Pathogenesis of Salmonella typhimurium and interactions with porcine neutrophils

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Pathogenesis of *Salmonella typhimurium* and interactions with porcine neutrophils

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and interactions with porcine neutrophils

by

Nancy Ellen Coe

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GENERAL INTRODUCTION AND EXPLANATION OF FORMAT

*Salmonella typhimurium* in swine has a major economic impact on the pork industry. Acute infections typically cause enterocolitis, which, although rarely fatal, cause increased production costs through temporary decreases in rate of weight gain and feed efficiency. Acute infection is typically followed by a persistent carrier state. Fecal shedding of *S. typhimurium* by carrier animals is variable and may be precipitated by stress, such as that encountered at slaughter. Thus, carrier animals pose a threat to food safety and provide a reservoir of infection for humans and other animals. Reduction or elimination of the carrier state is highly desirable, but little is known about the mechanisms that allow *S. typhimurium* to persist in porcine tissues.

The results of three scientific studies are presented here in alternate dissertation format. All of the studies describe host-bacterial interactions that may influence the establishment or maintenance of the carrier state of *S. typhimurium* in swine and/or evaluate potential prophylactic strategies that may reduce the prevalence of the carrier state. The objectives of these studies were 1) to evaluate alterations in porcine neutrophil function associated with *S. typhimurium* infection, 2) to evaluate the effect of prior exposure to a relatively avirulent mutant of *S. typhimurium* on the subsequent colonization and persistence of wild-type *S. typhimurium* in swine, and 3) to evaluate factors that affect the bactericidal efficiency of porcine neutrophils against *S. typhimurium*. The scientific studies are preceded by a literature review and followed by a general summary. References cited in the literature review and general summary are listed following the general summary. The studies presented in papers I and II have been accepted for publication in *Veterinary Immunology and Immunopathology* and *Veterinary Microbiology*, respectively.
LITERATURE REVIEW

Salmonellosis in swine

*Salmonella typhimurium* is a gram-negative bacterium that causes enteritis and/or septicemia in a wide range of animal hosts, including domestic livestock and man (Clarke and Gyles, 1986). It is a member of the enterobacteriaceae and is considered a facultative intracellular pathogen (Scanlan, 1988). It is readily transmitted between animals and man and can survive fairly well on fomites and in the environment, making it an important zoonotic agent (Wilcock, 1986).

Salmonellosis in swine is generally attributable to *S. choleraesuis* or *S. typhimurium*. *S. choleraesuis* causes a septicemic syndrome in swine that is closely analogous to *S. typhimurium* infections in mice (Mackaness et al., 1966; Wilcock, 1986). *S. choleraesuis*, however, is highly host adapted and poses less zoonotic threat than *S. typhimurium*. *S. typhimurium* infection in swine causes transient or recurrent enterocolitis and readily establishes a carrier state with variable shedding. Clinical disease is most common between weaning and four months of age, but chronic infections can persist at least until slaughter age. Although morbidity may be high, mortality associated with *S. typhimurium* in swine typically is low (Wilcock, 1986; Wood et al., 1989). This review will focus on *S. typhimurium* infections in swine.

Carrier animals provide a major reservoir of infection for *S. typhimurium* (Clarke and Gyles, 1986), yet little is understood about the carrier state. A number of factors probably contribute to the establishment of the carrier state, including the age of the animal, the isolate of *S. typhimurium* involved, and the infecting dose. Clinical severity does not appear to correlate with the magnitude or duration of bacterial shedding; even subclinical infections may lead to the carrier state. Shedding of *Salmonella* into the feces is highly
variable, but it may be precipitated by stress or concurrent disease (Williams and Newell, 1970; Clarke and Gyles, 1986). Antibiotics, such as oxytetracycline, do not influence the prevalence of carrier swine or the quantity of bacterial shedding (Evangelisti et al., 1975).

**Immunity to *Salmonella* spp.**

The relative importance of cell-mediated vs. humoral factors in immunity to salmonellosis is not clear. It is generally accepted that cell-mediated immunity is important for resistance to facultative intracellular organisms, such as *Salmonella*. Studies demonstrating superior efficacy of live vaccines over killed bacterins in mice support this concept (Hobson, 1957; Mitushashi et al., 1961; Collins and Mackaness, 1968). Other studies report that killed and subcellular *Salmonella* preparations offer substantial protective immunity (Jenkin and Rowley, 1965; Herzberg et al., 1972) and that susceptible mice can be protected with immune serum (Marecki et al., 1975), which suggests that humoral factors are important in host resistance to *Salmonella*. In all likelihood, both cell-mediated and humoral immunity are important in resistance to salmonellosis. The divergent results of the aforementioned studies may be due to the method of vaccine production (Jenkin and Rowley, 1965) or administration (Herzberg et al., 1972) or due to the relative natural resistance of the strains of mice used (Eisenstein and Sultzer, 1983).

The understanding of immunity to *Salmonella typhimurium* is incomplete, even in mice where research has been ongoing for decades. Specific literature pertaining to swine immunity to *S. typhimurium* is very scarce. Most published studies in swine describe only the acute phase of infection; even less is known about chronic infections and carrier states. Because the pig typically has a localized response to *S. typhimurium* which differs from the septicemic disease seen in *S. typhimurium*-infected mice, data from murine studies contribute little to the understanding of porcine immunity to *S. typhimurium*. 
Regardless of the relative importance of cell-mediated and humoral factors in resistance to *Salmonella*, it is accepted that macrophages (reviewed by Hsu, 1989) and neutrophils (Guo et al., 1986) are capable of ingesting virulent *Salmonella*. Ingestion and removal of *Salmonella* by professional phagocytes (i.e., neutrophils, macrophages) is the primary mechanism for clearing *Salmonella* from the infected host.

**Neutrophil physiology and function**

The neutrophil content of swine blood ranges from 3-15 x 10^3 per mm^3 blood and typically constitutes 30-50% of the circulating white blood cells (Fraser et al., 1991, p. 967). Swine neutrophils have a multilobular nucleus and pale staining ("neutral") cytoplasm. Granules in neutrophil cytoplasm contain enzymes and other substances involved in bactericidal activity, inflammation, and tissue destruction. Primary, or azurophilic, granules contain myeloperoxidase, cathepsins, neutral proteases, acid hydrolases, cationic proteins, and lysozyme. Secondary, or specific, granules contain lysozyme, alkaline phosphatase, collagenase, vitamin B\textsubscript{12} binding protein, and lactoferrin (Smolen, 1989).

Neutrophils have a short half-life in the circulation (approximately 10 hours) and do not return to the circulation once they have egressed through the vascular wall (Duncan and Prasse, 1984, pp. 33). They are the first cells to arrive at the site of inflammation and play a role in the early defense against most bacterial pathogens.

