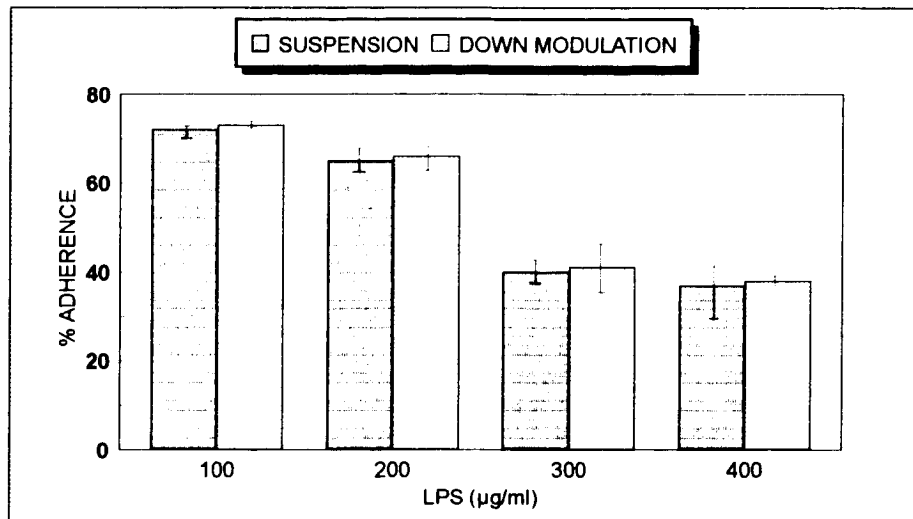


Fig. 2.7. Effect of LPS on bacterial adherence to MØ by down-modulation and by suspension assays.

compared to lipid A and $p < .004$ compared to KDO). Fluorescence histograms shown in Fig.2.8 B indicate that each preparation reduced the mean fluorescence intensity compared to the untreated control, yielding data similar to the results from visual binding assay.

Effect of 2-deoxy-glucose on bacterial adherence to MØ.

The chemical 2-deoxy-glucose (2-DG) has been shown to disrupt the uptake of bacteria by MØ via the Fc and complement receptors (Sung-Sang *et al.*, 1985) and to inhibit adherence of group B streptococci to MØ (Sloan and Pistole, 1993). It was tested in this study to determine whether complement receptors are involved in the adherence of *S. typhimurium* to MØ. Binding of these bacteria was reduced in a dose-dependent manner over the range of 100 to 500 µg/ml (see Fig. 2.9 A; $p < .0001$ compared to untreated control). A similar study was carried out with flow cytometry in which MØ pretreated with 2-DG were compared to control MØ. As shown in Fig.2.9 B, the treated sample exhibited reduced mean fluorescence, indicating that fewer labeled bacteria were binding to the MØ.

The effect of Fc receptor

Inhibition by 2-DG on adherence of *S. typhimurium* to mouse peritoneal MØ suggests that Fc receptors as well as complement receptors are involved in the uptake of bacteria (Sung-Sang *et al.*, 1985). The role of the Fc receptor was examined using a mouse irrelevant MAb as well as mouse Fc fragments that were made from mouse polyclonal antibodies. None of these components reduced bacterial binding (Fig 2.10).

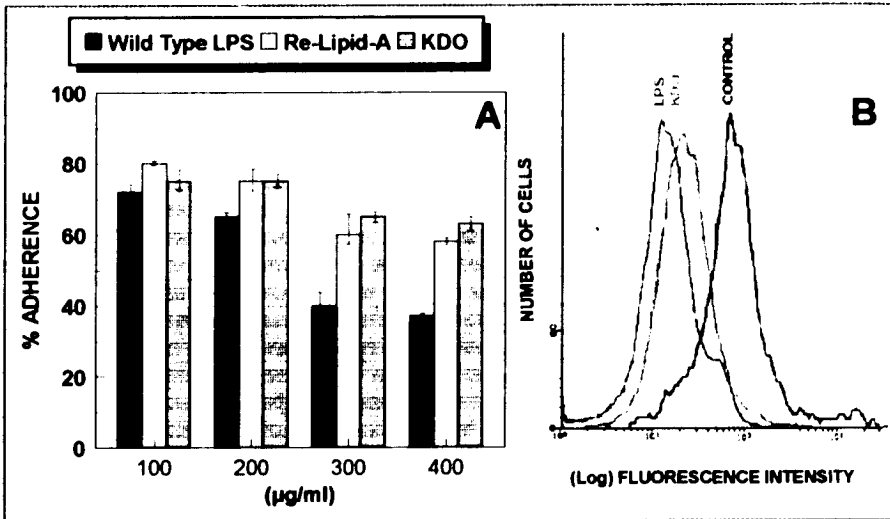


Fig. 2.8. The effect of LPS and its derivatives on adherence of *S. typhimurium* to mouse peritoneal MØ, as measured by (A) visual binding assays with MØ monolayer and (B) flow cytometry.

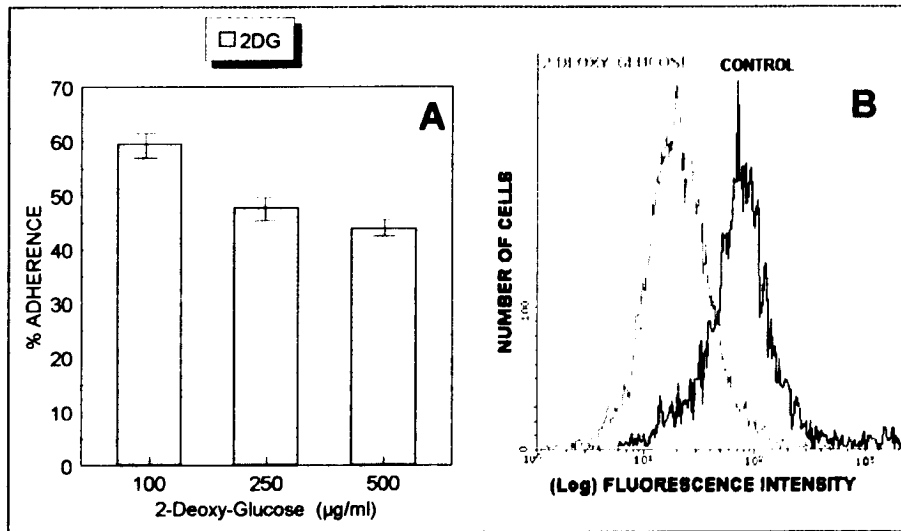


Fig. 2.9. The effect of 2-deoxy glucose on the adherence of *S. typhimurium* to mouse peritoneal MØ, as measured by (A) visual binding assays with MØ monolayer and (B) flow cytometry.

Effect of neutrophil elastase on bacterial adherence to MØ.

The enzyme neutrophil elastase has been shown to specifically cleave complement receptor type 1 (Fig 2.2) (CR1; Tosi *et al.*, 1990). MØ pretreated with this enzyme were tested for their ability to mediate adherence of *S. typhimurium*. A dose-dependent reduction in bacterial adherence was observed over the concentration range tested (see Fig. 2.11 A; $p < .0001$ compared to untreated control). At the highest concentration tested, 30 µg/ml, adherence was reduced to 60% of the control. This was confirmed in flow cytometry studies by the reduction of mean fluorescence in the treated samples, indicating that fewer bacteria were binding to MØ (Fig. 2.11 B).

Effect of MAb against β_2 integrins on bacterial adherence to MØ.

Three MAb, with distinct epitopes on this family of leukocyte membrane markers, were studied. Included in the β_2 integrin family is the complement receptor, CR3 (CD11b/CD18), so these experiments were designed to determine whether adherence of *S. typhimurium* is mediated through this receptor. Two methods were used: direct binding and down-modulation. As shown in Fig. 2.12, MAb M1/70, directed toward the iC3b binding site of the CD11b chain of CR3, and M18/2, which recognizes an epitope on CD18, the common subunit in this integrin family (Fig. 2.1), each inhibited adherence of *S. typhimurium* at concentrations of 75 and 100 µg/ml (Fig 2.12 A; $p = .025$ for M1/70 compared to 5C6, $p = .0024$ for M18/2 compared to 5C6). There was no significant difference in the inhibitory effect of M1/70 and M18/2 ($p = .65$). At the highest

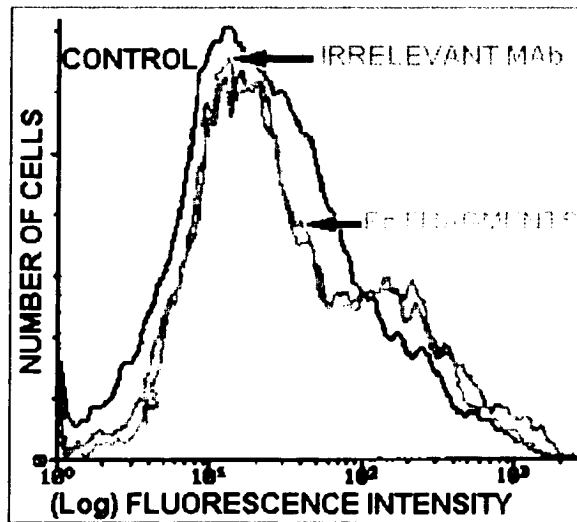


Fig. 2.10. Effect of Fc fragments and an irrelevant MAb on the adherence of *S. typhimurium* to MØ, as measured by flow cytometry.

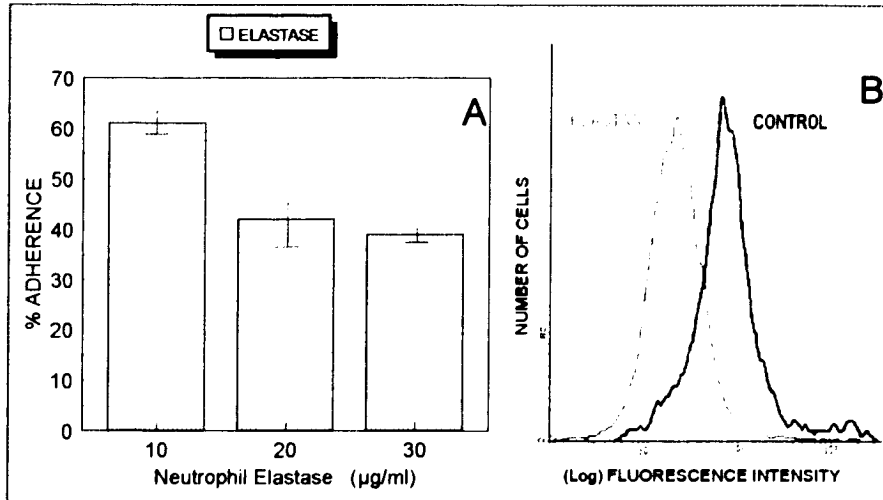


Fig. 2.11. The effect of neutrophil elastase on the binding of *S. typhimurium* to mouse peritoneal MØ, as measured by (A) visual binding assays with MØ monolayers and (B) flow cytometry.

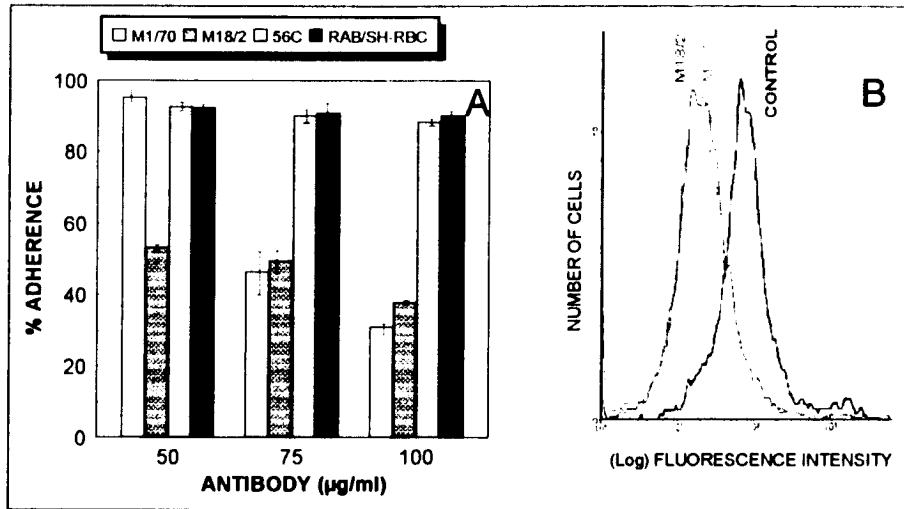


Fig. 2.12. Effect of pre-exposing MØ to anti-CR3 MAb on the ability of these cells to bind to *S. typhimurium*. (A) Down modulation studies in which MØ monolayers were developed on slides precoated with appropriate MAb. (B) Flow cytometry studies with MØ in suspension.

