Porcine neutrophil function in the presence of virulent and avirulent Salmonella choleraesuis

by

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EXPLANATION OF THESIS FORMAT

The following thesis consists of a general introduction, a review of the literature, a manuscript, a general summary, literature cited, and acknowledgements. The master's candidate, Michael Bruce Roof, is the senior author and principal investigator for the manuscript.

GENERAL INTRODUCTION

Salmonella are members of the family Enterobacteriaceae. Salmonella cause a wide array of diseases ranging from gastroenteritis to septicemia and pneumonia. They may also cause local infection in virtually every organ of the body because of spread during bacteremia (57).

The classification of Salmonella species has been controversial for several years. There are approximately 1500 serologically distinct serotypes, most of which are named by the site of geographical isolation (100). At the present time the Centers for Disease Control reports three biochemically distinct species of Salmonella; S. typhi, S. choleraesuis, and S. enteritidis. However, all salmonellae appear to be closely related. The Centers for Disease Control recommend the use of biochemical tests and polyvalent somatic (O) and capsular (Vi) antiserum to determine serogroups. Specific somatic (O) and flagellar (H) antisera are used to further serotype Salmonella. This is desirable because of their host adaptation, clinical significance, and ability to cause life-threatening infections (57).

Salmonella choleraesuis var. kunzendorf is the most common serotype associated with disease in swine. This serotype of Salmonella is a major economic concern to pork producers, and can also be a source of food-borne salmonellosis.

Salmonella are facultative intracellular pathogens. Macrophage infection has been the most studied aspect of cell mediated immunity. Facultative intracellular pathogens such as Salmonella grow within

phagocytic cells and are protected from extracellular factors such as complement, specific antibody, and antibiotics.

The purpose of this study was to investigate virulent and avirulent Salmonella choleraesuis and their interaction with porcine neutrophils.

LITERATURE REVIEW

Pathogenesis

Salmonella choleraesuis var. kunzendorf has traditionally been the most frequent serotype of Salmonella associated with disease in swine (100). Infection with S. choleraesuis usually does not occur in suckling pigs because of lactogenic immunity, but is found in weaned pigs and adults (75,100). The major source of S. choleraesuis in swine is shedding by infected pigs (81,100). During acute phases of the disease, pigs will shed up to 10^6 S. choleraesuis/gram of feces. The number of S. choleraesuis required to initiate infection depends on the virulence of the particular strain and the condition of the host. An oral dose of 10^8-10^{11} cells will usually cause infection. The ingestion of Salmonella in substances that increase the pH of gastric acid will reduce the number of cells required to initiate infection (68,81). Conditions such as high animal density, nutritional deficiencies, and infectious diseases are assumed to increase the shedding by carriers and susceptibility of exposed pigs (100).

Disease in swine is usually caused by S. choleraesuis and S. typhimurium, but swine are sometimes exposed to other Salmonella serotypes in feed, water, and feces of carriers. The salmonellae establish an infection of an unknown duration which is not noticed unless the pigs are subjected to disease or stress.

The ability of S. choleraesuis to initiate infection depends on a variety of factors, such as serotype, virulence, natural and acquired

immunity, route of infection, and the infective dose. Much of the research on the pathogenesis of S. choleraesuis has been done in mice, rabbits, and other laboratory animals and are only assumed to be valid in swine. The development of S. choleraesuis infection in swine can be divided into 4 steps: 1) growth and proliferation in the intestine, 2) invasion of mucosal epithelium and lamina propria, 3) stimulation of fluid secretion, and 4) dissemination from the intestine to mesenteric lymph nodes, spleen, liver, and blood.

The event leading to S. choleraesuis infection in pigs usually begin with ingestion of the bacteria followed by passage through the stomach into the bowel where the organism attaches and multiplies. The gastric activity of the stomach is bactericidal, but if given in a large enough dose, or if the ingesta raise the pH of the stomach, bacteria will pass through and initiate infection (68,81).

In most instances, the intestinal phase is associated with rather subtle degenerative and proliferative changes in the villi, crypt glands, and lamina propria of the small intestine. Although the cellular immune reaction involves mostly mononuclear cells when S. typhi is involved, other Salmonella infections involve primarily polymorphonuclear leukocytes (PMNL) (81). The organisms attach to the top of villi where they invade and multiply. There is no evidence of cellular damage or inflammation at the time of initial infection, but in response to chemotactic factors, inflammatory cells, such as PMNL, appear and may cause mucosal damage (75).

Intraluminal replication occurs, followed by invasion of the intestinal mucosa, particularly at the level of the ileum (75,81,88,100). The bacteria must penetrate the mucus and attach to the absorptive columnar epithelial enterocytes. These cells are joined by junctional complexes which prevent penetration of unwanted substances. Despite this, it has been shown that inert particles and bacteria can penetrate the intestinal epithelium (98). Passage occurs by receptor-mediated endocytosis (RME) by enterocytes and by M cells which are specialized in uptake of antigen for introduction into the Peyer's patches and other lymphoid follicles (31,71,73,98,103). The epithelium rests on the basement membrane which excludes bacteria from the lamina propria. If the basement membrane is altered, it may allow bacteria to pass through. Salmonellae penetrate the basement membrane using host phagocytic cells as transport vehicles (73).

The mechanism of bacterial translocation across the intestinal barrier appears to be by means enterocyte RME. Once the bacteria enter the lamina propria, they are ingested by phagocytic cells and are transported to extraintestinal sites such as the mesenteric lymph nodes, where they are liberated. IgM and IgG promote translocation by facilitating opsonization and ingestion by phagocytes (98). Resistance to bacterial translocation is dependent on the proper function of the phagocyte. Facultative intracellular pathogens such as Salmonella have been shown to survive and multiply outside host immune cells as well as in host phagocytic cells.

Microvilli surrounding the Peyer's patches and dome cells are affected more than at other locations and have increased enterocyte degeneration. Intraluminal replication by S. choleraesuis is probably not as important as for other serotypes due to the fact that it is inherently more invasive.

After passage through the basement membrane, S. choleraesuis are phagocytosed by macrophages and neutrophils in the lamina propria. Numerous studies have been performed that demonstrate the ability of salmonellae to survive macrophage killing (2,12,30,50,59,64,65,89), but little attention has been directed toward defense by the PMNL. Approximately 24 hours after challenge, the bacteria are found in macrophages as well as gut-associated lymphoid tissue (33). The bacteria spread to the lamina propria and submucosa and cause acute inflammation with microvascular damage and thrombosis. The organism multiplies in the lymph nodes, enters the thoracic duct, and finally reaches the systemic circulation. Once bacteremia occurs, there is invasion of the liver, spleen, gall bladder, and bile. There may be hemorrhagic lesions in the cortices of the kidneys. Mesenteric lymph nodes are enlarged, congested, and edematous. The liver may contain white typhoid nodules on its capsular surface and parenchyma, and the spleen may be enlarged. Also associated with infection of S. choleraesuis are button-shaped ulcers in the colon (75,81,88).

Microscopic lesions are first seen approximately 16 hours after inoculation. The gastric mucosa is congested and infiltrated with macrophages and PMNL. Multifocal necrosis of mucosal columnar cells

overlies thrombosed vessels in the lamina propria. The villi of the small intestine are atrophic and fused, and apical enterocytes are eroded. The lamina propria is infiltrated with PMNL, and small blood vessels are filled with PMNL and fibrin. At 24 hours, there is increased necrosis of ileal mucosal surfaces, exposing the lamina propria and large numbers of infiltrated PMNL (75).

Septicemia may occur by 72 hours and may be fatal. Mucosal invasion is a required step in the disease process, but depending on the initial dose, intraluminal replication may be minimal with rapid and frequent invasion of the blood (81).

S. choleraesuis is invasive and regularly causes septicemia 24-72 hours before the onset of diarrhea. Diarrhea is caused by decreases in sodium reabsorption and increases in chloride secretions in response to increased levels of mucosal cyclic adenosine monophosphate (cAMP). The invasion of enterocytes by Salmonella causes the release of prostaglandins which stimulate adenylate cyclase (100).

Mucosal inflammation occurs simultaneously with diarrhea, but by a separate mechanism. Microvascular thrombi and endothelial necrosis in the lamina propria are consistently seen in porcine salmonellosis due to the presence of endotoxin. Salmonellae do not directly stimulate the vessels but do so while remaining protected intracellularly in the macrophage. Other contributing factors in mucosal necrosis are chemical products of inflammation produced by PMNL (75,100).

Because inflammation increases vascular permeability, and aided by macrophages as a transport vehicle, S. choleraesuis are able to enter the

bloodstream. This usually occurs in pigs less than 4 months old and leads to sudden death. The systemic signs and lesions of septic salmonellosis are caused by endotoxemia (81).

The endotoxin molecule is a heat-stable toxin which is associated with the cell wall of gram-negative bacteria. The endotoxic lipopolysaccharide (LPS) consists of an inner lipid A, a core oligosaccharide, and an O-polysaccharide on the outer surface. Lipid A has been purified and was determined to be the toxic portion of the molecule (6,66,82).

Virtually every organ as well as the immune system are affected by endotoxin. These effects include fever, leukopenia, hypoglycemia, hypertension, hypotension, shock, diarrhea, and death. Both in vitro and in vivo, endotoxin has at least four pathophysiologic effects. These include activation of complement, coagulation, fibrinolysis, and triggering of a series of enzymatic reactions causing the release of bradykinins and other vasoactive peptides (6,66,82).