Particulate matter, including bacteria, are internalized by neutrophils through phagocytosis. Neutrophils can ingest *Salmonella* alone, but phagocytic efficiency is enhanced when the bacteria are opsonized with antibody and/or complement (Baron and Proctor, 1984; Mukkur, 1989). Once internalized, bacteria-containing phagosomes fuse with lysosomes within the neutrophil cytoplasm, forming phagolysosomes. Bacteria within
phagolysosomes can be killed by a number of oxidative or nonoxidative mechanisms or they can survive by escaping the phagolysosome, inhibiting phagosome-lysosome fusion, or resisting the microbicidal action of lysosome granules (reviewed by Finlay and Falkow, 1989).

Neutrophils are activated by specific receptor-ligand interactions or by perturbation of the cell membrane (Smolen, 1989). Within seconds after activation, neutrophils undergo a respiratory burst, which generates a variety of bactericidal products. Without this burst, intracellular microbicidal activity is very weak. Activation stimulates an oxidase transport system in the neutrophil membrane; the net reaction generates superoxide anions from NADPH and molecular oxygen. The superoxide anion ($O_2^-$) possesses short range antimicrobial activity and can generate a chemotactic factor. The direct toxicity of $O_2^-$, however, is low, and its major role is probably as a reactive intermediate in the formation of hydrogen peroxide, hydroxyl radicals, and singlet oxygen (Klebanoff, 1982).

The conversion of superoxide anion to hydrogen peroxide is catalyzed by superoxide dismutase, which is a component of many aerobic microorganisms. It also occurs spontaneously at low pH (e.g., in phagolysosomes). Hydrogen peroxide combines with halide ions ($Cl^-$, $Br^-$, and $I^-$) in a myeloperoxidase-catalyzed reaction to form hypohalites, such as hypochlorous acid, which are toxic to many bacteria, viruses, and protozoa. Hydrogen peroxide alone is also a powerful oxidant, but higher concentrations of hydrogen peroxide are required for direct microbicidal action than for halogenation reactions.

Hydroxyl radicals and singlet oxygen are powerful oxidants. They initiate chemical reactions that produce cellular damage, but the significance of their contribution to the microbicidal action of neutrophils is unclear.

Although the oxygen-dependent reactions associated with the respiratory burst produce potent microbicidal products in the neutrophil, other non-oxidative processes contribute to
the microbiostatic and microbicidal properties of the cell. Lysozyme, contained in primary and secondary granules, cleaves β1,4 linkages between N-acetylglucosamine and N-acetylmuramic acid in bacterial cell walls. Lysozyme is most effective against Gram-positive bacteria. Gram-negative bacteria are relatively resistant to the action of lysozyme because of their protective outer lipoprotein-rich membrane, but resistant organisms become more susceptible in the presence of hydrogen peroxide, proteases, low pH, or specific antibody and complement. Lysozyme may enhance the action of the complement membrane attack complex on Gram-negative bacteria (Martinez and Carroll, 1980).

Lactoferrin in its unsaturated form is microbiostatic because it binds trace iron needed by the microorganism (Wright, 1982). It is effective against Gram-positive and Gram-negative organisms and often acts synergistically with other antimicrobial mechanisms.

Defensins are small cationic peptides found in the neutrophil granules of several animal species (Lehrer et al., 1990). Defensins alter microbial membrane permeability and function and are effectively microbicidal against many bacteria, fungi, and enveloped viruses.

Bactericidal/permeability increasing protein (BPI) is found in the primary granules of human and rabbit neutrophils and may be present in other animal species (Elbsbach and Weiss, 1988). BPI is specific for Gram-negative bacteria; it increases the permeability of the outer membrane while having little effect on the inner membrane. It selectively activates bacterial phospholipids and causes alterations in porin synthesis.

Neutrophils and K-cells mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (Roitt et al., 1989, p. 9.6). Although the phenomenon has not been reported in human neutrophils, bovine neutrophils also mediate a significant level of antibody-independent neutrophil cytotoxicity (AINC) (Lukacs et al., 1985). ADCC is an effective mechanism for destroying virus-infected cells and certain tumor cells, but it may also play a role in the
control of intracellular bacterial infections. The mechanism of action of ADCC is unclear, but it appears to involve target binding, disruption of target membranes, and cell death (Roitt et al., 1989, p. 9.5).

Assays of neutrophil function

Numerous assays have been developed to measure specific aspects of neutrophil function, including motility, phagocytosis, bactericidal activity, oxidative metabolism, and cytotoxicity. When the results of several different assays are combined, an assessment of overall neutrophil function can be made. The following assays have been adapted for use with porcine and bovine neutrophils.

Agarose assays measure neutrophil movement on tissue culture plates beneath a layer of agarose and have been used to measure neutrophil random migration and chemotaxis. Neutrophils are placed in wells that have been punched through the agarose. The plates are incubated at 37°C, and the neutrophils, by random movement, migrate radially under the agarose away from the well. The area of random migration can be calculated from the measured radius of migration and is an estimate of neutrophil motility (Roth and Kaeberle, 1981a; Smith et al., 1985).

A modification of the agarose assay is used to measure neutrophil chemotaxis (Nelson et al., 1975; Smith et al., 1985). Chemotaxis is the directed movement of neutrophils toward an attractant substance and is important in vivo for recruiting neutrophils to sites of inflammation or infection. In the chemotaxis assay, three colinear, equidistant wells are punched into an agarose layer. Neutrophils are placed in the center well. A chemoattractant is placed in one outer well, and a nonattractant substance, such as saline, is placed into the remaining outer well. Numerous chemoattractants have been used in chemotaxis assays, most commonly N-formyl methionyl peptides (FMLP) or zymosan-
activated serum (ZAS). Pig neutrophils, however, lack FMLP receptors (Chenoweth et al., 1980; Fletcher et al., 1990). Therefore, ZAS is used as the chemoattractant in assays of porcine neutrophil function. The distance that the neutrophils have migrated toward the chemoattractant well is measured, as is the distance neutrophils have migrated toward the saline-containing well. The chemotactic response of neutrophils can be expressed in several ways: by the distance migrated toward the chemoattractant, by the distance migrated toward the chemoattractant minus the distance migrated toward saline, or by the chemotactic index, which is the distance migrated toward the chemoattractant divided by the distance migrated toward the saline. Each measure may be useful for detecting different alterations in chemotaxis or motility (Nelson et al., 1975).