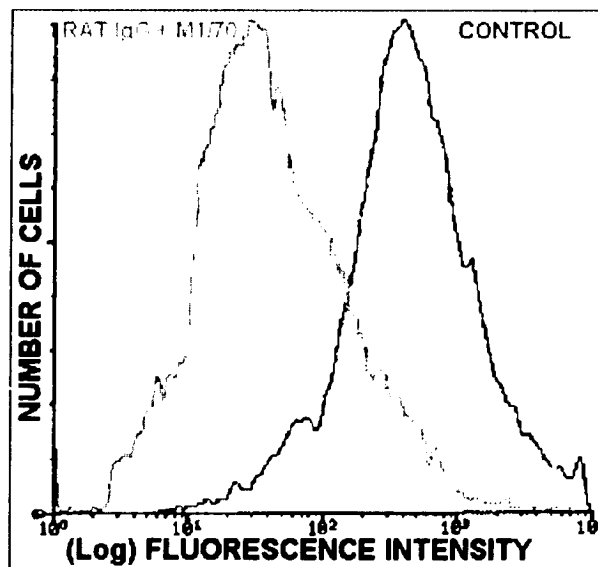


Fig 2.13. Effect of pre-exposing MØ to rat IgG prior to M1/70 treatment, as measured by flow cytometry.

concentration of each MAb tested adherence was inhibited more than 60%. MAb 5C6, which is directed toward the Mg^{2+} -dependent anchorage site on the CD11b molecule, yielded no inhibitory effect (Fig. 2.12 A). As seen in Fig. 2.12 B, MØ exposed to either M1/70 or M18/2 prior to the addition of *S. typhimurium* exhibited reduced mean fluorescence, compared to untreated controls. This supports the findings of the down-modulation assay and suggests the involvement of both the CD18 and CD11b chains in adherence of *S. typhimurium* to murine MØ. Blocking of Fc receptors with rat IgG prior to the addition of MAb did not show any effect on the adherence of bacteria to MØ (Fig 3.13).

Effect of combined MAb and neutrophil elastase.

A combination of MAb M1/70 and M18/2, each at a concentration of 100 µg/ml, was tested using both visual binding assay as well as flow cytometry assay to determine whether the inhibition to bacterial adherence seen with each antibody could be increased. The results, shown in Fig. 2.14 A and B, indicate that the combination was no more effective than each individual MAb. When neutrophil elastase was combined with these two MAb (Fig 2.14 A and C), again no increased inhibition of bacterial adherence was seen ($p = .28$), suggesting a role for as yet unidentified MØ receptors in this process.

Other factors

Since residual binding remained even when both CR1 and CR3 were blocked the involvement of other receptors and other components was investigated (Table 1).

Although one of the two antibodies against C1q receptor exhibited no effect on bacterial binding, pretreatment of MØ with anti-C1q receptor (R112) yielded enhanced adherence of *S. typhimurium* in flow cytometry studies (Fig 2.15). Antibodies against the C1q molecule did not show any effect on bacterial adherence to MØ (Fig 2.16).

MAB to two other MØ receptors, Mac-2 and Mac-3, were also tested for their ability to interfere with the adherence of *S. typhimurium* to MØ. As shown in Fig 2.17, neither MAB had inhibitory ability under the conditions used in these assays.

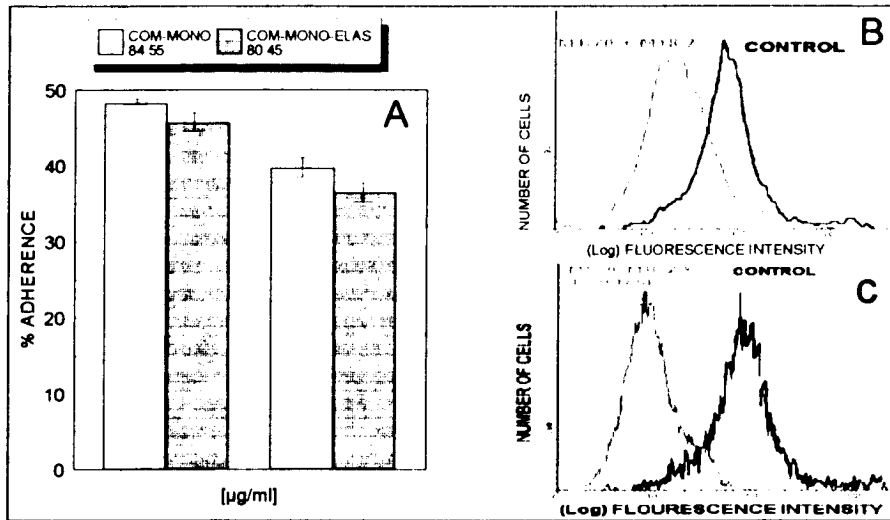


Fig. 2.14. Effect of pre-exposing MØ to combinations of anti CR3 MAb and neutrophil elastase and their ability to bind to *S. typhimurium*. (A) Visual binding assays on MØ monolayers, (B and C) flow cytometry studies of MØ in suspension.

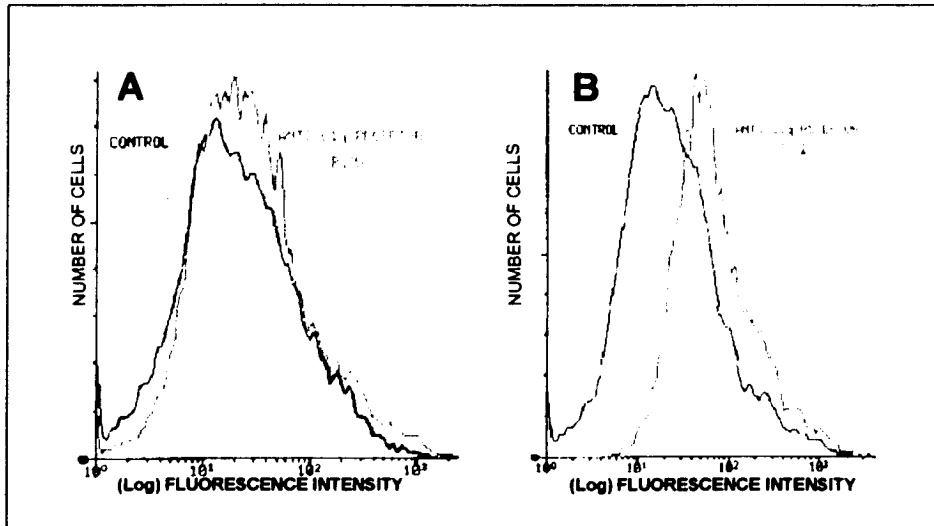


Fig. 2.15. Effect of pre-exposing MØ to one of two polyclonal anti-C1q receptors on the ability of these cells to bind to *S. typhimurium*. (A) Antibody lot R235, antibody lot R112 as measured by flow cytometry.

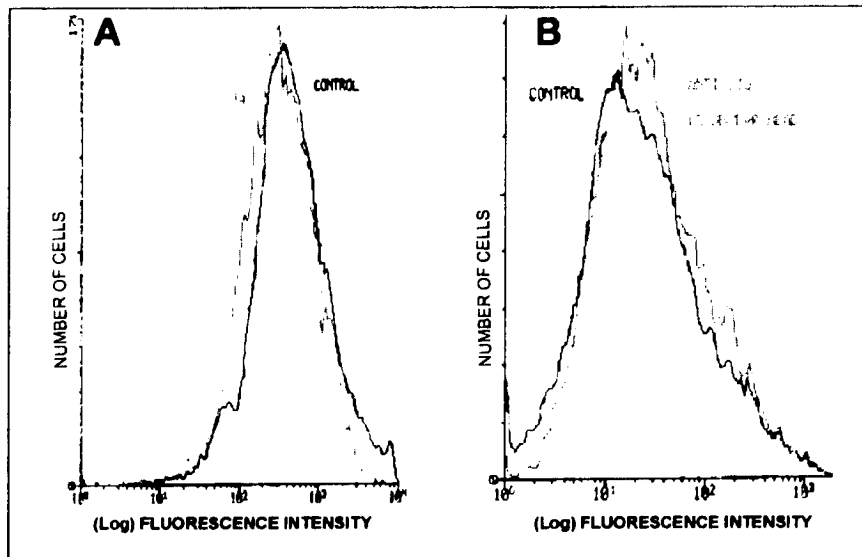


Fig. 2.16. Effect of polyclonal to C1q molecule on attachment of *S. typhimurium* to MØ, as measured by flow cytometry. (A) antibody made against the whole protein, (B) antibody against the globular head.

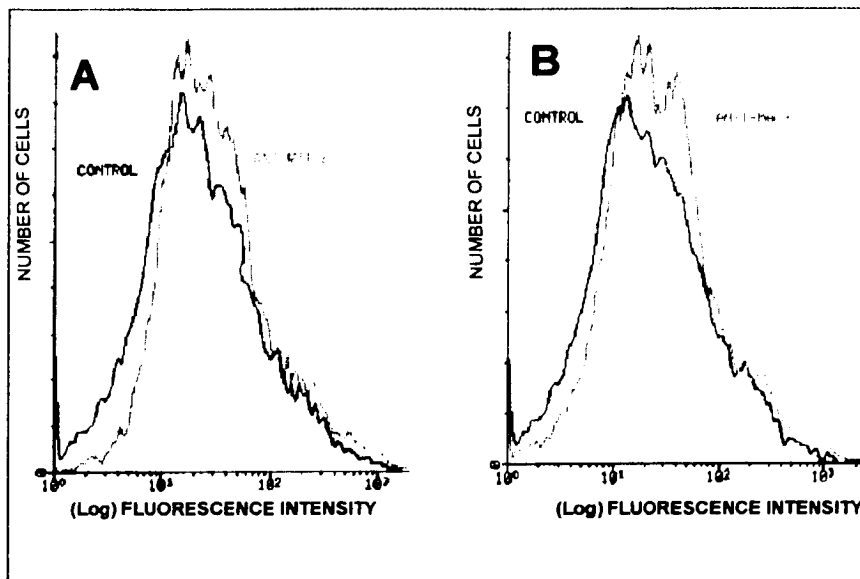


Fig. 2.17. Effect of pre-exposing MØ to MAb to Mac-2 (A) or (B) Mac-3 on their ability to block adherence of *S. typhimurium*, as measured by flow cytometry.

DISCUSSION

Adherence of *S. typhimurium* to mouse peritoneal MØ was determined by three methods: the visual binding assay, down-modulation studies, and FACS assays. The adherence studies were initially conducted using the visual binding assay. The results obtained from this method were confirmed by FACS techniques. Concentrations of test materials for which the maximum inhibition were shown in the visual binding assays were used for FACS studies. Flow cytometry allows the investigator to study adherence without the need to wash away unbound bacteria, a step that can affect the results in conventional adherence assays [(Fig 2.4), Bjerknes *et al.*, 1989]. The effect of each treatment was quantified by comparison to a control system containing MØ and bacteria but no potential inhibitor. Lucifer yellow is a sulfonated 4-amino naphthalimide with spectral qualities similar to fluorescein isothiocyanate, FITC (Sveum *et al.*, 1985). Unlike FITC, however, lucifer yellow is stable over a wide pH range (pH 2-10). As a result, adherent bacteria and phagocytosed bacteria can be readily detected without the need to use phagocytosis-inhibiting chemicals, such as cytochalasin B. MØ treated with cytochalasin B produced a fluorescence histogram comparable to the control system, indicating no demonstrable effect. All subsequent flow cytometry studies were performed in the absence of this chemical.

Bacterial adherence to eukaryotic cells requires viable cells (Lee *et al.*, 1990). MØ treated with 1% formaldehyde for 5-30 min or with higher concentration (200 µg/ml) of

cytochalasin B (Chapter 1) prior to the addition of bacteria exhibited a reduced level to bind bacteria compared to untreated controls.

