Interactions between Brucella abortus and phagocytic leukocytes

James Richard Birmingham
Iowa State University

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INTERACTIONS BETWEEN BRUCELLA ABORTUS AND PHAGOCYTIC LEUKOCYTES

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Interactions between *Brucella abortus* and phagocytic leukocytes

by

James Richard Birmingham

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Major: Immunobiology

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INTRODUCTION

Brucellosis is a disease with serious health consequences for man and a disease of animals with major economic impact worldwide. Bovine brucellosis, caused by Brucella abortus, is the most important economically, with world losses estimated in billions of dollars annually (1). Abortion is the significant clinical manifestation of the disease. The organisms preferentially localize in the gravid uterus, spread to placental-associated tissues, and cause abortion principally in the last three months of pregnancy.

Infections with Brucella abortus generally result in chronic lesions, with the organism residing mainly in cells of the reticulo-endothelial system and associated tissues (7, 29). However, in brucellosis and other microbial infections, polymorphonuclear leukocytes are the initial cells to arrive at the site of invasion (30, 41). These cells are among those blood cells termed "professional phagocytes" (31), and phagocytosis is central to nearly all the activities of this cell type. Granulocytic cells are short-lived, end-stage cells and in the evolution of an inflammatory reaction, polymorphonuclear cells soon give way to an influx of cells of the mononuclear phagocyte lineage (40). Mononuclear phagocytes are the dominant cell in chronic inflammatory lesions. Macrophages are considered to be the ultimate effector cells in these infections once they have reached a state of activation. Activation of macrophages can occur directly upon contact
with the organism and/or products of the organism, or indirectly, after stimulation by soluble mediators released by specifically sensitized T-lymphocytes (27). Once activated, macrophages display enhanced rates of endocytosis, antimicrobial and antitumor activity, increased production and secretion of a variety of lysosomal enzymes and neutral proteases, release of potentially toxic oxygen metabolites, and secretion of a variety of immunoregulatory factors (2). Thus, activated macrophages have the potential to mediate not only resistance to pathogens, but to modify the local environment and regulate immune responses.

Much of our understanding of the nature of the immunity in brucellosis derives from studies in laboratory animals. The in vivo and in vitro investigations of Mackaness in the 1960s (21, 22, 23) showed that immunity to intracellular bacteria such as Brucella, Listeria, and Mycobacteria depended on collaboration between lymphocytes and macrophages, and provided the concept of the activated macrophage as the final effector mechanism. Once activated, macrophages can also express non-specific resistance. Non-specific resistance to various viruses, unrelated bacteria, protozoa, and tumors has been demonstrated in animals infected with Brucella (5, 8, 13, 22). These studies have also emphasized the important role cellular responses (primarily macrophages) play in resistance to Brucella. Antibodies are not generally considered as important as cellular reactions in resistance to Brucella infection; however, some investigators have suggested that the presence of serum
factors which activate macrophages (32) or act as opsonins (4) could be involved in resistance. Several years ago *Brucella* was shown to be capable of inducing interferon production (37, 42). Recently, interferon has been shown to be a potent activator of macrophages (34), thus this activity may play an important role in resistance to *Brucella*.

Most research on bovine immune reactions to *Brucella* has concentrated on the improvement of vaccination schemes and development of effective diagnostic procedures (6, 39). Serological evaluation provides the basis for detection and diagnosis of brucellosis in cattle; however, it is widely accepted that circulating antibody has little relationship to the degree of resistance a cow may manifest upon challenge exposure (28). Because cellular reactions are believed to provide the basis for immunity to *Brucella*, much emphasis is currently placed on examining the bovine cellular immune system. Lymphocyte stimulation tests have been developed in recent years and although these procedures are proving to be a valuable adjunct in diagnosis, their relationship to resistance and mechanisms of immunity is presently uncertain (16, 17, 18). Direct investigation of interactions between *Brucella abortus* and bovine phagocytic cells is limited primarily to the work of Smith and colleagues in the 1960s (10, 35), These studies claimed that mononuclear phagocytes from immunized animals were able to inhibit replication of virulent strains of *B. abortus*, whereas the organism multiplied in normal cells. These studies can be criticized for several reasons: 1) the authors utilized an antibiotic
to eliminate extracellular bacteria and it is now known that these substances can penetrate cells and contribute to intracellular killing, 2) the cells they isolated required cultivation for nearly 2 weeks to purify mononuclear adherent cells and yet they still were bactericidal. This persistence of antimicrobial activity in vitro is contrary to presently accepted knowledge concerning the maintenance of activated states in macrophages, 3) the cells these authors claimed to be macrophages possessed characteristics not generally associated with this cell type, e.g. trypsin sensitivity. These authors later provided evidence for a fraction of the cell wall of virulent brucellae that contributed to resistance to intracellular destruction by macrophages (12). These studies are interesting, but should be accepted with caution.

Considerable effort has been devoted to the isolation and characterization of biologically active fractions of Brucella (9, 11 14, 15, 38). Lipopolysaccharides (LPS) represent major components of the cell walls of Gram-negative bacteria and purified LPS preparations elicit an impressive array of biological responses (26). A large number of investigations have dealt with the structure and biological activities of LPS from Brucella (3, 19, 20, 24, 25, 33). It appears that Brucella LPS is rather unique chemically and structurally. Many of the typical biological responses to enterobacterial lipopolysaccharides are elicited by Brucella LPS only after massive amounts are administered. Thus, the potential significance of Brucella LPS has been questioned.
The purpose of this investigation was to examine the interactions between *Brucella abortus* and/or components derived from *B. abortus* and phagocytic leukocytes, especially macrophages. It was recognized that the bovine presents a number of problems regarding manipulation for experimentation, but at the same time would provide the most relevant information for understanding the true nature of the host-parasite relationship. Thus, most of these studies deal, where possible, with bovine leukocytes.

Central to any investigation was the development of a technique for isolating and cultivating bovine mononuclear phagocytes. The first section of this study deals with the description of this technique and details of the differentiation of bovine mononuclear phagocytes in vitro.

Mobilization of phagocytic leukocytes to the site of microbial invasion is an integral component in the initiation of successful immune responses (36). *In vivo* and *in vitro* studies have revealed that chemotactic factors play an essential role in recruitment and mobilization of leukocytes (36). The second section of this study deals with the ability of a number of fractions of *Brucella abortus* to generate chemotactic factor for granulocytes and monocytes from serum.

As discussed, lipopolysaccharides are implicated as significant products in the pathogenesis of disease. *Brucella* LPS had generally been characterized as being a relatively feeble LPS, thus raising questions regarding its biological significance. Macrophages have
been proposed as central in the mediation of many of the effects of LPS, thus a study of the effects of *B. abortus* LPS on bovine macrophages was undertaken. This represents the third section of this dissertation.

Considerable disagreement exists in the literature regarding the induction, duration, and expression of macrophage function during *Brucella* infections. Most of these studies were conducted in mice and dealt with one particular function of these cells, leading to confusing conclusions. Thus, a study was undertaken to examine a broad range of macrophage activities during the course of a chronic infection with *B. abortus*. This is the subject of the final section of this investigation.

This thesis consists of an introduction, four separate manuscripts, general summary and discussion, general references and acknowledgements. The Ph.D. candidate, James R. Birmingham, was the senior author and principal investigator for each of the manuscripts.
PART I: THE ISOLATION, LONG-TERM CULTIVATION AND CHARACTERIZATION OF BOVINE PERIPHERAL BLOOD MONOCYTES

This manuscript has been accepted for publication by Immunology
The isolation, long-term cultivation and characterization of bovine peripheral blood monocytes

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ABSTRACT

Bovine peripheral blood monocytes were isolated from buffy coats of ACD-anticoagulated whole blood. Cells cultivated on plastic surfaces for 20 h were judged to be 90% monocytes based on non-specific esterase-1 staining, uptake of latex particles and surface receptor characteristics. Bovine monocytes were maintained up to 80 days in vitro in Dulbecco's modified Eagle medium with 15% horse serum and 15% fetal bovine serum. Several morphological and physiological features of bovine monocytes were examined during the course of culture. Cell size and cytoplasmic spreading, granulation and vacuolization increased progressively. Multinucleated giant cells predominated during the latter stages of in vitro culture. A high percentage of bovine monocytes possessed C3 and IgG Fc receptors, whereas IgM Fc and sheep erythrocyte receptors were not detected. Phagocytosis was mediated by the IgG receptor, but not by the C3 receptor. Peroxidase activity declined in a linear fashion, with cells essentially negative after 8 days of culture. Total cell protein and acid phosphatase increased during cultivation. Lysozyme activity was undetectable in both lysates and supernatants of bovine monocyte cultures. These findings are consistent with the concept of maturation of mononuclear phagocytes. The procedures for isolation and cultivation described in this paper will provide methodology for detailed study of bovine mononuclear phagocytes.
INTRODUCTION

The role of macrophages as effector cells against microbial pathogens and tumor cells has been extensively investigated (23, 29). In addition, macrophages are known to play a critical role in the induction of immune responses (32, 33). Recently a large body of evidence has established the macrophage as a major secretory cell (14). The above mentioned studies have been conducted primarily with rodent models.

Peripheral blood monocytes are generally thought to be the immature precursors of tissue macrophages (36). The differentiation of monocytes in vitro into macrophage-like cells appears to be related to maturational events that occur in vivo (12). Examination of the maturation process in vitro has only been conducted with peripheral blood monocytes from horses (3) and from humans (21, 41).

Detailed study of bovine peripheral blood monocytes has been hindered by difficulties in obtaining and cultivating these cells. Bovine erythrocytes cannot be separated from leucocytic elements by sedimentation techniques standard to most animal species and humans (10) and bovine monocytes survive poorly in culture after separation on sucrose or Ficoll-diatrizoate gradients (34). A recent paper described procedures for establishing continuous macrophage cell lines from peripheral blood monocytes of 7 animal species, however, the authors indicated an inability to establish continuous lines using bovine cells (40). Thus, previous investigations have been
limited to short-term studies on freshly isolated bovine cells or have utilized procedures requiring at least 6-12 days of culture in order to purify mononuclear phagocytes (16, 34). In this paper, we present a procedure for isolating monocytes from whole blood and describe optimal conditions for maintaining long-term primary cultures. In addition, we have examined a number of the morphological and physiological characteristics of bovine monocytes and followed the development of these cells during in vitro cultivation.
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MATERIALS AND METHODS

Reagents  Hank's balanced salt solution without Ca\textsuperscript{++} and Mg\textsuperscript{++} was obtained from Microbiological Associates (Walkersville, MD), Dulbecco's modified Eagle medium, Medium 199, RPMI-1640, Neuman-Tytell serumless medium, fetal bovine serum, and horse serum were from Grand Island Biological Co. (Grand Island, NY), gentamycin sulfate was from Schering Corp. (Bloomfield, NJ), α-naphthyl butyrate, p-nitrophenyl phosphate, 0.09 M citrate buffer, bovine serum albumin, and Micrococcus lysodeikticus were from Sigma Chemical Co. (St. Louis, MO), Triton X-100 was from BioRad Laboratories (Richmond, CA), IgG anti-sheep erythrocyte and IgM anti-sheep erythrocyte antibody were from Cordis Corp. (Miami, FL) and 0.81 μm latex particles were from Difco (Detroit, MI).