Host defense

A variety of non-specific factors control the proliferation of S. choleraesuis. These include: a) the pH of the stomach contents, b) the physical barrier formed by mucus over the epithelium, c) the cleansing of the bowel by peristaltic movement, d) lysozyme secretions, e) reduced free iron levels due to lactoferrin, f) B_2-glycoprotein of gastric acid which activates the alternate pathway of complement, g) the nutritional state of the host, and h) competing intestinal microflora. These non-specific

control mechanisms also play an important role in maintaining a complete population of normal flora.

The normal flora contributes to host defense by competing for substrates, blocking receptors, changing the environment, producing bacterocins, producing short chain fatty acids, stimulating peristalsis, and stimulating development of the immune system. These factors have been clearly defined in germ-free animals. The study of specific factors that determine resistance to infection by salmonellae is usually focused on the extensive population of plasma cells and lymphocytes in the lamina propria, Peyer's patches, lymphoid follicles, and mesenteric lymph nodes. The first line of defense is the phagocytic PMNL and macrophages. The functions of these cells have been extensively studied; they kill bacteria by oxygen-dependent and oxygen-independent mechanisms (15,23,24,25,27,32,49,56,70,74,76,80,84,90). Numerous investigators have demonstrated that Salmonella survive and multiply within the macrophages of the RES. Cell mediated responses to control Salmonella invasion requires lymphokine activation of macorphages, although antibody also plays an important role in phagocytosis. Once bacterial ingestion occurs, the phagocyte protects the bacteria from the effects of humoral antibody and administered antibiotics. Because of the invasive nature of salmonellae and their ability to survive phagocytic killing, a cell mediated response is usually required to eliminate bacteria.

Immunization against Salmonella

Numerous investigations have been completed on various aspects of Salmonella pathogenesis, virulence mechanisms, and Salmonella-host

interactions. The study of immunity to Salmonella is divided between those who believe that humoral immunity induced by killed vaccines are the method of choice for protective immunity, and those who place Salmonella in a group of facultative intracellular parasites with cell mediated immunity as the most important immune defense (26).

There is no indication that humoral factors alone can prevent salmonellosis or other diseases caused by infectious intracellular or facultative intracellular pathogens. The ineffectiveness of passive antibody protection in mice has been demonstrated by showing that almost as much growth occurs in the tissues of serum-protected animals as in controls. Passive immunization of animals usually prolongs survival after challenge, but bacteremia and extensive multiplication within the hosts' own phagocytes occur (4,16,19).

Bacteria can usually be killed by normal phagocytes, but some virulent bacteria require an "activated" phagocyte. This generation of specialized phagocytic cells in the infected host is a significant part of the cell-mediated immune response. Once facultative intracellular pathogens have established themselves within a tissue, powerful chemotactic stimuli attract large numbers of PMNL. The PMNL enter the lesion and rapidly ingest all foreign particles. These cells can inactivate large numbers of bacteria because of their large numbers and multiple killing mechanisms. Only bacteria with capsules or smooth LPS can evade this host defense (17). Macrophages from individuals vaccinated with live salmonellae have an increased initial bactericidal activity and an ability to inhibit intracellular replication. Other intracellular

pathogens such as Brucella, Mycobacteria, and Haemophilus, inhibit phagocyte function which allows them to survive in non-activated phagocytes. It was then determined that induction of the immune response was specific, but expression of immunity was non-specific (17). It was proposed that T lymphocytes recognize the antigen and through lymphokine production and release, activate macrophages and neutrophils stimulating increased bactericidal activity.

In contrast to the view presented above, numerous investigators have worked toward the use of killed vaccines to induce immunity to Salmonella. Heat killed and deoxycholate extracts resulted in 100% protection of vaccinated mice against a 100 LD_{50} challenge dose (48). Multiple inoculations of heat-killed cells resulted in protection against as many as 10,000 LD_{50} challenge doses (26). Eisenstein and Sultzer (26) have examined the use of subcellular ribosome-rich extracts, acetone-killed bacteria, and phenol-purified LPS. These provided acceptable levels of protection against Salmonella infection.

The O-antigen is the major protective immunogen (26). Vaccination with strains of Salmonella identical except in their O-antigen composition, induced specific protection against challenge from the same, but not different O-antigen types (60). Additional support for the role of O-antigen in immunity is the fact that purified LPS itself can be protective (26). Humoral recognition of the LPS core antigens of gram-negative bacteria permits binding by cross-reacting immunoglobulins, complement mediated cytotoxicity, and may increase phagocytosis (93).

Contradiction exists among reports describing protection given by live versus killed vaccines (26). Because of this contradiction between the contribution of CMI and humoral immunity, the efficacy of various vaccines remains uncertain.

Eisenstein and Sultzer (26) determined that both live and killed vaccines were effective in promoting immunity to Salmonella, although their relative effectiveness differed. These differences were attributed to the differences in the method of vaccine preparation, route of infection, dose of vaccine, and genetics of the host.

Non-living vaccines induced humoral immunity, living vaccines induced both CMI and humoral immunity, and differences existed in host susceptibility. Therefore, Eisenstein and Sultzer (26) hypothesized that: 1) immunity to Salmonella is mediated by both arms of the immune system, 2) the genetics of the host plays a major role in the host response to vaccination, 3) a host inherently resistant to salmonellae needs only antibody for protection, but inherently susceptible animals also require CMI, and 4) the route of infection is a variable affecting the contribution of each arm of defense (26).

Bacterial virulence

To understand the pathogenesis of salmonellosis, it is important to identify the virulence factors associated with the bacteria. Once an organism has invaded the body, the host responds with a variety of specific and non-specific humoral defense mechanisms. The two most important humoral factors are immunoglobulins and complement, although

other factors such as fibronectin, lysozyme, conglutinin, and transferrin play a significant role in host defense (35,61,92,94).

Gram-negative organisms possess a variety of resistance mechanisms to overcome the action of complement. As a general rule, smooth bacteria are more resistant than rough strains to bactericidal activity. A direct correlation exists between the virulence of smooth S. choleraesuis in pigs and resistance to the bactericidal activity of serum (38).

The structure of the bacterial cell wall appears to be responsible for differences in complement sensitivity. Resistance may be due to rendering the membrane attack complex (MAC) impotent, by preventing complement activation, or by diminishing complement activation. The bacterial components which are known to affect complement function include LPS, capsules, and cell wall proteins (91).

The O or lipopolysaccharide (LPS) somatic antigens are important determinants in the virulence of Salmonella. Rough mutants of the organism lack O-sidechains of LPS and are non-pathogenic, semi-rough strains with shortened LPS polymers are only moderately invasive, and smooth strains with intact sidechains are highly pathogenic. This effect of LPS plays an important role in masking recognition of the organism by the immune system, especially complement and phagocytes (36,37).

Salmonella strains which differed in the structure of their O antigen side chains differed in complement consumption. Those that differed in the lenght of their O side chains had no effect, but differences in structure did affect complement consumption. These changes affect the rate of complement deposition, opsonization, and phagocytosis (40,85).

Not only does LPS affect complement activation, but it may also inhibit its function. The O antigen of Salmonella may allow activation, binding, and formation of the MAC and then shed the LPS-MAC complex from its surface or because of the long length of some LPS, it may prevent insertion of the MAC into the lipid bilayer (35). C3b binds preferentially to long LPS over shorter strands. The MAC then forms at a site where it is unable to insert into the membrane and cause lysis (41).

Bacterial capsules are external cell surface components and may prevent opsonization and phagocytosis. Capsules are present on most bacteria responsible for invasive infections, and are therefore an important protective mechanism against host defense. The K1 capsule of E. coli is composed of N-acetylneuraminic acid polymers. The capsule may be responsible for covering cell components which would normally activate complement. This capsule is also weakly immunogenic and prevents immune recognition of the bacterium (20). It was observed that several passages of an avirulent strain of S. typhi through mice resulted in the appearance of a virulent strain which was not agglutinated by O antiserum. There was an inverse relationship between virulence and the ability of the bacteria to be agglutinated by O antiserum; the virulent strains were not agglutinated by O antisera due to the Vi capsular antigen. This antigen which is a highly polymerized acid polysaccharide consisting of repeating units of N-acetyl-d-galactosaminuric acid is found in some strains of S. typhi, S. paratyphi, S. dublin, and Escherichia and Citrobacter spp. The Vi antigen is neither toxic nor virulent alone, but bacteria with both O

and Vi antigens are virulent. The Vi antigen prevents phagocytosis and protects against the bactericidal activity of serum (101).

Other components of the cell wall which may contribute to serum resistance are the outer membrane proteins (OMP). Plasmids such as Col V in invasive E. coli encode for the production of a serum-resistant OMP. Col V may be similar to an 11 Kd cryptic plasmid in S. typhimurium which also codes for an OMP responsible for serum resistance (44).

Resistance to immunoglobulins is another mechanism that bacteria use to establish infection. Staphylococci have cell walls composed of peptidoglycan, teichoic acid, and protein A. Protein A is capable of binding IgG by its Fc fragment. This interferes with the ability of immunoglobulins to opsonize, and also interfers with complement activity. This leads to diminished availability of complement for activation at the cell surface (99,104).

Some group B streptococci are capable of binding host proteins such as IgA, fibrinogen, and haptoglobin to camouflage themselves and avoid an immune response (104).

Another mechanism that bacteria use to protect themselves is through the production of trypsin-like enzymes that cleave antibody. These proteases are produced by Neisseria, Haemophilus, and Streptococcus. They are secreted into the culture supernatants, are effective against IgA and are very antigenic (62).