The ability of *Salmonella* to escape opsonization and to activate MØ suggests that this microorganism binds directly to the MØ. LPS is a major component on the surface of many Gram-negative bacteria and is an important virulence factor in salmonellae. Wright *et al.* (1986) have shown that β_2 family of integrins is involved in the adherence of *Escherichia coli* to human MØ. Our results suggest that LPS is indeed recognized by murine MØ and are similar to those of Mroczenski-Willey *et al.*, (1989), who studied adherence of *S. typhi* to HeLa cells. In each case LPS appears to be an important but not exclusive contributor to microbial adherence to eukaryotic cells.

LPS derived from the mutant strain R595 lacks the O-side chain and most of the core sugar. This glycolipid and KDO each blocked adherence of *S. typhimurium* to MØ, but less effectively than wild-type LPS. Using *E. coli* and human MØ, Wright and Jong (1986) proposed that β_2 integrins bind to the hydrophilic portion of LPS, possibly the glucosamine disaccharide backbone of lipid A. They were able to block adherence of *E. coli* to leukocytes completely. Our findings are consistent with theirs; however, our data suggest that the O-side chain of *Salmonella* LPS is also an important contributor to this recognition, since wild-type LPS was more effective in blocking bacterial adherence than were LPS components. These findings are in agreement with those of Mroczenski-Willey *et al.* (1989) and Finley *et al.* (1989), who showed that *Salmonella* species with disrupted O-side chains or core structures adhered poorly to epithelial cells. Despite the similarities

therefore it may lack some of necessary factors for adherence to MØ. *S. typhimurium* on the other hand, is a facultative intracellular organism and has evolved different virulence factors that will enable bacteria to adhere to eukaryotic cells and subsequently to invade the cells.

Non-opsonin-mediated binding of *Mycobacterium avium* (Roecklein *et al.*, 1992) and *E. coli* (Salmon *et al.*, 1987) was found to occur via complement receptors and Fc receptors. Also, the work of Sung-Sang and Silverstein (1985) demonstrated the inhibitory effect of 2-deoxy glucose on the uptake of bacteria via the Fc receptors as well as both complement receptors CR1 and CR3. Similarly, Sloan and Pistole (1993) showed that this chemical blocked adherence of group B streptococci to murine peritoneal MØ. In the present study, 2-DG was found to block adherence of *S. typhimurium*, suggesting that one or more of these MØ receptors is involved in the binding.

The role of the Fc receptors was examined by using Fc fragments that were prepared from mouse polyclonal antibodies, and by irrelevant mouse MAb. Reduction of adherence levels was not detected suggesting that these receptors are not involved in direct recognition to *S. typhimurium*. The involvement of CR1 and CR3 receptors in the adherence process was confirmed by neutrophil elastase, an enzyme that specifically cleaves CR1 (Tosi, 1990), and MAb that recognize CR3 α - and β -subunits. MØ treated with neutrophil elastase exhibited reduced adherence of *S. typhimurium* suggesting a role for the CR1 receptor in recognition of salmonellae. Three different monoclonal antibodies (M1/70, M18/2, and 5C6), which bind to complement receptor CR3 α - and β -subunits were used. Blocking of the Fc receptors with rat IgG prior to blocking of those receptors

with the above antibodies did not affect the inhibition activity. Thus, all of the proceeding experiments were conducted without prior blocking of the Fc receptors. The individual monoclonal antibodies M1/70 and M2/18 blocked adherence almost equally, confirming the role of both α - and β -subunits in the adherence process. These results were similar to those of Wright *et al.* (1986) in which they showed the role of the β -subunit in recognition of *E. coli* LPS and confirmed that adherence of this organism occurred at an epitope distinct from C3bi-binding site (Wright *et al.*, 1989). However, in our study adherence of bacteria was also reduced significantly by blocking the α -subunit of CR3, suggesting that CD11b is also an important determinant on M ϕ in recognizing of *Salmonella*. Blocking both CR3 subunits by both MAb gave results similar to that obtained with the individual MAb. These data suggest that both subunits participate in the binding process. The monoclonal antibody 5C6, which recognizes a distinct epitope on CD11b (Cooper *et al.*, 1988) had no effect in blocking adherence of *S. typhimurium* to M ϕ . These data confirm that the inhibition seen with M18/2 and M1/70 MAb was specific.

In no case could we completely inhibit adherence even by blocking both receptors simultaneously. This suggests that there are additional mechanisms involved in this recognition process. It is possible that if one pathway is blocked, another becomes activated, thereby achieving adherence of *Salmonella* to M ϕ by multiple mechanisms. Also it is likely that several surface glycoproteins collaborate to promote specific, stable adhesion between macrophages and *Salmonella* that will ultimately lead to phagocytosis. Gordon *et al.* (1988) reported that receptors for complement and other components on

phagocytic cells can synergize in the binding and ingestion of microorganisms and other particulates. Strong support of this concept was provided by Bermudez *et al.* (1991) 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, FcR, transferrin receptor, mannose receptor, and possibly a separate β -glucan receptor were also found to be involved in nonopsonic uptake of *M. avium* complex by both blood monocytes and alveolar MØ (Roecklein *et al.*, 1992). It is likely that those receptors act in concert to mediate binding of bacteria and ultimately ingestion of the bound bacteria. For example, although salmonellae can adhere to and invade a wide variety of cells, they cannot invade erythrocytes (Finlay *et al.*, 1989_b) even though these cells express CR1 (Stewart *et al.* 1981), suggesting that these cells may lack other receptors that are used in the invasion process by *Salmonella*. Accordingly, the role of other factors on MØ was investigated (Table 1).

C1q receptor has a multifactorial activity (Ghebrehiwet *et al.*, 1988) and it is expressed on a variety of cells including MØ (Latsch *et al.*, 1990), Raji and U937 monocytic cells (Erdei *et al.*, 1989), B and T cells (Erdei 1990), fibroblasts (Bordin, 1990) and endothelial cells (Peerschke *et al.*, 1990). In addition to its role as a receptor for C1q, it has also been proposed to mediate adherence of microorganisms (B. Ghebrehiwet, personal communication). To explore this possibility for our system, we tested the effect of two anti-C1q receptor antisera on adherence of *S. typhimurium* to MØ (Table 1). Although one of the antisera (R235) exhibited no effect, the other (R112) produced enhanced bacterial adherence to MØ. The antibody probably binds to a specific epitope in

the C1q receptor, which in turns regulates other receptors on MØ. This kind of regulation has also been observed by Brown *et al.* (1988); when he blocked CR3 with monoclonal antibody, he was also able to block phagocytosis via the Fc-receptor. However, both of the antisera used in our study were rabbit polyclonal antibodies against C1q receptor (B. Ghebrehiwet, personal communication). It is possible that anti-C1q-R112, contains antibodies to *Salmonella* epitopes, which can serve as opsonins. Whether this antibody acted as an opsonin or a regulator can be confirmed by treating MØ with Fc fragments prior to the addition of anti-C1q-R112 to block opsonization of bacteria via Fc receptors.

C1q is a complex structure consisting of 18 similar but distinct polypeptide chains (Ghebrehiwet, 1987). Each chain consists of a C-terminal globular region and a collagen-like N-terminus. Local secretion of C1q by MØ has been suggested (Ezekowitz *et al.*, 1984; Grossman *et al.*, 1984). One of the functions of C1q is to recognize and to bind to a variety of particles including viruses and bacteria (Ghebrehiwet, 1986). Alvarez-Dominguez *et al.* (1993) found direct evidence for the role of C1q in phagocytosis of *Listeria monocytogenes* by a murine macrophage-like cell line. C1q has also been shown to be involved in binding of *Salmonella* in the absence of antibodies, to mediate the uptake of this organism by MØ, and probably to mediate intracellular survival of this bacteria within the phagocytic cell by preventing the fusion of phagosomes and lysosomes (Latsch 1990). Therefore, the role of C1q in our system was tested by specific antibodies. None of those antibodies showed any effect on bacterial adherence to MØ.

A MØ surface marker Mac-2 is highly expressed after elicitation of MØ with thioglycollate (Ho *et al.*, 1982) but at a lower level after elicitation with LPS (Springer *et*

al., 1984). Mac-2 is widely distributed throughout the body and is also expressed in some intestinal epithelium (Springer *et al.*, 1984). Since thioglycollate-elicited MØ are active phagocytically, and certain epithelial cells expressing this receptor are highly active endocytically, Mac-2 may play a role in *S. typhimurium* adherence to these cells.

Mac-3 is a glycoprotein that is also expressed in macrophages (Springer *et al.*, 1984) and is also found on some epithelial cells. Because both of these receptors are expressed on MØ, it is possible that *Salmonella* may bind to MØ via these receptors to establish their infection. Therefore adherence of *Salmonella* to those receptors was investigated using monoclonal antibodies to Mac-2 and Mac-3. However, reduction of adherence levels of *Salmonella* was not seen, suggesting that epitopes in those receptors that are blocked by those antibodies are not recognized by *S. typhimurium*. As we have shown above, only one (MI/70) of the two MAb against CD11b blocked adherence while the other (5C6) did not. Therefore, this does not rule out the activity of these two receptors in this adherence process.

In conclusion, adherence of *S. typhimurium* to murine macrophages can occur in the absence of exogenous opsonins. Results from inhibition studies suggest that multiple mechanisms are involved. LPS is an important ligand, both the O-side chain and the core glycolipid appear to be involved. Adherence is mediated by both CR1 and CR3 on the MØ; other, as yet unidentified, receptors are also involved.

CHAPTER 3

IDENTIFICATION OF BACTERIAL AND MØ MEMBRANE PROTEINS INVOLVED IN ADHERENCE

ABSTRACT

Several new bacterial surface proteins are expressed during the adherence process. However, the role of those proteins in adherence is unclear. In order to identify bacterial protein(s) involved in binding to MØ, purified bacterial membrane proteins were resolved by gel electrophoresis and transferred to nitrocellulose paper. The blotted bacterial proteins were probed with biotinylated MØ. A 44-kDa protein was found to be recognized by MØ.

To identify additional receptor(s) on MØ, we purified MØ cell membranes and separated them electrophoretically. The electroblotted proteins were probed with either biotinylated or ³⁵S-labeled bacteria. In each case a low molecular weight protein (16 kDa) was detected. Electroelution of this sample and further resolution by polyacrylamide gel (15%) electrophoresis identified two proteins of apparent molecular weights of 13 and 16 kilodaltons. MØ membrane preparations pretreated with phorbol esters were probed as above and these proteins plus an additional protein at the position of the α -chain (CD11b) of the Mac-1 β_2 integrin were detected. These results indicated that, depending on the state of activation, different MØ receptors are involved in bacterial adherence, supporting our hypothesis that multiple MØ receptors are involved in the adherence of *S. typhimurium*.

INTRODUCTION

Adherence of *S. typhimurium* to epithelial cells occurs at two stages: a reversible phase (bacteria can be removed by washing), and an irreversible phase (bacteria are resistant to washing) (Jones *et al.*, 1981). During this adherence process *S. typhimurium* induces global changes in both the bacterial cell and the host cell (Miller *et al.*, 1992). These changes facilitate firmer adherence of bacteria and uptake by specific receptor(s) usually involved in phagocytosis. If bacteria are incubated with a monolayer of eukaryotic cells, then transferred to another monolayer, they adhere much faster to the second monolayer (Finlay *et al.*, 1989_a). The presence of chloramphenicol inhibited the irreversible phase of bacterial binding to eukaryotic cells (Lindquist *et al.*, 1987) and others have indicated that *Salmonella* species require *de novo* synthesis of RNA and proteins to adhere to epithelial cells (Finlay *et al.*, 1989_a).

Several new bacterial surface proteins are synthesized in the second phase of adherence while the synthesis of some other bacterial proteins is inhibited. The former are essential for adherence (Finlay *et al.*, 1989_a), invasion (Finlay *et al.*, 1991; Portnoy *et al.*, 1992) and intracellular survival (Buchmeier, 1991; Alpuche 1992). Ten mutants of *Salmonella* have been isolated that do not adhere to epithelial cells (Finlay *et al.*, 1989_b). The mutants were unable to produce some of the induced proteins, confirming a role for those proteins in the initial adherence to eukaryotic cells. Another mutant was found to adhere to MØ but invaded MØ poorly compared to parental strains, even when the

bacteria were opsonized. This suggests that *Salmonella* invades MØ by two pathways: classical phagocytosis and a specific invasion pathway that is used to invade other nonphagocytic animal cells.