Phagocytosis or ingestion assays measure the ability of the neutrophil to internalize particulate matter, such as latex particles (Sanchez-Vizcaino et al., 1981), yeast (Patterson-Delafield and Lehrer, 1977; Buschmann and Winter, 1989), or bacteria (Roth and Kaeberle, 1981a; Kuypers et al., 1989). Successful phagocytosis requires several distinct events, including particle recognition, neutrophil activation, pseudopod extension around the particle, and phagosome formation. Altered phagocytosis may reflect abnormalities in any or all of these events. In assays of phagocytosis, neutrophils are incubated with particulate matter for a specified amount of time, the noningested particles are washed away, and the ingested particles are quantitated. A difficulty encountered with many phagocytosis assays, especially those evaluated by microscopic examination, is the differentiation of internalized particles from those that are adherent with the neutrophil surface. Failure to differentiate between internalized and surface-associated particles limits the usefulness of these phagocytosis assays.

An ingestion assay using $^{125}$I-labeled *Staphylococcus aureus* overcomes the difficulty of differentiating ingested from adherent particles. In this assay, neutrophils are incubated
with opsonized, radiolabeled *S. aureus* (Roth and Kaeberle, 1981a). At the end of the incubation period, extracellular *S. aureus* are lysed by the addition of lysostaphin and washed away; internalized bacteria are protected from the action of lysostaphin. The radioactivity associated with the washed sample is quantitated in a gamma counter, and the percentage of *S. aureus* ingested by the neutrophils is calculated.

Neutrophil oxidative metabolism involves a complex series of events. Several assays have been developed to measure specific aspects of the neutrophil oxidative burst that occurs immediately after activation. The cytochrome C reduction assay (Roth et al., 1986) measures superoxide anion production by stimulated (ZAS-treated) or resting neutrophils. Cytochrome C is reduced by superoxide anion, a reaction that is characterized by an increase in light absorbance (A550-650). Neutrophils are incubated with cytochrome C, and the sample A550-650 is read spectrophotometrically. The magnitude of the sample A550-650, corrected for background, is proportional to the amount of superoxide anion production by the neutrophils. Because other substances besides superoxide anion can reduce cytochrome C, the specificity of the response is often checked by the addition of superoxide dismutase (SOD). SOD catalyzes the conversion of superoxide anion to hydrogen peroxide, effectively inhibiting cytochrome C reduction by superoxide anion (Lord, 1989). Thus, increases in A550-650 that are inhibitable by SOD are due to superoxide anion.

The iodination assay measures the ability of the neutrophil myeloperoxidase to catalyze the incorporation of inorganic iodine into the tyrosine residues of proteins (Lord, 1989). It can also reflect alterations in phagocytosis and oxidative burst, which also are required for iodination to occur, but when evaluated in combination with assays of phagocytosis and oxidative burst, the iodination assay can be a specific indicator of myeloperoxidase activity. In this assay, $^{125\text{I}}$iodine, in an excess of unlabeled sodium iodide, is added to
neutrophil samples. After incubation, the samples are treated with cold 10% trichloroacetic acid, which precipitates the proteins in the sample. The radioactivity associated with the washed precipitate is counted in a gamma counter and is used to quantitate the amount of sodium iodide incorporated into the neutrophils per hour (Roth and Kaeberle, 1981a).

A chromium release assay is the standard for measuring antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-independent neutrophil cytotoxicity (AINC). $^{51}$Cr$^{6+}$ is readily taken up by cells and reduced to its 3+ valence form. The 3+ form does not cross the cell membrane, so the internalized, radiolabeled chromium is retained intracellularly until the cell membrane is destroyed, thereby releasing chromium into the surrounding medium (Kimball, 1986, pp. 346-348). In a chromium release assay, neutrophils are incubated with $^{51}$Cr-labeled target cells, such as chicken red blood cells (CRBC), with (ADCC) or without (AINC) specific antiserum. After incubation, the cells and cellular debris are pelleted. The radioactivity associated with the cell-free supernatant is proportional to the amount chromium released from lysed target cells. Increased cytotoxicity is associated with increased chromium release (Roth and Kaeberle, 1981b).

Assays of bactericidal activity evaluate the overall ability of the neutrophil to kill bacteria. They are nonspecific assays in that alterations in phagocytosis, oxidative metabolism, and motility all may affect the bactericidal ability of the neutrophil, but bactericidal assays are useful to detect changes in neutrophil function that cannot be attributed to any of these more specific aspects of function.

The MTT bactericidal assay is a colorimetric assay based on the principle that live bacteria, such as $S.\ typhimurium$, reduce yellow MTT to purple formazan (Stevens et al., 1991). Neutrophils and bacteria are incubated together at 37°C. After incubation, the neutrophils are lysed with saponin to release any internalized bacteria. MTT is added, and
the live bacteria remaining in the samples reduce the MTT to formazan. The samples are centrifuged to pellet the precipitated formazan, and the supernatants are discarded. The precipitated formazan is resolubilized in isopropyl alcohol, and the light absorbance of the samples are read spectrophotometrically. The light absorbance of each sample is proportional to the amount of formazan precipitated, which, in turn, is proportional to the number of live bacteria remaining in the sample. The percentage of bacteria killed in a sample can be determined by comparing the sample absorbance to a standard curve prepared with known numbers of bacteria.

**Salmonella survival within neutrophils**

It is unclear exactly how *Salmonella* survive and replicate within professional phagocytes (i.e., neutrophils, macrophages). It has recently been shown that *S. typhimurium* inhibits phagosome-lysosome fusion in murine macrophages (Buchmeier and Heffron, 1991), but the mechanism responsible is unknown. Fusion inhibition requires viable *Salmonella*, is not affected by bacterial opsonization, and is not due to lipopolysaccharide.

The lipopolysaccharide (LPS) located in the outer membrane of *Salmonella* increases resistance to host microbicidal mechanisms. Many strains of *Salmonella* are resistant to complement-mediated lysis. Complement is activated by LPS as well as by porins (outer membrane proteins) (Galdiero et al., 1984), but the membrane attack complex (C₅b-9) does not insert properly into the bacterial outer membrane. The majority of the complement complexes attach to the subset of LPS containing long O-chains, which sterically inhibits access to the sensitive hydrophobic domains of the outer membrane. Therefore, the complement complexes are easily shed and are not bactericidal (reviewed by Joiner, 1988). The 100 kb virulence plasmid found in *S. typhimurium* may also be
involved in this resistance, separate from the effects of LPS, but the mechanism is unclear (Groisman and Saier, 1990).

LPS increases resistance to cationic proteins of the neutrophil (Shafer et al., 1984; Stinavage et al., 1989). Cationic proteins appear to alter bacterial membrane permeability. The lipid A component of LPS binds several cationic proteins and blocks their bactericidal action, probably by restricting access to the sensitive domains of the outer membrane.