Monocyte isolation  Normal 8-24 month old Hereford-Angus heifers were selected at random from the herds at the National Animal Disease Center, Ames, IA (provided by Dr. B. L. Deyoe) and the Veterinary Medical Research Institute. Blood was collected by jugular venipuncture using 2X acid-citrate-dextrose (ACD) as anticoagulant (1 part 2X ACD: 10 parts whole blood). Mononuclear leucocytes were isolated by a modification of the procedure of Carlson and Kaneko (9). Briefly, the anticoagulated blood was centrifuged at 1000 g for 20 min and the buffy coat collected. Contaminating red cells were lysed by the addition of 2 volumes of cold phosphate-buffered (0.013 M) deionized H\textsubscript{2}O and isotonicity
restored with 1 volume of phosphate-buffered (0.013 M) 2.7% NaCl. The suspension was centrifuged (150 g, 10 min) and washed 4X with Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{++}. The washed leucocytes were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 50 μg/ml gentamycin sulfate, 15% fetal bovine serum (FBS) plus 15% horse serum (HS), and counted in a hemocytometer. All sera were previously heated at 56 C for 30 min. Cell concentrations were adjusted to 10^7 cells/ml and 2 ml was seeded into 35 x 10 mm plastic tissue culture dishes (Falcon, Los Angeles, CA). The cell cultures were incubated at 37 C in a humidified incubator with 5% CO₂ in air. Non-adherent cells were removed by washing with phosphate-buffered saline (PBS), pH 7.2, containing 5% FBS. Adherent cells were cultivated in 1.5 ml DMEM-15% FBS-15% HS. The culture medium was changed every 2 days. Viability was determined by trypan blue dye exclusion.

**Phase-contrast morphological studies** For phase-contrast microscopy, adherent cells were washed, overlaid with a cover slip, and examined in the living state with a Zeiss microscope equipped with a Zeiss polaroid attachment.

**Cytochemistry** Non-specific esterase-activity was determined by the method described by Li et al. (25), with α-naphthyl butyrate as substrate. Peroxidase activity was determined by the method described by Kaplow (22). Activity was determined on a minimum of 200 cells per culture.
**Latex phagocytosis**  For phagocytosis of latex particles by cells in suspension, 2 x 10^7 buffy coat leucocytes were incubated in 9 ml of Medium 199 (M199) containing 20% FBS and 1 ml of a 1:50 dilution of 0.81 μm latex beads. The suspension was incubated for 1 h at 37 C on a continuous rotator at 8 rev/min (Fisher Scientific Co., Itasca, IL). The cells were then centrifuged at 150 g for 10 min and washed 3X with HBSS. Wet mounts were prepared and examined by phase contrast microscopy. For phagocytosis by adherent cells latex particles were added to cultures at a ratio of 40:1, incubated 1 h at 37 C, washed 3X with HBSS and examined under phase contrast microscopy (630X). Two hundred cells were examined per test.

**Surface receptor enumeration and function**  Sheep erythrocytes (E) were stored in Alsever's solution at 4 C and used within 2 weeks of collection. The erythrocytes were centrifuged and washed 5X with M199. A 0.5% concentration of sheep erythrocytes in M199 was used as the source of E. Equal volumes of 1% E and the highest sub-agglutinating dilution of either IgG anti-E (EA_{IgG}) or IgM anti-E (EA_{IgM}) in M199 were incubated at 37 C for 30 min, followed by 30 min at 4 C. The cells were centrifuged and resuspended to the original volume (0.5% concentration). Complement-coated erythrocytes (EA_{IgM}C) were prepared by incubating 0.2 ml of fresh serum with 3 ml of 0.5% EA_{IgM} for 30 min at 37 C. C5-deficient DBA/2 mouse serum (preabsorbed with E) was used as the source of complement.

Adherent cells in plastic dishes were washed and covered with 1.5 ml of the appropriate indicator particle. The dishes were
incubated 30 min at 37 C and the monocytes carefully washed 4X with PBS. The cells were fixed for 5 min with 2.5% glutaraldehyde in PBS and examined under an inverted microscope or stained with Wright's for high power examination (970X). A positive rosette was considered to be a cell with 4 or more erythrocytes attached to the surface. At least 10 replications were performed in testing for each receptor, with 200 cells examined in each test.

Receptor-mediated phagocytosis was determined by incubating adherent cells with either 1 ml of EA\textsubscript{IgG} or EA\textsubscript{IgM} for 45 min at 37 C, followed by 4 washes with PBS. Erythrocytes attached to the surface of monocytes were lysed with distilled H\textsubscript{2}O and the monocytes examined for internalized erythrocytes.

**Lysozyme assay** Washed adherent cells were lysed by incubation for 30 min on ice with 1 ml of 0.2% Triton X-100 in PBS. Lysozyme activity was determined spectrophotometrically at 550 nm in cell lysates and supernatants according to the procedures described by Gordon et al. (20).

**Acid phosphatase assay** Acid phosphatase was determined by the method described by Beck et al. (1). Two-hundred \( \mu l \) of cell lysate was incubated for 30 min at 37 C with 200 \( \mu l \) of p-nitrophenyl phosphate in 0.09 M citrate buffer, pH 4.8. The reaction was stopped with 0.1 N NaOH and formation of p-nitrophenol was measured at 410 nm on a Gilford 250 spectrophotometer. Specific activity was expressed as nanomoles of p-nitrophenol liberated per h per mg cell protein.
Protein determination: Protein was measured by the method of Lowry et al. (26) as modified by Wang and Smith (39). Bovine serum albumin was used as a standard.
RESULTS

**Monocyte isolation and cultivation**  Centrifugation of ACD-anticoagulated bovine blood for 20 min at 1000 g results in a buffy coat containing an average of $2 \times 10^8$ leucocytes/100 ml blood (average of 30 determinations). Ninety-two percent of the buffy coat cells were mononuclear based on Wright's stained smears. Approximately 30% of the buffy coat cells were non-specific esterase-1 positive and 33% phagocytized latex particles. Viability was consistently 95-99% after 4 washes.

Monocytes were isolated by adherence to plastic dishes. Plastic surfaces resulted in more cells adherent to the substratum and better viability than glass surfaces. Non-specific esterase-1 reactivity and uptake of latex particles by adherent cells increased from 80-85% at 2 h to 95% after overnight incubation.

A variety of basal media and sources and concentrations of serum were examined in experiments designed to establish optimal cultural conditions for bovine monocytes. Basal media RPMI-1640, M199 and DMEM were all satisfactory for establishing and maintaining monocyte cultures. Serumless medium (Neuman-Tytell) did not support bovine monocytes adequately beyond 48 h. Fetal bovine serum and HS were determined to support growth better than swine serum. Higher concentrations of FBS and HS resulted in greater cell growth (30% > 20% > 10%). The effects of variability in lots of HS and FBS could be reduced by combining 15% HS and 15% FBS. Autologous serum resulted in some
clumping of cells and inconsistently supported monocytes. Acid-treatment of serum (2 h at pH 3.2) did not impair capacity to support monocyte cultures. Lactalbumin hydrolysate (LH, 0.2%) was unsatisfactory as a serum substitute for periods exceeding 2-3 days.

**Culture characteristics and morphology** Cultivation of bovine monocytes in DMEM with 15% FBS and 15% HS as described above reproducibly have been established (success rate >95%) in our laboratory for periods up to 80 days. Detachment of cells from the substratum was minimal after 2 days in vitro cultivation. Mitotic figures were not observed during the cultivation period under study. Incubation of cultures for periods up to 24 h in (3H)-thymidine consistently resulted in less than 1% uptake.

The morphology of bovine monocytes during in vitro cultivation was observed by phase-contrast microscopy. Initially, the cells were small (7-9 μm in diameter), rounded and homogeneous in morphology. In 3 day cultures the cells appeared to be more heterogeneous with some cells appearing circular to oblong in shape and others displaying cytoplasmic spreading characteristic of mononuclear phagocytes (Fig. 1.1A). After 1 week of cultivation, the cells were approximately twice the size of freshly isolated monocytes. Fusiform shaped cells, often with long, slender cytoplasmic extensions were mixed with cells that retained an oval appearance (Fig. 1.1B). Sixteen day old cultures contained cells that showed further increases in mass and granulation of the cytoplasm (Fig. 1.1C). At 24 days, the cells had increased to an average of 50 μm in diameter and prominent
Figure 1.1. Morphology of bovine monocytes during cultivation.

A–E: phase-contrast light microscopy; bar indicates 5 μm. Final magnification x 1260. (A) Day 3 culture; small monocytes showing some cytoplasmic spreading. (B) Day 6 culture; larger monocytes with long cytoplasmic extensions. (C) Day 16 culture; large cells with prominent granulation of cytoplasm and long cytoplasmic extensions. (D) Day 24 culture; large spread cells with peripheral vacuoles. (E) Day 47 culture; cell shown has diameter >100 μm, numerous granules and vacuoles. (F) Day 47; Wright's stain, light microscopy. Multinucleate giant cell, prominent nucleoli; bar indicates 5 μm. Final magnification x 1000.
peripheral vacuoles were evident (Fig. 1.1D). Multinucleate cells were infrequently seen at this time. Forty-seven day cultures were heterogeneous in size, some exceeding 100 μm in diameter (Fig. 1.1E). Staining revealed that nearly all cells were multinucleated at 47 days, some containing 10–20 nuclei (Fig. 1.1F).

**Surface receptor studies** A high percentage of bovine monocytes were positive when examined for IgG Fc (98.7 ± 2.1%) and C3 (93.3 ± 6.3%) receptors. Receptors for the Fc portion of IgM and for E were not detected. Receptor-mediated internalization of erythrocytes was apparent with IgG-coated particles, but not with C3-coated particles. For the period studied (up to 14 days), no significant differences were noted for either the percentage of rosette-positive cells or for ingestion characteristics.

**Peroxidase reactivity** Figure 1.2 illustrates granule-associated myeloperoxidase activity of bovine monocytes during maturation in vitro. Approximately 75% of newly isolated monocytes are positive for peroxidase. Activity declines in a linear manner, reaching nearly 0 after 8 days culture.

**Lysozyme activity** Newly established and long-term (up to 30 days) monocyte cultures were studied under a variety of conditions in attempts to detect lysozyme activity. The cells were cultivated in acid-treated serum, medium with no serum, in Neuman–Tytell medium, with 0.2% LH, and after phagocytosis of zymosan or latex particles. Lysozyme activity was not detectable in lysates of bovine monocytes,
Figure 1.2. Granule-associated peroxidase activity of bovine monocytes during culture. Each point represents at least 3 determinations, ± one standard deviation.
nor in their culture supernatants. Similarly maintained mouse peritoneal macrophages or porcine alveolar macrophages were positive when tested for lysozyme activity (data not shown).

**Cell protein and acid phosphatase** Table 1.1 shows that cellular protein increased at a linear rate from day 5 to day 15 and then leveled off. Total acid phosphatase activity increased early in culture, leveled off between days 10-15, and then increased sharply at 20 days culture. The specific activity of acid phosphatase decreased from day 5 to day 15, followed by a sharp increase at 20 days of culture.
Table 1.1. Cell protein and acid phosphatase activity during cultivation

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<td>Cell protein/dish $^b$</td>
<td>25.8± 7.4</td>
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<td>Total acid phosphatase/dish $^c$</td>
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<tr>
<td>Acid phosphatase, specific activity $^d$</td>
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</table>

$^a$n=6 (day 5); n=3 (days 10-20) for all determinations.