Other mutants were found to adhere to and invade eukaryotic cells but were incapable of surviving intracellularly (Field *et al.*, 1986; Groisman *et al.*, 1989). These data indicate that the induced proteins are imperative for *Salmonella* virulence and appear to be a part of a complex regulatory network that is induced during bacterial infection of epithelial cells (Finlay *et al.*, 1989), or MØ (Buchmeier, *et al* 1990) and MØ-like cells, e.g., U937 (Abshire *et al.*, 1993). Protein profiles from the target cells differ slightly following infection of epithelial cells or of MØ (Buchmeier *et al.*, 1990). How these proteins are induced is not yet known.

Treatment of the eukaryotic cell with trypsin, neuraminidase or periodic acid prevents the induction of these proteins and lowers adherence and invasion levels (Finlay *et al.*, 1989). The growth of *Salmonella* under anaerobic conditions can mimic the stress seen when bacteria interact with epithelial cells or are internalized by MØ (Lee *et al.*, 1990). Other conditions that may induce those proteins include oxidative stress and a sudden increase of temperature (Christman *et al.*, 1985; Stinavage, *et al.*, 1990), nutrient limitation (Fang *et al.*, 1992), or acidic conditions (Foster *et al.*, 1990; Leyer *et al.*, 1993; Garcia-del *et al.*, 1993).

The complete mechanism involved in the early events of the innate defense process remains unclear. Activation of CR1 and CR3 by LPS may allow *S. typhimurium* to use these receptors in binding to macrophages. Whether these receptors are the only ones

involved in the adherence of this bacterium to macrophages needs to be determined. We observed that even by blocking both CR1 and CR3 of peritoneal macrophages adherence of *S. typhimurium* continues to exist. The goal of this study was therefore to identify induced outer membrane *Salmonella* protein(s) that are involved in the adherence mechanism and to identify other receptors on MØ that play a role in this process.

MATERIALS AND METHODS

Induction of bacterial stress proteins and radiolabeling

S. typhimurium was grown in LB broth (Difco Inc, Detroit, MI) to late logarithmic phase (Lee *et al.*, 1990). The cultures were incubated at 37 °C in anaerobic chamber. About 0.4 ml of the cultures was washed with 1 ml PBS and resuspended in 80 µl of 0.1% methionine-free glucose medium. The medium was supplemented with 15 µCi of [³⁵S] methionine (DuPont, Massachusetts) and the cultures were incubated at 37 °C for an additional hour. Labeling was terminated by the addition of 10 µl of 0.1 M L-methionine. Bacteria were harvested by centrifugation for 5 min and were washed 3 x.

Bacterial outer membrane protein (OMP) preparations

Bacterial OMP preparations were prepared according to the method of Achtman *et al.* (1978). Bacterial cells were resuspended in 10 ml of a sonication buffer [10 mM Tris-HCl, pH 7.8, containing 5 mM Mg²⁺, 50 µg/ml containing Rnase, 1.0 mg/ml Dnase (Sigma, St. Louis, MO), 1.0% aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin (Boehringer-Mannheim, Indianapolis, ID)]. The remainder of the procedure was conducted at 4 °C. The bacterial suspension was sonicated 4 x for 30 seconds on ice. The contents were centrifuged at 10,000 x g (Beckman, J2-MI; California) for 10 minutes to remove unbroken cells. The suspension, which contained bacterial membranes, was centrifuged at 40,000 x g (Beckman

ultracentrifuge, L8-70) for 60 minutes. The supernate was decanted and the pellet was washed 3 x with sonication buffer. After the third wash, membranes were solubilized with two volumes of 2% (v/v) sodium dodecyl sulfate (SDS), and incubated at 70 °C for one hour. The SDS-insoluble components were removed by centrifugation at 50,000 x g for 60 minutes and the supernate containing the OMP was stored at -70 °C for later use.

Modified method for MØ membrane protein collection

Mouse peritoneal MØ were obtained as described in Chapter 1. The cells were washed 3 x with PBS. Two sets of MØ were used; one set served as a control and the other was treated with 50 ng of phorbol-12-myristate-13-acetate (PMA, Sigma Chemical Co) following the method of Shaw *et al.* (1990). MØ were incubated with PMA at 37 °C for 1 h and then washed 3 x. After the last wash the cells were resuspended in 5 ml of 5 mM Tris-HCl, pH 7.2, 1 mM MgCl₂ for 20 min on ice to allow cells to swell (Paganelli *et al.*, 1987). The remainder of the experiment was conducted at 4 °C unless otherwise noted. The following protease inhibitors were added: 1.0% aprotinin, PMSF (1 mM), leupeptin (0.01 mg/ml), pepstatin [(0.01 mg/ml)Boehringer Mannheim, Indianapolis, ID]. The cells were sonicated and the contents were brought up to 35 ml with 0.25 M sucrose containing 5 mM Tris-HCl and 1 mM MgCl₂. The suspension was centrifuged at 500 x g (Beckman, TJ-6) for 5 min. The pellet was discarded and the supernate was centrifuged at 18,000 x g (Sorvall, RC-58; Dupont Instrument, Massachusetts) for 30 min. The supernate was decanted and the membrane fraction was resuspended in 4 ml PBS without calcium or magnesium and the above protease inhibitors were added. Approximately 1

ml of 1% SDS detergent was added to solubilize membrane and the contents were incubated for 30 min. The suspension was centrifuged at 197,000 x g for 60 min to remove the insoluble materials and the supernate that contained solubilized membrane proteins was stored at -70 °C for later use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated according to their molecular weights following the method of Laemmli (1970). The stacking gels were made with 5% acrylamide and the running gels with 12% acrylamide. The buffers used were filtered through a 45- μ m filter to remove insoluble materials and were degassed by vacuum for 20 min. Molecular weight (m.w.) standards [lysozyme, β -lactoglobulin, carbonic anhydrase, ovalalbumin, bovine serum albumin, phosphorylase B, myosin (H-chain), with molecular masses of 14.3, 18.4, 29, 43, 68, 97, 200 kiloDalton (kDa) respectively; Gibco BRL, New York] were used to determine the m.w. of the unknown membrane proteins. Each protein sample was mixed with 10 μ l of tracking dye (0.025% bromophenol blue, 50% glycerol, and 50 mM Tris) and 10 μ l sample buffer (10 mM Tris pH 6.8, 2% beta-mercaptoethanol, 10% glycerol, and 4% SDS). The specimens were boiled in a water bath for 5 min to denature the proteins. About 90 μ l of protein were loaded into each well and the proteins were separated electrophoretically for either 2 h at 100 mA or at 10 mA overnight. The gels were removed from the glass plates and either visualized by silver staining or transferred to nitrocellulose sheets.

Silver stain

The silver staining of the gels was carried out according to the method of Wray (1981). Gels were soaked in 50% methanol overnight and then washed 2 x for 1 h. The gels were stained for 15 min with silver stain solution (0.8 g silver nitrate, 0.36% sodium hydroxide and 1.4 ml of concentrated ammonium hydroxide). The silver stained gels were washed and developed by soaking them in developing solution (2.5 ml of 0.05% citric acid and 0.012 ml of 37% formaldehyde in 500 ml water). After the protein bands appeared, the developing process was terminated with 10 ml of 50% methanol and the gels were washed with water.

Western Blot analysis

Western blot analysis was performed according to the procedure of Towbin *et al.* (1979). Polyacrylamide gels with separated proteins were used to transfer proteins electrophoretically onto a sheet of nitrocellulose paper by the use of a Western blot apparatus (Trans Blot, Bio-Rad, Richmond, CA) containing a transfer buffer (40% methanol, 192 mM glycine, and 25 mM Tris). Transfer of proteins onto nitrocellulose paper was carried out overnight at 100 mA. The nitrocellulose paper with the transferred proteins was washed in PBS for 30 min to remove methanol. Gelatin (3%) was used to block unoccupied areas on the nitrocellulose paper and the system was incubated for 1 h at room temperature. After additional washing the blotted proteins were used for probing and other analyses.

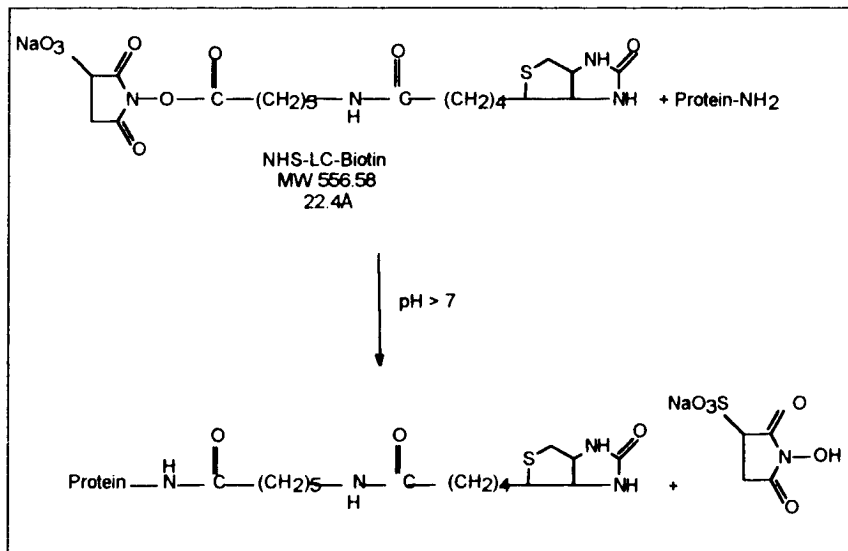


Fig 3.1. The reaction of NHS-LC-Biotin with a protein. Pierce ImmunoTechnology Catalog and Handbook, 1992.

Method for detection of S. typhimurium adhesins

The procedure of Prakobphol *et al.* (1987) for determining *S. typhimurium* adhesins from a mixture of OMP was modified in our laboratory. Bacterial OMP were resolved by SDS-PAGE, then transferred onto nitrocellulose paper as explained above.

Mouse peritoneal MØ were lavaged and prepared as explained in Chapter 1. The cells were surface-labeled with biotin using the method of Hurley *et al.* (1985). This reaction of biotin is shown in Fig 3.1. MØ (10^6 /ml) were resuspended in cold PBS (pH 7.4) and the cells were treated with biotin (Pierce Chemical Co, Illinois) at a final concentration of 5 mM. This concentration was shown in flow cytometry studies not to interfere with bacterial adherence. The reaction mixture was tumbled mixed for 1 h at 4 °C and then washed with cold DPBS for 3 x. The cells were resuspended in 6 ml DPBS and added to a nitrocellulose sheet containing transferred *S. typhimurium* outer membrane proteins. The system was incubated at 37 °C without agitation for 30 min to allow MØ to settle on the transferred proteins followed by agitation for 30 min. Nitrocellulose sheet blots were washed 4 x with cold PBS, 1 min each, to remove unbound MØ. Bound MØ were then fixed with 0.5% glutaraldehyde for 5 min and the sheet was washed 4 x with DPBS. Avidin-conjugated HRP (Pierce Chemical Co, Illinois), at a concentration of 2 µg/ml in 10 ml of DPBS, was added to probe for biotin. The system was incubated for 1 h at room temperature. The system was developed using the HRP developer (20 ml methanol, 60 µl of 30% H₂O₂ in Tris buffer saline, pH 7.5). This developed nitrocellulose sheet was compared with a corresponding silver stained polyacrylamide gel to locate *S. typhimurium* OMP band (adhesin).

Detection of MØ binding receptors

The procedure of Prakobphol *et al.* (1987) for detecting the MØ receptor recognized by *S. typhimurium* from a mixture of membrane proteins was used. These proteins were resolved by electrophoresis and transferred to the nitrocellulose membrane as explained above. Two types of labeled *S. typhimurium* strain 14028 were used, biotinylated and ³⁵S-radiolabeled bacterial cells. Labeled bacteria were washed and suspended in PBS and were permitted to attach to Western blots of the MØ for 18 h at 4 °C. The blots were then washed 3 x with PBS, 1 min each, to remove unbound bacteria. For the biotin-labeled bacteria, the blots were treated with avidin as explained above. The blots containing radiolabeled bacteria were dried under vacuum at 80 °C for 1 h. The blots were air-dried and exposed to X-ray film for three days prior to developing.