At least 20 *Salmonella* genes have been associated with intracellular survival (Fields et al., 1986). Among these is the phoP gene, which may be responsible for resistance to defensins (Fields et al., 1989). PhoP mutants are unable to survive in murine macrophages and are hypersensitive to the lethal effects of crude extracts of neutrophils and macrophages. The phoP gene is part of the regulatory network for a number of genes, including those encoding nonspecific acid phosphatases for which the phoP gene was named. Nonspecific phosphatases do not appear to be involved in virulence, though, and it is probably another, as yet uncharacterized, gene under phoP regulation that encodes resistance to defensins (reviewed by Groisman and Saier, 1990). The phoP gene also regulates pagC, which has been associated with virulence but probably does not affect resistance to defensins. Variability in intracellular survival depends, in part, on the source of macrophages (peritoneal vs. splenic) (Buchmeier and Heffron, 1989).

Little is known about the resistance of *Salmonella* to oxidative killing. *Salmonella* spp. contain superoxide dismutases and catalases that may have protective effects against oxidative activity, but direct evidence of such protection is lacking (reviewed by Fridovich, 1978). A 59 kD outer membrane protein has recently been described that may act as a catalase or superoxide dismutase (Stinavage et al., 1990). Mutants lacking this protein are highly sensitive to hydrogen peroxide and have decreased resistance to killing by neutrophils.
Flagella have been implicated in intracellular survival. Although the presence or absence of flagella does not appear to influence invasion or dissemination of *Salmonella*, flagellated strains survive longer in macrophages (Weinstein et al., 1984). The mechanism of action is unknown; flagella may act as a protective barrier.

Porins extracted from *Salmonella* have several effects on human neutrophil morphology and function. The binding of porins to specific neutrophil domains lowers surface hydrophobicity, decreases neutrophil adherence and chemotaxis, and decreases surface expression of Fc and C3 receptors (Tufano et al., 1989).

**Biological response modifiers**

Biological response modifiers (BRM), or immunomodulators, are substances that enhance host immune defense mechanisms. Most do not have direct antimicrobial action. BRMs can be grouped into two categories, exogenous and endogenous. Exogenous BRMs are not native to the host and include bacterial products and pharmacological compounds. Endogenous BRMs are substances produced by the host and include cytokines (Roth, 1988).

*Bacillus Calmette-Guerin* (BCG) is a strain of *Mycobacterium bovis* with immunomodulatory properties. It has been used in conjunction with surgery and cytotoxic drugs in the treatment of certain tumors (Crispen, 1986), but BCG also affects nonspecific resistance to bacterial infections. Mice treated with BCG or its analogues, cord factor and muramyl dipeptide, have increased resistance to infection by *S. typhimurium* (Senterfitt and Shands, 1970; Yarkoni and Bekierkunst, 1976), *Klebsiella pneumoniae*, *Escherichia coli*, and *Streptococcus pneumoniae* (Fogler, 1984). BCG induces an increase in the respiratory burst, phagocytosis, migration and bactericidal activity of murine macrophages. It can increase antibody responses to unrelated antigens in a dose-dependent manner.
(Brown et al., 1978). BCG activates T-lymphocytes, affects lymphocyte homing and distribution, and enhances killer and natural killer cell activities (Crispen, 1986; Koychev et al., 1986). The mechanism of action for BCG is unknown, but it probably modulates immune function indirectly by stimulating the release of cytokines, such as interferon, by the host (Crispen, 1986).

Tumor necrosis factor (TNF) is a 17-kD cytokine produced by host cells in response to inflammatory, infectious, or neoplastic stimuli. It was so named because it was initially characterized by its ability to induce the regression of certain tumors (Carswell et al., 1975). There are two forms of TNF: TNF-α and TNF-β. TNFα is produced mainly by macrophages and TNF-β by activated T-lymphocytes (reviewed by Steinbeck and Roth, 1989); both have immunomodulatory activity. Monoclonal antibodies to human TNF are active against TNF from dogs, pigs, and monkeys (Moller et al., 1990), suggesting that the structure of TNF is somewhat conserved among animal species. Treatment with TNF enhances phagocytosis, degranulation, oxidative metabolism, and ADCC by human neutrophils (Shalaby et al., 1985; Shalaby et al., 1987). TNF is one of the earliest cytokines produced in response to inflammation or infection; its primary immunomodulatory role in vivo may be as a regulatory cytokine to initiate the production and release of secondary inflammatory mediators and to initiate systemic responses to infection (Fong and Lowry, 1990; Nacy et al., 1991). TNF has a protective effect in mice challenged with *S. typhimurium* (Nakano et al., 1990; Tite et al., 1991), but the exact mechanism of protection is unclear.

There is potential veterinary clinical application for BRMs in the treatment and/or prevention of bacterial infections (reviewed by Blecha, 1990). Insight into the protective mechanisms of BRMs might also enhance our understanding of bacterial pathogenesis and aid in the development of strategies to reduce or eliminate carrier states.
PAPER I. ALTERATION OF NEUTROPHIL FUNCTION IN BCG-TREATED AND NONTREATED SWINE AFTER EXPOSURE TO SALMONELLA TYPHIMURIUM
Alteration of neutrophil function in BCG-treated and nontreated swine after exposure to *Salmonella typhimurium*

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Salmonella typhimurium infection in swine causes enterocolitis followed by a persistent carrier state, but little is known about the mechanisms that allow this organism to colonize and persist in host tissues. Neutrophils provide a first line of defense against invading pathogens such as Salmonella typhimurium. The purpose of this study was to evaluate porcine neutrophil function after in vivo exposure to Salmonella typhimurium and to determine if the immunomodulator, bacillus Calmette Guerin (BCG), exerts any effect on neutrophil function or on the colonization and persistence of S. typhimurium in the pig. Compared to negative controls, neutrophils from pigs exposed to S. typhimurium exhibited significantly decreased iodination, cytochrome-C reduction, antibody-dependent cell-mediated cytotoxicity, random migration, and chemotaxis (p≤0.05). Neutrophil bactericidal activity against S. typhimurium was significantly enhanced. Most of the significant differences were noted in the first two days after exposure to Salmonella. Often the functional alterations were biphasic, peaking again 7-10 days after exposure. BCG alone significantly depressed random migration and cytochrome-C reduction in unstimulated neutrophils. The clinical course, colonization pattern, and persistence of Salmonella were similar between pigs receiving BCG and untreated pigs. These data suggest that S. typhimurium infection causes a depression in oxidative metabolism and motility, yet an increase in overall bactericidal activity against S. typhimurium in circulating porcine neutrophils. It also appears that BCG treatment, as reported here, does not enhance resistance of pigs to S. typhimurium colonization or reduce the number of persistent organisms in the porcine ileum.
INTRODUCTION

*Salmonella typhimurium* causes enterocolitis in swine and can persist within the intestinal wall and associated lymph nodes for at least several months (Wood et al., 1989). *Salmonella typhimurium* is also a leading cause of food-borne illness in humans (Todd, 1989). The ability of infected swine to persist as subclinical carriers of *S. typhimurium*, yet shed large numbers of *Salmonella* during periods of stress (Hansen et al., 1964; Williams and Newell, 1970; Morgan et al., 1987), makes them an important reservoir of infection.