Antigenic mimicry and antigenic variation are additional techniques employed by some bacteria to avoid immune clearance. The polysaccharide capsule of E. coli K1 is similar to a glycoprotein oligosaccharide of the

human and rat brain. This antigenic similarity prevents recognition of E. coli as being foreign and inhibits the development of a humoral response (67,104). Antigenic variation consists of changes of surface antigens. Therefore, the antibody produced is not directed toward the current bacterial antigens being expressed. The antigens of Vibrio cholerae and of Camplybacter fetus adjust in vivo to immunologic pressure in gnotobiotic mice. A rough strain was introduced into a host, later became smooth, and was not recognized by the antibody produced (83).

Lactoferrin and transferrin in fluids, and ferritin and hemoglobin in tissues and cells make iron unavailable to invading bacteria. Therefore, an important survival and virulence mechanism of bacteria is their ability to obtain iron. They do this either by production of high affinity iron chelating agents which compete successfully with host iron-binding proteins, or by direct removal of iron by bacterial surface receptors, or by lysis of erythrocytes and degradation of heme containing proteins (39,67). The soluble, low-molecular weight, high affinity iron chelators are known as siderophores. Bacteria such as Salmonella, Escherichia, Klebsiella, and some Shigella secrete siderophores into their surroundings. Several of these bacteria produce a phenolate iron chelator, enterobactin, as well as a hydroxamate type siderophore, areobactin. Once secreted, siderophores acquire iron and then interact with specific OMP receptors produced by the cell. The iron-siderophore complex is taken up by a vesicle and iron is released to the cell. Enterobactin is produced in high quantities but is degraded in the vesicle. In contrast, aerobactin is produced in smaller quantities, has a

higher affinity for iron, and is recycled so it requires less energy (39,67).

Salmonella strains carrying plasmids which produce aerobactin as well as enterobactin not only are resistant to antibiotics, but produce disease with a higher incidence of septicemia. Mutation which causes the loss of siderophore production significantly reduces the virulence of S. typhimurium for mice and its ability to multiply in human serum (106).

Virulence factors are often associated with plasmids. Plasmids may code for a variety of cellular properties such as antibiotic resistance, serum resistance, outer membrane proteins, siderophores, adhesins, and invasive capabilities. Each serotype of Salmonella has a serotype-specific plasmid. S. typhimurium, S. enteritidis, and S. choleraesuis possess a 60 Md, 37 Md, and a 30 Md plasmid respectively. Strains with plasmids required a 10^6 fold lower dose to cause 50% lethality in mice than plasmid-free strains. Strains with plasmids were also resistant to the bactericidal activity of 90% guinea pig serum and were able to invade the livers of orally infected mice (47). The importance of plasmids in cell invasion has been demonstrated using HeLa cells as a model. S. typhimurium was significantly less adhesive and invasive when cured of its 60 Mdal plasmid than when the plasmid was present (53). This plasmid was then examined by other investigators and shown to be responsible for resistance to phagocytic killing rather than colonization of the Peyer's patches (45).

In vitro examination of S. typhimurium indicates the importance of a 100 Kb plasmid in bacterial virulence. Plasmid cured strains showed

increased LD_{50} doses, but did not affect adherence or invasion of Chinese Hamster Ovary (CHO) cells. Phagocytosis and killing by macrophages was also not affected. It appears that the role of this plasmid is primarily to invade mesenteric lymph nodes and the spleen after oral inoculation (43).

Bacteria-CMI interaction

Although humoral protection is a significant barrier to microbial infection, true protection against invasive bacteria requires a cellular response as well. A successful cellular response requires directed migration of phagocytes, attachment, ingestion, stimulation of oxidative burst, phagosome-lysosome fusion, activation of specific immune responses, phagocyte response to cytokines, and removal of bacterial debris (21,32,56).

The initial defense is provided by local mononuclear phagocytic cells found in tissues. If the inoculum is too large or if the organism is virulent, other macrophages and PMNL from the blood will be recruited to the area. The directed migration of phagocytes may be due to bacterial components such as N-formylated tripeptides, or to host components such as C5a (70).

Once the phagocytes have reached the focus of infection they must attach to the bacteria. This attachment is usually mediated by opsonins such as IgG and C3b. After attachment is complete, the phagocyte will attempt to internalize the bacteria into a plasma membrane-lined vesicle called a phagosome. Reportedly, C3b receptors of non-activated phagocytes mediate binding, but not ingestion of some particles (56).

Changes in the plasma membrane initiate an intracellular cascade that results in the activation of cytochrome oxidase on the inner surface of the phagosome and initiate movement of lysosomes to the phagosome. Phagosome-lysosome fusion occurs thus forming a phagolysosome and allowing an influx of lysosomal granule contents. There is a decrease in the pH of the phagolysosome and an increase in the oxygen uptake and glucose utilization via the hexose-monophosphate pathway. This leads to the production of highly reactive oxygen intermediates such as hydroxyl radicals, superoxide anion, and hydrogen peroxide (32,56).

The phagolysosomal environment is extremely toxic to the bacteria due to a combination of low pH, presence of oxygen intermediates, peptides, and hydrolases released from the granules during fusion. In most instances the bacteria are killed within minutes and there is no need for an immune response. In some cases bacteria survive ingestion and the host must initiate the proper immune response.

Facultative intracellular parasites invoke a humoral immune response. The antibodies produced interact with smooth somatic antigens and capsular antigens resulting in opsonization and subsequent phagocytosis. At mucosal surfaces, antibodies play a protective role by blocking bacterial attachment. Despite these humoral defense mechanisms, some highly virulent pathogens still invade the tissues and establish septic infection. Elimination of this type of infection requires induction of a specific T cell mediated immune response (18). The T cells activate mononuclear phagocytes, PMNL, and other T cells to eliminate these pathogens. Some pathogens may be able to survive this initial period of

ingestion by non-activated phagocytes, but most are eliminated once large numbers of activated phagocytes and T cells enter the site of infection (18).

Despite the potency of an activated CMI response, bacteria have developed methods to escape killing and in some cases use the host immune cells to their own advantage.

Once host leukocytes have detected bacteria and migrated toward a site of invasion, they must attach to and then internalize the bacteria. One avoidance mechanism of bacteria is through the production of antiphagocytic surface components such as capsules. These may be polysaccharide or protein in nature and prevent internalization by the phagocytes. The mechanisms by which encapsulated bacteria resist phagocytosis include decreased binding of serum opsonins, inaccessible ligands required for phagocyte ingestion, and decreased hydrophobicity of the bacterial surface (24,25,49,74,101).

Other bacteria, such as mycoplasmas and gonococci, readily attach to phagocytic cells but avoid internalization. They then proliferate on the phagocyte surface protected from host antimicrobial defenses. Specific antibodies against these bacteria do promote ingestion and killing by PMNL and macrophages, but some bacteria seem to be resistant to killing or alter the phagocytes so they cannot kill a second target (21).

A second, very effective mechanism that bacteria use to minimize their interaction with CMI is through the invasion of non-phagocytic cells. This mode of pathogenesis has been well studied in the infection of endothelial and epithelial cells by Rickettsia and Chlamydia

(5,8,22,57), but is used by other bacteria as well. In vitro studies of Shigella spp., Yersinia spp., some E. coli, S. typhimurium, and S. typhi, have shown that these bacteria invade eukaryotic cells (7,34,46,58,72,87,95,105,107). The exact mechanism of invasion appears to vary depending on the bacterium (7). Cellular invasion allows the bacteria to use host cell constituents for growth as well as protecting them from the host immune system.

The third survival mechanism used by some bacteria is survival and growth within phagocytic cells. Facultative intracellular pathogens capable of this include Mycobacterium, Yersinia, Brucella, Listeria, Haemophilus, and Salmonella. The mechanism of survival of each of these bacteria varies with species.

Perturbation of the phagolysosome is a technique used by some bacteria to avoid killing. Rickettsia and Mycobacteria have been reported to escape from the disrupted phagosome and grow in the cytoplasm (21).

M. tuberculosis survives in the phagosome by interfering with phagosome-lysosome fusion. Azurophilic granules become coated with strongly acidic sulfatides which then prevent fusion. Viable bacteria are able to avoid exposure to lysosomal contents whereas non-viable bacteria do allow degranulation (1,21,74).

Haemophilus somnus has been shown to have little effect on the respiratory burst of bovine PMNL as measured by nitroblue tetrazolium (NBT) reduction. There was a decrease in iodination, an indirect measure of primary degranulation, by low molecular weight nucleotides (14,51). A similar effect on degranulation has been studied in the survival of

Brucella abortus in bovine PMNL. No effect on PMNL respiratory burst was observed, but low molecular weight nucleotides did inhibit primary degranulation (3,10,11,79).

Phagocytes use a potent array of oxygen intermediates to kill ingested bacteria (21,23,27,32,57,76,80). Therefore, bacteria have developed various tactics to inhibit stimulation of an oxidative response. Bacterial enzymes prevent formation of oxygen radicals, or enzymatically neutralize oxygen radicals (102).

Non-opsonized B. abortus decreased the oxidative metabolism of phagocytes, allowing survival, whereas opsonized bacteria were killed (9). Other bacteria, such as Listeria monocytogenes, produce superoxide dismutase which converts oxygen radicals to hydrogen peroxide which can be broken down to oxygen and water by catalase (21). Human phagocytes ingested virulent and avirulent strains of S. typhi with equal avidity, but virulent strains significantly reduced the oxidative response. It has been postulated that the decreased oxidative response may be due to masking of receptors (57).

Finally, some bacteria such as S. typhimurium and M. lepraemurium are resistant to the lysosomal contents. This resistance appears to be associated with the LPS content of the cell wall (29,77,78,86,97).

