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THE ROLE OF HUMORAL AND CELLULAR FACTORS IN
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OF SALMONELLA TYPHIMURIUM

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THE ROLE OF HUMORAL AND CELLULAR FACTORS IN HEPATIC CLEARANCE AND KILLING OF *SALMONELLA TYPHIMURIUM*

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ABSTRACT

THE ROLE OF HUMORAL AND CELLULAR FACTORS IN HEPATIC CLEARANCE AND KILLING OF SALMONELLA TYPHIMURIUM

By

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Clearance and killing of Salmonella typhimurium was studied in normal, immunized and Corynebacterium parvum-treated animals in the presence and absence of plasma. The perfused liver was the major experimental model used. The initial objective was to evaluate normal and experimentally altered plasma in bacterial trapping and killing. Addition of 5% normal rat plasma to perfusion media increased bactericidal activity in the perfused rat liver. When complement activity was inhibited by heating at 57°C and 50°C, zymosan absorption or chelation with EDTA, bactericidal activity was significantly reduced. EGTA had no effect. Immune plasma heated to inhibit complement did not stimulate bactericidal activity but significantly increased bacterial trapping by the liver. Absorption of normal and immune plasma with a specific anti-rat-C3-Sepharose 4B immunoabsorbant inhibited bactericidal activity. These results demonstrate that complement is required for bactericidal activity and is activated via the alternate in normal and via the classical pathway in immune plasma.

A second objective was to examine experimentally altered livers. Livers from rats immunized with heat-killed S. typhimurium were not

statistically different from normal livers in bactericidal activity or distribution of recovered bacteria in the absence or presence of plasma. When the RES of mouse and rat livers was activated with C. parvum vaccine 22% of the perfused bacteria were killed in the absence of plasma. Silica and phenylbutazone inhibited in situ hepatic killing while EDTA had no effect suggesting that bactericidal activity reflected cellular killing. The addition of plasma did not significantly increase bactericidal activity over normal livers in the presence of plasma. Scanning electron microscopy of C. parvum-treated livers revealed large numbers of white blood cells mostly macrophages and lymphocytes, adhering to portal veins and sinusoids. These macrophages were morphologically similar to Kupffer cells. A relative peripheral blood monocytosis occurred simultaneously. Presumably the blood cell influx into the liver plays an important role in the enhanced nonspecific antimicrobial resistance observed in C. parvum-treated animals. In vivo, C. parvum-treated mice were able to clear and kill I.V. injected S. typhimurium better and had a ten-fold increased resistance.

Cumulatively the data shows that C. parvum vaccine enhances the ability of the liver to trap and kill bacteria in situ and increases clearance and resistance in vivo. Livers from immune animals behaved similar to normal livers.

To my wife, Ellen, for her love, perseverance and understanding during these past years. This degree is as much hers as it is mine.

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INTRODUCTION

The relative role of humoral and cellular factors in the antimicrobial properties of the liver is not well defined. The liver Kupffer cells make up a major percentage of the reticuloendothelial system in the body and are a key initial cellular defense barrier against microbial invasion of the blood (44, 92). Studies by Moon et al. (61) suggest that the removal of bacteria by hepatic tissue involves both phagocytic and non-phagocytic parameters of the liver and that plasma is obligatory for bactericidal activity. The initial observation that non-phagocytic cells play an important role in microbial filtration by hepatic tissue was extended by Friedman and Moon (35) who demonstrated that while treatment with the macrophage toxin, silica (4, 51), decreases bacterial trapping by 50% it did not abolish it.

The initial objective of this thesis is to confirm and extend earlier studies suggesting a role for both complement and antibody in the antimicrobial activities of the liver (13, 43, 59, 60, 117). Research progress, particularly in the complement system, warrants a more critical evaluation of the role of humoral elements in bacterial killing at this time.

A second objective is to describe the relative roles of antibody and complement in trapping of bacteria by both normal and immune livers.

A third objective involves a study of livers from C. parvum-treated animals. C. parvum is a macrophage activator (41, 98, 118) which in the course of our experiments, was found to induce bactericidal activities of liver in the absence of plasma. This observation led to additional investigation to determine more of the nature and biological significance of C. parvum induced changes in vivo, particularly with respect to bacterial infection.

LITERATURE REVIEW

The reticuloendothelial system (RES) is the major cellular defense barrier against microbes which enter the bloodstream (44, 92). It consists of fixed phagocytic cells including liver Kupffer cells, splenic macrophages, microglial cells of the brain, pulmonary alveolar macrophages and lymph node macrophages. In addition to clearance of microbes, the RES also functions in hemoglobin uptake and breakdown (39), protein transport (48), uptake and metabolism of steroids and lipids (9, 25), degradation of red blood cells (7), clearance of autologous tissue debris (116), endotoxin uptake and detoxification (32), phagocytosis of foreign colloidal and particulate material from the blood (11, 111), antigen processing (34) and immunity and destruction of tumor cells (8, 50).

As early as 1886 Wyssokowitsch (125) demonstrated that bacteria and fungal spores injected intravenously into rabbits were cleared by specific endothelial cells in the liver and spleen. Bull (15) in 1915 studied the fate of intravenously injected Salmonella typhi into rabbits. Fifteen minutes after injection the number of bacteria per ml of blood dropped from 10^7 to 40 bacteria per ml. Bull found that 21 minutes after injection, the majority of the S. typhi could be recovered from the spleen, liver and lung. He concluded that bacilli accumulated mainly in the liver and spleen and were taken up by assembled PMN (RES cells) and destroyed.

Rogers categorized bloodstream clearance of bacteria (92) into three phases. Initially, when large numbers of viable bacteria were injected I.V. into rabbits, 99.9% were cleared in the first ten minutes to 5 h, the rate depending on the organism being studied. This rapid clearance phase was followed by a period when microbes persisted in the circulation due to slower removal rates. A final phase occurred in which bacteria either disappeared or multiplied to high levels leading to death. Rogers states that the fixed phagocytic cells sequestered the majority of the bacteria during the initial rapid clearance phase. Kupffer cells and splenic macrophages were most active in this process. Sixty to 95% of the bacteria were sequestered in these organs. This thesis primarily evaluates Kupffer cell function during the early clearance phase.

Kupffer cells reside in the lumen of hepatic sinusoids adhering to the fenestrated endothelium by fine cytoplasmic filopodia (14, 62-64, 123). They occupy a large area in the lumen of the sinusoid and are usually found at sinusoidal junctions. The body of the Kupffer cell is covered by numerous folds, ruffles, invaginations and microvilli (60). Thirty-eight percent of the total number of liver cells are Kupffer cells (45).

The liver perfusion model offers a unique opportunity to study the interaction of cellular and humoral factors in bacterial trapping and killing by the liver. In 1916 Manwaring and Coe (59) did rabbit liver perfusions with pneumococci. Bacteria suspended in Ringer solution or Ringer plus 1-10% normal sera were not cleared by the liver. When 1% immune sera was added to the perfusion media, the liver retained all bacteria that were perfused. The serum component

involved was heat-stable at 60°C for 30 minutes (antibody) and was called an endothelial opsonin. Manwaring and Fritschen (60) did further perfused liver experiments using Staphylococcus aureus, E. coli and Bacillus anthracis with similar results. Howard and Wardlaw (43) found that human, rat and mouse sera increased clearance of E. coli by the perfused rat liver when added to the perfusion media. The opsonic activity of sera was reduced by heating at 56°C for 30 minutes, absorption with homologous strain of bacteria or with an antigen-antibody complex. Opsonic activity was abolished by homologous absorption followed by heating at 56°C or absorption with zymosan. They concluded that the serum factors involved in clearance of bacteria by the liver were specific antibody, complement and probably properdin. In the experiments, the actual number of viable bacteria in the perfused livers was not determined, so what effect these serum treatments had on actual bacterial killing is not known. Experiments were also done using other strains of gram negative and gram positive bacteria (117). The presence of serum enhanced bacterial clearance of all the gram negative organisms studied and heating serum reduced this activity. The gram positive bacteria (S. aureus, Strep. pyogenes, B. cereus and C. murium) were cleared in the absence of serum and its presence reduced their clearance.

Bonventre and Oxman (13) studied the role of humoral and cellular factors on clearance and killing of Staphylococcus aureus and Salmonella enteritidis by the perfused rat liver. Their results indicate the immunological status of the liver or of the serum had no effect on clearance or killing of S. aureus. Experiments with S. enteritidis showed that the presence of immune humoral and/or cellular factors

profoundly affected the removal of bacteria by the liver. When normal livers were perfused with immune serum the rate of clearance of S. enteritidis was greatly increased. In the non-immune liver perfusion system, 200% of the S. enteritidis perfused were recovered in the liver as determined by bacterial plate counts. In the presence of immune serum bacterial multiplication did not occur and with both immune liver and serum only 5% of the infused bacteria were recovered from the liver. The above mentioned studies suggest that antibody and complement play a role in bacterial trapping and killing in the liver. More specific treatments of normal and immune serum or plasma must be done to critically determine the role of these humoral factors in the liver's interaction with bacteria.

Complement is critically involved in immune processes and also interacts with the kinin, clotting and fibrinolytic systems (88). Complement is also involved in phagocytosis of foreign matter, neutralization of viruses by antibody, bactericidal reactions, immune adherence, chemotaxis and histamine-regulated vascular permeability (16, 38, 93). The proteins that make up the complement system account for 5-10% of the weight of all plasma proteins (20). Complement is synthesized by RES cells in the liver, intestine, bone marrow, spleen, lung, lymph nodes and macrophages (20).

The critical step in generation of the biological activities of the complement system is the production of the major cleavage fragment of C3, C3b. Both the classical and alternate complement pathways can cleave C3 to yield C3b but both do it by different mechanisms (31). In the classical pathway, activation of complement (C) is initiated by an antigen-antibody complex that binds and converts C1 to its activated

state $C\overline{1}$ (56) which then cleaves C4 (65) and C2 to form the classical C3 convertase, $C\overline{142}$ (66) which cleaves C3 to produce C3a and C3b. The alternate pathway of complement activation was discovered when it was observed that zymosan, an insoluble polysaccharide-containing derivative of yeast cell walls, inactivated serum C3 without depletion of C1, C4 or C2 (84). Alternate pathway activity requires low levels of C3b which is produced by interaction of C3 and B in the presence of $\overline{D}$ or P to form the amplification C3 convertase, C3bBb (22, 30, 31) which is stabilized by properdin (P) (30). C3b acts as a receptor for B in a Mg^{++}-dependent reaction that exposes a site on B to cleavage by $\overline{D}$ (31). $\overline{D}$ causes release of Ba fragment from B to uncover the C3-cleaving site in the Bb fragment that remains bound to C3b (31).

Once C3b is produced by either pathway it can be involved in a number of biological activities. Foremost of these is C3b interaction with C5 to generate the C5b6789 cytolytic complex (54) which can lyse red blood cells or bacteria. C3b is also involved in immune adherence (71), enhances phagocytosis of C3b coated particles (10), induces secretion of lysosomal enzymes by cultured peritoneal macrophages (101), secretion of a chemotactic factor by B lymphocytes (96) and is involved in lymphocyte differentiation (82).