Immune mechanisms involved in the establishment and maintenance of the *S. typhimurium* carrier state in swine are not clear. Most studies describing immunity to *Salmonella* have focused on other animal species and the role of the macrophage in resistance to *Salmonella* (Lowrie et al., 1979; Buchmeier and Heffron, 1989; Hatchigian et al., 1989). The neutrophil, however, is an important mediator of host defense, especially in the early stages of infection. Smith et al. (1981a,b, 1985) have described alterations in porcine neutrophil adhesion, chemotaxis and bactericidal activity after exposure to *S. typhimurium*, but no confirming reports are available. To our knowledge, there are no published reports describing the effect of *S. typhimurium* infection on the respiratory burst or cytotoxic activity of porcine neutrophils.

Biologic response modifiers have been proposed as a means of enhancing nonspecific immunity to bacterial infections. Studies have shown that bacillus Calmette-Guerin (BCG) administration increases the LD$_{50}$ of *S. typhimurium* in mice (Senterfitt and Shands, 1970; Yarkoni and Bekierkunst, 1976). Betstadin treatment in mice reduces persistent populations of *S. typhimurium* in the liver and spleen (Dickneite et al., 1984). To our knowledge, there are no reports of similar studies in swine.
In this study, we characterized eight parameters of neutrophil function during acute and convalescent stages of *S. typhimurium* infection in swine. We also characterized the effect of prior BCG administration on neutrophil function in *Salmonella*-free and -infected swine. We measured the influence of BCG on the carrier state by comparing persistent *Salmonella* populations in BCG-treated and nontreated swine.
MATERIALS AND METHODS

Pigs

Twenty-two hysterectomy-derived, colostrum-deprived pigs of both sexes were raised in isolation to 8 weeks of age. Periodic bacteriologic examinations of rectal swabs and feces were performed as described previously (Wood et al., 1989). All pre-exposure samples were negative for *Salmonella*.

Bacteria

*Salmonella typhimurium* strain 798 (Wood et al., 1989), originally isolated from a clinical case of porcine salmonellosis, was used throughout this study. Static cultures used for pig exposure were grown overnight in Luria broth at 37°C and contained approximately 1.5 x 10^9 cfu/ml. Bacteria for the bactericidal assay were grown to mid-log phase in Luria broth at 37°C in a shaking incubator (150 rpm). The bacteria were then centrifuged at 2500 x g for 15 minutes. The pellet was washed in 0.01 M phosphate-buffered saline (PBS), recentrifuged, and resuspended in tryptose soy broth at 1/20 original volume. Aliquots for the assay were stored at -70°C.

BCG (Calbiochem, San Diego, CA) was reconstituted in PBS (2 mg/ml) and killed by autoclaving. The killed suspension was mixed 1:1 with Freund’s incomplete adjuvant and stored at 4°C prior to use.

Exposure of pigs

The pigs were divided into four isolated groups. Group 1 (n=6) received BCG and *Salmonella*, group 2 (n=6) received only *Salmonella*, group 3 (n=5) received only BCG, and group 4 (n=5) served as a nontreated, noninfected control according to the following schedule.
Each pig received two subcutaneous injections, 15 days apart (days -17 and -2), of either 2 ml BCG in Freund’s incomplete adjuvant or 2 ml sterile saline. Two days after the second injection (day 0), each pig received a 10 ml oral dose of either \textit{S. typhimurium} or sterile culture medium.

**Evaluation of clinical response**

Rectal temperatures were obtained on days -5 through -3 and days -1 through 7. Rectal swabs were obtained from each pig on days 1-4. Fecal samples were collected weekly throughout the study. Total white blood cell (WBC) counts were performed on each day that neutrophil assays were run (see below).

**Collection of blood and separation of neutrophils**

Each pig was bled via the cranial vena cava 1 and 3 days prior to the first subcutaneous injection and on days 1, 2, 3, 4, 7, 10, and 14 after exposure to \textit{S. typhimurium} or culture medium. Thirty-five milliliters of blood were drawn into syringes loaded with 1 ml 7.5\% EDTA in PBS. Most of the erythrocytes were removed by hypotonic lysis. The remaining cells were centrifuged through a Ficoll-diatrizoate density gradient. The neutrophil-rich pellet was harvested, and residual erythrocytes were removed by an additional hypotonic lysis step. The remaining cells were washed and resuspended in PBS and adjusted to $5 \times 10^7$ cells/ml. The purity of the neutrophil preparation, as determined by microscopic morphologic examination, was >90\% granulocytes; viability, as determined by trypan blue exclusion, was >95\%.

**Assays of neutrophil function**

The following assays of neutrophil function were performed: antibody-dependent cell-mediated cytotoxicity (ADCC), iodination, \textit{Staphylococcus aureus} ingestion, stimulated and unstimulated cytochrome-C reduction, random migration, chemotaxis, and an MTT [3-,4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-tetrazolium bromide] bactericidal assay.
The *S. aureus* ingestion and iodination assays were performed as described by Roof and Kramer (1989) except that the harvested neutrophils were not incubated with *Salmonella*.

The cytochrome-C reduction assay was performed using a modification of the procedure described by Roth et al. (1986). Neutrophils were incubated with cytochrome-C containing (stimulated) or without (unstimulated) opsonized zymosan for 10 minutes prior to reading the absorbance of the solution at 550/650 nm.

Random migration under agarose was performed as described by Roth and Kaeberle (1981a), except that the agarose contained M199 medium with Hank's salts and porcine serum, and the plates were fixed with 8% glutaraldehyde. The area of migration in square millimeters was calculated from the measured radius of migration.

The chemotaxis assay was performed in a manner similar to that described by Smith et al. (1985) with the following modifications: using the same agarose mixture as described above, 2 mm diameter wells, spaced 3.5 mm apart, were loaded with 7.5 μl (3.75 x 10^5) neutrophils. After incubating for 3 hours, the plates were fixed and stained as described for random migration plates. Results were reported as the chemotactic index, the distance migrated toward the chemoattractant (zymosan-activated serum) divided by the distance migrated toward the control.