Salmonella-phagocyte interaction

Investigations of the interaction of Salmonella and phagocytic cells have largely focused on the macrophage because of its longer life, ability to regenerate granules, and continued metabolism. However, the PMNL

comprise 37% of the blood phagocytes in pigs (63) and therefore may be the most important phagocyte in preventing invasion by Salmonella. Phagocyte-Salmonella interactions were studied in two separate ways: 1) investigations of phagocytic efficiency against Salmonella, and 2) investigations of the mechanisms Salmonella use to alter the phagocyte.

As early as the 1960s, researchers were aware of different interactions between phagocytes and various salmonellae. Smooth wild type and rough cell wall mutants of S. typhimurium and S. enteritidis were examined for their sensitivity to ingestion and killing by peritoneal macrophages. Rough strains were ingested easier than smooth strains and were sensitive to early intracellular killing. It was shown that a complete bacterial wall core was essential for resistance to ingestion and intracellular survival. The O-side chains further enhanced this resistance, but were not essential. This was determined to have occurred in both the macrophage and PMNL, but killing rates were effected more in the PMNL (29).

The differences in survival rates between smooth and rough Salmonella inside phagocytes were attributed to differences in the interaction between lysosomal enzymes and the LPS of the cell wall. Studies of S. typhimurium and cell wall mutants indicated that a complete LPS core was essential for resistance to bactericidal activity, but the O-side chain was not (28).

It had been known that phagocytes can kill bacteria by oxygen-dependent mechanisms, but not until a series of elaborate experiments by Okamura and Spitznagel (69) was the importance of oxygen-

independent mechanisms realized. The evaluation of the ability of human PMNL to phagocytose under aerobic and anaerobic conditions, as well as the effect of oxygen-dependent and oxygen-independent mechanisms were examined. S. typhimurium and its LPS mutants stimulated production of superoxide anion by phagocytes under aerobic, but not under anaerobic conditions. Although no oxygen intermediates were produced under anaerobic conditions, the PMNL killed these bacteria in an order that appeared to be dependent on their LPS content. As the LPS content decreased, the bacteria became more sensitive to oxygen-independent bactericidal activity (69).

The importance of the oxygen-independent granules was further defined when the various granule extracts were examined. Extracts of both subpopulations of azurophilic granules and the specific granules were examined for bactericidal activity against S. typhimurium. As the bacterial cell LPS content decreased, the bacteria became increasingly susceptible to all the granule extracts. Bactericidal activity of the extracts ranged from the most potent mixed population of azurophilic and specific granules, followed by azurophilic only, and finally specific granules only. This activity was temperature and pH dependent (77,78).

The granule extracts appeared to possess anti-bacterial activity due to their ability to bind to lipid A of the cell wall. Bacteria with smooth LPS block the binding of lipid A, as does polymyxin B in S. typhimurium mutants. In each case, blocking of lipid A binding caused resistance of the bacteria to the granule contents (86).

Although oxygen-independent mechanisms of phagocytosis have been thoroughly studied with Salmonella in humans and laboratory animals, few investigators have examined the interaction of S. choleraesuis with porcine phagocytes. Griffith and Kramer (37) examined numerous S. choleraesuis strains with known sensitivities to antibody-complement lysis. Susceptibility in vitro to the action of porcine PMNL paralleled the sensitivity to antibody-complement. These differences were undoubtedly related to the compositions of the cell walls (37).

Furness (30) compared the interaction of avirulent and virulent S. typhimurium with mouse macrophages. No difference was observed in the efficiencies of phagocytosis, but some virulent Salmonella survived, multiplied, and then lysed the macrophages (30). The opsonized virulent strains were then able to multiply under conditions which killed avirulent Salmonella. Contrary to this, others have reported that opsonized virulent S. typhimurium were not phagocytosed as well as avirulent strains by mouse macrophages. Macrophages prestimulated with Mycobacterium tuberculosis (BCG) killed Salmonella better than normal macrophages (52).

Vaccination of mice with live S. enteritidis resulted in rapid clearance from the blood as well as inhibition of intracellular growth. This was not dependent on the presence of antibody. The virulent strain showed rapid intracellular multiplication and macrophage death within three days (64).

Normal macrophages exposed to opsonized S. typhimurium and S. typhosa of varied virulence killed all strains almost immediately. S. typhimurium

only required opsonization with normal serum for ingestion, whereas the S. typhosa required opsonization by specific antibody (65).

Virulent and avirulent S. typhimurium and their interactions with guinea pig macrophages were examined. One percent of the avirulent strains survived ingestion compared to 8% survival for the virulent strains. After the initial killing, there was intracellular growth of both strains (50). Twice as many virulent bacteria per macrophage were required to obtain equal numbers of internalized bacteria by comparison to the avirulent strain. This indicates that virulent strains resist not only killing, but also ingestion (50).

Although comparative data on ingestion and killing of virulent and avirulent Salmonella often are confusing and contradictory, most authors agree that Salmonella are resistant to lysosomal enzymes. Studies involving S. typhimurium and mouse macrophages indicated that there was no inhibition of granular fusion or increased levels of cAMP. There does seem to be a direct correlation between the amount of LPS and the level of resistance (12).

The ability of Salmonella to resist phagocytic killing has been well established, but the mechanism of resistance is not certain. Human PMNL were evaluated in the presence of virulent and avirulent S. typhi. Ingestion was equivalent for both strains. The virulent strain caused a significant decrease in oxygen metabolism by PMNL. This may be due to direct inhibition of oxygen metabolism or diminished stimulation of the proper receptors, possibly by masking. Despite the decrease in cellular oxygen metabolism caused by ingestion of virulent strains, both strains

were killed in equal numbers indicating death due to low levels of oxygen radicals or sufficient killing by oxygen independent mechanisms (57).

The role of flagella as a virulence factor and its interaction with phagocytes has recently been investigated by using S. typhimurium in mice. Flagellated and non-flagellated strains of S. typhimurium were used. The presence of flagella, not their function, was in fact a virulence factor. Flagella did not affect colonization, but did allow increased replication of bacteria in the liver and spleen. Flagellated bacteria were then shown to be more resistant than non-flagellated strains to macrophage killing in these organs. It appears that flagella either protect the Salmonella from intracellular killing or enhance their ability to multiply intracellularly. Hypothetically this could be explained in several ways: 1) flagellated Salmonella may not stimulate the respiratory burst due to masking of receptors, 2) flagellated strains and non-flagellated strains may be sequestered in different compartments in the macrophage, and 3) flagella may protect Salmonella from microbicidal enzymes (13,96).

The possible role of PMNL in host defenses against Salmonella has received little attention. According to Jubb and Kennedy in Pathology of Domestic Animals (54),

> "the cellular defense against (Salmonella) is conducted by fixed and sessile phagocytes of the reticuloendothelial system and the polymorphs have little or nothing to do with (defense)".

In contrast, Baskerville et al. (2) showed that PMNL had the ability to kill some Salmonella. Furthermore, the PMNL may actually promote the spread of virulent strains resistant to killing (2). This was again evaluated using PMNL from pigs and their interaction with S. anatum and S.

typhimurium. It was determined that PMNL from animals infected with S. typhimurium had enhanced bactericidal activity. This was attributed to increased maturation of immature cells and enhancement of functional capabilities of PMNL in the presence of Salmonella (89).

The purpose of this study was to investigate the interaction of virulent and avirulent Salmonella choleraesuis with PMNL from the host most commonly infected, the pig.

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PORCINE NEUTROPHIL FUNCTION IN THE PRESENCE OF VIRULENT AND AVIRULENT *SALMONELLA CHOLERAESUIS*

Porcine neutrophil function in the presence of virulent and avirulent *Salmonella choleraesuis*

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ABSTRACT

Roof, M.B. and Kramer, T.T., 1989. Porcine neutrophil function in the presence of virulent and avirulent Salmonella choleraesuis. Vet. Immunol. Immunopathol.,

Porcine polymorphonuclear leukocytes (PPMNL) may be activated by bacteria to begin phagocytosis followed by oxidative and non-oxidative mechanisms of killing. The purpose of this study was to identify differences between virulent and avirulent Salmonella choleraesuis (S. choleraesuis) strains, 38 and 9 respectively, in their interactions with PPMNL using five different assays. 1) Staphylococcus aureus (S. aureus) ingestion was determined by exposure of PPMNL to a mixture of S. choleraesuis and ^{125}I labeled S. aureus. There were 3.0% and 22.2% decreases in S. aureus ingestion by mouse-avirulent S. choleraesuis 9 and mouse-virulent S. choleraesuis 38, respectively. 2) Proteins were iodinated by exposing zymosan-stimulated PPMNL to S. choleraesuis in the presence of ^{125}I and measuring the incorporation of the label into PPMNL proteins. Iodination of PPMNL proteins was decreased by 37.5% and 38.5% by S. choleraesuis 9 and S. choleraesuis 38, respectively. 3) Cytochrome c reduction was performed by using PPMNL, zymosan, and S. choleraesuis to determine the bacterial effect on superoxide anion production. S. choleraesuis 9 and S. choleraesuis 38 inhibited superoxide anion production by 40.6% and 53.4%, respectively. 4) Lactoferrin release from PPMNL was measured by an ELISA, using the supernatant from the cytochrome c assay. Lactoferrin release was increased by 5.0% and 6.0% by

S. choleraesuis 9 and 38, respectively. 5) A bactericidal assay was performed by counting CFUs of S. choleraesuis after preliminary incubation with PPMNL, followed by killing of extracellular S. choleraesuis and lysis of PPMNL. Survival of S. choleraesuis 9 and E. coli (control) were 7.5% and 1.4% respectively, in contrast to 52.6% survival of the virulent S. choleraesuis 38. These results indicate that both strains inhibited protein iodination, decreased superoxide anion production, and caused a slight increase in lactoferrin release, but the virulent S. choleraesuis 38 inhibited S. aureus ingestion and survived PPMNL killing more effectively than the S. choleraesuis 9.