Electroelution of protein bands from SDS-PAGE

MØ and bacterial membrane proteins were resolved by SDS-PAGE as explained above and the gels were reverse-stained following the method of Fernandez-Patron *et al.* (1992). The gels were rinsed with water for 5 s and soaked in 100 ml of 0.2 M imidazole (Sigma) with gentle shaking for 5 min. The gels were rinsed again with water and were stained with 100 ml of 0.3 M zinc sulfate (Sigma) until the background became deep white and protein bands appeared transparent. The bands of interest were sliced from the gels using a clean, sharp razor blade (Harrington 1990). Fragments containing the proteins of interest were destained with 2% citric acid, washed and equilibrated in the electroelution buffer (15 mM NH₄CO₃ and 0.1% SDS, pH 8.2). The fragments were then placed in a

sample chamber of an elutrap (Schleicher & Schuell, New Hampshire) containing electroelution buffer. Prior to the addition of gel fragments, the system was placed in a horizontal electrophoresis chamber which was connected to a cooling device to prevent heating. The system was allowed to run overnight at 100 V. Eluted proteins migrate from the sample chamber through a BT2 filter and become trapped in a trap chamber. After elution was accomplished, the polarity was reversed for 20 s at 200 V to remove proteins that might be attached to a BT1 membrane. The eluted proteins were collected and stored at -70 °C.

Functional assay

Eluted proteins were washed from the electroelution buffer and were concentrated using Centricon-10 concentrators (Amicon, Beverly, MA). The proteins were resolved in 12% and 15% gels and were either silver stained or transferred to nitrocellulose paper as explained above. The activity of these proteins were confirmed following the method Prakobphol *et al.* (1987) as described above. These proteins were then used for FACS studies in competitive inhibition assay (Materials and Methods, Chapter 2). Prior to the competitive inhibition assay SDS detergent was removed from the proteins (Suzuki *et al.*, 1988). They were precipitated with 1 ml of ice cold 20 mM KCl for 30 min. The precipitate was collected by centrifugation for 30 min at 10,000 rpm at 5 °C using a Beckman microfuge II. The supernate was removed and the precipitate was resuspended in PBS. The samples were transferred into the Centricon-10 and washed with PBS 3 x.

EDTA treatment

MØ, suspended in 300 µl of RPMI, were treated with 5, 25, or 50 µg/ml of EDTA for 5 min. Suspension of *S. typhimurium* containing the corresponding concentration of EDTA in RPMI were added and the standard binding assay was completed as discussed earlier (Chapter 1). These results were assessed using factorial analysis of variance.

For studies with isolated MØ membrane proteins, radiolabeled bacteria in DPBS containing 50 µg/ml of EDTA were added onto nitrocellulose sheets containing the blotted proteins and the experiment was completed as described above.

RESULTS

Blocking studies with various receptors (see Chapter 2) yielded only partial inhibition of bacterial binding and MØ. This suggested that the initial interaction of *S. typhimurium* to MØ is a multifactorial process. Attempts were made to identify protein factors expressed on the surface of *Salmonella* and MØ that are involved in this interaction.

The role of bacterial stress proteins

S. typhimurium was grown under anaerobic conditions to late logarithmic phase to induce the expression of stress proteins. The outer membrane proteins were isolated and were used for adherence studies using biotinylated whole mouse peritoneal MØ. The concentration of biotin used for surface labeling of MØ did not affect the adherence process (Fig 3.2). The proteins were resolved according to their molecular weights by SDS-PAGE electrophoresis and blotted on nitrocellulose papers. Of the various *Salmonella* outer membrane proteins, only one protein, with a molecular mass of 44 kDa (P44), was shown to bind to MØ (Fig 3.3).

The protein band was isolated from SDS-PAGE gels by electroelution and SDS was removed prior to the competitive binding inhibition assay using flow cytometry. Adherence levels of *S. typhimurium* to mouse peritoneal MØ dropped drastically in the presence of this protein (Fig 3.4). Reduction of adherence levels of *S. typhimurium* dropped further in the presence of both P44 and LPS (Fig 3.4).

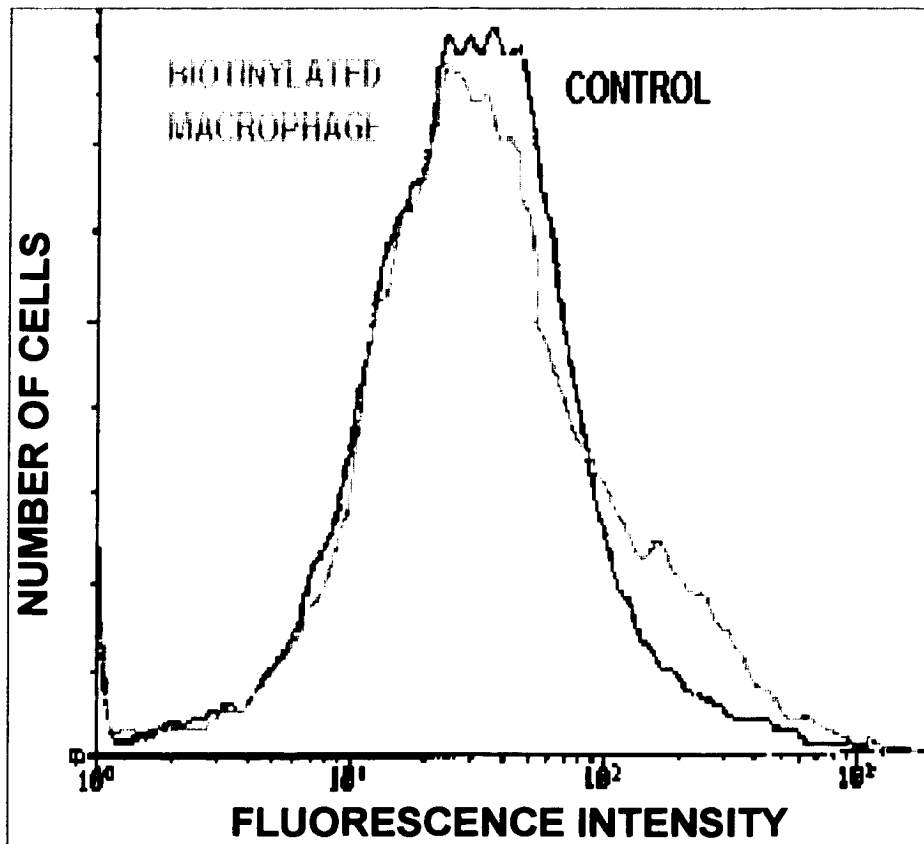


Fig 3.2. Adherence of *S. typhimurium* to biotinylated MØ and control, non-biotinylated MØ, as measured by flow cytometry.

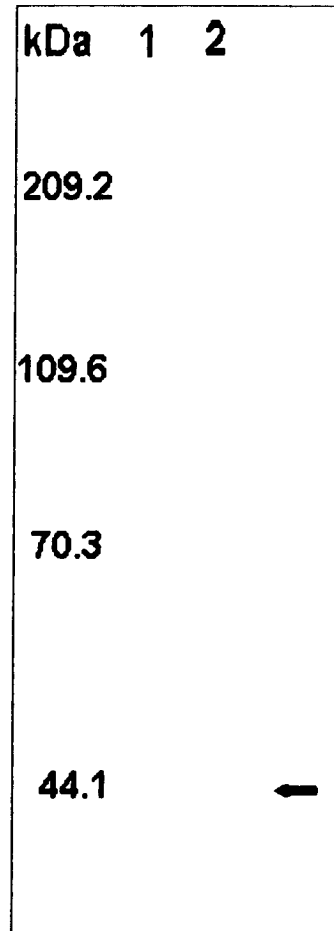


Fig. 3.3. Western blot of *S. typhimurium* outer membrane proteins resolved by SDS-PAGE and probed with biotinylated MØ (lane 2). Lane 1. m.w. markers.

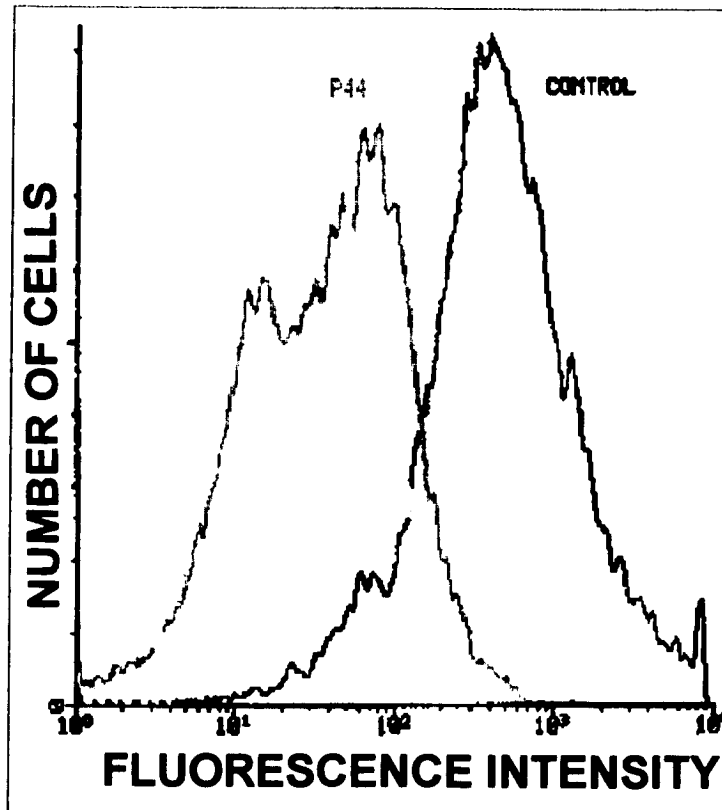


Fig. 3.4. The effect of pre-exposing MØ to *S. typhimurium* outer membrane protein P44, lane (A) or in combination with LPS (B) on their ability to bind to these bacteria as measured by flow cytometry.

Detection of MØ receptors

Detection of the MØ bacterial binding receptors was accomplished by extracting MØ membrane proteins. These proteins were electrophoresed on 12% SDS acrylamide gel and were blotted onto nitrocellulose paper. The proteins were initially probed with biotinylated *Salmonella*. One protein band of low molecular weight was detected (Fig 3.5, lane 2). Adherence of *S. typhimurium* to this protein was confirmed using radiolabeled bacteria (Fig 3.5, lane 3). This protein was shown to be expressed on the surface of MØ (Fig 3.6) by biotinylating MØ, recovering their cytoplasmic membranes, resolving them by electrophoresis, blotting them on nitrocellulose, and probing with avidin. Since we initially had shown that adherence of these bacteria involves other receptors, we also attempted to detect additional bacterial binding proteins from these MØ. We found that treatment of MØ with phorbol ester amplified MØ binding activity to *Salmonella* compared to controls (Fig 3.7). Treatment of MØ with phorbol ester prior to membrane protein isolation and Western blot analysis resolved an extra band of higher molecular weight at the position of β_2 integrins (Fig. 3.5, lane 4). No additional bands were detected.

The MØ membrane protein band of low molecular weight was electroeluted and the activity of the two proteins was confirmed with a 12% acrylamide gel. Adherence levels of *S. typhimurium* to whole MØ dropped significantly in the presence of EDTA [Fig 3.8, ($p < .0001$ for EDTA concentration of 25 and 50 $\mu\text{g/ml}$ compared to untreated controls)]. However, binding of *S. typhimurium* to this band on nitrocellulose paper occurred even in the presence of EDTA (Fig 3.9).

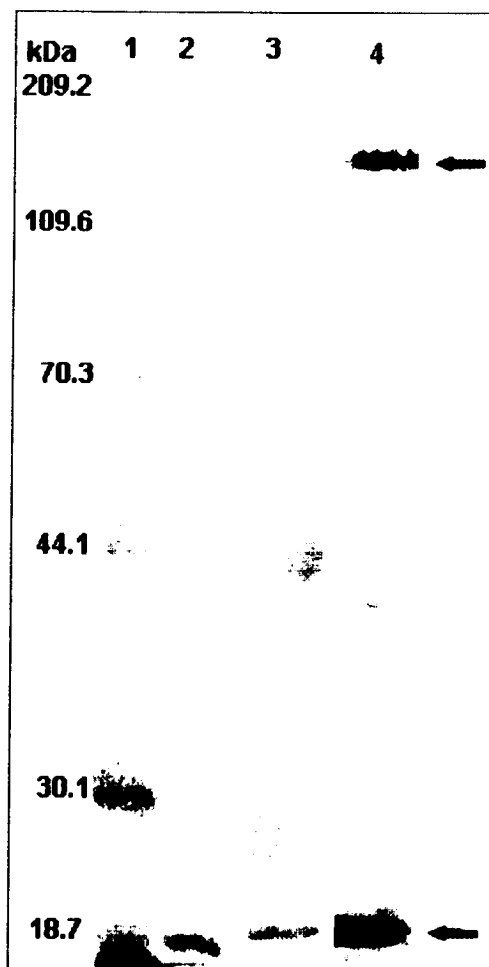


Fig. 3.5. Western blot of MØ membrane proteins probed with biotinylated *S. typhimurium* (lane 2, or with ³⁵S-radiolabeled bacteria (lane 3). On lane 4 membrane proteins were derived from MØ pretreated with phorbol ester, then probed with radiolabeled bacteria; m.w. marker are shown in lane 1.