Data reported in this thesis point to a critical role of C3 in the bactericidal properties of perfused livers in the presence of plasma. Little is known about rat C3, while human (73), guinea pig (105) and murine C3 (83) have been isolated and characterized. Recently Daha et al. (23) isolated and characterized C3 from rat plasma by precipitation with polyethyleneglycol in the presence of benzamidine which inhibited proteolytic enzymes and kept C3 functionally active

during chromatographic purification. The percipitate was chromatographed on CM-cellulose, hydroxyapatite and QAEA50-Sephadex, and gel filtered on Sephadex G-200 superfine. Recovery of C3 was 18-26% and the material was homogeneous on SDS-PAGE analysis. Rat C3 has a molecular weight of 187,000, which is similar to human, mouse and guinea pig C3. It is composed of 2 disulphide linked polypeptide chains of 123,000 and 76,000 daltons. The isolated protein was proven to be rat C3 by its ability to react with EAC142 and cause subsequent blood cell lysis. In the assay rat and human C3 functional activities were similar. The plasma concentration of rat C3 in Wistar rats was calculated to be 581±49 μg/ml. Rat C3 was able to react with human components of the alternate pathway to form the C3 convertase, showing compatibility between rat and human C3.

In vitro studies on isolated Kupffer cells by Munthe-Kaas et al. (67, 68) demonstrated that they have C3b receptors. C3b-coated SRBC attached to Kupffer cells but less than 20% were phagocytosed in the absence of serum. When cultures were rinsed and incubated in media containing 30% newborn calf serum, the majority of the attached red blood cells were phagocytosed. Mouse peritoneal exudate cells did not phagocytose C3b-coated SRBC when incubated with newborn calf serum. The C3b receptor was sensitive to treatment with trypsin and pronase as was the C3b receptor of peritoneal cells. The ability of Kupffer cell C3b receptors to induce phagocytosis is unique and does not occur in other mononuclear cells (58). In vivo studies of clearance and destruction of erythrocytes by Schreiber et al. (75) suggest that Kupffer cell C3b receptors are involved.

Kupffer cells also have Fc receptors for IgG (47, 67-69). Huber et al. (47) using red blood cells sensitized with IgG, demonstrated attachment and phagocytosis of the RBC in more than 90% of the isolated human Kupffer cells. Munthe-Kaas et al. (67-69) demonstrated Fc receptors on rat Kupffer cells in vitro. Seventy-five percent of the cultured cells by two days and 96% by six days had ingested IgG-coated SRBC. At 4°C, IgG-coated SRBC attached but were not internalized and upon transfer to 37°C, phagocytosis occurred. Peak phagocytosis of IgG-coated SRBC occurred within 30 minutes after their addition to Kupffer cell cultures. Kupffer cell Fc receptors were sensitive to trypsin and mercaptoethanol.

The role of Kupffer cells in trapping and killing of bacteria in the liver has not been well characterized. Early work by Manwaring et al. (59, 60) and Howard and Wardlaw (43, 117), using the perfused liver, studied clearance of bacteria from perfusion media. Any bacteria not recovered from the media after perfusion through the liver were considered phagocytosed and killed by Kupffer cells, but no direct quantitation of viable bacteria in the liver was done.

Moon et al. (61) recently showed that clearance of S. typhimurium by the perfused mouse and rat liver was not synonymous with phagocytosis and killing by Kupffer cells. No killing of S. typhimurium occurred in the perfused liver when the perfusion media contained only M-199 but greater than 70% of the bacteria were cleared in a single pass. By phase, electron and scanning electron microscopy (35, 61), bacteria appeared trapped in the liver sinusoids giving a "log-jam" appearance. Fifty-percent of the trapped bacteria were killed when blood or plasma was added to the perfusion media. No killing occurred when bacteria

were incubated with blood or plasma alone. These experiments demonstrate that bacterial clearance in the perfused liver may not reflect phagocytosis but actually trapping of bacteria in liver sinusoids. These results suggest a number of research questions regarding the mechanism of bacterial trapping by Kupffer cells.

In an earlier study Friedman and Moon (35) evaluated the effects crystalline silica had on bacterial trapping by the perfused liver. The working hypothesis was that destruction of Kupffer cells should significantly decrease trapping. Crystalline silica is a specific macrophage toxin (4, 51). When taken up by macrophages it interacts with lysosomal membranes making them permeable. Lysosomal enzymes leak into the cytoplasm and cause death of the cell (4, 42, 70). Normal mouse livers trapped 63.5% of an infused dose of S. typhimurium with 42.3% recovered in the effluent. Silica-treated livers trapped only 31.1% with 65.9% recovered in the effluent (35). Scanning electron microscopy revealed that silica caused damage and destruction of Kupffer cells but had no other histotoxic effects on the liver (35). Destruction of Kupffer cells significantly decreased bacterial trapping by the liver, but still liver bacterial trapping occurred. These experiments indicate that maximal bacterial trapping by the liver devoid of most Kupffer cells can still trap bacteria in sinusoids, which demonstrates that bacterial trapping also involves non-Kupffer cell components of the liver.

Another tool used to study the role of Kupffer cells in bacterial-liver interactions is phenylbutazone (PB), an anti-inflammatory drug that inhibits phagocytosis (55, 108, 110) and intracellular killing (77, 108, 110). Whitehouse (121) observed that 2.5×10^{-4} M PB inhibited

phosphorylation coupled to succinate oxidation in rat liver mitochondria. PB uncouples oxidative phosphorylation (production of ATP) without inhibiting oxidative metabolism and cellular respiration. Strauss et al. (110) studied the effect of PB on phagocytosis and intracellular killing by guinea pig PMN. PB inhibited intracellular killing and uptake of E. coli by PMN. When 5 μ moles/ml of PB were added to PMN homogenates bactericidal activity was inhibited. When PB was added to PMN in the process of killing bacteria, killing was immediately stopped. These results demonstrate that PB inhibits killing by an effect on intracellular activities and shows that inhibition of phagocytosis and bactericidal activity are independent events. Metabolic studies showed that the drug inhibited glucose-1-^{14}C and ^{14}C-formate oxidation of both resting and phagocytizing PMN which suggest an effect on hexose monophosphate shunt and H_2O_2 formation. The study also showed that PB inhibits glucose-6-phosphate and 6-phosphogluconate dehydrogenase activity. Similar effects of PB on human PMN (108) and monocytes (77) have been observed.

Work by Leijh et al. (55) demonstrated that 1 mM PB inhibited the intracellular killing of C. albicans by human PMN and blood monocytes and had no effect on phagocytosis. Kjosen et al. (53) verified this by showing that low levels of PB inhibit intracellular killing of bacteria by PMN by blocking the hexose monophosphate shunt (required for bacterial killing) and not the Embden Myerhoff glycolytic pathway (required for phagocytosis).

Another method to evaluate the role of Kupffer cells in bacterial trapping and killing is to stimulate RES cell activity. Halpern et al. (41) while examining the genus Corynebacterium for species related to

mycobacteria in their ability to stimulate the RES, observered that C. parvum was a powerful macrophage activator. C. parvum causes a vast array of biological responses in vivo and in vitro. Injection of C. parvum I.V. causes enlargement of liver, spleen, and lung and increases carbon clearance (2, 17, 57), transient anaemia (57), adjuvant effect on antibody response (46, 79, 106) and stimulates RES activity (98, 118). C. parvum depresses T-cell-mediated immune phenomena which include delayed hypersensitivity (19, 99), phytohemagglutinins, mixed lymphocyte, graft-v.s.-host (100) and homograft responses (17). C. parvum vaccine also has anti-tumor activity (98, 113, 114, 124).

Treatment of experimental animals with C. parvum enhances their resistance to Listeria monocytogenes (6, 95, 112), Brucella abortus (1), Salmonella enteritidis (19), Staphylococcus aureus (103), Herpes Simplex virus (52), Plasmodium berghei (76), T. gondii (112) and C. albicans infection (103). C. parvum treatment reduces resistance of mice to Aspergillus nidulans infection and leads to fatal murine aspergillosis (87). C. parvum exerted an immunosuppressive effect on Trichinella spiralis infections in rats and prolonged the infection (94). Treatment of rabbits with C. parvum did not enhance resistance to infection with Treponema pallidum (6).

In vitro and in vivo studies show that C. parvum stimulates immunological activities via direct interaction with lymphocytes and RES cells (12, 18). In vitro, Christe and Bonford (18) showed that oil-induced CBA mouse peritoneal macrophages could be activated by contact with C. parvum alone, while normal macrophages required simultaneous exposure to C. parvum and spleen cells from mice immunized with C. parvum. Treatment of immune spleen cells with anti-theta serum and

complement inhibited activation. They observed *in vivo* that T-cell depleted mice were able to respond to *C. parvum*, in demonstrating macrophage activation and splenomegaly, but the ability of spleen cells to activate normal macrophages *in vitro* was reduced (12). From these experimental results Christe and Bonford concluded that *C. parvum* activated macrophages by both direct and immunological mechanisms, i.e. via T-cells.

The immunopotentiating activity of *C. parvum* seems to reside in the phospholipid of the bacteria (27). Fauve and Hevin found that bacterial phospholipid extracts of *C. parvum* injected into mice enhanced blood clearance of *S. typhimurium* (27). The extract also increased resistance to *L. monocytogenes* infection and inhibited multiplication of the organism in the liver and spleen (27).

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats, 300 to 350 g were obtained from Harlan Industries (Indianapolis, Ind.). Female Carworth CF-1 mice weighing 18 to 25 g were obtained from Charles River, Wilmington, Delaware. All animals were maintained under standard laboratory conditions with Purina Laboratory Chow and water available *ad libitum*.

Bacteria

Eighteen to 24 h cultures of *Salmonella typhimurium*, strain SR-11, were grown in brain heart infusion broth and centrifuged at 3,000 x g for 15 minutes. The bacteria were resuspended in either M-199 (Gibco, Grand Island, N.Y.), Ca^{++} and Mg^{++} free Hanks balanced salt solution or sterile saline.

Corynebacterium parvum

Corynebacterium parvum vaccine was supplied as a gift by Dr. Richard Tuttle, Burroughs Wellcome Co., Research Triangle Park, N.C. The two lots of vaccine (CA528A and CA580A) used throughout this study were formalin killed suspensions supplied at a concentration of 7 mg dry weight/ml containing thiomersal. Both lots were washed three times in sterile saline to remove the preservative and stored at 4C. No variation in response between vaccine lots was observed.

Rats were injected with 350 μg of C. parvum intravenously two days before examination. Mice were injected with 700 μg C. parvum nine days before examination.

Chemicals

Dörenturp crystalline silica (DQ12), particle size 5 μm or less, was supplied by Dr. Ing M. Reisner, Steinkohlenbergbauereiw, 43 Essen-Kray, Frillendolfer Strabe 351, W. Germany. Before injection it was autoclaved in powder form, suspended in sterile saline at a concentration of 20 mg/ml, and sonicated in a Bronsonic ultrasonic cleansor (no. B220, Branson Instruments Co., Sketon, CT). Silica was given to mice six days after C. parvum injection. A total of 10 mg was given intravenously over a three day period, i.e., 3 mg on days six and seven and 4 mg on day eight. Mice were studied on the ninth day after C. parvum injection.

Phenylbutazone (Lot #127C-0083, Sigma Chemical Co., Columbus, OH) was dissolved in 95% ethanol at a concentration of 10 mM and added to M-199 to yield a final concentration of 1 mM (pH adjusted to 7.3 by 1N NaOH). The solution was filter sterilized. Experimentally, 1 ml of 1 mM phenylbutazone was infused into C. parvum-treated mouse livers and the livers washed for 20 minutes with M-199 prior to infusion of bacteria.

EDTA (disodium ethylenediaminetetraacetate) (Matheson Coleman and Bell, Cincinnati, OH) was added to Ca^{++} and Mg^{++} free Hanks balanced salt solution (HBSS) at a concentration of 0.01 M and the pH readjusted to 7.3 by 1N NaOH.