INTRODUCTION

Salmonella choleraesuis var kunzendorf causes the septicemic disease swine paratyphoid and is the most frequent serotype associated with disease in swine. This is not only a major concern to swine producers, but can also be a source of food-borne salmonellosis (15). Polymorphonuclear leukocytes (PMNL) make up 37% of mature leukocytes in the blood of pigs (17) and are an important mechanism in preventing bacterial infection. In this report we will describe the interaction between Salmonella choleraesuis (S. choleraesuis) and porcine polymorphonuclear leukocytes (PPMNL).

Investigations of host defenses against salmonellosis have largely been focused on Salmonella as facultative intracellular pathogens, with cell mediated immunity as the main host defense. Studies on the pathogenesis of S. typhimurium have focused on the ability of intracellular salmonellae to evade killing by human, guinea pig, and mouse macrophages (2,3,5,11,14,16,18).

PPMNL can kill bacteria by oxygen-dependent and oxygen-independent mechanisms (4,6,13,22). A wide variety of virulence factors are known which alter these phagocytic cell functions. Included in these factors are: 1) inhibition of lysosome-phagosome fusion, 2) resistance to lysosomal enzymes, 3) exotoxin-induced cytotoxicity, 4) inhibition of oxidative response, and 5) inhibition of function by adenylate cyclase (19).

It has been shown that virulent S. typhimurium can survive better than avirulent strains when phagocytosed by macrophages (9) and can then multiply and spread to other sites.

The purpose of this study was to investigate the interaction of virulent and avirulent S. choleraesuis with PPMNL.

MATERIAL AND METHODS

Bacteria

Smooth clinical isolates of S. choleraesuis from the Iowa Veterinary Diagnostic Laboratory (VDL), and selected, virulence-defined strains (TTK) (7), were grown in brain heart infusion broth (BHI) (Difco, Detroit, MI, U.S.A.) at 37°C overnight. Glycerine was added to a final concentration of 15% and aliquots were frozen at -70°C.

These strains were streaked onto BHI agar and incubated at 37 C overnight. The cells were harvested, washed three times in 0.85% NaCl and resuspended in 0.85% NaCl to a concentration that, when diluted 1/10, produced an O.D. of 0.2 at 540 nm (2×10^8 cells/ml). These cell suspensions were used in all the PPMNL function assays described.

Sources and preparation of PPMNL

Eight healthy 40 lb pigs were used as a source of PPMNL. PPMNL were isolated by using a modification of procedures described previously (23). Briefly, blood was collected in an acid-citrate-dextrose solution and cells were sedimented by centrifugation. The resulting buffy coat was harvested. Contaminating erythrocytes were lysed twice with phosphate-buffered deionized water, and the remaining cells were separated on a stepwise Percoll gradient to isolate a relatively pure PPMNL sample. The PPMNL were washed three times and resuspended to a concentration of 5×10^7 cells/ml. All samples were >95% viable by Trypan blue exclusion staining.

PPMNL function assays

PPMNL function tests were performed using a modification of procedures previously described for bovine PPMNL (24).

S. aureus ingestion: This assay measured the effect of S. choleraesuis on ingestion of heat-killed S. aureus by PPMNL. Heat-killed S. aureus labeled with (^{125}I)iodo-deoxyuridine (UdR); (Amersham, Arlington Heights, Ill) were used to evaluate ingestion by PPMNL. The test was conducted in duplicate and the average of the values was used for calculation. The standard reaction mixture contained 0.10 ml of (^{125}I)UdR-labeled S. aureus in PBS (1.5 x 10^8 CFU/ml), 0.05 ml of PPMNL in PBS (2.5 x 10^6 PPMNL), 0.05 ml of 1:10 dilution of bovine antiserum to S. aureus, and 0.30 ml of Earles balanced salt solution (EBSS; GIBCO, Grand Island, NY). To determine the effects of S. choleraesuis on ingestion by PPMNL, we added 0.05 ml of S. choleraesuis (2 x 10^8 CFU/ml) previously prepared or 0.05 ml of phosphate buffered saline (PBS) (as a control) to the standard reaction mixture. All components except the PPMNL were added and the mixture allowed to equilibrate to 37 C. The reaction was started with the addition of PPMNL. After incubation at 37°C for 10 minutes with agitation (Microshaker^R; Dynatech, Alexandria, Va), extracellular S. aureus were lysed with lysostaphin (0.05 ml, 1 IU in PBS) for 30 min at 37°C. The PPMNL were washed twice with 2.0 ml of PBS. The final pellet was placed in a gamma counter to determine counts per minute (CPM) of radioactivity present. For each assay, a control of S. aureus without lysostaphin was prepared. Another control contained all reactants

except PPMNL. Results were expressed as the percent inhibition of labeled S. aureus ingested.

$$\text{Percent ingestion} = \frac{(\text{CPM in reaction tube}) - (\text{CPM in background tube})}{(\text{CPM in standard tube}) - (\text{CPM in background tube})} \times 100$$

Iodination assay: The myeloperoxidase ($MPO-H_2O_2$-halide) activity of PPMNL was determined by measuring the amount of ^{125}I incorporated into protein. The assay was performed in duplicate and the average of the values was used for calculations. The standard reaction mixture contained 0.05 ml of PPMNL (2.5×10^6 PPMNL), 0.1 uCi of ^{125}I in 0.05 ml of EBSS, 0.05 ml of NaI in EBSS (20 nmole), 0.05 ml of zymosan exposed to fresh porcine serum (7.5 mg/ml in EBSS) as prepared previously (23), and 0.30 ml of EBSS. Iodination by resting cells was determined similarly, but in the absence of preopsonized zymosan. To determine the effects of S. choleraesuis on $MPO-H_2O_2$-halide activity of PPMNL, 0.05 ml of S. choleraesuis (2×10^8 cfu/ml) or 0.05 ml of PBS (as a control) was added to the standard reaction mixture. The reaction was started by the addition of PPMNL. After incubation at 37°C for 20 min with agitation, the cells were washed twice with 2.0 ml of 10% trichloroacetic acid (TCA) and the amount of encorporated radioactivity was determined. Results were expressed as nmole of NaI per 10^7 PPMNL per hour.

Cytochrome c: The amount of superoxide anion produced by PPMNL during the oxidative metabolic burst was determined by measuring the change in optical density due to the reduction of cytochrome c by superoxide anion. The assay was conducted in triplicate, and the average

of the values was used for calculation. The standard reaction mixture contained 0.150 ml of cytochrome c solution (Gibco: 538 uM in EBSS without phenol red), 0.05 ml of PPMNL in PBS (2.5 x 10^6 PPMNL), and 0.05 ml of preopsonized zymosan (7.5 mg/ml in EBSS without phenol red). Superoxide anion production by resting PPMNL was determined similarly, except that preopsonized zymosan was replaced with 0.05 ml of EBSS without phenol red. To determine the effects of S. choleraesuis on superoxide anion production by PPMNL, 0.050 ml of S. choleraesuis (2 x 10^8 CFU/ml) or 0.050 ml of PBS (as a control) was added to the standard reaction mixture. Reagents were added and the reaction was started with the addition of PPMNL. After incubation at 37°C for 30 minutes with agitation, the culture supernatant was collected into a conventional 96-well plate and the O.D. of the solution at 550 nm was determined by using a Micro-plate autoreader (model EL 310;BioTech Inst).

Lactoferrin release: The supernatant from the cytochrome c assay was utilized in a sandwich ELISA to determine lactoferrin release. A microtitration plate was coated overnight with primary avian anti-porcine lactoferrin antibody diluted 1:500 in 0.05M carbonate-bicarbonate coating buffer (pH 9.6). Non-specific binding sites were blocked with 0.5M carbonate-bicarbonate buffer containing 2% bovine serum albumin (GIBCO, Grand Island, NY). Secondary rabbit anti-porcine lactoferrin antibody diluted 1:500 in coating buffer was added, followed by goat anti-rabbit antibody conjugate (Kirkegaard and Perry Lab., Inc.) diluted 1:1000 in coating buffer. Reagents were pre-calibrated for optimal concentration by using a checkerboard design. The plate was washed five times between each

step using 0.01M PBS containing 0.05% Tween 20 to remove all excess antibody. Peroxidase substrate (Kirkegaard and Perry Lab., Inc.) was added and the reaction was stopped after 30 minutes. The resulting color change was then examined at 504nm (Molecular Devices, Vmax kinetic microplate reader). The amount of bound lactoferrin from normal PPMNL were compared to the values obtained by zymosan and zymosan-Salmonella stimulated PPMNL values.

Bactericidal assay: To determine the survival of S. choleraesuis within PPMNL, overnight culture of S. choleraesuis and of E. coli K88 (control) were sedimented by centrifugation and suspended in 10 mM $MgCl_2$ to an O.D. of 0.20 at 540 nm (2 x 10^8 cells/ml). The test was conducted in 1.5 ml microcentrifuge tubes containing 0.20 ml of bacterial suspension, 0.20 ml PMNL (1 x 10^6 PPMNL), 0.10 ml of heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 0.10 ml porcine anti-S. choleraesuis and anti-E. coli sera respectively, and 0.40 ml of M199 (GIBCO, Grand Island, NY). After incubation with agitation at 37°C for 20 minutes, the mixture of PPMNL and bacteria was pelleted and the supernatant removed. The pellet was resuspended in 1.0 ml of 100 ug/ml gentamicin, incubated for 5 minutes, washed once with PBS and resuspended in 1.0 ml of PBS. Aliquots were removed at 0, 30, 45, and 60 minutes, and diluted in 0.1% SDS in PBS to lyse the PPMNL and plated. A control consisting of 0.20 ml bacteria in PBS in lieu of PPMNL was included in the assay to insure that extracellular bacteria were killed by gentamicin. A total viable bacterial count before addition to the test mixture was also done in duplicate to determine the total number of bacteria added.