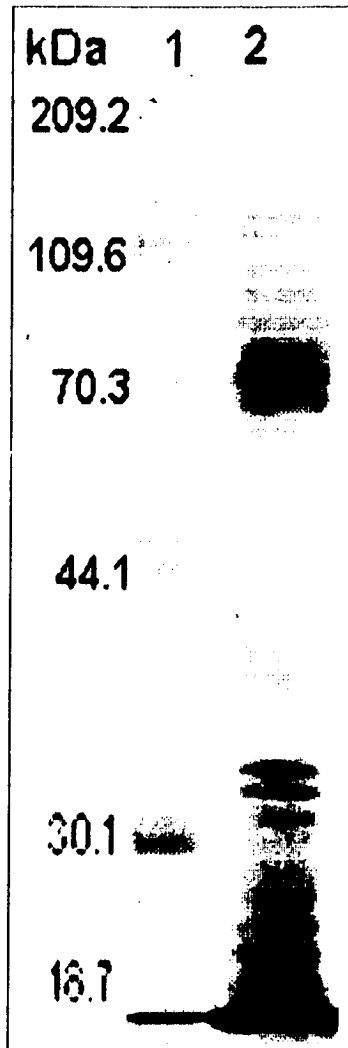


Fig. 3.6. Western blot of biotinylated MØ membrane proteins probed with avidin (lane 2). Lane: m.w. markers.

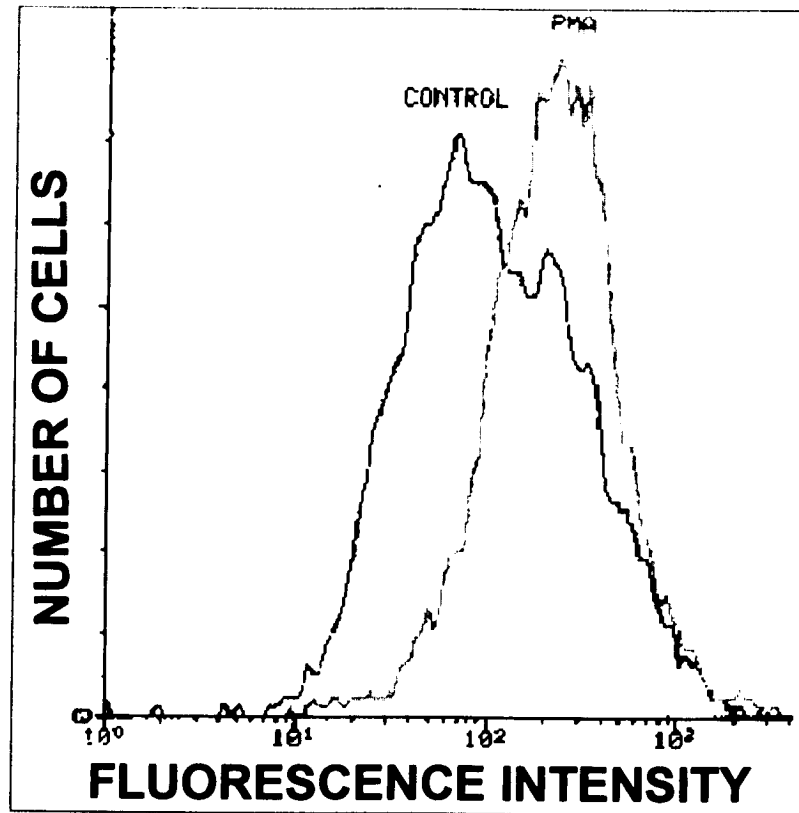


Fig. 3.7. The effect of phorbol ester treatment of MØ on adherence of *Salmonella typhimurium*, as measured by flow cytometry.

When the protein band was resolved with 15% gel, two bands were observed one with a molecular mass of 16 kDa and the other with a molecular mass of 12 kDa (Fig 3.10). However, no bacterial binding could be detected on the MØ protein bands resolved in the 15% gel. Additionally, competitive binding assays with these two proteins were negative. (Fig 3.11).

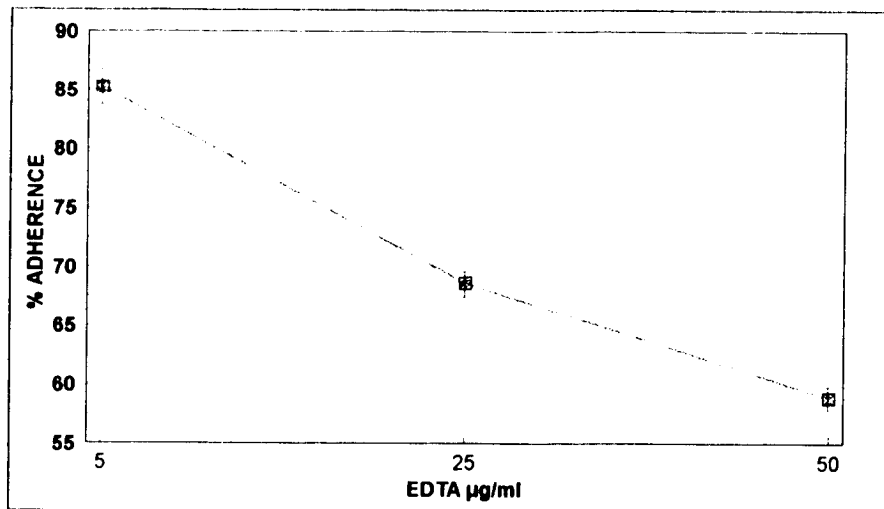


Fig. 3.8. Visual binding assay showing the effect of different concentrations of EDTA on bacterial adherence to mouse peritoneal MØ.

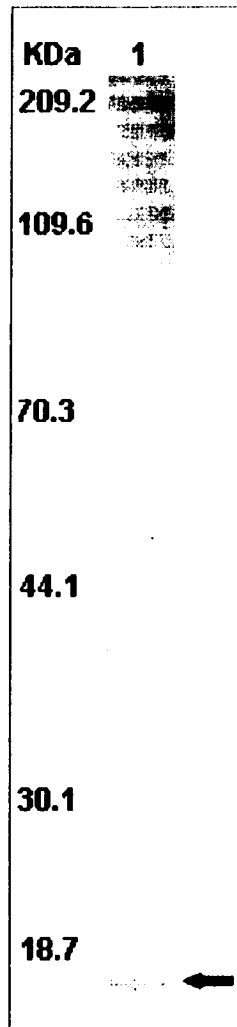


Fig. 3.9. Adherence of *S. typhimurium* to nitrocellulose-blotted MØ membrane proteins in the presence of EDTA (50 µg/ml).

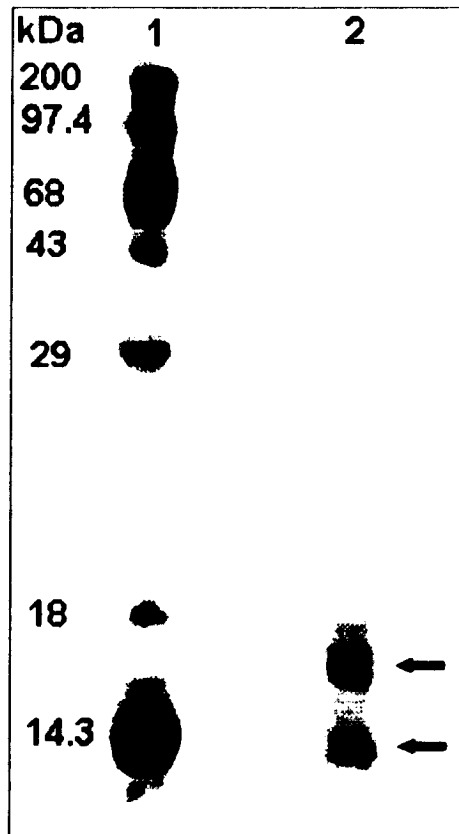


Fig. 3.10. Polyacrylamide gel (15%) with MØ receptor protein electroeluted from 12% gel [(lane 2), see Fig 3.5]. Lane 1: m.w. markers.

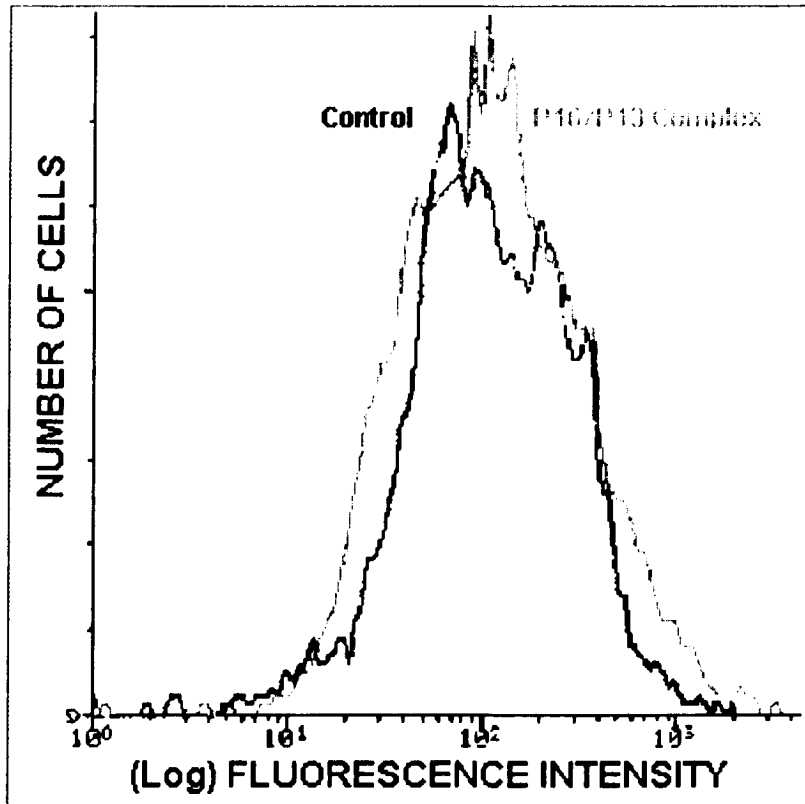


Fig. 3.11. Effect of P16/P13 complex on adherence of *S. typhimurium* to mouse peritoneal MØ, as measured by flow cytometry.

DISCUSSION

Previous attempts were made to block adherence of *S. typhimurium* to mouse peritoneal macrophages. However, complete inhibition was not achieved, suggesting that bacterial adherence to MØ is a multifactorial process. Here, we attempted to identify bacterial stress-induced outer membrane proteins and other MØ receptors that may be involved in the bacterial-MØ interaction following the method of Prakobphol *et al.* (1987).