Rat plasma

Blood was obtained from heparinized rats by cardiac puncture and centrifuged to obtain plasma. Plasma was pooled and stored at -70°C until use. Immune rat plasma was obtained from rats immunized with heat-killed S. typhimurium vaccine. The vaccine was made by heating BHI cultures of S. typhimurium at 65°C for 1.5 h, centrifuged and washed three times in sterile saline and resuspended in saline at 1 x 10^{10} bacteria/ml. Rats were immunized via IP injections of 1 ml of a 1/100 dilution of vaccine on day one and then injected with 1 ml of vaccine 4, 8, 12, and 16 days later. Immune plasma was pooled and used in experiments. The tube agglutination titer of pooled immune plasma to S. typhimurium was 6,400.

Rat plasma treatments

Zymosan Type A (Sigma Chemical Co., Cleveland, OH) was prepared by the method of Fine et al. (33). Zymosan was boiled in normal saline, centrifuged and washed and stored in saline at -20°C at a concentration of 2 mg/ml. Zymosan was added to plasma at 2 mg/ml and incubated for 1 h, 37°C and removed by centrifugation at 3000 x g for 15 minutes. EGTA (ethyleneglycol-bis-(β-aminoethyl ether) N, N'-tetraacetic acid) (Sigma Chemical Co., Cleveland, OH) and EDTA were used as chelating agents. EDTA and EGTA with $MgCl_2$ (Mg EGTA) were added to rat plasma at a concentration of 0.01 M and pH was adjusted to 7.3 with NaOH. EDTA inhibits the classical and alternate pathways of complement (107) while EGTA inhibits only the classical pathway (24, 33, 107). Rat plasma was heated to 57°C for 1 h to destroy complement activity (104) or to 50°C for 30 minutes to inhibit alternate pathway activity (40).

The effect of these treatments on hemolytic complement activity are presented in Tables 2 and 6.

In vitro liver perfusion

Procedures for animal surgery and liver perfusion have been described in detail (61, 97). Briefly, in both rats and mice, the portal system was exposed by a midline abdominal incision. The portal vein was cannulated efferently and the cannula secured by ligatures. The thorax was exposed and the inferior vena cava was cannulated above the hepatic vein. The inferior vena cava was closed by a ligature placed above the right renal vein and livers were perfused with sterile M-199 or HBSS to remove gross blood. After washing, 1 ml of S. typhimurium was slowly infused and followed immediately by perfusion media. The effluent was collected from the efferent cannula into a sterile graduated cylinder. Perfusions lasted 30 minutes and were done at room temperature. The liver was disconnected from the perfusion apparatus and homogenized in sterile distilled water and the collected effluent was also blended.

Quantitative tryptose agar pour plates were made from the homogenate to determine the number of trapped bacteria which remained viable in the liver and on the effluent to determine the number not trapped in the organ. The percentage of viable bacteria trapped in the liver plus the percentage of recovery in the effluent could then be subtracted from the percentage of bacteria infused (100%) to give the percentage of killing.

Distribution and survival of S. typhimurium in mice

Mice were killed 15 or 60 minutes after intravenous injection of 1.0×10^9 S. typhimurium. The liver, spleen and lungs were removed and homogenized with a Teflon and glass homogenizer in 9 ml of sterile distilled water. The carcass, excluding the stomach, intestinal tract, skin, paws and tail was homogenized in 99 ml of distilled water in a Waring blender for four minutes. Quantitative pour plates of all homogenates were prepared.

S. typhimurium infection in normal and C. parvum-treated mice

Normal and C. parvum-treated mice were infected by intravenous injection of 4.1×10^5 to 4.1×10^7 S. typhimurium. Survival was observed for two weeks. The LD_{50} for normal and C. parvum-treated mice was determined by the method of Reed and Muench (89).

Total (WBC) and differential white blood cell counts

Leukocyte WBC from rats and mice were performed using Becton, Dickinson Unopettes for manual white blood cell enumeration. Cells were counted in an improved Neubauer chamber. Differential white cell counts were determined on air dried peripheral blood smears. Blood was obtained by cardiac puncture in rats or from the retroorbital plexus of mice. Slides were stained with Wright stain. Monocytes, polymorphonuclear leukocytes and lymphocytes were expressed as a relative percentage of 100 total cells counted.

Anti-C3-Sepharose 4B immunoabsorbent

Goat anti-rat C3 sera was provided by Dr. Jeffrey Williams. Its specificity for rat C3 was verified by immunoelectrophoresis against

normal rat sera in which only a precipitin line to C3 was obtained. The anti-rat-C3 antibody fraction of the sera was isolated by precipitation with 50% $(NH_4)_2SO_4$ two times. The precipitate was resuspended in 0.1 M PBS and dialyzed until no $(NH_4)_2SO_4$ remained.

The Sepharose 4B immunoabsorbent was prepared by the method of Cuatrecasas and Anfinsen (21) with modification. The antibody fraction from the anti-C3 sera was added to CNBr-activated Sepharose 4B at a concentration of 10 mg protein/ml of gel in coupling buffer ($NaHCO_3$ buffer, 0.1 M, pH 8.3; containing 0.5 M NaCL) and mixed for 2 h at room temperature. The gel was washed with coupling buffer and excess active groups on the gel were blocked with 1 M ethanolamine (pH 9.0) and then the gel was washed with coupling buffer and acetate buffer (0.1 M, pH 4.0 with 0.5 M NaCl) three times. The prepared immunoabsorbant was stored at 4°C in 0.1 M PBS with 0.01% sodium azide. A control Sepharose 4B immunoabsorbant was also made with anti-Salmonella H antigen antibody in the same manner.

Normal and immune plasma was absorbed two times with the anti-C3-4B absorbant by adding 20 ml of plasma to 10 ml of centrifuged absorbant in a capped plastic centrifuge tube and mixed at room temperature for 1 h. The absorbed plasma was recovered by pelleting of the immunoabsorbant at 11,000 x g for ten minutes. The used anti-C3-4B was re-activated by washing with glycine-HCl (0.2 M, pH 2.8 and 0.2 M, pH 2.2) and used again.

Assay for hemolytic complement

Hemolytic C titers were measured in CH_{50} units following a modification of Kabat and Mayer (49) on untreated and treated rat

plasmas (Table 1). Sheep erythrocytes (Colorado Serum Co., Denver, CO) were sensitized with rabbit anti-SRBC hemolysin (Microbiological Assoc., Walkersville, MD). Sensitized calls (EA) in 0.5 ml of Veronal-buffered saline containing 1.5×10^{-4} M Ca^{++} and 5×10^{-4} M Mg^{++}, pH 7.4 were added to 0.5 ml of rat plasma dilutions. The tubes were incubated at 37°C for 1 h and then 2 ml of buffer were added to each and centrifuged at 1000 x g for ten minutes and the absorbance read at 541 nm.

TABLE 1. Bacterial trapping and killing by the perfused rat liver in the absence or presence of rat plasma.[a]

	Recovery (%)	
Experimental	M-199	Plasma
Liver	61.1 ± 9	27.8 ± 5[b]
Effluent	40.4 ± 8	36.1 ± 10
Total	101.5 ± 12	62.1 ± 14[b]
Killing	0	37.7[b]

[a]Average ± standard deviations from at least six separate experimental determinations.

[b]$P < 0.001$.

Immunodiffusion

Double diffusion experiments were used to detect levels of C3 in untreated and treated rat plasmas. The tests were done on microscope slides with a 3 ml layer of 1.5% agarose (L, Industrie Biologique Francaise) in barbital buffer (pH 8.6). In the test, anti-rat C3 sera was placed in the center well and dilutions of rat plasma were placed in the surrounding wells. The immunodiffusion plates were read at 24 and 48 h.

Scanning electron microscopy

Perfused livers from C. parvum-treated animals were prepared for SEM by the methods of Friedman and Moon (35) for mice and Sawyer et al. (97) for rats. After livers were washed free of blood the upper reservoir of the perfusion apparatus was filled with fresh warm 2% glutaraldehyde (Eastman Kodak, Rochester, NY) in sterile 0.2 M sodium phosphate buffer (2% GA-PB) at pH 7.4, and 100 ml (rats) or 50 ml (mice) was perfused in 30 minutes. Fixed livers were excised, cut into small blocks, and allowed to stand in 100 ml fresh cold 2% GA-PB overnight. Blocks were dehydrated in sequential 30 minute steps with 10, 20, 40, 70, 90, 95, and 100% ethanol. The blocks were allowed to stand overnight at 4°C in a fresh change of 100% ethanol. The dehydrated blocks were cryofractured in liquid nitrogen (78). Fractured tissue was placed in metal baskets under liquid nitrogen and dried in an Omar SPC 90/EX critical point dryer using CO_2 as the carrier gas. The specimens were coated with gold (200-300Å) using the EMS 41 Minicoater (Film Vac Inc., Englewood, NJ) and viewed in an ISI Super Mini II SEM. Micrographs were made using Polaroid Type 665 positive/negative film.

Statistics

Where appropriate, data were evaluated by the White rank order method (122).

RESULTS

Trapping and killing of S. typhimurium by perfused rat livers in the absence or presence of normal rat plasma

Normal rat livers (Table 1), trap an average of 61.1% of 1-2 x 10^9 dose of S. typhimurium on a single pass. An average of 40.4% of the bacteria were recovered in the effluent for a total recovery of over 100%. No bacterial killing occurred. When normal rat plasma was added to both bacteria and M-199 (5 ml plasma in 95 ml M-199) prior to perfusion, an average of 27.8% of the bacteria were recovered in the liver and 36.1% in the effluent for a total recovery of 62.1% (Table 1). It is presumed that the 37.7% of the bacteria not recovered were killed by the liver. No bacterial killing occurred when S. typhimurium was incubated with normal plasma and perfused through the perfusion apparatus without a liver attached.

Effect of heat, zymosan and chelators on bactericidal activity of plasma in the perfused liver

Normal plasma heated at 57°C or treated with zymosan or EDTA lost hemolytic complement activity (Table 2). Heating at 57°C for 1 h destroyed most of the ability of plasma to stimulate bactericidal activity in the perfused liver. A total of 92.4% of the bacteria were recovered in the liver and effluent, indicating only 7.6% of the bacteria were killed (Table 3). Zymosan treatment also diminished the ability of plasma to stimulate bactericidal activity. A total of 92.7%

TABLE 2. Percentage of depression of total hemolytic complement levels in treated normal rat plasma.

Treatment	% decrease in CH_{50}
Normal	0
57°C	100
Zymosan	69
EDTA	100
50°C	34
EGTA	93
Anti-C3 absorbed	54
Anti-SalH absorbed	0

[a]Average value of at least three separate experimental determinations.

TABLE 3. Role of complement in bacterial killing by perfused rat livers.[a]

	Recovery (%)			
Experimental	Normal Plasma	57°C	Zymosan	EDTA
Liver	27.8 ± 5	40.9 ± 8[b]	47.6 ± 8[b]	37.4 ± 10
Effluent	36.1 ± 10	51.5 ± 9[d]	45.1 ± 12	62.3 ± 10[b]
Total	62.1 ± 14	92.4 ± 7[d]	92.7 ± 18[c]	99.7 ± 8[b]
Killing	37.7	7.6[b]	7.3[c]	0.3[b]

[a]Average ± standard deviation from at least six separate experimental determinations.

[b]$P < 0.001$ vs. normal plasma.

[c]$P = 0.01$ vs. normal plasma.

[d]$P = 0.05$ vs. normal plasma.

of the bacteria were recovered in the liver and effluent, indicating only 7.3% of the bacteria were killed (Table 3). Addition of 0.01 M EDTA to rat plasma and the perfusion media (Ca^{++} and Mg^{++} free HBSS) also inhibited the ability of plasma to stimulate bactericidal activity. In this instance a total of 99.7% of the bacteria were recovered indicating negligable bacterial killing (Table 3). With EDTA treatment there were significantly increased numbers of bacteria recovered in the effluent. Perfusion of rat livers with HBSS gave the same bacterial distribution as the M-199 control (data not shown).