Bacterial viability assay: This assay was done to allow visual determinations of bacterial viability after ingestion by the PPMNL. The acridine orange staining was performed using a modification of the procedure described (20). The S. choleraesuis-PPMNL mixture used in the bactericidal assay was spread on a coverslip and incubated for 30 minutes to allow attachment. The coverslips were gently washed with PBS and then incubated 5 minutes with acridine orange (20 ug/ml). The preparation was washed with PBS and inverted onto a slide where it could be viewed with fluorescent microscopy. The live bacteria were green, whereas the dead bacteria were stained orange.

Statistical evaluation of data: All data were statistically analyzed at $P<0.01$ level by using the T test (LSD) which controls primarily the type I comparison error rate, and the Tukey's studentized range (HSD) which controls primarily for the type I experimentwise rate.

RESULTS

Results of function assays

Effect of S. choleraesuis on the ingestion of S. aureus: The results of studies of the ingestion of S. aureus are shown in Figure 1. Ingestion of S. aureus was reduced by 2.9 +/- 3.9% of control values by the avirulent S. choleraesuis 9 and by 22.2 +/- 4.5% of control values by the virulent S. choleraesuis 38. This reduction of S. aureus ingestion by S. choleraesuis 38 was significant at $P<0.01$.

Effects of S. choleraesuis on iodination: The effects of S. choleraesuis on the ability of PPMNL to iodinate protein are presented in Figure 2. Iodination of proteins was inhibited by both S. choleraesuis 9 (37.5%) and S. choleraesuis 38 (38.5%). The zymosan activated PPMNL were significantly stimulated ($P<0.01$) over the resting PPMNL.

Effect of S. choleraesuis on cytochrome c: The results of the cytochrome c reduction assay are shown in Figure 3. The ability of PPMNL to produce superoxide anion in response to opsonized zymosan, as measured by the reduction of cytochrome c, was decreased by both S. choleraesuis 9 (40.6%) and S. choleraesuis 38 (53.4%). The decrease in cytochrome c reduction by S. choleraesuis 9 was not significant from values obtained from opsonized zymosan, but S. choleraesuis 38 significantly ($P<0.01$) decreased cytochrome c almost to the level of the resting PMNL.

Effect of S. choleraesuis on lactoferrin release: The ability of S. choleraesuis to affect lactoferrin release is shown in Figure 4. The results indicate that S. choleraesuis 9 caused a 5.0% increase and

S. choleraesuis 38 a 6.5% increase in lactoferrin release compared to the zymosan activated PPMNL alone, but these differences were not significant.

Bactericidal assay: The results of the bactericidal assay (Figure 5), indicated that significant ($P<0.01$) numbers (52.6% +/- 10.3%) of S. choleraesuis 38 can survive intracellularly in the PPMNL after 60 minutes of phagocytosis. This contrasted with S. choleraesuis 9 and E. coli K88 which survived at rates of 7.5 +/- 1.8% and 1.4 +/- 1.0% respectively. Laboratory strains (TTK) and clinical samples from clinically ill swine (VDL) were examined (Figure 5). Virulent strains TTK-S. choleraesuis 38, 33-13, 33, CV-13, CV-16, VDL-S. choleraesuis 11, and VDL-S. choleraesuis 15 were all significantly ($P<0.01$) more resistant than E. coli K88 to intracellular killing, with values ranging from 27.9% to 41.3% survival 60 minutes after phagocytosis. The survival of three isolates was similar to E. coli; these were avirulent TTK-S. choleraesuis 9, 61, and VDL-S. choleraesuis 17. Figure 6 shows the viabilities of strains TTK-9, 38, 33-13, and E. coli K88 after 0, 30, 45, and 60 minutes of PPMNL exposure.

Bacterial viability assay: Salmonella-PPMNL preparations were stained with acridine orange to confirm that the PPMNL in the bactericidal assay ingested bacteria as expected, and enabled the visual identification of live versus dead intracellular bacteria. PPMNL phagocytosing S. choleraesuis appeared with brightly green nuclear fluorescence, under acridine orange fluorescent microscopy, whereas PPMNL phagocytosing E. coli exhibited a mixture of green and orange nuclear fluorescence. Numerous killed but unlysed extracellular E. coli were seen, whereas most

S. choleraesuis remained sequestered within the PPMNL. Although not a quantitative assay, it did allow confirmation of the results obtained in the bactericidal assay.

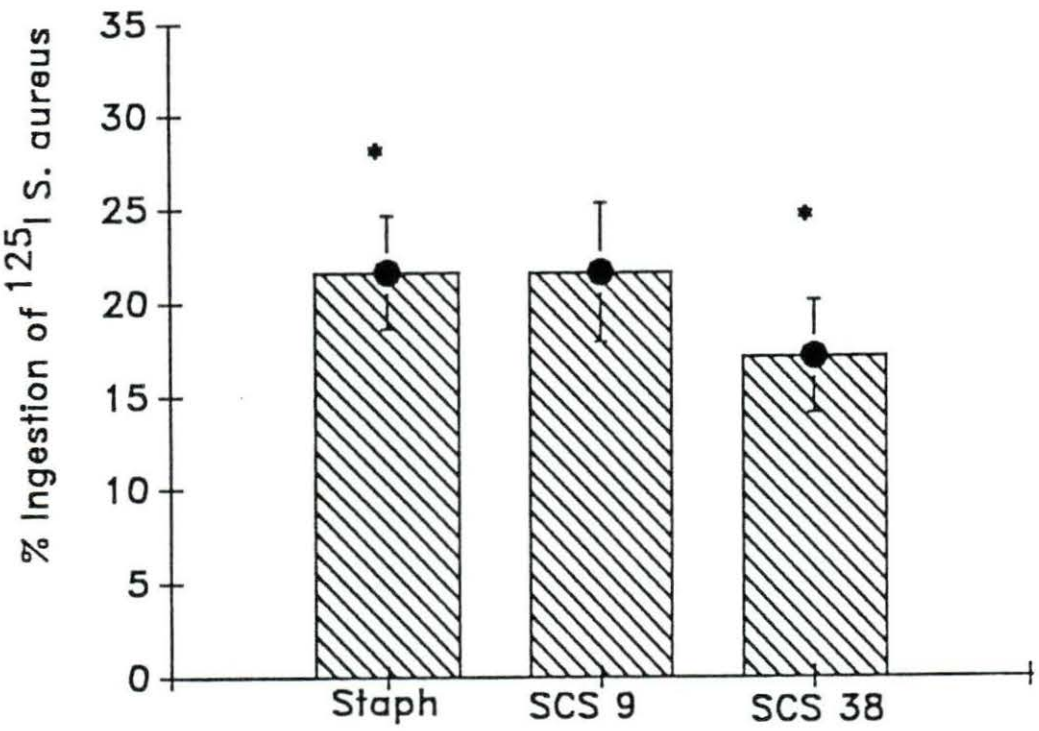

Figure 1. The effects of avirulent S. choleraesuis 9 (SCS 9) and virulent S. choleraesuis 38 (SCS 38) on S. aureus (Staph) ingestion by PPMNL.
* Level of significance P<0.01

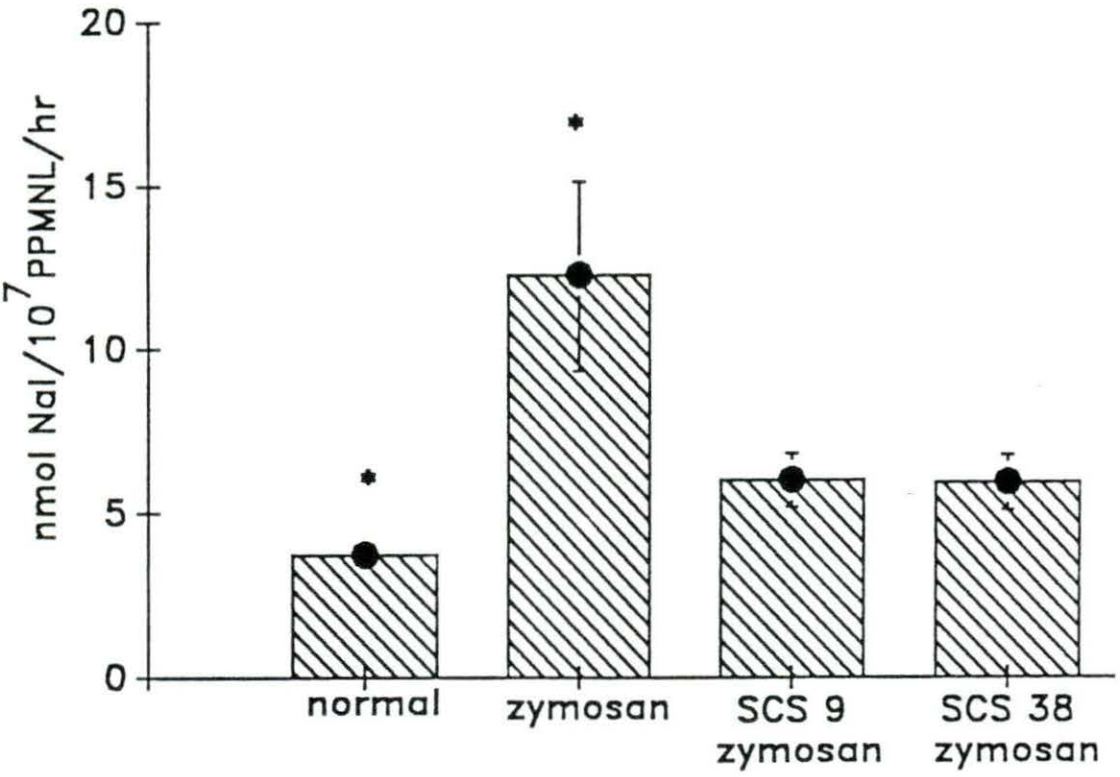

Figure 2. The effects of avirulent S. choleraesuis 9 (SCS 9) and virulent S. choleraesuis 38 (SCS 38) on iodination by PPMNL.
* level of significance $P<0.01$