$^b$mean µg/dish ± 1 standard deviation (S.D.)

$^c$mean nmoles p-nitrophenol liberated/hr/dish ± 1 S.D.

$^d$mean nmoles p-nitrophenol liberated/hr/µg cell protein ± 1 S.D.
DISCUSSION

The present paper describes procedures that can be used for reproducible isolation of fresh bovine peripheral blood monocytes, and for cultivation of purified cells for periods up to 80 days. Centrifugation of ACD-anticoagulated blood yields a buffy coat, which, after washing, contains primarily mononuclear leucocytes. Washing the buffy coat cells 3-4X is important because it removes platelets which cause cells other than monocytes to adhere to surfaces. Cultivation of the cells for approximately 20 h yields an adherent cell population that is 95% monocytes based on uptake of latex beads, non-specific esterase staining and surface receptor characteristics. Similar to human monocytes (5, 21), bovine monocyte yield and survival is better on plastic surfaces than on glass surfaces.

Dulbecco's modified Eagle medium plus 15% FBS and 15% HS was determined to be the optimal medium for long-term bovine monocyte cultivation. Fetal bovine serum and HS supported cell cultures equally well. Effects of variability in lots of these sera could be reduced by combining them in equal proportions as described by Zuckerman et al. (41). Swine serum, autologous serum and LH were found to be unsatisfactory in their ability to support monocyte cultures. Several basal media are suitable for bovine monocyte culture, but we used DMEM because it provides better control of pH than M199 (15). Human monocytes have recently been successfully cultured for long periods in Neuman-Tytell
serumless medium (21). Bovine monocytes contract and begin to detach from culture vessels after several days in this medium.

The procedures developed for isolation and long-term cultivation of freshly obtained bovine monocytes made it possible to study maturation and differentiation of these cells in vitro. A progressive enlargement of the monocyte occurs in culture with increasing granulation and vacuolization of the cytoplasm. Initially, the bovine monocytes are small (7-9 μm) and rounded with cytoplasmic spreading becoming apparent in some cells after 2 days. In contrast, Johnson et al. (21), described 90-95% of human monocytes to be well spread with extensive ruffled plasma membranes and mean cell diameters of 30-40 μm after 30 min culture. These differences may be related to exposure to activated complement and coagulation factors (7) during isolation of human peripheral blood monocytes.

In the present study, multinucleated giant cells were observed to develop between 24 and 47 days culture. Development of multinucleated giant cells, usually from long-term peripheral blood monocyte cultures, has been previously described (11, 18, 24). However, polynucleated cells have also been found as early as 2-3 days in human (41) and bovine (34) peripheral blood monocyte cultures. The factors influencing formation of multinucleated giant cells in vitro are poorly understood.

The presence of a surface receptor for the Fc portion of IgG has been previously described on 75-90% of bovine monocytes (35). Ninety-
nine percent of the bovine monocytes in this study were found to possess IgG Fc receptors. The use of different procedures for demonstrating IgG receptors may account for this discrepancy. The presence of complement receptors has also been described on bovine monocytes, although the percentage of cells bearing the receptor were not reported (34). We found that 93% of bovine monocytes possessed these receptors. IgG receptors are believed to mediate both attachment and ingestion of particles and complement receptors are considered to mediate primarily attachment to normal mononuclear phagocytes (7). In this study, we have found similar functions for these receptors on bovine monocytes.

Peroxidase activity, associated with a population of cytoplasmic storage granules, has been demonstrated in human (4, 28) monocytes. Tissue macrophages, apparently derived from blood monocytes (36), do not contain granule-associated peroxidase activity (13). It has been suggested that the loss of granule-associated peroxidase activity by monocytes in culture is related to maturation of these cells into macrophages (2, 8). We have examined the kinetics of peroxidase activity in bovine monocytes during cultivation. The progressive loss of granule-associated peroxidase activity by bovine monocytes resembles that reported for murine (37) and human monocytes (38).

Associated with a progressive enlargement in cell size bovine monocytes exhibited increases in total cell protein which leveled off beyond 15 days culture, although the cells continued to increase in size. Striking increases in acid phosphatase specific activity was
noted in bovine monocytes from 15-20 days in culture, at a time when cell protein values were constant. These changes are comparable to 5-10 day human monocyte cultures as reported by Zuckerman et al. (41).

In humans and mice, immature granulocytes, monocytes, and macrophages are the cells primarily responsible for production of lysozyme (27). Secretion of lysozyme is a constitutive function of mononuclear phagocytes (20). In the present study, bovine mononuclear phagocytes neither secreted lysozyme not contained it intracellularly. A previous investigation indicated the absence of lysozyme in bovine polymorphonuclear leucocytes, tears, saliva, and nasal exudates (31). These findings, in conjunction with the present report, suggest an inability of cattle to produce lysozyme. The bacterial role of lysozyme is well-established (17) and recent investigations have suggested that lysozyme plays a role in modulation of inflammation (19). Lysozyme may also play a role in responses to tumors and can interact with mammalian cell membranes (30). The identity of substances performing these functions in cattle remains largely unclear.

The techniques described in this paper should facilitate study of the physiology and differentiation of mononuclear phagocytes and provide an in vitro model for examination of the roles of these cells in inflammatory reactions.
REFERENCES


PART II: BIOLOGIC ACTIVITIES OF FRACTIONS ISOLATED FROM BRUCELLA

ABORTUS: GENERATION OF CHEMOTACTIC FACTOR FOR GRANULOCYTES

AND MONOCYTES FROM SERUM

This manuscript has been submitted for publication to
Infection and Immunity
Biologic activities of fractions isolated from *Brucella abortus*: generation of chemotactic factor for granulocytes and monocytes from serum.

James R. Birmingham, Louisa B. Tabatabai, Billy L. Deyoe, Edward L. Jeska, and Mary E. Nuessen

From the Immunobiology Program, Veterinary Medical Research Institute (Birmingham, Jeska, Nuessen), Iowa State University, Ames, Iowa, and the National Animal Disease Center (Tabatabai, Deyoe), Ames, Iowa
ABSTRACT

Several fractions isolated from *Brucella abortus* were examined for their ability to generate chemotactic factor from normal serum. Nucleic acid-rich, lipopolysaccharide-rich, and protein-rich hot aqueous phenol extracts were active. A carbohydrate-rich aqueous methanol fraction was inhibitory at high concentrations, but a non-dialysable component of this fraction contained a potent stimulator of chemotactic activity. Protein-rich fractions from both strain 19 and strain 2308 were inactive. Preheating the serum at 56°C for 30 min prevented generation of chemotactic activity by the various fractions, suggesting a role for serum complement. No chemotactic activity was produced by *Brucella* fractions in C5-deficient DBA/2J mouse serum.
INTRODUCTION

Accumulation of mononuclear and polymorphonuclear phagocytes at sites of microbial invasion is a critical component of host defenses. Since the introduction of quantitative in vitro methods for the study of leukotaxis by Boyden in 1962 (5), analysis of cellular and humoral aspects of leukocyte migration has progressed rapidly. Despite this progress, little information is presently available regarding pathogen and host factors involved in regulating migration of leukocytes for many important diseases.

Brucella is an intracellular pathogen of considerable importance to man and domesticated animals (1). Many studies have been concerned with possible antigenic or toxic effects of components of Brucella (9, 14, 15, 19, 28). To our knowledge, no studies have been reported dealing with the stimulation or inhibition of leukocyte migration by factors isolated from Brucella organisms.

Recently, we have isolated two fractions from Brucella abortus that are toxic when injected intradermally into guinea pigs (34). In the present study, we have examined the ability of these fractions to generate chemotactic factor(s) from serum for granulocytes and monocytes. In addition, several fractions were isolated using the hot aqueous phenol procedure of Westphal et al. (36). These were evaluated for their ability to generate leukotactic factor(s) from serum. The results indicate that several fractions from Brucella abortus
can generate chemotactic factor(s) from serum, and one fraction, a carbohydrate-rich extract, is markedly inhibitory for leukocyte migration.
MATERIALS AND METHODS

Preparation of bacterial factors  Hot aqueous phenol extracts from *Brucella abortus* strain 19 were obtained as outlined by Redfearn (25) and Leong et al. (15). Lyophilized fractions were dissolved in distilled water at 10 mg/ml and designated f3 (nucleic acid-rich), f5 (lipopolysaccharide-rich), and f6 (protein-rich), as proposed by Redfearn. Aqueous methanol extracts and protein-rich extracts from *Brucella abortus* strains 19 and 2308 were prepared as outlined by Tabatabai et al. (34). Fraction 1 is the carbohydrate-rich concentrated extract dissolved in distilled water before dialysis, and fraction 2 is the material remaining in the bag after dialysis of fraction 1 against distilled water. Fraction 3 is the protein rich extract from strain 19; fraction 4 is the protein-rich extract from strain 2308.

Chemical analysis  Protein was determined with the Folin-phenol method of Lowry et al. (17); bovine serum albumin (Armour Pharmaceutical Co., Tucson, Arizona) served as standard. Carbohydrate was determined as described by Dubois et al. (7); D-glucose served as standard. Total nitrogen was measured by the method of Ma and Zuasaga (18) and total phosphorus was measured as described by Ames (2). The 2-keto, 3-deoxy-sugar content was determined as described by Ashwell (3), except an 8 min hydrolysis step at 100 C in the presence of 0.02 N H_{2}SO_{4} was performed on the samples before the assay. Chemical analysis of the various fractions is summarized in Table 2.1.
Table 2.1. Chemical composition of bacterial extracts

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Carbohydrate</th>
<th>2-keto,3-deoxysugar</th>
<th>N</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot aqueous phenol^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f3</td>
<td>1.2</td>
<td>36.4</td>
<td>0.91</td>
<td>26.5</td>
<td>1.10</td>
</tr>
<tr>
<td>f5</td>
<td>33.8</td>
<td>10.4</td>
<td>0.52</td>
<td>15.0</td>
<td>0.14</td>
</tr>
<tr>
<td>f6</td>
<td>73.7</td>
<td>0.75</td>
<td>0.02</td>
<td>24.4</td>
<td>0.005</td>
</tr>
<tr>
<td>Aqueous methanol^b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.60</td>
<td>2.09</td>
<td>11.7</td>
<td>0.65</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.90</td>
<td>0.25</td>
<td>10.4</td>
<td>0.63</td>
<td>0.06</td>
</tr>
<tr>
<td>Protein-rich^b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.67</td>
<td>0.18</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>2.58</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

^a Composition given as percent dry wt.

^b Composition given in mg/ml except for 2-keto,3-deoxysugar (µg/ml). These samples were kept in solution rather than in dried form due to their toxicity (see reference 33).

^c Not determined, interfering substance present.