To evaluate the relative roles of the classical and alternate complement pathways, plasma was heated to 50°C for 30 minutes thereby destroying alternate pathway activity (37). Heating at 50°C diminished hemolytic complement activity by 34% (Table 2). This treatment also significantly inhibited the ability of plasma to stimulate bactericidal activity in the perfused liver (Table 4). A total of 87.2% of the bacteria were recovered in the liver and effluent, indicating only 12.9% were killed. By contrast addition of EGTA, which specifically inhibits the classical complement pathway (24, 33, 107), did not inhibit the bactericidal activity of the perfused liver (Table 4). The percent bacteria recovered in liver and effluent were not statistically different from perfusions with untreated plasma (Table 1).

Effect of immunoabsorption of C3 on the ability of normal rat plasma to stimulate bactericidal activity in the perfused liver.

Normal plasma was absorbed twice with an anti-rat-C3-Sepharose 4B immunoabsorbant (anti-C3-4B). By immunodiffusion anti-C3-4B absorption decreased the C3 titer of normal plasma from 32 to 8 (Figure 1). Anti-C3-4B absorbed plasma produced a 54% depression of hemolytic

TABLE 4. Differentiation of classical and alternate complement pathway activity in mediating plasma killing in the perfused liver.[a]

	Recovery (%)		
Experimental	Normal Plasma	50°C	EGTA
Liver	27.8 ± 5	44.0 ± 16	31.6 ± 9
Effluent	36.1 ± 10	44.3 ± 8[b]	41.9 ± 10
Total	62.1 ± 14	87.2 ± 19[b]	73.5 ± 10
Killing	37.7	12.9[b]	26.5

[a]Average ± standard deviations from at least six separate experimental determinations.

[b]P = 0.05 vs. normal plasma.

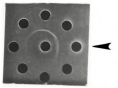
activity (Table 2). A control Sepharose 4B immunoabsorbant made with anti-Salmonella H antigen antibody (anti-Sal H-4B) did not alter C3 titer (Figure 1). Anti-Sal H-4B absorbed plasma had no loss of hemolytic complement activity (Table 2). Anti-SalH-4B absorbed plasma lost some of its ability to stimulate bactericidal activity in the perfused liver, decreasing killing from 37.7% to 23.6% (Table 5). Normal plasma absorbed with anti-C3-4B resulted in significant inhibition of bactericidal activity in the perfused liver when compared to both anti-SalH-4B absorbed and normal plasma (Table 5). A total of 93.6% of the bacteria were recovered with 47.3% from the liver and 46.3% in the effluent. Only 6.4% of the bacteria were killed while unabsorbed plasma killed 37.7%.

Bactericidal activity of perfused livers in the presence of immune plasma

Immune rat plasma, having a tube agglutination titer of at least 6,400, was raised in rats by immunization with heat-killed S.

FIGURE 1. Immunodiffusion slides of C3 titers from normal and immune plasma before and after anti-rat-C3 immunoabsorption. (A-E) Center wells contain goat-anti-rat-C3 serum. Outside wells contain serial two-fold dilutions of plasma starting at arrow going counterclockwise; 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256. (A) Normal plasma, titer 1/32, (B) Anti-C3-4B absorbed normal plasma, titer 1/8, (C) Anti-SalH-4B absorbed normal plasma, titer 1/32, (D) Immune plasma, titer 1/64, (E) Anti-C3-4B absorbed immune plasma, titer 1/16.

A



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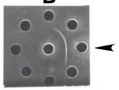

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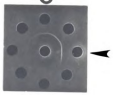

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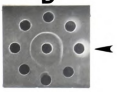

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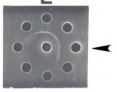


TABLE 5. Immunoabsorption of C3 from rat plasma and its effect on plasma's bacterial killing in the perfused liver.[a]

	Recovery (%)		
Experimental	Normal Plasma	Anti-SalH-4B Absorbed Plasma	Anti-C3-4B Absorbed Plasma
Liver	27.8 ± 5	57.1 ± 8[d]	47.3 ± 11[c,e]
Effluent	36.1 ± 10	19.3 ± 7[e]	46.3 ± 14[b]
Total	62.1 ± 14	76.4 ± 10[f]	93.6 ± 11[b,e]
Killing	37.7	23.6[f]	6.4[b,d]

[a]Average value ± standard deviation from at least six separate experimental determinations.

[b]$P = 0.01$ vs. anti-SalH-4B absorbed plasma.

[c]$P = 0.05$ vs. anti-SalH-4B absorbed plasma.

[d]$P < 0.001$ vs. normal plasma.

[e]$P = 0.01$ vs. normal plasma.

[f]$P = 0.05$ vs. normal plasma.

typhimurium. When immune plasma was either incubated in vitro with S. typhimurium for 30 minutes or perfused with bacteria through the perfusion apparatus in the absence of a liver, 70% of the bacteria were killed (Table 6). Heating immune plasma at 57°C for 1 h, treating with zymosan, EGTA or absorption with anti-C3-4B (Figure 1) blocked bactericidal activity in vitro and significantly depleted hemolytic complement activity (Table 6).

Since immune plasma alone could kill bacteria, perfusion experiments evaluating bactericidal activity of this material could not be interpreted with confidence. It did appear, however, that untreated immune plasma increased trapping. Table 7 shows that untreated immune

TABLE 6. Effect of immune plasma treatment on bactericidal activity *in vitro* and CH_{50} titers.[a]

Treatment	% viability *in vitro* after 30 min	% depression CH_{50} titers
None	30.0	0
57°C-1 h	99.9	100
Zymosan	100.0	84
EGTA	103.0	89
Anti-C3 Absorption	64.0	71

[a]Average of three separate experimental determinations.

TABLE 7. Bacterial killing in the perfused rat liver in the presence of immune rat plasma either untreated or treated by heat, zymosan, EGTA or C3-absorption.[a]

	Recovery (%)				
Experimental	Immune Plasma	57°C	Zymosan	EGTA	Anti-C3 Absorbed
Liver	25.0 ± 7	76.2 ± 15[b]	93.1 ± 15[b]	76.6 ± 12[b]	59.1 ± 8[b]
Effluent	3.6 ± 2	8.9 ± 7	5.6 ± 4	23.3 ± 6[b]	4.7 ± 3
Total	28.6 ± 8	85.1 ± 10[b]	98.7 ± 15[b]	99.9 ± 7[b]	68.8 ± 9[b]
Killed	71.4	14.9[b]	1.5[b]	0.1[b]	32.2[b]

[a]Average value ± standard deviations from at least six separate experimental determinations.

[b]$P < 0.001$ vs. immune plasma.

plasma significantly increased bacterial trapping since only 3.6% of the bacteria were recovered in the effluent. That enhanced trapping does actually occur is suggested by data in the remainder of the table. Immune plasma heated at 57°C for 1 h, which reduces its bactericidal activity in vitro to 0%, decreased killing in the perfused liver to 14.9% (Table 7). Over 85% of the bacteria were recovered with 76.2% from the liver and only 8.9% from the effluent. Zymosan treatment also diminished bactericidal activity with only 1.5% being killed. Ninety-eight percent of the bacteria were recovered with 93.1% in the liver and 5.6% in the effluent (Table 7). Likewise EGTA-treated immune plasma had no bactericidal activity in the perfused liver. Almost 100% of the bacteria were recovered with 76.6% in the liver and 23.3% in the effluent. While anti-C3-4B absorption of immune plasma did not reduce bactericidal activity as low as other treatments, it did significantly reduce killing to 32.2% as compared to 71.4% with untreated immune plasma (Table 7). A total of 68.8% of the bacteria were recovered with 59.1% in the liver and 4.7% in the effluent.

Trapping and killing of S. typhimurium by livers from humorally immunized rats in the absence and presence of normal plasma

Livers from rats immunized with heat-killed S. typhimurium trapped 72.0% of the infused bacteria with 26.4% being recovered in the effluent (Table 8). The 98% recovery of bacteria suggests no significant killing occurred. When normal rat plasma was added to the system, 36.5% of the bacteria were recovered in the liver and 20.7% in the effluent (Table 8). Only 57.2% of the bacteria were recovered indicating that 42.8% were killed. The results of these experiments

TABLE 8. Trapping and killing of S. typhimurium by livers of immunized rats in the absence and presence of normal rat plasma.[a]

Percent Recovery	M-199	Normal Plasma
Liver	72.0 ± 8	36.5 ± 9
Effluent	26.4 ± 5	20.7 ± 12
Total	98.4 ± 11	57.2 ± 16
Killed	1.6	42.8

[a]Average value ± standard deviations from at least six separate experimental determinations.

were not statistically different from normal rat livers perfused in the absence or presence of normal rat plasma (Table 1).

Trapping and killing of S. typhimurium by perfused livers from normal and C. parvum-treated mice

Livers from normal mice trapped an average of 67.6% of a 1-2 x 10^{10} dose of S. typhimurium on a single pass, with 38.5% being recovered in the effluent (Table 9). No bacterial killing occurred. Livers from C. parvum-treated mice yielded an average of 45.8% of the bacteria from the liver and 31.8% from the effluent. Total recovery was 77.4%, indicating that approximately 23% of the bacteria were killed (Table 9).

Effect of EDTA, silica and phenylbutazone on bacterial killing by perfused livers from C. parvum-treated mice

To determine whether residual complement and/or properdin systems might be playing a role in the bactericidal activity of C. parvum-treated perfused liver, perfusions were done using Ca^{++} and Mg^{++} free HBSS containing 0.01 M EDTA (HBSS + EDTA). This medium had no significant effect on bactericidal activity when compared with C. parvum

TABLE 9. Clearance of S. typhimurium by perfused livers from normal and C. parvum-treated mice.[a]

Experimental	Recovery (%) Normal	C. parvum	P
Liver	67.6 ± 14.9[a]	47.8 ± 5.4	0.001
Effluent	38.5 ± 5.9	31.8 ± 14.4	NSS
Total	106.1 ± 11.2	77.4 ± 14.1	0.05
Killing	0	22.6	0.001

[a]Average value ± standard deviations from at least seven separate experimental determinations.

TABLE 10. Effect of EDTA, silica and phenylbutazone on bacterial killing by perfused livers from C. parvum-treated mice.

Experimental	C. parvum	EDTA	Silica	PB[c]
Liver	45.8 ± 5.4[a]	43.8 ± 12.6	42.3 ± 12.8	45.9 ± 25.1
Effluent	31.8 ± 14.4	27.5 ± 8.4	54.7 ± 3.1[b]	58.4 ± 28.7
Total	77.4 ± 14.1	71.4 ± 17.4	100.4 ± 12.8[b]	104.4 ± 9.9[b]
Killing	22.6	28.6	0	0

[a]Average ± standard deviations from at least six separate experimental determinations.

[b]$P < 0.001$.

[c]Phenylbutazone.

controls using M-199 (Table 10). Control studies in normal livers using HBSS + EDTA showed no difference in either total recovery or distribution of bacteria between liver and effluent (data not shown).

To evaluate the contribution of the RES cells in bactericidal activity of C. parvum livers, crystalline silica and phenylbutazone were tested. In silica-C. parvum-treated livers 42.3% of the bacteria were recovered in the liver and 54.7% in the effluent for an average recovery of 100% (Table 10). In phenylbutazone-C. parvum-treated livers 45.9% of the bacteria were recovered in the liver and 58.4% in the effluent for an average recovery of 104.4%. Control experiments with normal livers showed phenylbutazone had no effect on distribution or viability of bacteria (data not shown).