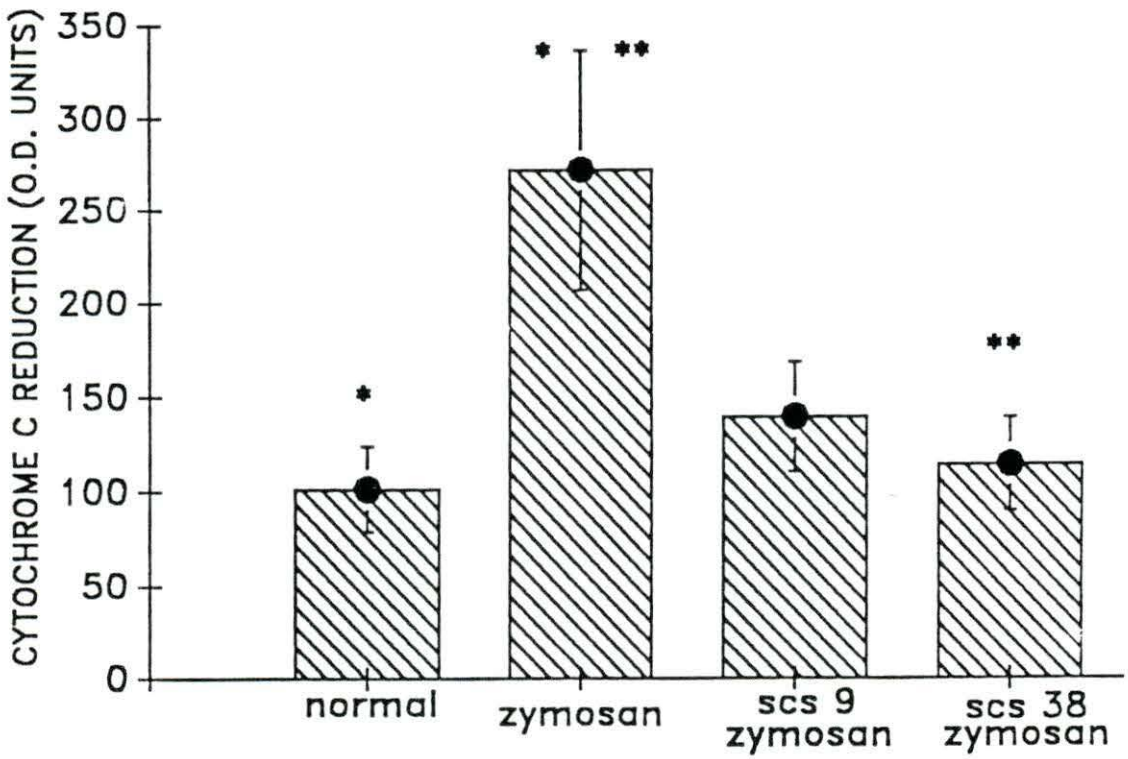

Figure 3. The effects of avirulent S. choleraesuis 9 (SCS 9) and virulent S. choleraesuis 38 (SCS 38) on the reduction of cytochrome c by PPMNL. Identical symbols (*) or (**) are significantly different. Level of significance $P<0.01$

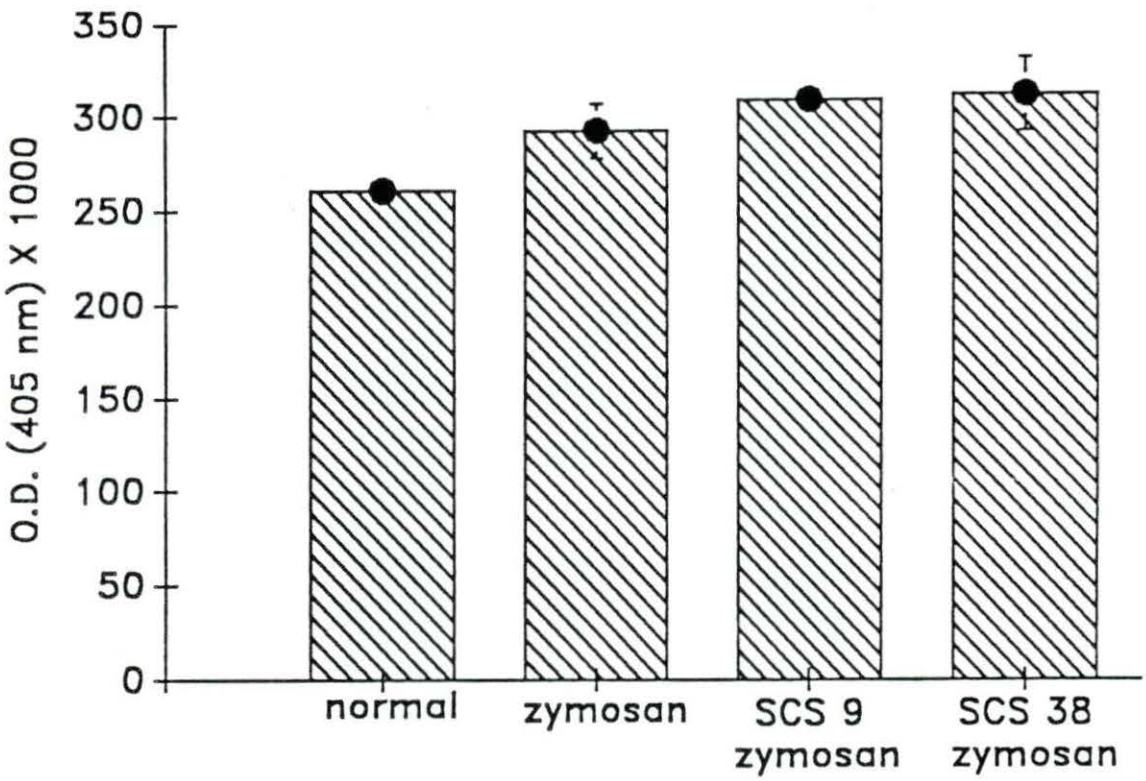

Figure 4. The effects of avirulent S. choleraesuis 9 (SCS 9) and virulent S. choleraesuis 38 (SCS 38) on lactoferrin release by PPMNL.

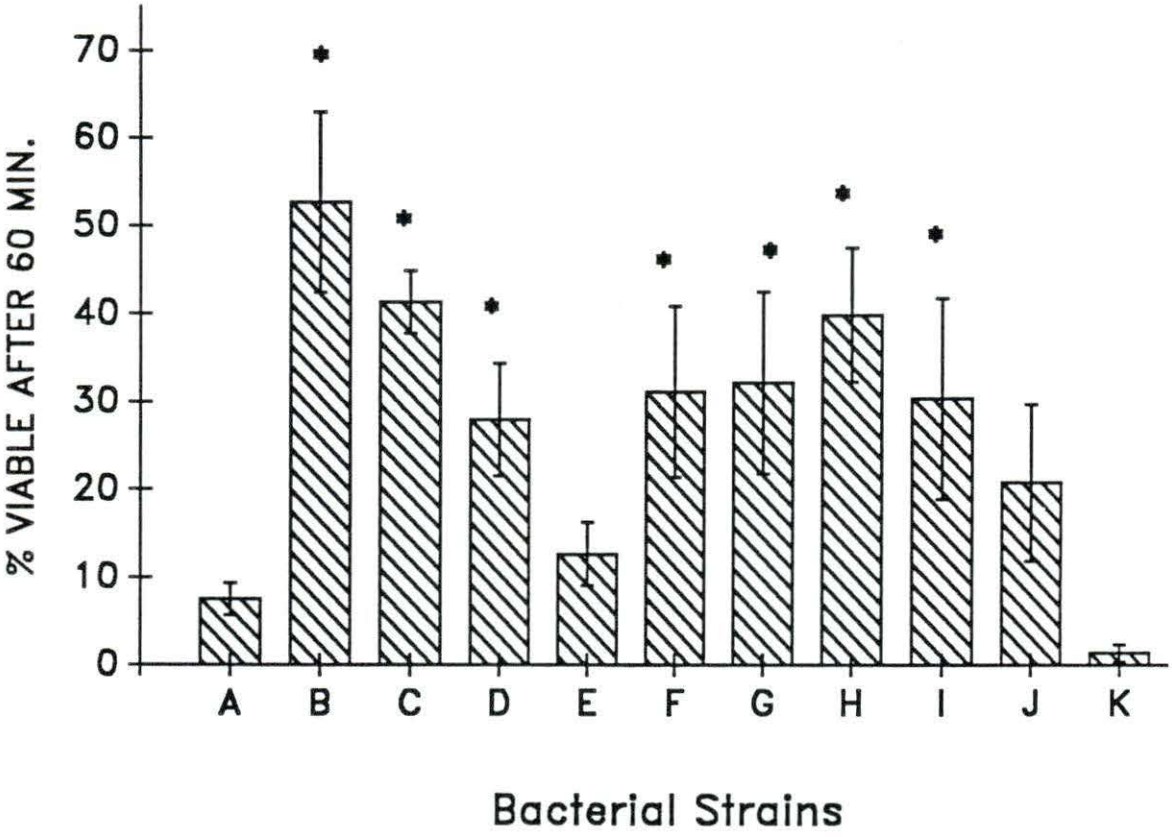

Figure 5. The comparison of 10 strains of S. choleraesuis (SCS) and their ability to survive PPMNL microbicidal activity after 60 minutes compared to E. coli K88 (control). Strains used: a) TTK-SCS 9, b) TTK-SCS 38, c) TTK-SCS 33-13, d) TTK-SCS 33, e) TTK-SCS 61, f) TTK-SCS CV-13, g) TTK-SCS CV-16, h) VDL-SCS 11, i) VDL-SCS 15, j) VDL-SCS 17, and k) E. coli K88.