Salmonella expressing stress proteins have enhanced bacterial adherence, invasion and intracellular survival; the specific role of these proteins, however, is still not clear. Thus we made an attempted to identify the role of stress proteins in the adherence process. The method of Prakobphol *et al.* (1987) was modified to probe for bacterial proteins by viable MØ. Bacterial outer membrane proteins were purified and blotted onto nitrocellulose paper and probed with MØ. Initially, we used antibodies to detect MØ bound to bacterial proteins on the western blots (Fig. 3.12. A). However, we were unable to detect any binding on the blots, probably due to the many washing steps. Better results were obtained when we minimized washing steps by prelabeling surface proteins of MØ with biotin (Fig 3.12. B). The cells need to be fixed prior to the detection process with avidin to prevent cell lysis in the following steps. From many outer membrane proteins blotted onto nitrocellulose paper, we were able to detect only one band of bacterial protein to which MØ bound with high affinity. This bacterial protein had a molecular mass

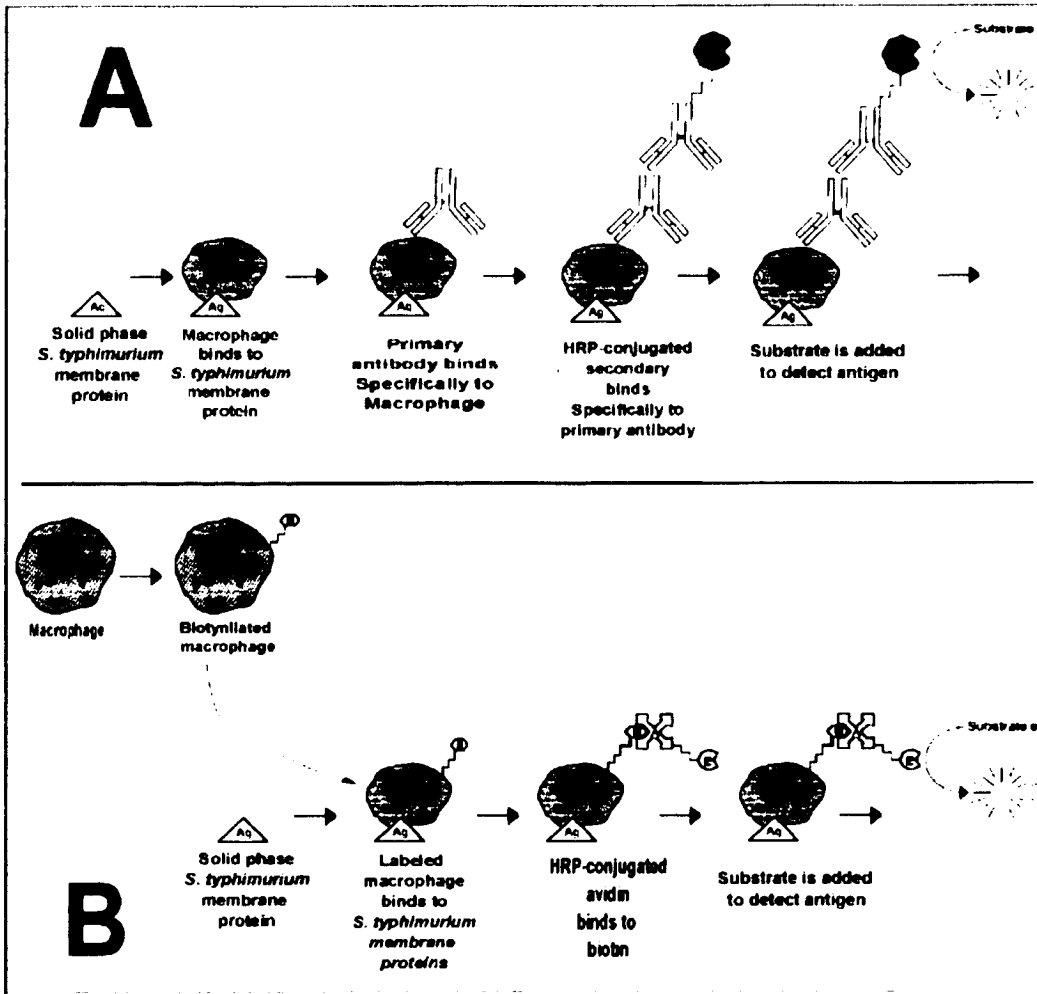


Fig. 3.12. Methods used to probe for bacterial proteins. A: Probing with antibodies, B: probing with biotinylated MØ.

of 44 kDa (P44). Higher concentrations of biotin used in labeling process interfered with bacterial binding. We confirmed that the concentration of biotin used in this assay did not interfere in the bacterial-MØ interaction.

P44 was electroeluted from acrylamide gels and freed of SDS detergent prior to its use in the functional assays. We observed reduced levels of bacterial adherence to MØ when P44 was used in competitive binding assays. These data confirm the role of this protein in the adherence of MØ to salmonellae. More reduction of adherence levels were observed when MØ were incubated with P44 in combination with LPS. However, complete inhibition was not achieved.

Because earlier studies had shown *Salmonella* adherence to MØ involves multiple mechanisms, the method of Prakobphol *et al.* (1987) was used to identify additional receptors on MØ membranes. Initial attempts to use antibodies as a probe to detect bacteria bound to MØ proteins on nitrocellulose papers were unsuccessful due to numerous washing steps required. Improved results were obtained when bacteria were biotinylated and were used to probe for MØ membrane proteins on Western blots. Interestingly, only one band, with a molecular weight of 16 kDa, was detected, although our previous studies suggested that there is more than one receptor involved in bacteria-MØ interaction. We confirmed these data by probing MØ membrane proteins with ³⁵S-metabolically-radiolabeled *Salmonella* and detected bacterial adherence at the same position where biotinylated bacteria bound. Using biotinylation techniques to outer surface label MØ we confirmed that this protein band is expressed on the surface of the MØ. About 4-5 bands with low affinity to bacterial binding were also detected when the

washing process to remove bacteria was minimized. However, the autoradiographs contained a high background making it difficult to resolve those bands from the background.

Additional attempts were made by treating MØ with PMA prior to the MØ membrane proteins isolation. PMA induces binding activity in certain lymphoid cells (Patarroyo, 1990). The enhancement in cell-binding activity occurs as a result of an increase in CR3 expression on the phagocytic cell surface (Majima *et al.*, 1990) derived from intracellular pools in the MØ (Bainton *et al.*, 1987). However, an increase in the number of cell surface CR3 molecules by itself is not sufficient for the elevated binding activity of this receptor (Hermanowski-Vosatka, *et al.* 1992). During this process CD18, a subunit of CR3, is phosphorylated, leading to an enhancement of cell adhesion activity (Valmu *et al.*, 1991). This activation also causes structural changes in CR3 (Wright *et al.*, 1988) that will augment CR-mediated phagocytosis (Newman *et al.*, 1991) via an opsonin-independent pathway (Gordon *et al.*, 1988). PMA also enhances phagosome-lysosome fusion (Park *et al.*, 1991) and stimulates oxygen consumption (Rist *et al.*, 1993), the respiratory burst (Meier *et al.*, 1990) and nitric oxide production in cultured peritoneal MØ, which occur as a result of activation of protein kinase C (Hortelano *et al.*, 1993).

In this study, treatment of viable MØ with PMA enhanced adherence of *Salmonella* markedly. As a result, this chemical was used to treat MØ prior to the membrane proteins isolation, resulting in the detection of an extra membrane protein band on the blots with radiolabeled bacteria. This band was detected at the position of α -subunit of CR3 (Springer, 1990).

The primary recognition and function of CR3 has been shown to require physiologic concentrations of divalent cation (Hynes, 1992; Michishita, 1993). These cations are essential for the function of the CR3 receptor (Wright *et al.*, 1986). They regulate the integrin function and are required to maintain binding integrity (Kirchhofer *et al.*, 1991). They are also essential for CR-mediated phagocytosis in human neutrophils and MØ (Fällman *et al.*, 1992). Treatment of viable MØ with EDTA diminished bacterial adherence to 50%, suggesting the role of these cations in the adherence of *Salmonella*, probably via CR3. Bacterial adherence remained even in the presence of EDTA suggesting that binding of *Salmonella* to other receptors does not require cations. Unlike the putative CR3, the presence of EDTA on the blots containing either whole MØ membrane proteins or electroeluted P16 did not affect adherence of *Salmonella* to this band. This indicates that adherence of *Salmonella* to this receptor did not require divalent cations.

When the P16 band was resolved with 15% acrylamide gel, two bands were detected, one with m.w. of 16 kDa and the other with 13 kDa. Interestingly, bacterial adherence on the blots did not occur when the two bands were separated by a 15% gel even though bacterial adherence continued to exist if the bands were resolved by the 12% gel. Also, when the two proteins were electroeluted from 12% gels and were used for competitive inhibition assay no reduction in the adherence of bacteria to whole MØ were observed. This suggests that the two proteins in a 12 % gel exist together in close proximity as a complex similar to the case when they exist naturally on the surface of a MØ. Separation of the two proteins as in a 15% gel or in suspension cause the proteins unable to bind to

bacteria.

Selective clustering of MØ receptors around an attached particle during the inflammatory response have been observed by other investigators (Weir, 1984). In another case, modulation experiments in which either CR3 or mannose-fucose receptor are rendered inaccessible demonstrated that both receptors must present in a given segment of the MØ membrane for effective binding of particles (Blackwell *et al.*, 1985). Also, similar to CR3, CR4 (p150/95; CD11c/CD18) in neutrophils is able to bind C3bi. However, in COS cells this receptor cannot bind to C3bi suggesting that COS cells lack other factors or receptors that are necessary to accomplish this activity (Kishimoto, 1988). All these data suggest that various receptors on a cell act in concert, and probably in a close proximity, to accomplish a particular physiologic activity.

This physiologic activity probably requires viable cells since, in our case, adherence of *Salmonella* to fixed MØ dropped drastically (Chapter 2). This may also explain our inability to detect more receptors using the Western blot technique. To prove the close proximity hypothesis, the two membrane proteins, P16 and P13, can be associated by a special cross linker prior to the competitive inhibition assay. The involvement of these two proteins in *Salmonella* adherence can also be confirmed by antibodies prepared especially to those two proteins, used individually or in combination.

In conclusion, adherence of *S. typhimurium* to MØ in the absence of exogenous opsonins is a complex process occurs via multiple mechanisms contributed by specific factor in *Salmonella* and MØ. Bacterial adherence occurs by both LPS and the stress-induced protein, P44. MØ to binds to P44 with high affinity. Depending on the state of

activation, different MØ receptors are involved in bacterial adherence and that those receptor act together to maintain this binding activity. In case of *Salmonella* our studies has demonstrated the involvement of protein complex composed of 13 kDa and 16-kDa subunits, and a larger protein whose properties suggest that it is the CR3.

GENERAL DISCUSSION

Salmonellosis is the most prevalent zoonosis in both developed and developing countries (WHO News and Activities). In some countries the *Salmonella* infection level in humans, mainly from *S. enteritidis*, is increasing by approximately 25-30% per year. *Salmonella* is a facultative intracellular pathogen that can survive and replicate in phagocytic cells (Groisman, 1989). Because of this property, some *Salmonella* strains, *i.e.*, *S. typhi*, may cause long and debilitating diseases that may lead to death if untreated. Macrophages (MØ) play a central role in host defenses against these diseases. Recent evidence has shown that MØ surface receptors, such as complement receptor 1 (CR1) and complement receptor 3 (CR3), are involved in direct recognition of such microorganisms as *Staphylococcus epidermidis*, *E. coli*, *M. avium* and *Histoplasma capsulatum* (Patarroyo *et al.*, 1989; Roecklein *et al.*, 1992). One likely ligand on *Salmonella* for these receptors is LPS.

LPS is a major component on the surface of many Gram-negative bacteria and is an important virulence factor in salmonellae (Finlay 1988). Exposure of MØ to purified LPS results in a dramatic increase in the cell surface expression of various receptors, including CR1 and CR3 (Marra *et al.*, 1991). The interaction of LPS and its derivatives to MØ in the adherence process was therefore studied. Initially, we monitored bacterial adherence by direct microscopic visualization, using competitive inhibition as well as down-modulation techniques. Both yielded similar results. We confirmed the results by flow

cytometry assays. We showed that homologous wildtype lipopolysaccharide effectively blocked bacterial binding. Our results suggest that LPS is indeed recognized by murine MØ. In contrast to Wright *et al.* (1986), we could not inhibit bacterial binding completely with LPS. Thus, LPS appears to be an important but not exclusive contributor to microbial adherence to eukaryotic cells.