Trapping and killing of S. typhimurium by perfused C. parvum-treated rat livers in the absence or presence of plasma

C. parvum-treated rat livers, perfused with M-199, trapped 66.3% of 1-2 x 10^9 dose of S. typhimurium on a single pass, with 14.1% being recovered in the effluent (Table 11). Only 80.4% of the bacteria were recovered indicating that 19.6% were killed. These data when compared to normal perfused rat livers (Table 1) were significant in all cases, except in the percent bacteria recovered in the liver. The addition of normal or C. parvum (plasma from C. parvum-treated rats) or heated immune plasma to C. parvum-treated liver perfusions (Table 11) did not significantly enhance bactericidal activity above normal rat liver perfusion experiment levels in the presence of plasma (Tables 1 and 7). C. parvum-treated livers in the presence of normal plasma did trap a greater number of bacteria when compared to normal, i.e. only 19.2% of the bacteria were recovered in the effluent as compared to 36.1% in

TABLE 11. Bacterial trapping and killing by C. parvum-treated and normal rat liver in the absence or presence of plasma.[a]

Experimental	C.P. Liver + M-199	C.P. Liver + Normal Plasma	C.P. Liver + C.P. Plasma	C.P. Liver + ΔImmune Plasma	Normal Liver + C.P. Plasma
Liver	66.3 ± 12	31.5 ± 7	31.3 ± 4	79.1 ± 11	34.5 ± 4
Effluent	14.1 ± 9[b]	19.2 ± 8[c]	27.7 ± 5	8.9 ± 5	23.2 ± 5
Total	80.4 ± 9[c]	50.7 ± 7	58.9 ± 8	88.0 ± 11	57.7 ± 8
Killed	19.6[c]	49.3	41.1	12.0	42.3

[a] Average value ± standard deviations from at least six separate experimental determinations.

[b] $P < 0.001$ vs. normal liver perfusions.

[c] $P = 0.01$ vs. normal liver perfusions.

normal liver perfusions in the presence of plasma. When C. parvum plasma was added to normal rat liver perfusions (Table 11), no significant difference was observed in bacterial killing or distribution between liver and perfusate when compared to perfusions using normal plasma (Table 1).

Perfusion data using livers from C. parvum-treated mice and rats were the first instance in our hands to demonstrate that hepatic tissue had the ability to kill Salomonella typhimurium in the absence of blood. This observation led to more detailed examination of the morphological and physiological alterations induced by C. parvum. Studies were performed in both mice and rats. Where comparable data were obtained, no significant differences were observed.

Scanning electron microscopy of livers from normal and C. parvum-treated animals

Rat livers were examined two days after treatment with 350 μg C. parvum while mouse livers were examined nine days after treatment with 700 μg C. parvum. The different treatments used in rats and mice represent the optimal dose and time for each species. Similar morphological alterations were observed in both animals.

Figure 2 shows low maginification SEM of normal and C. parvum-treated livers. In Figure 2A portal veins of normal liver are free of white blood cells. By contrast portal veins of C. parvum-treated livers have extensive accumulation of white blood cells adhering to portal veins (Figure 2B).

Figure 3 presents micrographs of the representative cell types which adhere to protal veins in C. parvum-treated livers. Figures 3A and 3B reveal cells morphologically similar to macrophages adhering to

FIGURE 2. SEM of normal and C. parvum-treated liver.

A. Normal liver with branching portal veins (PV), sinusoids (S), and central vein (CV) (X 42).

B. Portal vein (PV) and sinusoids (S) of C. parvum-treated liver showing adhering white blood cells (X 100).

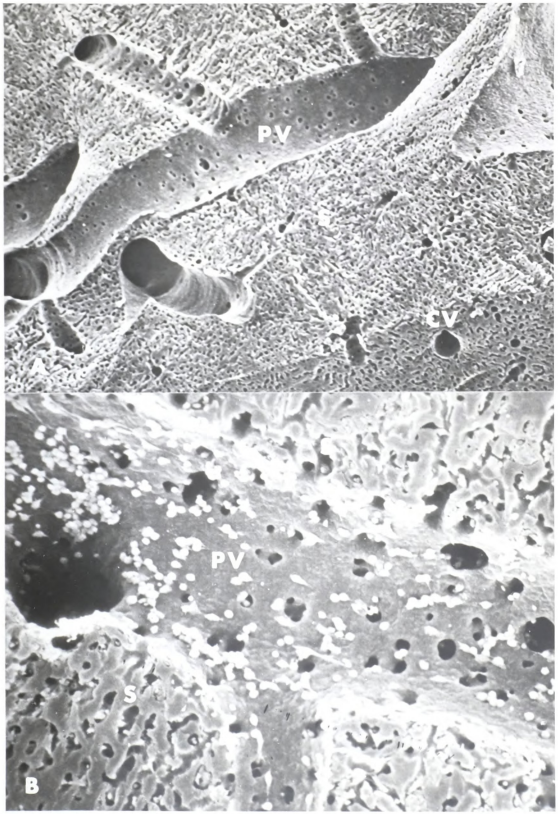

PV
EV
PV
S
B

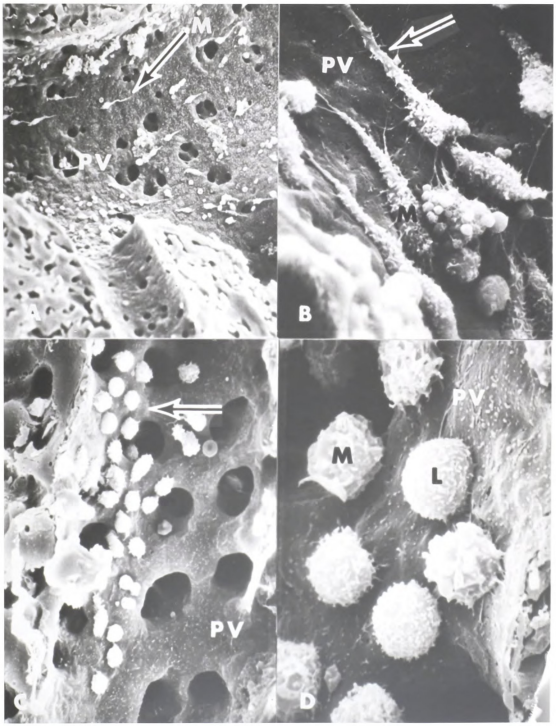
FIGURE 3. SEM of various white blood cell types observed in C. parvum-treated livers.

A. Portal vein (PV) with branches and adhering macrophages (M) with tails (arrow) (X 200).

B. Higher magnification of macrophages (M) with tails (arrow) adhering to a portal vein (PV) (X 1600).

C. Portal vein (PV) with adhering white blood cells enlarged in D (arrow) (X 700).

D. White blood cells adhering to portal veins (PV) in C, showing lymphocytes (L) and macrophages (M) (X 3000).


M
PV
A
PV
M
B
PV
C
PV
M
L
D

portal vein surfaces. Invaginated surfaces, ruffles and microvilli characteristic of macrophages are particularly evident in Figure 3B. These macrophages also display typical "tails" which extend away from the "head" of the macrophage. Figure 3C and 3D show other adherent cells morphologically similar to lymphocytes. Round cell bodies and numerous microvilli are readily observed.

Figure 4 shows sinusoidal areas from normal and C. parvum-treated livers. Normal liver sinusoids (Figure 4A) are lined by a layer of fenestrated endothelium, and contain Kupffer cells adhering to the endothelium by fine cytoplasmic filopodia. The Kupffer cells have a stellate appearance with folded and ruffled surfaces. They occupy a large area in the lumen of the sinusoid and are usually found at the junction of sinuoids. Sinusoids of C. parvum-treated livers were congested with numerous white blood cells identified as macrophages and lymphocytes (Figure 4B). Lymphocytes had round cell bodies and numerous microvilli. Macrophages were morphologically indistinguishable from Kupffer cells.

Figure 5 demonstrates the macrophage-macrophage interactions observed among cells adhering to portal veins and sinusoids of C. parvum-treated livers. In both instances (Figure 5A and 5B) filopodia extend between macrophages. Figure 5B shows three macrophages whose cell surfaces are in intimate contact.

Figure 6 shows macrophage-lymphocyte interactions within the sinusoids and portal veins of C. parvum-treated livers. Figure 6A through 6C presents a magnification series of such interactions. The macrophage surface had numerous invaginations or ruffles and was attached to the sinusoidal endothelium by filopodia. Lymphocytes have

FIGURE 4. SEM of sinusoidal areas of normal and C. parvum-treated livers.

A. Normal liver sinusoid (S) with fenestrated endothelial lining (E), parenchymal cells (PC), and Kupffer cells (KC) (X 2,200).

B. C. parvum-treated liver sinusoid (S) with macrophages (M) and lymphocyte (L) (X 1,960).

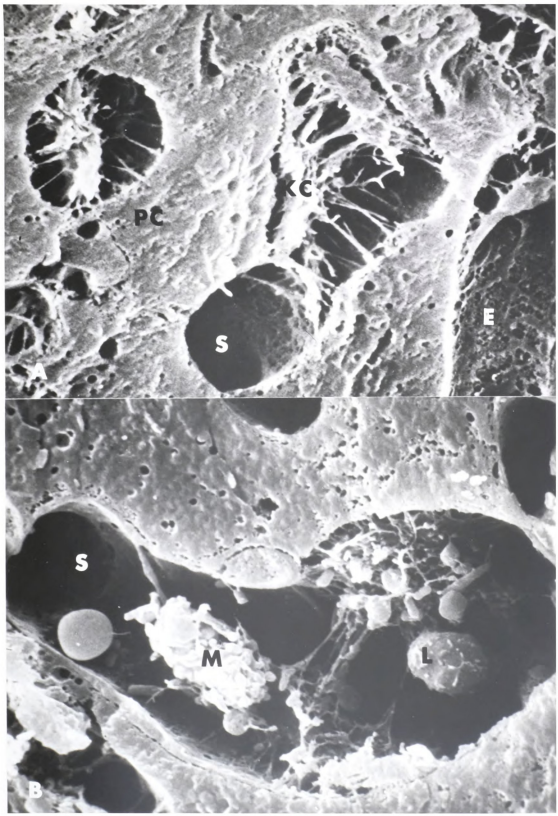

PC
KC
S
E
S
M
L
B

FIGURE 5. SEM of macrophage-macrophage interaction in C. parvum-treated liver.