Antibody-dependent cell-mediated cytotoxicity was measured by a chromium release assay as described by Roth and Kaeberle (1981b) except that the supernatant was harvested from 96-well plates using a SCS harvesting system (Skatron, Sterling, VA). The results were expressed as percent specific release of ^51^Cr.

The MTT bactericidal assay was performed as described by Stevens et al. (1991) except for the following modifications: The bacteria were adjusted to a concentration of 2.5 x 10^8 cfu/ml RPMI-1640 containing 5% porcine or rabbit immune serum and 2.5 % fresh normal porcine serum (complement source) and were incubated for 30 minutes at
room temperature. Neutrophils were adjusted to $1 \times 10^7$ cells/ml in PBS. Neutrophils and opsonized bacteria were incubated together for 60 or 90 minutes. Controls included wells receiving only neutrophils and wells receiving only PBS. After the addition of isopropanol, the plates were tightly covered and stored overnight. The optical densities were read in a microplate reader at 570-630 nm (Dynatec, Torrence, CA).

Results of the MTT bactericidal assays were expressed as percentage of bacteria killed by neutrophils. The average of quadruplicate wells was calculated for each sample and the background due to neutrophil debris was subtracted from each average. A linear regression was performed on the values of the wells containing the known reductions in bacterial cell number, and a best-fitting line was obtained. Corrected experimental averages were entered into the equation of the best-fitting line to calculate the percentage of bacteria killed by the neutrophils. Each plate was compared to its own standard curve to account for day-to-day and plate-to-plate variations.

Necropsy

Two pigs from each group were necropsied on days 8, 15, and 22 with the exception of groups 3 and 4 on day 8 where only one pig was necropsied from each group. The pigs were anesthetized, exsanguinated, and necropsied as described by Wood et al. (1991). The following tissues were collected aseptically from Salmonella-infected pigs: spleen, liver, ileocolic lymph node, terminal ileum, apex of cecum, and peritoneal fluid. The spleen, terminal ileum, and apex of cecum were sampled from the pigs not receiving Salmonella.

Bacteriologic examination

Liver, ileocolic lymph nodes, cecum, peritoneal fluid, rectal swabs, and feces were examined qualitatively for the presence of Salmonella as described by Wood et al. (1989). Quantitative estimations of S. typhimurium populations in the spleen and ileum were determined by a most probable number method (Wood and Rose, 1992).
Statistical analysis

The study was divided into four periods: period 1, pre-exposure; period 2, days 1 and 2 after exposure to *Salmonella*; period 3, days 3 and 4 after exposure; period 4, days 7, 10, and 14 after exposure. Analyses of variance were performed for each assay, using average values from each animal in each period. The data were blocked by date to minimize the effect of daily variation inherent in these assays. P-values were considered significant at the 0.05 level.
RESULTS

The clinical response was similar in groups receiving *Salmonella* with or without BCG and was characterized by soft feces and a mild, often biphasic, febrile response (data not shown). Noninfected pigs receiving BCG alone showed no systemic clinical signs of illness. All groups, except the noninfected, nontreated controls, demonstrated an increase in WBC count immediately after treatment with BCG and/or *Salmonella* (Fig 1, day 1). Those pigs receiving *Salmonella* with or without BCG usually demonstrated a second leukocytosis 7-10 days postinfection. However, because the timing of the second leukocytosis was variable among individual pigs, the group averages shown in Fig. 1 do not reflect this trend.

**Fig. 1.** White blood cell counts (mean ± SEM) in pigs receiving *S. typhimurium* and/or BCG. *Salmonella* was given on day 0. BCG was given on days -17 and -2.
Neutrophils from pigs receiving *Salmonella* and/or BCG exhibited a general depression in ADCC activity (Fig. 2) compared to control animals. ADCC activity was significantly reduced (p<0.05) in both groups receiving *Salmonella* during periods 2 and 4 and in pigs receiving only BCG during period 4.

Figure 2. Antibody-dependent cell-mediated cytotoxicity (mean ± SEM) of neutrophils from pigs receiving BCG (days -17 and -2) and/or *S. typhimurium* (day 0). Periods reflect average of values from days indicated in parentheses. *different from controls (p<0.05)

Cytochrome-C reduction was significantly decreased (p<0.05) in unstimulated (Fig. 3(a)) and stimulated (Fig. 3(b)) neutrophils from all three infected and/or treated groups in period 2 when compared to controls. However, a significant difference (p<0.05) was also detected between the group receiving only *Salmonella* and the control group before exposure (period 1).

Iodination was significantly reduced (p<0.05) in groups receiving *Salmonella* during periods 2 and 4 (Fig. 4) when compared to controls in the same period. There was no statistically significant depression in the group receiving only BCG.
Figure 3. Cytochrome C reduction (mean ± SEM) by unstimulated (a) and zymosan-stimulated (b) neutrophils from pigs receiving BCG (days -17 and -2) and/or S. typhimurium (day 0). Periods reflect average of values from days indicated in parentheses. *different from controls (p<0.05). **different from group receiving BCG only (p<0.05)
Figure 4. Iodination activity (mean ± SEM) of porcine neutrophils. Pigs received BCG on days -17 and -2 and *Salmonella* on day 0. Periods reflect average of values from days indicated in parentheses. *different from controls (p ≤ 0.05)

Neutrophil motility was inhibited in pigs receiving only *Salmonella* and in pigs receiving only BCG compared to controls. Random migration (Fig. 5 a) was significantly decreased (p ≤ 0.05) in these groups during period 2. The chemotactic index (Fig. 5 b) was significantly depressed (p ≤ 0.05) in pigs receiving only *Salmonella* during period 2 and in pigs receiving either *Salmonella* or BCG during period 3.

Neutrophil bactericidal activity against *S. typhimurium* was increased (p ≤ 0.05) after exposure to *Salmonella* (Figs. 6 and 7). Assay results are shown using rabbit or pig serum as an opsonin with 60- or 90-minute incubation periods, respectively. The original optimization of this assay was performed using neutrophils from random adult swine, and satisfactory killing rates were obtained with rabbit serum and a 60-minute incubation. The pigs in this study, however, had low killing rates using this protocol (Fig. 6, period 1). In the time interval between periods 1 and 2, a suitable immune pig serum source became available and various incubation intervals were assessed. For the remaining periods in this study, simultaneous assays using pig serum and a 90-minute incubation interval, which
were believed to yield more sensitive results, were performed along with the assays using the original protocol. Both sets of data show similar overall trends.

*Salmonella* infection and BCG administration had no significant effects on *S. aureus* ingestion by neutrophils (data not shown).