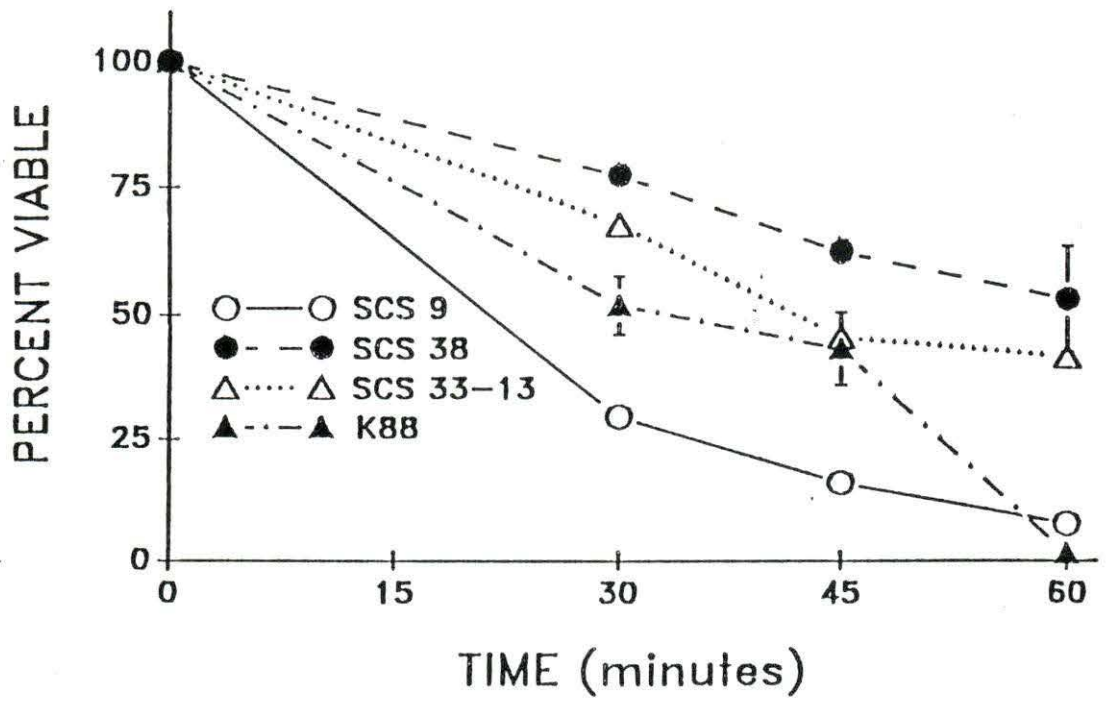

Figure 6. The survival rates of S. choleraesuis 38, 33-13, and 9 and E. coli K88 at times of 30, 45, and 60 minutes after exposure to PPMNL.

DISCUSSION

Results of the present study suggest virulent strains of S. choleraesuis can be differentiated from avirulent strains of S. choleraesuis on the basis of PPMNL function and bactericidal assays.

The bactericidal assay was performed with strains of S. choleraesuis that varyied in virulence: virulent S. choleraesuis 38, avirulent S. choleraesuis 9, and the control E. coli K88. S. choleraesuis 9 and E. coli K88 were readily killed in 60 minutes, with 7.5% and 1.4% survival respectively. S. choleraesuis 38 and S. choleraesuis 33-13 survived at 52.6% and 41.3% respectively, after 60 min. Seven additional strains were examined for degrees of PPMNL bactericidal effect, to determine the survival trends of other S. choleraesuis after PPMNL ingestion. Seven of 10 S. choleraesuis strains examined were significantly ($P<0.01$) more resistant than E. coli K88 to killing (Figure 5). In the bactericidal assay, a 0.1% SDS solution was used for lysing PPMNL instead of distilled water, because SDS lysed PPMNL and released all intracellular bacteria more effectively than distilled water. Incubation of PPMNL-S. choleraesuis in gentamicin, followed by SDS lysis of PPMNL assured that all extracellular S. choleraesuis were killed, and that all PPMNL were disrupted at the end of the assay, thus allowing an accurate count of viable ingested bacteria.

The other PPMNL function assays were performed on S. choleraesuis 9 and S. choleraesuis 38. Oxidative metabolism of PPMNL is an important requirement for oxygen-dependent bactericidal activities. When stimulated

by ingestion, oxidase enzymes in the PPMNL plasma membrane catalyze the conversion of oxygen to superoxide anion. Superoxide anion is reduces cytochrome c and this reaction is an efficient way to measure production of superoxide anions. Both S. choleraesuis 9 and S. choleraesuis 38 inhibited superoxide anion production, but only the 53.4% decrease of virulent S. choleraesuis 38 was significantly ($P<0.01$) different from zymosan activated PPMNL (Figure 3). At least three possible mechanisms are possible: 1) Diminished stimulation of the oxidative response due to masking of receptors (14); 2) S. choleraesuis may directly inhibit the oxidative pathway (12); and 3) S. choleraesuis may neutralize oxygen radicals by enzymes such as superoxide dismutase (12).

The iodination assay evaluates the ability of PPMNL to bind inorganic iodide to proteins through the action of the MPO-H_2O_2-halide bactericidal system. Iodination depends on the production of H_2O_2, on the release of MPO due to degranulation of primary granules, on the presence of iodide, and on the ability of the MPO enzyme to catalyze the reaction. S. choleraesuis 9 and S. choleraesuis 38 decreased iodination by 37.5% and 38.5% respectively, Thus both virulent and avirulent strains have similar capabilities to either prevent degranulation, inhibit H_2O_2 production, or prevent MPO enzyme activity. The exact mechanism is not known, but the important feature is that both virulent and avirulent S. choleraesuis affected iodination in similar manners.

The iodination and cytochrome c assays both used zymosan opsonized with fresh porcine serum to stimulate PPMNL. Initially, these assays were performed simultaneously with bovine PMNL as controls of species activity.

Human and bovine PMNL gave much higher values than PPMNL as reported earlier (12,24).

Staphylococcus aureus ingestion by PPMNL was inhibited by S. choleraesuis 9 and S. choleraesuis 38 by 2.9% and 22.2%, respectively (Figure 1). Only the virulent S. choleraesuis 38 reduced ingestion of S. aureus significantly ($P<0.01$). There may be several explanations for the inhibition of S. aureus by S. choleraesuis 38. 1) S. choleraesuis 38 is preferentially ingested over S. aureus. This may occur by a mechanism similar to the preferential endocytosis of Salmonella reported in Peyer's patches (8,10,21). 2) S. choleraesuis 38 is ingested and inhibits ingestion of other bacteria by altering PPMNL function. 3) Virulent S. choleraesuis has some form of "toxic" effect which inhibits ingestion of all bacteria. The most likely explanation may be a combination of the former suggestions. Virulent S. choleraesuis may use the PPMNL as an escape mechanism to prevent stimulating a humoral response and then to evade humoral factors such as antibody and complement. Once ingested, S. choleraesuis may excrete or have on its cell surface "toxic" substances which inhibit ingestion as well as function.

The results of this study corrborate with other reports on Salmonella-PMNL interactions. Human PMNL and S. typhi were investigated by other investigators (14). These authors have showed that ingestion was not altered by virulence, but virulent Salmonella decreased oxidative metabolism due to the failure of receptor stimulation. The interaction between S. choleraesuis and macrophages has also been examined by using porcine pulmonary macrophages and S. choleraesuis (1). The results

indicated that S. choleraesuis can survive, multiply, and even establish bacteremia after macrophage ingestion. This may indicate similar mechanisms by which S. choleraesuis are able to avoid PMNL and macrophages in establishing disease.

In conclusion, this study has shown that both virulent and avirulent S. choleraesuis inhibit iodination. The main difference between strains is their ability to be ingested and survive once inside the PPMNL. The virulent S. choleraesuis have a means to significantly inhibit superoxide anion production, prevent S. aureus ingestion, and resist the bactericidal mechanisms of PPMNL once ingested, and survive intracellularly in phagocytic cells.

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SUMMARY AND CONCLUSIONS

From this research it can be concluded that the interaction of Salmonella choleraesuis and PPMNL is a complex system that needs to be further investigated. The most important information gained through this investigation is the identification of differences between virulent and avirulent strains of S. choleraesuis and their interaction with PMNs in their natural host, the pig. Differences in cytochrome c reduction, S. aureus ingestion, and survival of PPMNL killing allow a basis for further research toward identifying the exact mechanisms of Salmonella infection and toward immune protection in the future.

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