^d None detectable.
Isolation of leukocytes

Blood was collected by jugular venipuncture from normal 12-14 mo old crossbred cows using 2X acid-citrate-dextrose as anticoagulant. Mononuclear cells were purified as recently described (J. R. Birmingham and E. L. Jeska, Immunology, in press). Briefly, the blood was centrifuged at 1000 x g for 20 min, the buffy coat was collected, and the contaminating red cells removed by hypotonic lysis with 0.013 M phosphate buffer. The remaining cells were washed 4X with Hank's balanced salt solution (HBSS) without Ca$^{++}$ and Mg$^{++}$ and resuspended at a concentration of $3 \times 10^7$ cells/ml in Dulbecco's modified Eagle medium supplemented with 2% bovine serum albumin (DMEM-BSA). Approximately 30% of the cells were monocytes as revealed by morphology and non-specific esterase staining (16), 5% were granulocytes, and the remainder were lymphocytes.

Granulocytes were obtained from the bottom fraction of the tube after centrifugation as described as above. The red cells were removed by lysis, remaining cells washed 2X with HBSS without Ca$^{++}$ and Mg$^{++}$ and resuspended to $2.5 \times 10^6$ cells/ml in DMEM-BSA. Ninety to 95% of the cells were granulocytes as determined on Wright's-stained smears.

Chemotactic factors

One ml of fresh normal bovine or murine serum was incubated with or without 0.1 ml of various concentrations of the fractions isolated from B. abortus. The mixtures were placed in a waterbath at 37 C for 60 min, followed by 30 min at 56 C and then centrifuged at 500 x g for 10 min. The supernatants were collected and diluted 1:1 in DMEM-BSA. In some experiments, the sera were
preheated at 56°C for 30 min before adding the Brucella fraction. Random migration was assessed against DMEM-BSA.

Murine serum was collected from C5-deficient (24), DBA/2J (M. A. Associates, Walkersville, MD) or C5-normal CF-1 (Veterinary Medical Research Institute colonies) mice.

**Chemotaxis assay** Chemotaxis was evaluated *in vitro* by a modification of the procedures described by Snyderman et al. (32), using Neuroprobe blind-well Lucite chemotaxis chambers (Bio-Rad Laboratories, Richmond, CA). The lower well was filled with 0.2 ml of the appropriate chemotaxin or control preparation and then covered with a polycarbonate membrane filter with 5 μm pores (Bio-Rad Laboratories). The upper well was filled with 0.2 ml of either the granulocyte or mononuclear leukocyte suspension. The chambers were incubated at 37°C in a humidified 5% CO₂-95% air incubator (3.5 h for mononuclear cells; 3 h for granulocytes). After the incubation period, the filters were removed, inverted on glass slides, air-dried, fixed in absolute methanol, and stained with Giemsa. Monocyte chemotactic responses were scored as the number of monocytes that completely migrated through the filter in 20 oil immersion (970X) fields. Granulocyte responses were scored as the number of granulocytes that completely migrated through the filter in 10 medium power (400X) fields. Results are expressed as the mean number of cells per field ± one standard deviation for a minimum of triplicate filters.
Statistical analysis  The F test with probability statements was used to compare untreated serum and serum treated with the various Brucella fractions (29). Duncan's multiple range test was performed to test for the number of different populations within each treatment group.
RESULTS

Production of chemotactic factor for leukocytes by hot aqueous phenol extracts in normal bovine serum

Three different hot aqueous phenol extracts, f3 (nucleic acid-rich), f5 (lipopolysaccharide-rich), and f6 (protein-rich), each generated chemotactic factor for granulocytes and monocytes from normal bovine serum (Table 2.2). However, it is apparent that considerably more migration was obtained against serum treated with f5 and f6 than with f3. According to Duncan's multiple range test, f5 and f6 do represent a different class of inducers of chemotactic activity than f3.

Production of chemotactic factor for leukocytes by carbohydrate-rich aqueous methanol extracts in normal serum

Fraction 1 manifested a marked inhibitory effect upon monocytes and especially granulocytes when incubated with normal bovine serum at high concentrations (Table 2.3). The inhibition was decreased at lower concentrations of fraction 1, and at 2 μg/ml serum no inhibition was observed. Fraction 2, the material remaining after dialysis of fraction 1, is a very potent generator of serum-derived chemotactic factor. Doses as low as 1.3 μg/ml normal bovine serum produced significant amounts of the chemotaxin (Table 2.3).

Failure to produce a chemotactic factor for leukocytes by protein-rich extracts in normal bovine serum

Fractions 3 and 4 were obtained and incubated with 1 ml of normal serum at dosages ranging from 0.0075 μg to 75 μg. In no instance did the serum treated with these fractions differ significantly from the untreated serum in its ability to attract leukocytes in vitro (data not shown).
<table>
<thead>
<tr>
<th>Fraction tested(^a)</th>
<th>Chemotactic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Granulocytes(^b)</td>
</tr>
<tr>
<td>Medium (random migration)</td>
<td>22.3 ± 0.2</td>
</tr>
<tr>
<td>Untreated serum</td>
<td>142.0 ± 50.7</td>
</tr>
<tr>
<td>f3</td>
<td>270.7 ± 32.0(^*)</td>
</tr>
<tr>
<td>f5</td>
<td>717.7 ± 15.0(^*)</td>
</tr>
<tr>
<td>f6</td>
<td>642.3 ± 56.4(^*)</td>
</tr>
</tbody>
</table>

\(^a\) 1.0 ml bovine serum was treated with 0.1 ml of each fraction (containing 1 mg material) for 60 min at 37 °C, followed by 30 min at 56 °C. The mixtures were then centrifuged at 500 x g for 10 min, diluted 1:1 with DMEM-BSA and tested for chemotactic activity.

\(^b\) Cells per medium power (400X) field. Mean of at least triplicate filters ±1 standard deviation.

\(^c\) Cells per oil immersion (970X) field. Mean of at least triplicate filters ±1 standard deviation.

\(^*\) P < 0.0001.

\(^**\) P < 0.005.
Table 2.3. Chemotactic activity of carbohydrate-rich aqueous methanol extracts

<table>
<thead>
<tr>
<th>Fraction tested^</th>
<th>Chemotactic Activity</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Granulocytes</td>
<td>Monocytes</td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td>22.3 ± 0.2</td>
<td>22.6 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>Untreated Serum</td>
<td>142.0 ± 50.7</td>
<td>38.3 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>Fraction 1 (µg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>209</td>
<td>2.3 ± 0.6*</td>
<td>14.7 ± 7.2*</td>
<td></td>
</tr>
<tr>
<td>20.9</td>
<td>38.0 ± 8.3*</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2.09</td>
<td>124.6 ± 35.6</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Fraction 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>127</td>
<td>935.7 ± 15.1*</td>
<td>99.9 ± 6.7*</td>
<td></td>
</tr>
<tr>
<td>12.7</td>
<td>578.7 ± 25.8*</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>1.27</td>
<td>363.9 ± 26.0*</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

^1.0 ml bovine serum was incubated (60 min at 37 C followed by 30 min at 56 C) with 0.1 ml of each fraction containing the indicated amount. Samples were then centrifuged at 500 x g for 10 min, diluted 1:1 with DMEM-BSA, and tested for chemotactic activity.

^bCells per medium power (400X) field. Mean of at least triplicate filters ±1 standard deviation.

^cCells per oil immersion (970X) field. Mean of at least triplicate filters ±1 standard deviation.

^dNot determined.

* P <0.0001.
Characterization of the chemotactic factor generated from serum by the various Brucella fractions

The involvement of an intact complement system in the generation of chemotactic factor was suggested by experiments in which the bovine serum was preheated at 56°C for 30 min and then incubated with the various Brucella extracts.

Table 2.4 presents the results of an experiment using aqueous methanol fraction 2. Preheating the serum before adding fraction 2 reduced the chemotactic responses to near control levels. Similar results were obtained with the hot aqueous phenol extracts. The migratory responses of monocytes were also significantly reduced by preheating the serum before treatment with Brucella fractions.

The role of the fifth component of complement was investigated by adding various Brucella fractions to normal mouse serum and to serum from mice deficient in C5. The results presented in Table 2.5. show that C5 is implicated since incubation of C5-deficient serum with several Brucella fractions fails to stimulate monocyte chemotactic responses. In contrast, monocyte chemotaxis is stimulated by Brucella fractions in C5-intact murine serum. Similar results were obtained when granulocytes were used instead of monocytes.
Table 2.4. Abrogation of chemotactic activity by preheating serum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chemotactic activity^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated serum</td>
<td>249.6 ± 13.6</td>
</tr>
<tr>
<td>Preheated serum</td>
<td>250.0 ± 20.7</td>
</tr>
<tr>
<td>Serum + 127 µg fraction 2^b</td>
<td>1211.3 ± 35.3*</td>
</tr>
<tr>
<td>Preheated serum + 127 µg fraction 2^c</td>
<td>341.3 ± 34.6</td>
</tr>
</tbody>
</table>

^aGranulocytes per medium power (400X) field. Mean of at least triplicate filters ±1 standard deviation.

^b1.0 ml of bovine serum was treated with fraction 2 and chemotaxis assayed as described in the legend for Table 2.

^cSerum was preheated at 56 C for 30 min before mixing with fraction 2 and assaying for chemotaxis as described.

^P <0.0001.
Table 2.5. Production of chemotactic activity in C5-deficient mouse serum

<table>
<thead>
<tr>
<th>Serum tested</th>
<th>Chemotactic Activity^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF-1 serum</td>
<td>13.7 ± 1.8</td>
</tr>
<tr>
<td>CF-1 serum + f5</td>
<td>23.7 ± 1.5^*</td>
</tr>
<tr>
<td>CF-1 serum + f6</td>
<td>21.5 ± 0.4^*</td>
</tr>
<tr>
<td>CF-1 serum + fraction 2</td>
<td>25.8 ± 3.5^*</td>
</tr>
<tr>
<td>DBA/2J serum</td>
<td>9.2 ± 0.2</td>
</tr>
<tr>
<td>DBA/2J serum + f5</td>
<td>10.1 ± 1.7</td>
</tr>
<tr>
<td>DBA/2J serum + f6</td>
<td>8.7 ± 2.0</td>
</tr>
<tr>
<td>DBA/2J serum + fraction 2</td>
<td>12.1 ± 3.6</td>
</tr>
</tbody>
</table>

^1.0 ml of mouse serum was treated with 1 mg (f5 and f6) or 127 μg fraction 2 and chemotaxis assayed as described in the legend for Table 2.

^bMonocytes per oil immersion (970X) field. Mean of at least triplicate filters ±1 standard deviation.

^P < 0.0001.
DISCUSSION

Many bacterial components have been described which possess the capacity to evoke chemotactic responses in macrophages and/or granulocytes (reviewed in 37). Some of these substances are capable of directly stimulating leukocyte migration in the absence of serum. The best characterized of this group of substances (termed cytotaxins), are the culture filtrates of E. coli (27). Most bacterial factors that evoke leukotactic responses do so by interacting with serum to generate chemotactic factor(s) from the complement system. Bacterial components acting in this manner are termed cytotaxinogens (37). Included in this class of chemotactic agents are whole cells or filtrates of staphylococci (12, 35), cell walls of Listeria monocytogenes (4), cell walls and glycolipids of Mycobacterium tuberculosis (13), endotoxins from a large number of species (20, 30, 31), and enterotoxin B from staphylococci (6). Recently, Russell et al. (26) found that staphylococcal cells and their culture filtrates evoked weak direct leukotactic responses. However, these same substances, incubated with serum, induced strong chemotactic activity. The cytotaxigenic substances could be separated into at least 10 distinct factors based on isoelectric focusing patterns. Thus, it appears that many structurally and chemically distinct fractions of bacteria have the ability to act as cytotaxinogens.