Infected pigs receiving BCG and nontreated pigs had similar patterns of *S. typhimurium* colonization and persistence in internal organs. Rectal swabs were culture-positive for *Salmonella* 24 hours after exposure in all but one pig, and with the exception of one different pig each day, rectal swabs remained positive throughout the period of collection. The ileocolic lymph nodes, ileum, and cecum were infected in all exposed animals at necropsy. *Salmonella* was cleared from the spleen by day 8 post-exposure in three of four pigs necropsied. From one pig in the *Salmonella*/BCG group, less than 5 organisms/gram spleen were recovered. *Salmonella* was recovered from the liver of all pigs necropsied on day 8. By day 15, we recovered *Salmonella* from one out of four livers. Both organs were culture-negative in all pigs necropsied on day 22. We did not culture *Salmonella* from the peritoneal fluid of any animal at any necropsy. Feces from *Salmonella*-infected pigs were culture-positive in all post-exposure samples. Feces from pigs not receiving *S. typhimurium* did not contain *Salmonella*.

Populations of *S. typhimurium* in the ileal wall were similar between the BCG-treated and nontreated infected groups (data not shown). The numbers of bacteria isolated were fairly stable throughout the study, with the log10 of the population averaging 6.0 on day 8 and 6.7 on day 22.
Figure 5. Random migration (a) and chemotaxis (b) (mean ± SEM) of neutrophils from pigs receiving BCG (days -17 and -2) and/or S. typhimurium (day 0). Periods reflect average of values from days indicated in parentheses. *different from controls (p<0.05); **different from groups receiving Salmonella only or BCG only (p<0.05); ***different from group receiving Salmonella only (p<0.05)
Figure 6. Bactericidal activity (mean ± SEM) of porcine neutrophils against *S. typhimurium* pre-incubated with rabbit antibody and complement. Neutrophils and bacteria were incubated together for 60 min. Pigs received BCG on days -17 and -2 and *Salmonella* on day 0. Periods reflect average of values from days indicated in parentheses. *different from controls (p < 0.05)

Figure 7. Bactericidal activity (mean ± SEM) of porcine neutrophils against *S. typhimurium* pre-opsonized with porcine antibody and complement. Neutrophils and bacteria were incubated together for 90 min. Pigs received BCG on days -17 and -2 and *Salmonella* on day 0. Periods reflect average of values from days indicated in parentheses. *different from controls (p < 0.05)
DISCUSSION

Functional changes in circulating neutrophils occur in swine after oral exposure to *S. typhimurium*. Iodination and ADCC were depressed in infected pigs compared to controls immediately after infection (period 2). Activity normalized 3-4 days after infection (period 3), but was depressed again 7-14 days after infection (period 4). A waxing and waning clinical nature of *S. typhimurium* has been previously described (Wilcock, 1986). Our study demonstrates that alterations in certain neutrophil functions follow a similar pattern.

Cytochrome-C reduction was also depressed in infected pigs during period 2. The significance of this depression in the group receiving only *Salmonella* must be interpreted with caution, as pre-exposure assays from this group were also significantly different from controls. The depression evident in the *Salmonella*/BCG group during period 2, however, is considered to be a result of the treatment protocol.

Both cytochrome-C reduction and iodination measure oxygen-dependent bactericidal activities of the neutrophil. Alteration of neutrophil oxidative metabolism is a common pathogenic mechanism among many infectious agents. Depression of the oxidative burst has been demonstrated in bovine neutrophils after infection with bovine viral diarrhea virus (Roth et al., 1986), parainfluenza virus (Briggs et al., 1988), and *Brucella abortus* (Canning et al., 1988).

*Salmonella typhimurium* infection enhances the bactericidal activity of porcine neutrophils against *S. typhimurium*. This enhancement was evident throughout the duration of the experiment (to 15 days after infection), especially in the assay using porcine serum. These findings are in partial agreement with those of Smith et al. (1981b), who reported
increased porcine neutrophil bactericidal activity against *Staphylococcus aureus* 48 hours after infection with *S. typhimurium* but detected no increases at 96 hours or 6 days.

It seems paradoxical that neutrophil bactericidal activity should be increased when parameters of neutrophil oxidative metabolism are depressed. Oxidative bactericidal mechanisms are considered more potent than non-oxidative mechanisms (Smolen, 1989). Our data suggest a possible role for non-oxidative killing of *S. typhimurium* by porcine neutrophils. Cationic proteins, which comprise the major portion of neutrophil and macrophage cytoplasmic granule contents, have non-oxidative bactericidal action on *S. typhimurium* in other animal species (Shafer et al., 1984; Stinavage et al., 1989; Groisman and Saier, 1990) and may be important in swine. Alternatively, oxidative bactericidal mechanisms not evaluated by cytochrome C reduction or iodination may be enhanced. Similar apparently conflicting data were reported by Eisenstein et al. (1988), wherein vaccination of mice with aroA mutants of *S. typhimurium* provided good immunity despite depression of specific macrophage functions.

*Salmonella typhimurium* infection reduced neutrophil motility immediately after infection. Both random migration and chemotaxis were inhibited during period 2. Inhibition of chemotaxis continued through days 3 and 4 post-infection (period 3). These findings support those of Smith et al. (1985), who reported decreased chemotaxis at 72 hours after infection. Smith et al., however, did not detect an alteration in chemotaxis 24 hours after infection. Other infectious agents, such as *Bacteroides spp.* (Adamu and Sperry, 1981) or infectious bovine rhinotracheitis virus (Briggs et al., 1988), decrease chemotaxis of neutrophils in other animal species.

BCG has been used as a non-specific immunoenhancer in the treatment of human malignancies (Crispen, 1986). Its effects on the immunomodulation of bacterial infections have been investigated in mice. BCG or its purified components increase murine resistance
to *S. typhimurium* (Senterfitt and Shands, 1970), *Klebsiella pneumoniae*, *Escherichia coli*, and *Streptococcus pneumoniae* (Fogler, 1984).

In our study, BCG had no statistically significant main effect on any of the neutrophil function parameters examined. That is, the average of both groups receiving BCG was not statistically different from the average of groups not receiving BCG.

The effects of BCG observed in this study differ from those seen in murine macrophages treated with BCG (Fogler, 1984). While decreased migration was observed in both studies, treated murine macrophages exhibited increased oxidative metabolism and ingestion in contrast to the decreased oxidative burst and unaltered ingestion seen in our treated porcine neutrophils.