A. Filopodia (F) extending between three macrophages (M) which adhere to a portal vein (PV) (X 1,900).

B. Several macrophages (M) adhering to a portal vein (PV) showing cell-cell surface interaction (arrow) (X 2,000).

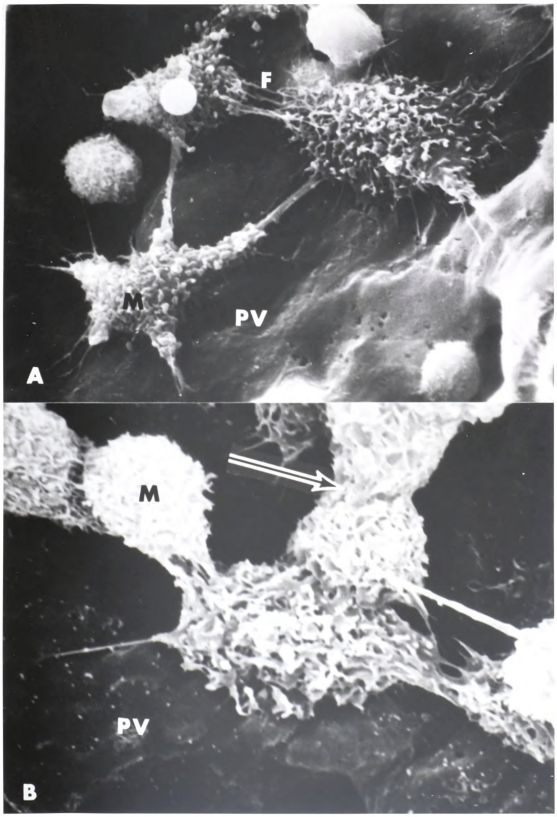

F
M
PV
A
M
PV
B

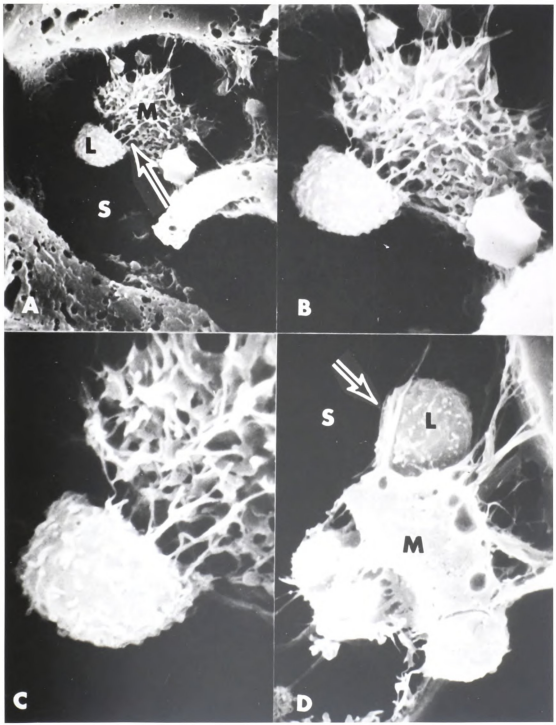
FIGURE 6. SEM of macrophage-lymphocyte interaction in C. parvum-treated liver.

A. Macrophage (M) in a sinusoid (S) and lymphocyte (L) attached by several appendages (arrow) (X 1,900).

B. Higher magnification of A showing the ruffled invaginated surface of the macrophage (X 4,000).

C. Higher magnification of A showing the macrophage appendages contacting the lymphocyte surface (X 7,000).

D. Macrophage (M) in a sinusoid (S) with an attached lymphocyte (L) which is surrounded by cytoplasmic filaments (arrow) (X 5,000).


M
L
S
A
B
C
S
L
M
D

round cell bodies and numerous microvilli. Cytoplasmic filaments extend from the macrophage contacting the lymphocyte surface. Figure 6D shows a sinusoidal macrophage which has trapped a lymphocyte in a cup-like depression on its surface. Filopodia have extended around the lymphocyte from the macrophage.

Scanning electron microscopy of C. parvum-treated mouse livers perfused with S. typhimurium

Figures 7, 8 and 9 are micrographs exemplifying selected types of bacterial-host cell interactions observed in C. parvum-treated livers perfused with bacteria. Figure 7 shows a sinusoidal macrophage, stellate in appearance, fixed to the sinusoid endothelium by numerous fine cytoplasmic filopodia. The macrophage has many microvilli on its surface. Bacteria are interacting with the surface microvilli primarily at one end of the cell.

The macrophages in Figures 8 and 9 (cells labeled M) are actively phagocytizing bacteria. The surfaces of these macrophages have many folds, ruffles, invaginations, microvilli, blebs and micropliae. They have no observable cytoplamic filopodia attached to sinusoid endothelium as seen in the sinusoidal macrophage in Figure 7. The area of the macrophage involved in phagocytosis is highly blebbed and invaginated (Figures 8A and 9A). Microvilli are not present in these areas but are observed in areas of the cell not interacting with the bacteria (Figures 8B and 9B). Figure 8A also shows a lymphocyte in the sinusoid (L). The lymphocyte is round bodied with finger-like microvilli on its surface. Figure 9A and 9B shows a macrophage phagocytizing bacteria with a cytoplasmic filament extending out to a bacteria in the

FIGURE 7. Scanning electron micrograph of a sinusoidal macrophage from a C. parvum-treated liver. The cell is attached to the sinusoidal endothelium by many fine filopodia (arrow A) bacteria and macrophage interactions via surface microvilli can be observed (arrow B) (X 2,970).

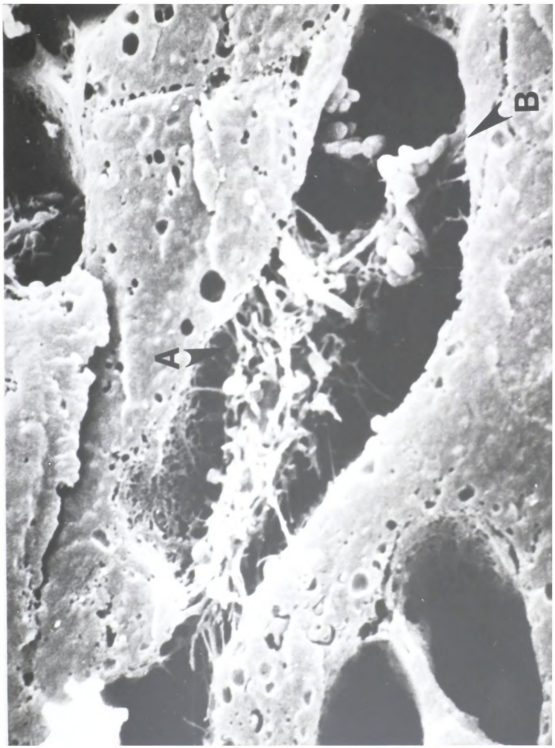

A
B

FIGURE 8. Scanning electron micrographs of macrophage phagocytizing S. typhimurium in C. parvum-treated liver. (A) Liver sinusoid with a lymphocyte (L) and a macrophage (M) phagocytizing bacteria (arrow) (X 5,524), (B) higher magnification of macrophage in A showing area of phagocytosis. Microvilli are not present in the area of phagocytosis (arrow A) while they are present in areas not interacting with the bacteria (arrow B) (X 11,712).

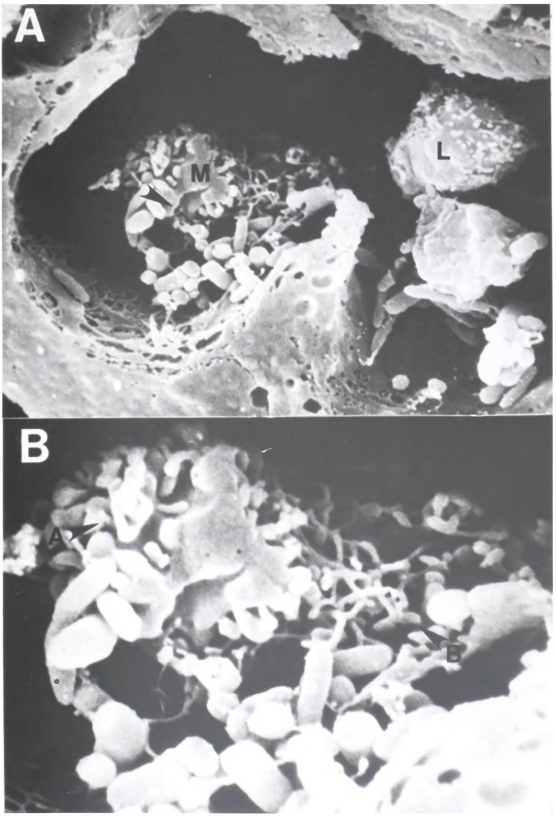
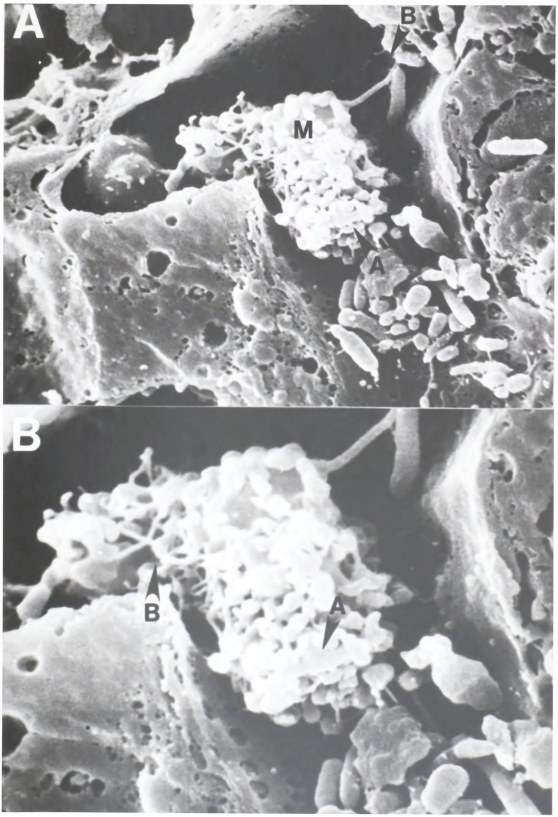

A
M
L
B
A
B

FIGURE 9. Scanning electron micrographs of macrophage phagocytizing S. typhimurium in C. parvum-treated liver. (A) Area of the macrophage (M) involved in phagocytosis is highly blebbed and invaginated (arrow A). Note the cytoplasmic filament extending out to a bacteria (arrow B) (X 5,020). (B) Higher magnification of macrophage in A. In the blebbed area of the cell, rod-shaped forms of bacteria can be observed (arrow A). Microvilli are present in the area of the cell not involved in phagocytosis (arrow B) (X 7,614).


A
B
M
A
B
B
A

sinusoid. In the highly blebbed area of the macrophage, rod-shaped forms of phagocytosed bacteria can be observed (Figure 9B).

White blood cell kinetics in normal and C. parvum-treated rats and mice

The total white cell count for normal rats was 10,092 cells/mm^3. On differential, 5% were monocytes, 14% PMN and 81% lymphocytes (Table 12). Forty-eight hours after C. parvum injection, the total WBC increased to 16,819 cells/mm^3 with the monocyte count increasing from 5 to 19% of the differential count. Ten days after C. parvum injection the relative monocytosis was still evident. Although a relative decrease in PMN existed in C. parvum-treated rats, the absolute numbers of PMN in the peripheral blood remained constant at approximately 1,000 cells/mm^3.

The total white cell count for normal and C. parvum-treated mice did not increase at three and nine days after C. parvum injection. Peripheral blood monocyte counts increased 12% above normal mice at three days and remained elevated after nine days (Table 12). The percent PMN decreased from 40% to 31% three days after injection and to 25% nine days after injection of C. parvum.

In vivo distribution and survival of S. typhimurium in normal and C. parvum-treated mice

Table 13 compares the distribution and rate of killing of a 1 x 10^9 dose of S. typhimurium after 15 and 60 minutes in normal and C. parvum-treated mice. In normal mice approximately 58% of the bacteria were recovered as viable cells at both time periods with only minor differences observed in organ distribution. After 15 minutes 47.4% of the organisms were recovered from the liver, 4.9% from the carcass,

TABLE 12. Total and differential white blood cell counts in normal and *C. parvum*-treated rats and mice.[a]

Experimental	Total WBC	Differential (%)		
	Cells/mm^3	Monocytes	PMN	Lymphocytes
Normal Rats	10,092 ± 320	5 ± 1	14 ± 2	81 ± 2
350 µg/Two Days[b]	16,819 ± 6,945	19 ± 3	6 ± 3	75 ± 3
350 µg/Ten Days	11,913 ± 1,593	18 ± 3	7 ± 2	75 ± 6
Normal Mice	5,499 ± 1,005	6 ± 2	40 ± 10	54 ± 12
700 µg/Three Days[b]	4,517 ± 1,570	18 ± 10	31 ± 6	50 ± 14
700 µg/Nine Days	4,331 ± 1,197	14 ± 5	25 ± 8	61 ± 18

[a]Data are expressed as counts or differential ± standard deviation. Each value represents at least six separate experimental determinations.