In the present study, it was found that several distinct fractions of Brucella abortus can act as cytotaxinogens. These consisted of
three different hot aqueous phenol extracts and a carbohydrate-rich aqueous methanol extract. An intact complement system appeared to be required for the generation of chemotactic factor since migration of leukocytes was not obtained when preheated bovine serum was used. Previous studies have indicated that the major serum-derived chemotactic factor for leukocytes is C5a (8, 10, 11). In this study, the inability to produce chemotactic activity when C5-deficient mouse serum was used with the various Brucella fractions, strongly suggests C5a is the chemotactic factor generated.

It was of considerable interest to examine the ability of Brucella lipopolysaccharide to generate chemotactic factor from normal serum. Many studies (10, 20, 30, 31) have utilized enterobacterial LPS to generate chemotactic factor from serum, however, Brucella LPS has several characteristics distinct from LPS isolated from the enterobacteria. Brucella LPS (f5) is found primarily in the phenol rather than the aqueous phase after extraction with the Westphal procedure (15, 25). Unlike enterobacterial LPS, massive doses of LPS are required to elicit pyrogenic responses in rabbits and lethality in chick embryos, and Brucella LPS is only 1/75 as potent as E. coli LPS in non-specific protection tests (15). Recently, Brucella lipopolysaccharide was found to be mitogenic for splenocytes of the endotoxin-resistant C3H/HeJ mouse strain (21, 33). Our present understanding of the structural and chemical composition of Brucella lipopolysaccharide indicates an unusual tightly bound protein(s) and significant differences in fatty acid composition from E. coli LPS (22). It has
been suggested that these differences may account for some of the
distinct biological activity of *Brucella* LPS (21, 33). Enterobacterial
lipopolysaccharides can activate both the classical (via Lipid A) and
alternative (via polysaccharides) complement pathways, with subsequent
liberation of chemotactic factors for leukocytes (reviewed in 23). The
strong cytotaxigenic capacity of *B. abortus* LPS observed in this study
suggests that Lipid A and/or complement-activating polysaccharides
of *Brucella* are intact and functional for interaction with complement.

An LPS is also found in f3, a nucleic acid-rich hot aqueous phenol
extract of *B. abortus*. This fraction acts as a cytotaxinogen, although
it does not appear to be as active as f5. These results are
consistent with reports showing that the f3 LPS is considerably less
active biologically than f5 (15, 25).

Chemotactic responses were also seen upon interaction of serum
and f6, a protein-rich aqueous phenol extract. This fraction is
essentially free of LPS, indicating a protein, polysaccharide, or some
minor contaminant is responsible for the observed activity. Fraction 3,
a protein-rich extract from attenuated strain 19, and fraction 4, a
protein-rich extract of virulent strain 2308, failed to produce serum-
derived chemotactic factor over a wide range of concentrations. These
fractions induced dermal lesions in guinea pigs (34), indicating *in
vitro* cytotaxigenic activity and *in vivo* skin reactivity may not be
strictly related.
Fractions 1 and 2, carbohydrate-rich aqueous methanol extracts, have previously been shown to elicit necrotic dermal lesions in guinea pigs (33 and L. Tabatabai and B. Deyoe, unpublished observations). When tested for cytotoxicigenic activity in vitro, fraction 1, at high concentrations, manifests a strong inhibition of leukocyte migration. Fraction 1 did not decrease leukocyte viability at the dosages used in this study (J. Birmingham, unpublished observation). The ability of polysaccharides to inhibit chemotaxis has received little attention, but Wilkinson recently proposed that polysaccharide-induced inhibition of leukocyte migration may be a significant and common mechanism of bacterial virulence (36). After dialysis of fraction 1, a potent cytotoxicigenic substance remains (fraction 2). The role of fraction 1 in masking the activity of fraction 2 or other cytotoxicigenic components of Brucella abortus in vivo deserves further study.

These results indicate that Brucella abortus contains a number of stimulatory factors and at least one inhibitory factor of leukotaxis in vitro. Further investigations of the roles these factors play in vivo may increase our understanding of the pathogenesis of brucellosis and contribute to improved prophylactic and/or diagnostic measures.
REFERENCES


PART III: **IN VITRO** INTERACTION OF BOVINE MONONUCLEAR PHAGOCYTES AND LIPOPOLYSACCHARIDE FROM *BRUCELLA ABORTUS*

This manuscript has been submitted for publication to the African Journal of Clinical and Experimental Immunology
In vitro interaction of bovine mononuclear phagocytes and lipopolysaccharide from *Brucella abortus*

James R. Birmingham and Edward L. Jeska

From the Immunobiology Program, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa
ABSTRACT

The in vitro interaction between lipopolysaccharide from Brucella abortus and bovine peripheral blood monocyte-derived macrophages was examined. Established primary cultures of bovine macrophages were incubated for 48 h in serumless medium with or without 20 μg/ml B. abortus LPS. No significant effect of LPS was noted on the percentage of cells capable of forming rosettes with IgG-coated erythrocytes, but a marked increase in the number of cells forming rosettes with complement-coated erythrocytes was found. The percentage of cells capable of phagocytosis by IgG Fc receptors was increased somewhat by LPS treatment and a very significant enhancement of C3 receptor-mediated internalization was noted. In addition, the number of particles internalized per cell was increased for both IgG Fc and C3 receptors. The rate of pinocytosis of horseradish peroxidase was stimulated by B. abortus LPS. No significant differences were noted for protein values of non-treated and LPS-treated macrophage cultures, however, the lysosomal enzymes, acid phosphatase and beta-glucuronidase were increased in LPS-treated cultures.
INTRODUCTION

Brucellosis is a disease of major economic impact worldwide (1). Considerable effort has been devoted towards isolating and characterizing biologically active components of Brucella. A number of studies have examined the effects of lipopolysaccharide preparations of Brucella. It appears that Brucella LPS is unusual in a number of respects. For example, Brucella LPS is found primarily in the phenol rather than the aqueous phase after extraction with the Westphal procedure (15, 23). Unlike enterobacterial LPS, the induction of pyrogenicity in rabbits and lethality in chicken embryos requires massive doses of Brucella LPS (15). Brucella LPS is also mitogenic for spleen cells of endotoxin-resistant C3H/HeJ mice (17, 24). Examination of the structural and chemical composition of Brucella lipopolysaccharide has revealed an unusual tightly bound protein(s) and significant differences in fatty acid composition from E. coli and Salmonella LPS (3, 5, 18). It has been suggested that these differences may account for some of the distinctive biological activity of Brucella LPS (3, 18, 24).

It has been proposed that macrophages may play a central role in mediating many of the responses LPS can elicit in vivo (21). These cells are extremely sensitive to minute amounts of LPS. The interaction of LPS and macrophages results in marked alterations in the activities of these cells and elaboration of an impressive range of soluble mediators (21, 22).
Since Brucella LPS reportedly differs markedly from entero-
bacterial LPS, we considered that a study of their interaction with 
bovine mononuclear phagocytes would be of interest. This paper 
describes the in vitro effects of B. abortus LPS on bovine monocyte-
derived macrophage surface receptors, phagocytosis, pinocytosis, and 
lysosomal enzyme activities.
MATERIALS AND METHODS

Isolation and cultivation of peripheral blood monocytes

Blood was obtained by jugular venipuncture from normal 12–14 mo old crossbred heifers (provided by Dr. B. L. Deyoe, National Animal Disease Center, Ames, IA). Acid-citrate-dextrose (2X) was used as anticoagulant. Mononuclear leukocytes were isolated as previously described (7). Briefly, the blood was centrifuged at 1000 x g for 20 min, the buffy coat collected, and contaminating red cells lysed with 0.013 M phosphate buffer. The remaining cells were centrifuged, washed 4X with Hank’s balanced salt solution without Ca<sup>++</sup> and Mg<sup>++</sup>, and resuspended in Dulbecco’s modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 50 µg/ml gentamycin sulfate (Schering Corp., Bloomfield, NJ). The cells were counted in a hemocytometer, resuspended to 10<sup>7</sup> cells/ml, and 1.5 ml seeded into 35 x 10 mm plastic tissue culture dishes. The cell cultures were incubated overnight in a humidified 5% CO<sub>2</sub>-95% air incubator. The cultures were then washed 3X with phosphate-buffered saline (PBS), pH 7.2, containing 5% FBS, and overlaid with 1.5 ml DMEM-10% FBS. After 24 h incubation, these cultures were washed with PBS and covered with 1.5 ml Neuman-Tytell serumless medium (Grand Island Biological Co., Grand Island, NY) supplemented with 0.2% mononuclear phagocytes as determined by morphology, uptake of latex and zymosan, and non-specific esterase staining. At this time 20 µg/ml
Brucella abortus lipopolysaccharide was added to some of the cultures. After 48 h further incubation, the cultures were processed for use in the tests described below.

Brucella abortus LPS was provided by Dr. L. Tabatabai, National Animal Disease Center. This material was obtained in the phenol phase after hot aqueous phenol extraction as previously described (15, 23). The chemical composition of this preparation (as percent dry weight) is 33.8% protein, 10.4% carbohydrate, 0.52% 2-keto,3-deoxysugar, 15% nitrogen, and 0.14% phosphorus (18).

Surface receptor enumeration and function Sensitized sheep red blood cells were prepared as previously described (7). The various reagents were designated E (sheep erythrocytes), EA\textsubscript{M} (sheep erythrocytes sensitized with the highest subagglutinating dilution of rabbit IgM anti-E), EA\textsubscript{G} (sheep erythrocytes sensitized with the highest subagglutinating dilution of rabbit IgG anti-E), and EA\textsubscript{M}C (EA\textsubscript{M} incubated with C5-deficient DBA/2J mouse serum as a source of complement). The anti-erythrocyte antibody preparations were obtained from Cordis Corp. (Miami, Florida). The presence of surface receptors on macrophages was determined by adding the appropriate indicator to adherent cells for 30 min at 37 C. The dishes were then washed gently in a beaker of PBS and immediately examined for rosetting cells. For each assay, 200 cells were scored (a positive rosette was considered a cell with 4 or more erythrocytes attached). A minimum of three replications were performed for each test.
Receptor-mediated phagocytosis was determined by incubating the adherent cells and appropriate erythrocyte reagent for 60 min at 37 C. The dishes were then gently washed in PBS, dipped in a beaker of distilled water for 20 sec to lyse erythrocytes attached to the monocyte surface, washed with PBS, and overlaid with a coverslip. These preparations were examined with an inverted microscope to ensure all surface-associated erythrocytes were lysed. The cells were then examined under a phase-contrast microscope (630X) for internalized erythrocytes. Two hundred cells were examined in each dish with a minimum of three replications per test.