LPS derived from a mutant strain R595 that lacks the O-side chain, and the core sugar KDO each blocked adherence of *S. typhimurium* to MØ, but less effectively than wild-type LPS. This suggests that both the O-side chain and the glycolipid core of LPS is an important virulence factor that contributes to the adherence of these bacteria. Using *E. coli* and human MØ, Wright *et al.* (1989) proposed that β_2 integrins bind to the hydrophilic portion of LPS, possibly the glucosamine disaccharide backbone of lipid A. Our findings were consistent with those; however, our data suggested that other parts of *Salmonella* LPS are also important contributor to this recognition. Those findings were in agreement with those of Tahri-Jouti *et al.* (1990) who showed that MØ recognize multiple sites on LPS, since attempts to completely block binding of LPS to MØ by different types of anti-LPS antibodies were unsuccessful. Our data suggest that the O-side chain of LPS is also recognized by MØ since wild-type LPS was more effective in blocking bacterial adherence than were LPS components. Those data were similar to those of other researchers who found that *Salmonella* species with disrupted O-side chains or core structures adhered poorly to eukaryotic cells (Mrockzenski-Willey *et al.*, 1989).

Since LPS activates complement receptors on MØ, we tested the involvement of these receptors in bacterial attachment. Two-deoxyglucose has been previously shown to

disrupt the uptake of bacteria by MØ via Fc and complement receptors (Sung-Sang and Silverstein, 1985) and to inhibit adherence of group B Streptococci to MØs (Sloan and Pistole, 1992). In our study only 48% of MØ exhibited bacterial adherence in the presence of 250 µg/ml of this compared to untreated controls, as determined by visual binding assays. We then confirmed these data by flow cytometry assays. These results suggest that Fc or complement receptors may be involved in the adherence process. Adherence of other microorganisms to the Fc receptors in the absence of opsonins was reported by Roecklin *et al.* (1992) and Salmon *et al.* (1987). However, when we blocked Fc receptors with Fc fragments, bacterial adherence remained similar to our control. This indicates that Fc receptors are not involved in direct recognition of *Salmonella*.

The next step was to study the contribution of complement receptors. Neutrophil elastase enzyme has been shown to specifically cleave complement receptor type 1 (CR1) but not CR3 (Tosi *et al.*, 1990). We tested the effect of this enzyme on direct adherence of *S. typhimurium* to MØ to determine whether CR1 is involved. The tests were initially done by a visual binding assay. The maximum reduction of bacterial adherence to MØs occurred at a concentration of 30 µg/ml of neutrophil elastase. At this concentration, the number of MØ with adherent bacteria was reduced to 60 % of untreated controls. We confirmed reduction of adherence of neutrophil elastase by flow cytometry studies.

CR3 is a multifactorial receptor that adheres to an array of ligands and the binding of those ligands involves different sites in the receptor (Larson *et al.*, 1990). Three types of MAb were used and confirmed the direct involvement of the CR3 receptor in the adherence of MØ to *S. typhimurium* (Table 1). The MAbs recognize unique epitopes on

the CR3 molecule (Cooper *et al.*, 1988). Both M1/70 and 5c6 recognize α subunit (CD11b). However, M1/70 binds to the C3bi binding site (Springer 1979) and 5C6 binds to the Mg^{2+} -dependent binding site (Rosen 1987). M18/2 identifies the β subunit (CD18) of CR3 (Sanchez 1983). Although 5C6 did not have any effect on the adherence process, we found that both M1/70 and M18/2 reduced adherence of *Salmonella* similarly compared to untreated controls. This suggests that both α and β subunits play a role in the adherence process. A combination of these two antibodies M1/70 and M18/2 exhibited no enhanced effect compared to that obtained when MØ treated with individual MAb.

Residual bacterial binding activity remained even when both CR1 and CR3 were blocked. We hypothesize that the initial recognition of *S. typhimurium* by murine MØ is therefore a multifactorial process involving receptors for bacterial LPS (as well as other *Salmonella* stress proteins) and both CR1 and CR3 plus other as yet unidentified MØ structures.

In addition to LPS, *Salmonella* synthesizes over 30 proteins which are selectively induced during the 4-8 hr of infection of macrophages (Buchmeier *et al.*, 1989). During the process of infection *Salmonella* faces undesirable environmental conditions. Among those stresses are starvation, shifts between aerobic and anaerobic conditions, heat shock, oxidative stress, and fluctuations in external pH (Foster, 1992). To cope with these stresses, *Salmonella* develop a wide range of molecular defense tactics that will inhibit or repair injuries, including expression of a group of stress proteins.

After the initial contact between bacteria and eukaryotic cell, bacteria synthesize novel

proteins that contribute to stable adherence, necessary for successful invasion of the host cells (Lee *et al.*, 1990). This binding triggers events in the eukaryotic cell inducing signal transmission and global changes involving cellular metabolism, physical and/or chemical changes in the plasma membrane and its receptors (Weir, 1984). Those changes will allow bacterial uptake by additional receptor(s) distinct from those used in the initial binding process (Galán *et al.*, 1992). One of those receptors was found to be an epidermal growth factor receptor. It has been proposed that bacterial invasion via such receptors may preclude triggering microbicidal activity, thus allowing bacterial survival within MØ compartment (Miller *et al.*, 1992).

Portnoy *et al.* (1992) has indicated that unlike *Yersinia pseudotuberculosis*, *S. typhimurium* adherence and invasion is a 2-step model involving two different loci. In fact two different mutants of *Salmonella* lacking some of the stress proteins were isolated: one mutant was unable to adhere to eukaryotic cells and the other was unable to invade eukaryotic cells (Finlay 1989_b). Other mutants lacking those proteins were also unable to survive intracellularly suggesting the role of those proteins to protect bacterial cells against intracellular killing by the macrophages (Groisman *et al.*, 1990).

Growth of *Salmonella* under anaerobic conditions can mimic stress seen when bacteria are incubated with epithelial cells or internalized by MØ (Lee *et al.*, 1990). Those bacteria synthesize stress proteins and have up to a 70-fold increase in adherence capabilities. Direct evidence that these, or other outer membrane proteins, are involved in binding to MØ surface is lacking.

Of the various *Salmonella* OMP, we have identified only one protein band, P44, that

binds to biotinylated MØ. We confirmed that this protein is one of many stress OMP that are induced under anaerobic conditions. *Salmonella* that were grown under aerobic conditions did not express this protein very effectively. The purified P44 was shown to competitively inhibit bacterial adherence to MØ confirming the role of this protein in the adherence process. However, residual bacterial adherence continued even when MØ were preincubated with both LPS and P44 suggesting the role of other factors involved in the adherence process.

Tahri-Jouti (1990) suggested the existence of multiple LPS receptors on the MØ surface. Others found that adherence and uptake of another intracellular bacteria, *M. avium*, by MØ requires more than one receptor (Bermudez *et al.*, 1991; Roecklein *et al.*, 1992). It is likely that several surface factors collaborate to promote specific, stable adhesion between MØ and bacterial cells. Receptors for complement and carbohydrate structures or for complement and immunoglobulin can synergize in the binding and ingestion of microorganisms and other particulate ligands by macrophages (Gordon *et al.*, 1988).

Since the evidence mentioned above indicated that multiple mechanisms are probably involved in *Salmonella*-MØ interaction, we attempted to identify other MØ receptors that are highly expressed by thioglycollate-elicited MØ. Thioglycollate- elicited MØ highly express Mac-2 (Ho *et al.*, 1982) and Mac-3 surface markers (Springer *et al.*, 1984). The two markers are also expressed widely by other epithelial cells. Since *Salmonella* binds to both type of cells and since activated cells expressing those markers are active phagocytically and endocytically, binding inhibition studies using MAb to those receptors

were conducted. However, bacterial adherence was similar to the controls suggesting that epitopes in those receptors identified by the MAbs used in our assays are not involved in bacterial recognition. This does not confirm that those two receptors are not involved in *Salmonella*-MØ interaction. For example, we have shown above that one of the two MAb against CD11b (M1/70) decreased bacterial adherence although another (5C6) did not.

Local secretion of C1q and other regulatory proteins by MØ has been described (Lemerrier *et al.*, 1992). Secretion of C1q caused by *Salmonella* has also been observed (Grossman *et al.*, 1984). The lipid A portion of LPS as well as some of the porins found in the bacterial outer membrane have been shown to be involved in direct binding to C1q (Latsch *et al.*, 1990). Binding and uptake of microorganisms via the C1q receptor has been proposed (B. Ghebrehwet, personal communication). The involvement of C1q and its receptor in adherence of *S. typhimurium* to MØ was tested using polyclonal antibodies directed to those molecules. Bacterial adherence remained similar to the controls in all cases with the exception of one of the two antibodies (R112) directed towards the C1q-receptor. This antibody enhanced binding of *Salmonella* to MØs suggesting that C1q receptor plays a regulatory role in the adherence process, probably by activating or upregulating other receptors on MØ. Depending on the activation component, the adhesion receptors undergo various conformational changes that will shroud or unveil the ligand binding sites (Kishimoto *et al.*, 1988).

Andrew *et al.* (1993) described an antibody that recognizes an epitope on CD18 which caused conformational changes and induced adherence of neutrophils by a

CD11b-dependent mechanism probably by opening more epitopes. On the other hand, Pytoski *et al.* (1988) demonstrated that MAb that recognized a 157-kDa cell surface protein on neutrophils suppressed the C3bi-binding activity of CR3 receptor. However, Brown *et al.* (1988) showed that blocking of CR3 with monoclonal antibody can block phagocytosis of particles via the Fc-receptor. Those data strongly suggested that the activity of one receptor on the surface a MØ can be regulated by other receptors.

We used electrophoresis and Western blot techniques to identify additional MØ receptors that contribute to the adherence process. Only one protein band, P16, was detected that bound either radiolabeled or biotinylated *S. typhimurium* with high affinity. The P16 band is expressed on the surface of MØ. Treatment of MØ with phorbol ester amplified MØ binding activity to *Salmonella* compared to control systems. Treatment of MØs with phorbol ester prior to membrane protein isolation and Western blot analysis resolved an extra band of higher molecular weight at the position of β_2 integrins.

Use of the Western blot method to identify other purified protein components involved in adherence may not be sufficient. Purified components may act different from those on the viable cells. Initial cell-cell communication can take place in the absence of cellular metabolism via some receptors on the plasma membrane of MØ (Weir, 1984). This initial contact is not stable and can be easily breached (Jones *et al.*, 1981). After initial contact the cells undergo various changes that trigger signal transmission. This involves cellular metabolism and leads to chemical and physical changes of the cells (Weir, 1984) that in turn lead to firmer adherence that is crucial for phagocytosis and invasion (Finlay *et al.*, 1989_b). Adherence of bacteria to P16 did not require metabolic activity and therefore is

likely to be involved in early recognition of *Salmonella*.

Whether P16 by itself is the only receptor involved in this initial interaction is not clear. In our studies with Western blots using radiolabeled bacteria we were also able to probe for other MØ proteins where *Salmonella* bind with low affinity. However, washing of the blots rendered those bands undetectable. Reducing the number of washings caused higher background of radioactive labeled bacteria. Other receptors that are involved in the adherence process are probably activated after the initial contact takes place and probably require metabolic activity of MØ. A similar concept was confirmed by Fällman *et al.* (1993). They showed that CR1 primarily promotes the adhesion of particles and that in turns signals CR3 to mediate subsequent engulfment. Such receptors that are activated later on in the adhesion process will be difficult to detect by immunoblotting techniques. Our findings that *Salmonella* adhere poorly to high concentration (200 µg/ml) of cytochalasin B treated MØ or formalin-fixed MØ support this concept.

Since P16 has not been previously described, we have electroeluted this protein from SDS-PAGE gels for further studies and characterization. Two bands were resolved when the purified P16 band was separated with a 15% gel. However, bacterial binding did not take place when those bands were separated suggesting that both of the two bands are required.

Bacterial LPS causes activated MØ to undergo calcium fluxes (Raddassi *et al.*, 1994). Treatment of MØ with EDTA reduced, but did not eliminate bacterial adherence. These data suggest that some MØ receptors involved in the adherence process require divalent cations. Characterized MØ receptors, such as CR3 require divalent cations (Wright *et al.*,

suggesting that the mechanism used to bind to this protein differs from that of CR3.

We conclude that the *S. typhimurium*-MØ interaction is a complicated process involving more than one surface cellular component on both MØ and *Salmonella*. LPS is an important, but not an exclusive, factor that is involved in a very initial phase of the adherence process and that different parts of LPS are recognized by MØ. In addition to LPS, a *Salmonella* stress protein, P44, is involved in the second phase of adherence since this protein is expressed after the initial contact of bacteria to MØ. Multiple MØ receptors are involved in the adherence of *Salmonella* to MØ. These include three recognized MØ receptors, CR1, CR3, and possibly the C1q receptor, as well as a newly described receptor complex P13/P16.

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