[b]*C. parvum* dose/time of exposure to vaccine.

TABLE 13. Recovery of *S. typhimurium* 15 and 60 minutes after I.V. injection into normal and *C. parvum*-treated mice.[a]

	Percent Recovery After			
	15 Min		60 Min	
Organ	Normal	*C. parvum*	Normal	*C. parvum*
Liver	47.4 ± 13.8[a]	19.4 ± 2.6[b]	56.4 ± 5.5	4.5 ± 2.2[b]
Lung	4.6 ± 5.5	1.0 ± 0.3	0.7 ± 0.3	0.2 ± 0.1
Spleen	1.3 ± 0.7	3.6 ± 1.2	0.4 ± 0.2	1.0 ± 0.3
Carcass	4.9 ± 2.1	1.8 ± 1.1[b]	1.1 ± 0.2	0.6 ± 0.6[b]
Total Recovery	58.2 ± 9.9	25.8 ± 1.0[b]	58.5 ± 5.7	6.5 ± 2.4[b]
Percent Killing	41.8	74.2	41.5	93.5%

[a]Average value ± standard deviations from at least six separate experimental determinations.

[b]$P < 0.001$.

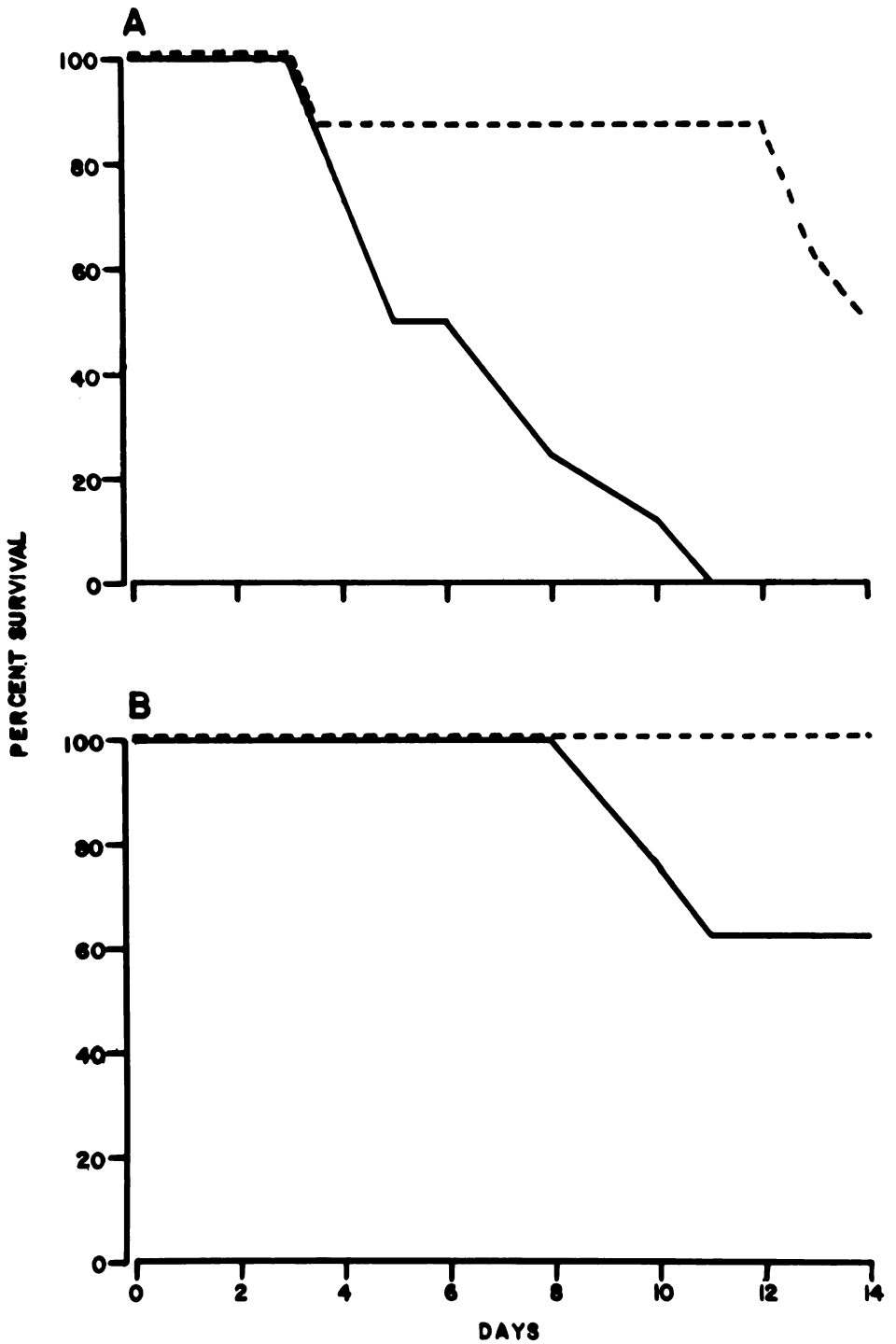
4.6% from the lungs and 1.3% from the spleen. After 60 minutes 56.4% were recovered from the liver, 1.1% from the carcass, 0.7% from the lungs and 0.4% from the spleen. In C. parvum-treated mice after 15 minutes only 26% of the bacteria were recovered as viable cells with 19.4% recovered from the liver, 3.6% from the spleen, 1.8% from the carcass and 1.0% from the lungs. At 60 minutes only 7% of the bacteria remained viable with 4.5% recoverd from the liver, 1.0% from the spleen, 0.6% from the carcass and 0.2% from the lung. The data for C. parvum-treated mice show a significant decrease ($P < 0.001$) in the recovery of viable bacteria in all tissues examined.

Survival of normal and C. parvum-treated mice infected with S. typhimurium

Normal and C. parvum-treated mice were injected intravenously with either 4.1×10^5 or 4.1×10^6 S. typhimurium and survival was monitored for 14 days. Figure 10A shows that at the 10^6 dose all normal mice died by day 11 while only 50% of the C. parvum-treated mice died after 14 days. Survival ratios were signficantly different by chi-square analysis on and after day five.

At the 10^5 dose (Figure 10B) 62.5% of the normal mice survived while 100% of the C. parvum-treated mice survived. Survival ratios were significantly different on and after day 11. The LD_{50} for normal mice was 6.4×10^5 and for C. parvum-treated mice was 4.1×10^6, representing about a ten-fold increase in resistance to S. typhimurium infection.

FIGURE 10. Susceptibility of normal and C. parvum-treated mice to infection by 4.1×10^6 (A) and 4.1×10^5 (B) S. typhimurium. Normal (———); C. parvum-treated (-----).


A
B
100
80
60
40
20
0
PERCENT SURVIVAL
0
2
4
6
8
10
12
14
DAYS

DISCUSSION

An initial objective of this thesis was to define the role of complement in the early events in liver-bacterial interactions. The pivotal role of complement in mediating bactericidal activity is clearly demonstrated by these data. No antibody to S. typhimurium could be detected in normal plasma using tube agglutination titrations. Rat plasma was treated to inhibit complement activity by heat inactivation at 57°C, zymosan absorption and chelation with EDTA. Heating at 57°C destroys complement activity by denaturing $C\overline{1}$ of the classical pathway and Factor B of the alternate pathway (31). Zymosan activates the alternate pathway and thereby depletes plasma C3, inhibiting both complement pathways (29, 31). All treatments inhibited the ability of plasma to stimulate bactericidal activity in the perfused liver (Table 3). These results clearly demonstrate the need for complement activity in bacterial killing, presumably because complement is required for bacterial phagocytosis.

To determine which complement pathway was required for bactericidal activity, plasma was heated at 50°C for 30 minutes or EGTA was added. Heating plasma at 50°C denatures Factor B thereby inactivating the alternate pathway but has minimal effect on the classical pathway. Table 2 shows that this treatment has no effect on hemolytic complement activity while it significantly inhibited bactericidal activity in the perfused liver (Table 4). EGTA chelates Ca^{++} which is required

by the classical pathway for activity but has only minimal effect on the alternate pathway which utilizes Mg^{++} (24, 33, 107). EGTA depressed hemolytic complement activity 93% (Table 2) but had no inhibitory effect on liver bactericidal activity (Table 4). Further, the percent distribution of bacteria in the liver and effluent was not statistically different from normal plasma experiments. Taken together these results demonstrate that the alternate pathway is the key pathway for mediating bactericidal activity of normal plasma in the perfused liver. The results are not surprising in that gram negative bacteria can activate complement via the alternate pathway in the absence of specific antibody via the lipopolysaccharide present in their outer membrane (31, 37).

To further demonstrate the critical role of C3 in plasma's bactericidal activity in the liver, normal plasma was absorbed with a specific anti-C3-Sepharose 4B immunoabsorbant. Absorbtion with an anti-SalH-4B immunoabsorbant was used as a control. C3 absorption significantly inhibited the ability of plasma to stimulate bactericidal activity in the liver (Table 5). These results confirm and extend the data obtained by heating normal plasma at 57°C or 50°C, and zymosan absorption (Tables 3 and 4).

These experiments suggest the possible role of C3b receptors in phagocytosis of bacteria by liver Kupffer cells. Munthe-Kaas (67) *in vitro* and Schreiber and Frank (102) *in vivo* demonstrated the presence of Kupffer cell C3b receptors. Munthe-Kaas showed C3b coated-RBC were ingested by Kupffer cells in the absence of IgG (67), while C3b receptors of other mononuclear cells are only involved in attachment (58,

90). Direct phagocytosis of C3b coated-RBC occurs only in activated peritoneal macrophage (10).

Heating plasma at 57°C or 50°C and chelation with EDTA significantly decreased hepatic bacterial trapping, demonstrated by the increased numbers of bacteria recovered in the effluent (Tables 3 and 4). Since EGTA treatment had no effect on liver bacterial trapping (Table 4), Ca^{++} is not required for this process. Chelation with EDTA, on the other hand, inhibited liver bacterial trapping, suggesting that Mg^{++} may play a role in the process. Heating plasma at 57°C and 50°C also decreased bacterial trapping. This may be due to heat denaturation of plasma proteins which coat the bacteria and decreases their interaction with liver tissue. The results of the EDTA experiments may also suggest the requirement of Mg^{++} for bacterial phagocytosis by Kupffer cells.

Immune plasma is bactericidal in vitro (Table 6), probably due to activation via specific antibody of the classical complement pathway which causes bacterial killing (31). By heating immune plasma to inhibit both complement pathways one can study the role of specific antibody in bacterial trapping and killing in the liver. When immune plasma was heated at 57°C, absorbed with zymosan or chelated with EGTA, in vitro bactericidal activity was inhibited (Table 6). Cumulatively these data indicate that the bactericidal activity in vitro of immune plasma was due to activation of complement.

While heat inactivation, zymosan and EGTA treatment blocks the bactericidal activity of immune plasma in the perfused liver (Table 7), it also causes a significant increase in the number of bacteria trapped in the liver and no change in the number of bacteria recovered in the

effluent except in the case of EGTA (Table 7). C3 absorption of immune plasma, while not decreasing bactericidal activity as greatly as other treatments, still significantly decreased its activity. This is probably due to the remaining C3 being activated via the classical pathway. These data show that the presence of specific antibody does not mediate killing in the perfused liver but significantly increases bacterial trapping over that observed in liver perfusion using M-199 alone or normal plasma (Tables 1 and 7).