**Measurement of pinocytic rate** Pinocytic rate was determined using the horseradish peroxidase (HRP) technique (25). When assayed for pinocytosis, the adherent cell populations (both LPS-treated and non-treated) contained no detectable peroxidase as determined biochemically (25) and morphologically (13). Adherent cells were incubated with 1 mg/ml HRP in DMEM-20% FBS, washed thoroughly at various times, and cell lysates assayed for peroxidase at 460 nm on a Gilford spectrophotometer. Controls included dishes without cells and cells incubated with HRP on ice. Type II HRP and O-dianisidine were obtained from Sigma (St. Louis, MO), \( \text{H}_2\text{O}_2 \) from Mallinckrodt (St. Louis, MO), and Triton X-100 from Bio-Rad laboratories (Richmond, CA). Results were expressed as ng HRP/100 µg cell protein.

**Enzyme assays** Cell lysates were prepared by incubating washed adherent cells with 1 ml of 0.05% Triton X-100 for 45 min on ice. The
dishes were scraped with a rubber policeman and the lysates were collected. Acid phosphatase was determined by a modification of the method of Beck et al. (4) as described (7). Results were expressed as nmoles p-nitrophenol liberated/hr/dish. Beta-glucuronidase was measured by a modification of the procedure described by Fishman et al. (10). Two hundred µl of cell lysate was incubated for 60 min at 56°C with 200 µl of 0.03 M phenolphthalein glucuronic acid in 0.2 M acetate buffer, pH 4.5. The reaction was stopped with 2-amino-2-methyl-1-propanol buffer, pH 11, and released phenolphthalein measured at 410 nm on a Gilford spectrophotometer. Results were expressed as µg phenolphthalein liberated/hr/dish. Protein was measured by the method of Lowry et al. (16) as modified by Wang and Smith (27). Bovine serum albumin was used as standard. All reagents were obtained from Sigma.
RESULTS

Effect of *B. abortus* LPS on cell surface receptor activity

Receptors for E and EA<sub>M</sub> were not detected on either LPS-treated or non-treated bovine monocyte-derived macrophages (Figure 3.1). A high percentage of these cells expressed receptors for EA<sub>G</sub>, but no major differences were detected between treated and non-treated cells. However, marked differences were noted when the cells were examined for their ability to form rosettes with complement-coated erythrocytes. Approximately 35% of normal bovine macrophages reacted with EA<sub>M</sub>C, whereas >75% of LPS-treated cells formed rosettes.

Effect of *B. abortus* LPS on receptor-mediated phagocytosis

Figure 2 shows the effects of *B. abortus* LPS on the percentage of macrophages capable of ingesting erythrocytes by either IgG Fc or C3 receptors. Normal bovine mononuclear phagocytes are capable of internalizing erythrocytes via the IgG Fc receptor. This activity is increased 20% by treatment with *B. abortus* LPS in vitro. In contrast, *B. abortus* LPS dramatically increased C3 receptor-mediated internalization (Figure 3.2). Approximately 4% of the normal macrophages phagocytized sheep erythrocytes via C3 receptors, but this was increased to 43% after treatment of macrophages with LPS.

In vitro treatment of bovine macrophages with *B. abortus* LPS influences not only the percentage of cells that can phagocytize particles, but also the phagocytic capacity of individual cells. The
Figure 3.1. Effect of B. abortus LPS on bovine macrophage rosette-forming ability. Dark bars, non-treated macrophages; light bars, LPS-treated macrophages.
Figure 3.2 Effect of *B. abortus* LPS on the percentage of phagocytizing bovine macrophages. Dark bars, non-treated macrophages; light bars, LPS-treated macrophages.
data presented in Table 3.1 shows increases in the number of erythrocytes ingested by populations of cells treated with LPS, and increases in the number of erythrocytes ingested on a per cell basis.

**Effect of *B. abortus* LPS on pinocytic rate of bovine macrophages**

Incubation of bovine macrophages with *B. abortus* LPS in vitro results in a considerable elevation in the pinocytic rate (Table 3.2). Controls designed to investigate the efficiency of washing (dishes without cells), gave background HRP accumulations for 120 min (10 ng). Incubation of macrophages with HRP at 0°C resulted in HRP measurements similar to background values.

**Effect of *B. abortus* LPS on bovine macrophage protein and lysosomal enzyme activity**

No significant differences were observed in protein values between untreated and LPS-treated macrophages (Table 3.3). The lysosomal enzymes acid phosphatase and beta-glucuronidase each increased approximately 2X in LPS-treated cultures.
Table 3.1. The effects of *B. abortus* LPS on receptor-mediated phagocytosis

<table>
<thead>
<tr>
<th>Receptor</th>
<th>LPS treatment</th>
<th>#RBC internalized/100 macrophages</th>
<th>#Internalized RBC/phagocytic cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG Fc</td>
<td>-</td>
<td>96.5 ± 20.5</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>220 ± 2.8</td>
<td>3.5</td>
</tr>
<tr>
<td>C3b</td>
<td>-</td>
<td>4 ± 0.7</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>102 ± 11.3</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Table 3.2. Effect of *B. abortus* LPS on bovine macrophage pinocytosis of horseradish peroxidase

<table>
<thead>
<tr>
<th>Time of incubation^b</th>
<th>Pinocytic rate^a</th>
<th>Untreated macrophages</th>
<th>LPS-treated macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>25</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td>57</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>37</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td>120 min, 0 C</td>
<td>12</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

^a ng HRP/100 µg cell protein.

^b 1 mg/ml HRP incubated with untreated or LPS-treated bovine macrophages at 37 C.
Table 3.3 The effect of *B. abortus* LPS on bovine macrophage lysosomal enzymes and protein

<table>
<thead>
<tr>
<th>Activity measured</th>
<th>Untreated macrophages</th>
<th>LPS-treated macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein$^a$</td>
<td>91.7 ± 13.8</td>
<td>120 ± 13.2</td>
</tr>
<tr>
<td>Acid phosphatase$^b$</td>
<td>153.5 ± 47.4</td>
<td>329 ± 125.2</td>
</tr>
<tr>
<td>beta-glucuronidase$^c$</td>
<td>11.5 ± 8.4</td>
<td>20.1 ± 13.7</td>
</tr>
</tbody>
</table>

$^a$ mean μg/dish ± S.D.

$^b$ mean nmol p-nitrophenol liberated/hr/dish ± S.D.

$^c$ mean μg phenolphthalein liberated/hr/dish ± S.D.
DISCUSSION

Brucella LPS has been described as differing from enterobacterial LPS in several respects (14, 15, 23). It is soluble in the phenol rather than the aqueous phase after hot aqueous phenol extraction, is required in massive doses to produce pyrogenic and lethal effects, induces mitogenic responses in splenocytes of C3H/HeJ mice, and differs in chemical structure and composition from enterobacterial LPS. In addition, Hinsdill and Berman (12) found the levels of B. abortus LPS required to induce cytotoxicity in guinea pig macrophages was 10-100X higher than levels reported for E. coli LPS (11).

The results in our study indicate that Brucella abortus LPS is capable of inducing enhanced activities in bovine monocyte-derived macrophages. The dosage of Brucella LPS used to stimulate cells in vitro (20 µg/ml), is similar to dosages of enterobacterial LPS used in previous investigations (26, 28). The effects of B. abortus LPS on bovine mononuclear phagocytes appears to be direct because >95% of the adherent cells were non-specific esterase positive phagocytes, none of which were seen to possess morphological characteristics of polymorphonuclear cells.

The ability of B. abortus LPS to induce modulation of bovine macrophage cell surface receptor activity was examined. No receptors for either sheep erythrocytes or heterologous IgM were detected on normal or LPS-treated macrophages. A high percentage of both normal and LPS-treated cells reacted with IgG-coated erythrocytes. In
contrast, *Brucella abortus* LPS induced a significant increase in the number of cells forming rosettes with complement-coated erythrocytes. Morland and Kaplan (19) reported *E. coli* LPS-induced increases in C3 receptor activity in murine peritoneal macrophages if the cells were cultured in the presence of 20% serum. However, no increase was seen if the cells were cultivated with LPS in the absence of serum. A synergistic action of serum and LPS on macrophages has been reported (19, 28), thus we chose to cultivate cells in *Brucella* LPS and serumless medium. Although *Brucella* LPS induced C3 receptor modulation, the results may not be directly comparable to those of Morland and Kaplan since the macrophages were initially cultivated in serum for 36 h before being placed into serumless medium.

Normal mononuclear phagocytes will bind particles via C3 receptors but will not internalize them (6). Suitably stimulated macrophages will phagocytize particles via the C3 receptor and it has been suggested that this criterion could be used to distinguish resting and 'activated' macrophages (6). In our study, *Brucella abortus* lipopolysaccharide was able to induce C3 receptor-mediated attachment and ingestion of sheep erythrocytes by bovine mononuclear phagocytes.

The rate of pinocytosis of horseradish peroxidase by macrophages was increased by *in vitro* *B. abortus* LPS treatment. Edelson et al. (9) initially observed an elevated pinocytic rate in murine peritoneal macrophages after *in vivo* injection of thioglycollate or *E. coli*
endotoxin. The basis for an elevated pinocytic rate is unknown, but it has been suggested to be characteristic of the state of activation of macrophages (9).

In vitro treatment of murine peritoneal macrophages with enterobacterial LPS has been shown to stimulate a rise in the levels of certain cellular lysosomal enzymes (2, 20, 28). In this report, Brucella LPS was found to be capable of stimulating an increase in the levels of bovine monocyte-derived macrophage acid phosphatase and beta-glucuronidase.

This study shows that Brucella LPS, at doses similar to lipopolysaccharides from enterobacteria, can stimulate a number of functions in bovine macrophages. We conclude that Brucella LPS, acting on mononuclear phagocytes, may play a more significant role in the course of disease than previously believed. Further investigation of the interactions of mononuclear phagocytes and Brucella LPS is required.
REFERENCES


PART IV: CHARACTERIZATION OF MACROPHAGE FUNCTIONS IN MICE INFECTED WITH BRUCELLA ABORTUS

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Characterization of macrophage functions in
mice infected with *Brucella abortus*

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ABSTRACT

Macrophage spreading, surface receptor density/avidity, phagocytosis, random migration, chemotactic responsiveness, and serum lysozyme were examined during the course of infection (up to 60 days) of mice with Brucella abortus strain 19. Markedly enhanced in vitro spreading activity was observed throughout the period of study. The density/avidity of cell surface IgG Fc receptors was increased for up to 60 days post-infection. Internalization of sheep erythrocytes via C3 receptors was significantly enhanced. Random locomotion and chemotactic responsiveness to casein, lymphocyte-derived chemotactic factor, and N-formyl-L-methionyl-L-leucyl-L-phenylalanine were markedly stimulated. Serum lysozyme was also elevated in infected animals. These changes are indicative of significant and prolonged enhancement of macrophage activity during Brucella infection.
INTRODUCTION

Brucellosis is a disease of considerable importance for man and domesticated animals worldwide (1). Brucella is a facultative intracellular pathogen that resides primarily in cells of the reticuloendothelial system and associated tissues (10,34). Resistance is generally believed to depend upon successful collaboration between specifically sensitized T-lymphocytes and the ultimate effector cell, the macrophage (29).

The development of non-specific antibacterial resistance *in vivo* in Brucella-infected animals has been described in several studies (11, 19, 28). These reports have assumed that activated macrophages are responsible for the antibacterial activity. There are also studies showing heightened antibacterial activity *in vitro* by macrophages collected from Brucella-infected animals (16,28,37). However, there is not general agreement in the above *in vivo* and *in vitro* studies regarding the kinetics of induction or the duration of enhanced macrophage antibacterial activity.

A large number of reports have described increased resistance to various viruses (5, 23, 45), protozoa (20), and tumors (12, 12, 43) in animals infected with Brucella. In these studies, the enhanced non-specific resistance has usually been attributed to macrophage activation. However, few of these investigations have directly examined the effects of Brucella infection on macrophage function.

In this study, we have characterized a number of activities of macrophages during the course of infection with *B. abortus*. The
results indicate significant and prolonged enhancement of a variety of macrophage functions, including surface receptor activity, phagocytic capacity, and locomotor function. These enhanced functional capacities may play a direct role in mediating resistance to Brucella, to other unrelated organisms, and to tumors.
MATERIALS AND METHODS

**Infection of animals and collection of cells**  
Mice of the C57BL/6 strain (originally obtained from M. A. Associates, Walkersville, MD), 6-12 weeks of age were used in this study. Animals were matched for sex and age in each experiment.

*Brucella abortus* strain 19 (provided by Dr. B. L. Deyoe, National Animal Disease Center, Ames, IA) was maintained at 4°C and passaged monthly on tryptose agar (Difco, Detroit, MI). For animal inoculations, the bacteria were grown at 37°C in tryptose broth (Difco), centrifuged at 12,000 x g for 20 min, washed 2X in phosphate-buffered saline (PBS), pH 7.2, and resuspended in PBS. The number of bacteria were estimated by spectrophotometric techniques and confirmed by plating on tryptose agar. Mice were injected i. p. with 1 ml PBS containing 3 to 5 x 10^7 bacteria. Control animals were injected with PBS.

At various times post-infection, the mice were killed by cervical dislocation and the peritoneal cavities lavaged with 5 ml Dulbecco's modified Eagle medium (DMEM) containing 5% fetal bovine serum (FBS, Grand Island Biological Co., Grand Island, NY) and 5 U/ml heparin. The peritoneal cells were centrifuged at 150 x g for 10 min and resuspended in DMEM-20% FBS for culturing or DMEM-2% bovine serum albumin (BSA) for chemotaxis studies. Cell differentiations were determined by morphology and non-specific esterase staining (26).
Enumeration of bacteria in the spleen    Spleens were aseptically removed from mice and immediately weighed. The spleens were then homogenized in 10 ml of PBS with a Ten-Broeck tissue grinder. Dilutions were prepared in PBS and duplicate tryptose agar plates inoculated. Colony-forming units (CFU) were counted after 4-5 days of incubation at 37 C.

Spreading assay    Peritoneal cells, in 35 x 10 mm plastic tissue culture dishes, were incubated for 2.5 h at 37 C in a 5% CO_2 in air incubator. The dishes were then removed from the incubator, washed vigorously 5X with PBS, overlaid with a cover slip, and examined under a phase-contrast light microscope at 630X magnification. Spreading was assessed as described by Rabinovitch and DeStefano (38), and results expressed as the percentage of spread cells. Two hundred cells were counted for each determination with at least three replications.

Cell surface receptor enumeration and function    Sensitized sheep red blood cells (E) were prepared as previously described (6). The reagents were designated EAg (E coated with the highest subagglutinating dilution of rabbit IgG anti-E) and EAMC (E coated with the highest subagglutinating dilution of rabbit IgM anti-E and C5-deficient DBA/2J mouse serum as a source of complement). The rabbit anti-E reagents were obtained from Cordis Corp. (Miami, Florida). For studies of the density/avidity of IgG Fc surface receptors, E were sensitized with 1:4, 1:9, 1:19, and 1:49 dilutions
of the highest rabbit IgG subagglutinating preparation (1:1000). The sensitized erythrocytes were added to adherent cells in Lab-Tek multi-well tissue culture chamber/slides and the slides incubated for 30 min at 37 C. At the end of this period, the superstructure of the slide was removed, the slides gently washed in a beaker of PBS, and immediately examined for rosetting cells. For each assay, 200 cells were scored (a positive rosette was considered a cell with 4 or more erythrocytes attached). A minimum of three replications were performed for each test. Complement receptor-mediated phagocytosis was determined by incubating macrophages with EA\textsubscript{M}C for 60 min at 37 C. The culture dishes were washed gently in PBS, dipped in a beaker of distilled water for 20 sec to lyse erythrocytes attached to the macrophage surface, washed with PBS, and overlaid with a coverslip. These preparations were examined with an inverted microscope to ensure cell surface-associated erythrocytes were lysed. The cells were then examined under a phase-contrast microscope (630X) for internalized erythrocytes. Two hundred cells were examined in each test.

Chemotactic factors

Macrophage migratory activity was assayed against three materials: casein, lymphocyte-derived chemotactic factor (LDCF), and the chemotactic peptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP). Casein (Sigma) was suspended at 0.5% (w/v) in DMEM-BSA. This concentration was determined to give optimal migratory responses of normal peritoneal macrophages in preliminary
experiments. Lymphocyte-derived chemotactic factor was produced by stimulation of C57BL/6 mouse spleen cells with phytohemagglutinin (Sigma) as described by Meltzer and Stevenson (30). The 72 h supernatant fluids of these cultures were collected, centrifuged at 500 x g for 10 min, and diluted to 20% in DMEM-BSA. The chemotactic peptide FMLP (Sigma) was diluted in DMEM-BSA to the concentrations described in the tables.

**Chemotaxis assay**  
Chemotaxis was assayed essentially as described by Boetcher and Meltzer (7) and Snyderman et al. (42), using Neuroprobe blind-well chemotaxis chambers and polycarbonate membrane filters with 5 μm pores (Bio-Rad Laboratories, Richmond, CA). Cell suspensions were standardized to 500,000 macrophages/0.2 ml. After 3.5 h of incubation in a humidified 5% CO₂-95% air incubator, the filters were removed, inverted on glass microscope slides, air-dried, fixed with absolute methanol, and stained with Giemsa. Filters were scored as the number of macrophages that had completely migrated through the filter in 10 high power fields (1000X). Random migration was determined against DMEM-BSA. Each assay was performed in triplicate. Results are expressed as the mean total cells per 10 high power fields (hpf) ± one standard deviation.

**Lysozyme assay**  
Sera from 5 mice were pooled and the concentration of serum was estimated turbidometrically by determining the rate of lysis of *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, MO) as described (17). Egg white lysozyme (Sigma) served as standard and the results were expressed as μg lysozyme/ml serum.
RESULTS

Course of B. abortus infection in C57BL/6 mice

The course of infection of C57BL/6 mice, based on viable bacterial counts in the spleen and spleen weights, is shown in Figure 4.1. Bacterial multiplication in the spleen reaches a peak at 5-7 days and is followed by a rapid decrease in the number of viable organisms. Over 90% of the brucellae in the spleen are killed in 10 days. A chronic infection is established, with approximately $10^4$ organisms recoverable from the spleen 60 days after the initial injection of bacteria. The increase and decline in the weight of the spleen parallels the number of viable bacteria in this organ, although it is delayed by several days.

Macrophage spreading activity in vitro

The percentage of peritoneal macrophages showing spreading (veils or aprons greater than the initial cell diameter) after 2.5 h of in vitro cultivation is shown in Table 4.1. Significantly enhanced spreading behavior is seen throughout the course of infection, even in cells collected 60 days post-infection.

Macrophage Fc receptor activity during Brucella infection

The ability of macrophages from normal and infected mice to form rosettes with erythrocytes sensitized with various dilutions of rabbit IgG antibodies was studied. As shown in Figure 4.2, a markedly increased percentage of cells from infected animals formed rosettes with erythrocytes sensitized with dilutions of antibody as high as 1:50,000.
Figure 4.1. Viable bacteria in the spleen (●) and weight of spleen
(o) in C57BL/6 mice given 3 to 5 $\times 10^7$ B. abortus i.p.
Each point represents the mean for at least 4 mice.
Log$_{10}$ Brucella/spleen

Days post-infection

Spleen weight (g)
Table 4.1. Macrophage spreading activity during *Brucella* infection

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>% Macrophages spread$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>10.5 ± 4.6</td>
</tr>
<tr>
<td>4</td>
<td>86.0 ± 4.1</td>
</tr>
<tr>
<td>17</td>
<td>73.0 ± 2.7</td>
</tr>
<tr>
<td>30</td>
<td>70.6 ± 8.3</td>
</tr>
<tr>
<td>60</td>
<td>41.2 ± 6.6</td>
</tr>
</tbody>
</table>

$^a$Percentage of macrophages exhibiting spreading after 2.5 h cultivation *in vitro*. Each point represents the mean ± one standard deviation for triplicate determinations. Two hundred cells were examined for each determination.
Figure 4.2. Macrophage IgG Fc receptor density/avidity during *B. abortus* infection. Each point represents the mean of three determinations. Within groups, each determination varied less than 15%. Uninfected (o), 4 day infection (●), 10 day infection (■), 20 day infection (■), 40 day infection (▲), 60 day infection (▲).
(1000 was the highest subagglutinating titer of the antibody preparation used). The greatest increase in the number of rosette-forming cells at the various dilutions was seen early in infection (4 days), although notable differences are still detectable at 60 days post-infection. These studies do not distinguish between the number of receptors on an individual cell or changes in the avidity of a particular receptor for its ligand.

The effect of B. abortus infection of macrophage C3b receptor-mediated internalization

The results shown in Figure 4.3 indicate that a low percentage of normal peritoneal macrophages internalize erythrocytes by means of this receptor, whereas 65-80% of macrophages from B. abortus-infected mice have this capacity. The enhanced receptor activity was apparent throughout the 30 day infection period studied.

Macrophage migratory responses during B. abortus infection

Significantly enhanced responsiveness was observed for macrophages from infected mice against several chemoattractants, including lymphocyte-derived chemotactic factor (Figure 4.4), casein (Table 4.2), and a range of concentrations of the chemotactic peptide, FMLP (Figure 4.5). Notable in these studies was the markedly enhanced random migration of macrophages from infected animals (Table 4.2 and Figure 4.5). Enhanced responses to LDCF persisted through the 30 day period of study, whereas responses to FMLP returned to near control values by 30 days.
Figure 4.3 Macrophage C3b receptor-mediated internalization during *B. abortus* infection. Bars represent the mean percent phagocytosis-positive cells ± one standard deviation for triplicate determinations. Two-hundred cells were examined for each determination.
Figure 4.4 Macrophage chemotactic responses to lymphocyte-derived chemotactic factor during *B. abortus* infection.

Supernatants of Con A-stimulated spleen cells were diluted to 20% in DMEM-BSA. Bars represent mean total cells/10 hpf ± one standard deviation for triplicate filters.
Table 4.2. Macrophage chemotactic responses to casein during *B. abortus* infection

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>Chemotactic stimulus&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diluent</td>
<td>Casein&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>17.9 ± 9.3</td>
<td>25.3 ± 10.6</td>
<td></td>
</tr>
<tr>
<td>4 days</td>
<td>54.7 ± 13.6</td>
<td>218.7 ± 26.6</td>
<td></td>
</tr>
<tr>
<td>8 days</td>
<td>85.0 ± 10.6</td>
<td>175.3 ± 62.1</td>
<td></td>
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<tr>
<td>12 days</td>
<td>182.7 ± 33.1</td>
<td>359.3 ± 18.3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are the mean total cells/10 hpf ± one standard deviation for triplicate filters.