The ability of EGTA to inhibit the bactericidal activity of immune plasma was surprising since the alternate pathway was still available for activation of complement. Normal plasma treated with EGTA still stimulated bacterial killing in the liver (Table 4). A probable explanation is that bacteria in immune plasma are coated with specific antibody thereby preventing the bacterial surface from interacting with the alternate pathway and hence killing does not occur. These experiments demonstrate that in the presence of specific antibody, the critical mode of complement activation is via the classical pathway and that such activation is required for bacterial killing by the liver.

The results of immune plasma experiments suggest that Kupffer cells may be able to trap bacteria more efficiently in the presence of specific antibody due to Fc receptors on these cells interacting with antibody coated bacteria. Munthe-Kaas et al. (67-69) and Huber et al. (47) reported the presence of IgG Fc receptors on Kupffer cells in vitro. Alveolar and peritoneal macrophages also have IgG Fc receptors (58, 90). Munthe-Kaas demonstrated that IgG-coated RBC attached to isolated Kupffer cells and were ingested by peritoneal macrophages only when coated with IgG.

Experiments were done to determine the effect of stimulation of hepatic RES on the liver's ability to trap and kill bacteria. The methods used were 1) to specifically immunize rats with heat-killed S. typhimurium vaccine and 2) to non-specifically activate hepatic RES by C. parvum vaccine. Immunized rats developed humoral titers of 6,500 to the bacteria, but livers from these animals behaved as normal in their ability to trap and kill bacteria in the absence or presence of plasma (Tables 1 and 8). This demonstrates that Kupffer cells cannot be activated by a heat-killed vaccine and strongly suggests that macrophage cytophilic antibody does not play a role in the bactericidal and trapping activity of hepatic tissue.

The hepatic RES was non-specifically activated with C. parvum vaccine, a macrophage stimulator (41, 98, 118) and the resultant effects on trapping and killing of S. typhimurium in situ in mice and rats and in vivo in mice were studied. Data presented in previous papers (35, 61, 92) and in this thesis demonstrate that both the mouse and rat models yield similar data in correlative experiments.

The initial C. parvum studies were in the absence of plasma. In normal perfused mouse livers, over 100% of the bacteria were recovered (Table 9) while in C. parvum-treated livers only 77.4% were recovered indicating 22.6% of the bacteria were killed. The bactericidal activity correlates with the decrease in percent viable bacteria recovered from the liver, i.e. 45.8% in C. parvum-treated livers versus 67.6% in normal. In both normal and C. parvum-treated livers the effluent contained the same percent bacteria (Table 9). These data represent the first successful demonstration of bactericidal activity in perfused liver in the absence of plasma and suggest that activated RES cells

populating C. parvum-treated mice are functionally different from normal Kupffer cells.

To be certain that cellular and not residual humoral factors were the active component in bactericidal activity, perfusion experiments using EDTA, silica and phenylbutazone were performed. For EDTA studies, Ca^{++} and Mg^{++} free HBSS containing 0.01 M EDTA was used as perfusion medium instead of M-199. EDTA at this concentration inhibits both the classical and alternate complement pathways (107). The data show (Table 10) that EDTA did not alter the distribution or bactericidal activity of C. parvum-treated perfused livers indicating that the classical and/or alternate pathways were not participating in the bactericidal activity.

Silica treatment blocked the ability of C. parvum to stimulate bactericidal activity in livers due to destruction of RES cells. Silica treatment also decreased the ability of the perfused livers to trap bacteria. These results are similar to those previously reported (35) and reaffirm that viable RES cells are required for optimal bacterial trapping in the perfused liver even though they alone are not the only factor involved.

Phenylbutazone (PB) is an anti-inflammatory drug that inhibits phagocytosis (55, 108, 110) and intracellular killing (77, 108, 110). Infusion of PB into C. parvum-treated livers both inhibited bactericidal activity (Table 10), and decreased bacterial trapping. Since both silica (a specific RES toxin) and PB inhibited killing to the same extent, it can be concluded that the bactericidal activity of C. parvum-treated livers resides in activated liver RES cells.

C. parvum-treated rat livers were used to study the effect of plasma on bacterial trapping and killing in the activated organ. C. parvum-treated rat livers had similar bactericidal activity as C. parvum-treated mouse livers (Tables 11 and 9). C. parvum treatment also enhanced rat livers ability to trap bacteria as demonstrated by the decreased number of bacteria recovered in the effluent (Tables 11 and 1). This enhanced trapping ability was not observed in C. parvum-treated mouse livers (Table 9).

C. parvum-treated rat liver in the presence of plasma did not have greater bactericidal activity over normal liver, but significantly enhanced bacterial trapping did occur in the presence of normal plasma (Tables 11 and 1). C. parvum treatment had no effect on plasma factors involved in liver bacterial trapping and killing. This is shown by results of normal liver perfusion experiments using C. parvum-treated liver and plasma (Tables 11 and 1). These results suggest that bacterial killing by liver Kupffer cells, whether they are normal or activated, in the presence of plasma can kill only a certain percentage of bacteria that are cleared by the organ in the time period investigated (30 minutes).

SEM was used to morphologically study C. parvum-treated livers to determine why these livers were able to kill bacteria in the absence of plasma. Observations made by SEM revealed that C. parvum treatment in both rats and mice caused an influx of white blood cells into the liver. White blood cells consisting mainly of macrophage and lymphocytes were observed adhering to portal veins, portal venules and sinusoids (Figures 2-4). This white cell influx into the liver occurred concomitant with a relative peripheral blood monocytosis (Table 12).

This study corroborates the observation by Warr and Sljivic (118) that C. parvum treatment results in the accumulation of extra-hepatic mononuclear cells in the liver. Similar results have been obtained by North (75) and Volkman (115) using Listeria monocytogenes which showed that cells emigrating into the liver were peripheral blood monocytes. This response is not surprising since the major portion of an intravenous C. parvum injection is cleared by the liver (26).

The morphological features of T and B lymphocytes, blood monocytes and polymorphonuclear leukocytes (PMN) have been studied in detail by SEM (3, 5, 74, 85, 86, 120). While there is general agreement that macrophages and lymphocytes may be differentiated, Polliack et al. (85, 86) showed that in vitro lymphocytes of known T and B cell identity display a considerable amount of surface variation. Barber and Burkholder (5) and Albrecht et al. (3) found that macrophage morphology is extremely variable and may reflect the functional state of these cells. By SEM alone this study did not reveal the histological type of white blood cell adhering to portal vasculature in anything other than general terms.

It is unlikely that cells identified as macrophages adhering to portal veins represent Kupffer cells emigrating from liver sinusoids though their morphological features were strikingly similar to those of Kupffer cells and macrophages of extra-hepatic origin were morphologically very similar (Figure 4). A difference in attachment to the sinusoidal wall in Figure 4B can be observed between the two macrophages. One macrophage has no observable filopodia attached to the sinusoidal endothelium while the other macrophage is attached to the endothelium by numerous cytoplasmic filopodia. This morphological difference may

suggest that the highly attached macrophage is a Kupffer cell while the other cell is a blood derived macrophage. No direct evidence supporting this contention is presently available. North (75) could only make this distinction by the use of autoradiographic studies of characteristic labeling patterns of Kupffer cells and blood monocytes.

It was found that macrophages characteristically adhere in a "head-tail" orientation in portal veins (Figure 3A and 3B). This head-tail orientation was not observed in smaller portal venules or in sinusoids. The dynamics of fluid movement in portal veins of larger diameter may influence such cell orientation. This orientation might also be due to macrophage motility (5).

A considerable amount of cell-cell contact or interaction between adhering white blood cells was observed in C. parvum-treated liver. These interactions occurred in both portal veins and sinusoids. Macrophages were observed interacting with macrophages (Figure 5) and lymphocytes (Figure 6) by direct cell surface contact and by the extension of cytoplasmic appendages between cells. Frost and Lance (36) showed that C. parvum treatment results in the sequestering of lymphocytes in lympoid tissue, a process termed lymphocyte trapping. This study demonstrates that lymphocyte trapping also occurs in C. parvum-treated rat and mouse livers. Neilsen et al. (72) and Werdelin et al. (119) used SEM to study macrophage-lymphocyte cluster formation during the in vitro induction of the immune response to soluble protein antigens. Roelants (91) reviews the significance of such a model in some detail. It is unknown if similar macrophage-lymphocyte interactions observed in this study have functional significance pertinent to these models,

although, the immunopotentiating ability of C. parvum is a function of its direct stimulation of both lymphoid and RES cells (12, 18, 41, 98, 118).

SEM of C. parvum-treated mouse livers perfused with S. typhimurium revealed numerous macrophage-bacterial interactions. The host cells were stellate in appearance with surface invaginations, ruffles and microvilli (Figures 7, 8 and 9). In C. parvum-treated livers it was difficult to differentiate morphologically between blood-derived macrophages and liver Kupffer cells.

The macrophage in Figure 7 is attached to the sinusoidal endothelium by numerous cytoplasmic filopodia while the macrophages in Figures 8 and 9 have no observable filopodia attached to the endothelium. This difference in attachment to the sinusoidal wall may be significant, possibly suggesting that the macrophage in Figure 7 is a Kupffer cell while those in Figures 8 and 9 are blood-derived macrophages.

The macrophages actively phagocytizing bacteria in Figures 8 and 9 show specific surface areas demarcated into regions involved or not involved in phagocytosis. Regions of the macrophage directly involved in bacterial phagocytosis are highly blebbed and invaginated (Figures 8B and 9B). In these areas no surface microvilli are present. Parakkal et al. reported similar loss in surface microvilli on macrophages during phagocytosis in vitro (80). Regions of the macrophage not directly involved in bacterial phagocytosis retain the surface microvilli (Figures 8B and 9B).

While SEM fail to distinguish Kupffer cells from extra-hepatic macrophages in C. parvum-treated liver it demonstrates the accumulation of white blood cells in the liver. This study also suggests that

several immunologically significant phenomena such as macrophage-macrophage and macrophage-lymphocyte interactions occur in C. parvum-treated liver. The anatomical alterations in liver most likely are an integral part of the increased microbial resistance observed in the C. parvum-treated host (1, 6, 19, 52, 76, 103, 112) and is the reason for the perfused liver's ability to kill bacteria in the absence of plasma.

The effect of C. parvum on the ability of mice to clear and kill microbes in vivo were investigated to determine if the perfused liver was a true indicator of RES activity in the whole animal. Recovery of S. typhimurium 15 and 60 minutes after intravenous injection varied significantly between normal and C. parvum-treated mice. In normal mice approximately 42% of the bacteria were killed after 15 minutes. No additional killing occurred after 60 minutes (Table 13). There was a slight rise in the percent recovery in liver after 60 minutes, possibly due to some bacterial multiplication. In C. parvum-treated mice 74.2% of the bacteria were killed by 15 minutes and 93.5% by 60 minutes. The majority of viable bacteria were recovered in the liver and carcass in normal mice while the majority were recovered in the liver and spleen in C. parvum-treated mice. The increased splenic trapping in C. parvum-treated mice may be due to its increased size. Cumulatively these data demonstrate that activation of the RES by C. parvum significantly increases the ability of liver and spleen to clear and kill S. typhimurium in vivo and that the perfused liver is an accurate indicator of RES function as previously shown (35).

C. parvum treatment increased the LD_{50} of S. typhimurium in mice from 6.4×10^5 to 4.1×10^6 (Figure 10). This difference represents roughly a ten-fold increase in resistance to S. typhimurium infection

and is the first demonstration of the ability of C. parvum vaccine to enhance resistance to this organism. Previous reports have demonstrated the ability of C. parvum vaccine to enhance resistance to bacterial (1, 6, 19, 95, 112), protozoan (76, 112), fungal (103) and viral infections (52).

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LITERATURE CITED

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