<sup>b</sup>0.5% casein in DMEM-BSA.
Figure 4.5. Macrophage chemotactic responses to FMLP during *B. abortus* infection. Each point represents mean total cells/10 hpf ± one standard deviation for triplicate filters. Diluent (○), $10^{-5}$ M FMLP (●), $10^{-6}$ M FMLP (■), $10^{-7}$ M FMLP (▲).
Serum lysozyme in Brucella-infected mice

Serum lysozyme, an index of macrophage activity, was found to be elevated throughout the 30 day period of study (Table 4.3).
Table 4.3. Serum lysozyme during *Brucella* infection

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>Serum lysozyme&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>3.0</td>
</tr>
<tr>
<td>4</td>
<td>4.2</td>
</tr>
<tr>
<td>6</td>
<td>7.5</td>
</tr>
<tr>
<td>7</td>
<td>9.6</td>
</tr>
<tr>
<td>11</td>
<td>9.5</td>
</tr>
<tr>
<td>14</td>
<td>7.1</td>
</tr>
<tr>
<td>30</td>
<td>7.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>μg lysozyme/ml serum.
DISCUSSION

Injection of 3 to 5 x 10^7 B. abortus i.p. into C57BL/6 mice results in the establishment of a chronic infection, with 10^4 organisms still recoverable from the spleen at 60 days. The course of infection in this study is similar to that described by others (11, 19, 28), although the early phases occurred somewhat more rapidly. Based on spleen CFU, the infection can be divided into three phases: (i) bacterial multiplication, which peaks at 2.5 x 10^7 organisms in the spleen at 7 days, (ii) bactericidal phase, lasting until approximately day 15 to day 20, (iii) so-called latent phase with slowly declining bacterial numbers. Some reports have indicated that macrophage activation in experimental brucellosis is first detectable only during the bactericidal phase and then wanes as the bacterial numbers fall (19, 28), some have indicated that macrophage activation can be demonstrated as early as three days post-infection (11), whereas others have indicated macrophage activation persists for several months (20, 37). Macrophage activation in each of these studies was measured by heightened non-specific resistance in vivo and/or in vitro antimicrobial activity. In the present study, a wide range of macrophage functions were found to be elevated very early in infection (4 days). Additionally, although not all parameters of macrophage function were followed for the entire testing period, some activities (spreading and receptor density/avidity) were still elevated at 60 days, far into the latent phase.

Previous studies have shown enhanced IgG Fc receptor activity in inflammatory macrophages (3, 39). Recently, Amsden and Boros (2)
found elevated IgG Fc receptor activity in macrophages isolated from a number of different experimentally induced granulomas. The greatest increases in receptor density/avidity induced with Brucella infection was observed at 4 days. This activity decreased as infection progressed, but enhanced macrophage IgG Fc receptor density/avidity was still apparent at 60 days. Enhanced receptor density/avidity could play a direct role in controlling various infections, because binding and internalization of particles may then be triggered at lower concentrations of opsonin. In addition, the enhanced activity may facilitate a large number of macrophage functions, including clearance of antigen–antibody complexes (15), release of lysosomal enzymes (9), release of toxic oxygen metabolites (21), and enhanced antibody-dependent cytotoxicity against tumor cells or pathogens (27, 32).

Normal macrophages bind particles via C3 receptors but do not ingest them, whereas appropriately stimulated macrophages have the capacity to internalize via this receptor (4). Our results indicate that macrophages from Brucella-infected mice possessed a markedly enhanced capacity to internalize sheep erythrocytes via the C3 receptor. Clearance of IgM-coated particles in vivo is absolutely dependent upon complement (8, 40), thus C3 receptor activity could play an important role, especially early in infection. Activated C3 receptor activity may have special relevance for brucellosis because an elevated and persistent IgM response reportedly is a feature of this infection (33).
It was found that macrophages from Brucella-infected mice have greatly increased random migration and chemotactic responses to casein, LDCF and FMLP. The increased mobilization of macrophages to a site of infection is considered to play an important role in resistance. Meltzer et al. (31) showed that increased chemotactic activity is a property of activated macrophages. Agents that activated macrophages for tumoricidal activity (i.e., BCG, endotoxin, Concanavalin A, poly I:C) also increased their chemotactic responses. On the other hand, agents that were stimulatory for macrophages and did not induce tumoricidal responses (i.e, starch, mineral oil, thioglycollate) also did not produce enhanced chemotactic activity. Macrophages from Brucella-infected mice fit this criterion of activated cells.

The mechanism of enhanced locomotor activity in activated macrophages is presently unknown. One intriguing possibility may be that activated macrophages express an increased number and/or affinity for chemoattractants on their cell surface. Receptors have recently been described on macrophages for casein (22) and for N-formylated peptides (41). Pike et al. (36) demonstrated that the development of increased chemotactic responsiveness after stimulation by lymphokines in the monocyte-like cell line, U937, is accompanied by the appearance of an increasing number of specific binding sites for N-formylated peptides. These findings indicate that surface receptor activity is an important event in the development of responsiveness to chemotactic agents.
Our studies indicate that chemotactic responsiveness was high towards LDCF at 30 days, yet, responsiveness to FMLP at 30 days returned to near control levels. These findings could be explained if specific surface receptor activity for LDCF persisted at 30 days whereas surface receptor activity for FMLP declined. The enhanced random locomotion of activated macrophages seen in the present study and by others (31) suggests other metabolic events may also have been involved. It may be that activation of macrophages for enhanced random migration involves stimulation of as yet undefined metabolic events, whereas enhanced chemotactic activity requires this effect plus development of cell surface receptor activity.

Serum lysozyme was found to be markedly elevated during *Brucella* infection. Studies (14, 25) have suggested that serum lysozyme is primarily produced by macrophages and is a sensitive indicator of the functional state of these cells. The elevated serum lysozyme in *Brucella*-infected mice may be an important mediator of non-specific resistance, because lysozyme can stimulate phagocytosis (24). Lysozyme may also interfere with tumor cell surface activities (35) and can dampen inflammatory activity (18).

The findings in this study indicate that a number of activities of macrophages are markedly enhanced during murine *Brucella* infection. These activities could function in various ways against *Brucella*, unrelated pathogens, or tumors. This fact, coupled with the dissociation of some of the characteristics of the activated macrophage seen in this
report and by others (30, 44), may help to explain apparently con¬
dictory findings in the literature (11, 20, 28, 37) regarding the length
of time required to activate macrophages and the duration of that
activation in \textit{Brucella}-infected animals.
REFERENCES


A method for obtaining bovine peripheral blood monocytes was developed. Optimal conditions for long-term cultivation in vitro were determined and this permitted cells to be maintained up to 80 days. Several features of the normal differentiation of bovine mononuclear phagocytes were examined during the course of culture. Peroxidase activity declined in a linear manner, with no cells positive by the 8th day. During cultivation, cell size, spreading, granulation, and vacuolization increased. Multinucleated giant cells dominated the culture by the 45th day. Cell surface receptors for IgG Fc and C3 were detected and their roles in phagocytosis elucidated. Cell protein and acid phosphatase increased during cultivation. Bovine mononuclear phagocytes were found to be devoid of lysozyme activity.

Several fractions isolated from Brucella abortus were examined for their ability to generate chemotactic factor(s) from normal bovine serum for granulocytes and monocytes. Three different hot aqueous phenol extracts (nucleic acid-rich, carbohydrate-rich, and protein-rich) were active. A carbohydrate-rich aqueous methanol fraction inhibited leukocyte migration, whereas the non-dialysable component of this fraction contained a potent stimulator of leukotaxis. Protein-rich fractions from both attenuated strain 19 and virulent 2308 were inactive. Preheating bovine sera abolished the ability of the active fractions to generate chemotactic activity. Normal mouse serum could
serve as the source of chemotactic factor for granulocytes and monocytes, whereas C5-deficient mouse serum was inactive. These experiments strongly suggest C5a is the chemotaxin generated by the various Brucella fractions from normal serum.

The in vitro interaction between lipopolysaccharide from Brucella abortus and bovine mononuclear phagocytes was studied. Brucella LPS exerted a major effect on macrophage complement receptor activity, increasing rosette forming capacity from 37% to 73% of the cell population, the percentage of C3 receptor-mediated phagocytosis from 4% to 43%, and the phagocytic capacity of individual cells 2.5 times. Less marked effects were observed for IgG Fc receptors. Pinocytosis of horseradish peroxidase was stimulated significantly by Brucella LPS. The lysosomal enzymes acid phosphatase and beta-glucuronidase were increased two-fold. These characteristics are all indicative of activation of bovine macrophages by B. abortus LPS. An important point in these studies was that B. abortus LPS acted in concentrations similar to that reported effective for enterobacterial lipopolysaccharides. These studies indicate the Brucella LPS may play a more significant role in disease than previously thought.

A number of activities of murine peritoneal macrophages were studied during the course of infection of mice with Brucella abortus. Significant alterations in macrophage spreading, IgG Fc receptor density/avidity, C3 receptor-mediated phagocytosis, random and chemotactic locomotion and serum lysozyme were observed. These studies are
indicative of significant early and prolonged activation of macrophages during *Brucella* infection. Some areas for future research include:

1. Probably the most pressing need for an understanding of the role of bovine macrophages in brucellosis is the development of a reliable, quantitative assay for phagocytosis and intracellular killing of *Brucella abortus*. Personal experience indicates this will be a rather technically demanding procedure and probably will not be suitable for mass screening of animals.

2. Once a phagocytosis/bactericidal assay is established, the effects of various serum factors on the assay could be investigated, including serum from normal, infected, or vaccinated animals, purified immunoglobulin preparations, and complement.

3. The effects of various states of activation of the macrophages on the performance of the phagocytosis/bactericidal assay should be investigated. The present study suggests that significant alterations of macrophage activities occurs during brucellosis or after interaction with LPS, and it is anticipated these changes would also be reflected in a phagocytosis/bactericidal test.

4. Bactericidal mechanisms of the bovine macrophage that have relevance for *Brucella* should be studied. In recent years, a most exciting development in the study of macrophage physiology has been the demonstration that suitably stimulated macrophages can produce and secrete a number of potentially toxic oxygen metabolites, including hydrogen peroxide, superoxide anion, hydroxyl radical and perhaps
singlet oxygen. The present study has indicated bovine mononuclear phagocytes are deficient in their ability to produce at least one important mediator, lysozyme, and an investigation of their ability to produce oxygen metabolites and other factors would be of interest. In addition, one recent study indicated Brucella may escape bactericidal activity in neutrophils by not stimulating oxygen-dependent pathways, and the relevance of this finding to macrophage-Brucella interactions may be important.

5. The effects of Brucella infection on the ability of macrophages to release a number of factors that could modify the local micro-environment would be interesting. Included would be plasminogen activator, elastase, collagenase, colony stimulating factor, interferon, and prostaglandins. These mediators could play an important role in the pathogenesis of disease. The effects of Brucella LPS on these activities would also be instructive.

6. The effects of a number of the factors described in the second manuscript of this study on macrophage functions, including cytotoxicity for macrophages, would be of interest.
REFERENCES


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