Overall, BCG treatment had few significant effects on normal porcine neutrophil function and did not reverse the effects of *Salmonella*-induced neutrophil functional depression. It did not further enhance the increased bactericidal activity detected in *Salmonella*-infected pigs. BCG did not alter the porcine clinical response to *S. typhimurium* nor did it affect colonization of and persistence of *S. typhimurium* in internal organs of pigs. It appears, therefore, that although BCG may exert minor influences on neutrophil function, it is not an effective immunomodulator of *S. typhimurium* in swine when used as reported here.
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PAPER II. THE EFFECT OF EXPOSURE TO A ΔCYAΔCRP MUTANT OF SALMONELLA TYPHIMURIUM ON THE SUBSEQUENT COLONIZATION OF SWINE BY THE WILD-TYPE PARENT STRAIN
The effect of exposure to a ΔcyaΔcrp mutant of *Salmonella typhimurium* on the subsequent colonization of swine by the wild-type parent strain

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This study characterizes the clinical response and colonization pattern of caesarean-derived, colostrum-deprived swine exposed to a ΔcyDAcrp mutant (x4233) of S. typhimurium and challenged with the wild-type parent strain. x4233 was mildly virulent in swine and induced transient fever and soft stools. x4233 colonized the ileum, cecum, liver, spleen, tonsils, and mandibular and ileocolic lymph nodes of swine in a manner similar to the parental wild-type, but the numbers of S. typhimurium (x4233) in the ileum were 100- to 1000-fold less than those of pigs exposed to the parental wild-type. Pigs exposed to x4233 21 days before parental wild-type challenge demonstrated a milder clinical response to challenge than did pigs that did not receive x4233. The wild-type populations in the ilea of x4233-exposed pigs after challenge were 100- to 10,000-fold less than those in pigs not receiving x4233. The liver, spleen, and ileocolic lymph nodes were cleared of wild-type S. typhimurium more quickly after challenge in x4233-exposed pigs. The populations of x4233 in the ilea of exposed pigs after wild-type challenge were also less than would have been expected in unchallenged pigs. Thus, exposure of swine to a ΔcyDAcrp mutant of S. typhimurium modulated the subsequent response to parental wild-type challenge and reduced carrier populations of wild-type S. typhimurium in infected swine.
INTRODUCTION

*Salmonella typhimurium* infections in swine cause enterocolitis of varying severity followed by a persistent carrier state (Wood et al., 1989). *S. typhimurium* may persist in low numbers in infected swine for at least 28 weeks, especially in the gastrointestinal tract, tonsils, and associated lymph nodes (Wood and Rose, 1992). Increased shedding of *S. typhimurium* may occur during conditions of stress, such as during transport or at slaughter (Williams and Newell, 1970; Morgan et al., 1987), providing a potential source of *Salmonella* infection for other animals and humans.

Several strategies have been developed to inhibit the colonization of *S. typhimurium* in poultry and therefore reduce *Salmonella* populations in infected birds (Pivnick et al., 1981; Bailey et al., 1988; Oyofo et al., 1989). Oral administration of cecal contents decreases subsequent intestinal colonization of chickens by *Salmonella* (reviewed by Mead and Impey, 1986), but widespread use of this practice is limited by the incomplete characterization of such products.

Current research has focused on the identification of specific microbes than could be used to reduce *Salmonella* populations in poultry. Recently it has been shown that the oral administration of avirulent *Salmonella* mutants, such as ΔcyΔcrp mutants, inhibits the colonization of chickens by virulent *S. typhimurium* (Berchieri Jr. and Barrow, 1990; Hassan and Curtiss, 1990). ΔcyΔcrp mutants of *S. typhimurium* are avirulent but immunogenic in mice (Curtiss and Kelly, 1987). They are similar to wild-type strains in their attachment to and invasion of gut-associated lymphoid tissue. ΔcyΔcrp mutants contain deletions in their genes encoding adenylate cyclase and cyclic AMP receptor protein. Cyclic AMP, produced via the catalytic action of adenylate cyclase, is necessary...
for the regulation of genes involved in glucose utilization, amino acid metabolism, the synthesis of flagella and pili, and the synthesis of certain outer membrane proteins.

Reduction of the virulent *S. typhimurium* population in carrier swine would reduce the potential reservoir of infection that they represent to other animals and humans. It is possible that an avirulent mutant of *S. typhimurium* could reduce the colonization of swine by wild-type *S. typhimurium*.

In this study, we investigated the effect of a ΔcyaΔcrl mutant of *S. typhimurium* on the colonization of the porcine gastrointestinal tract and extraintestinal organs by the wild-type parent strain. First, the virulence and colonizing ability of a ΔcyaΔcrl mutant in swine was compared to that of wild-type *S. typhimurium*. Next, swine previously exposed to a ΔcyaΔcrl mutant were challenged with the wild-type parent strain. The colonization patterns and intestinal populations of wild-type *S. typhimurium* in these pigs were compared with those of challenged swine not previously exposed to the ΔcyaΔcrl mutant.
MATERIALS AND METHODS

This study consisted of two serial experiments.

Swine

Crossbred caesarian-derived, colostrum-deprived pigs, six weeks of age, were used. For each experiment, the pigs were raised in isolation as described previously (Wood et al., 1989). Rectal swabs and fecal samples were obtained at periodic intervals during the six-week growing period and cultured for the presence of indigenous Salmonella, also as described previously (Wood et al., 1989). Salmonella was not isolated from any of the samples cultured.

Bacteria

S. typhimurium strain 798 (Wood et al., 1989), used in experiment 1, was isolated from a case of porcine salmonellosis and was obtained from the National Veterinary Services Laboratories, Ames, IA. S. typhimurium χ4347 and χ4233 were kindly provided by Dr. Roy Curtiss III and Sandra Kelly, Washington University, St. Louis, MO. χ4347 is a tetracycline-resistant derivative of S. typhimurium strain 798 and was constructed specifically for experiment 2 to facilitate retrieval from mixed bacterial cultures. χ4233 is a nalidixic acid-resistant ΔcyADcrp mutant of strain 798. Cultures of strain 798 and χ4233 were grown to mid-log phase in Luria broth, centrifuged at 2000g for 20 minutes, and the bacterial pellets were resuspended in one half volume of buffered saline gelatin (0.85% NaCl, 0.03% KH₂PO₄, 0.06% Na₂HPO₄, 0.01% gelatin). Cultures of χ4347 were grown to mid-log phase in Luria broth and were fed to the pigs without further modification.
Exposure

Experiment 1. Each pig in group 1 (n=10) received $3.7 \times 10^{10}$ cfu of x4233 p.o.; pigs in group 2 (n=10) each received $2.28 \times 10^{10}$ cfu of strain 798 p.o., and those in group 3 (n=5) each received 10 ml sterile culture medium p.o. (unexposed control) at 7-8 weeks of age.

Experiment 2. Each pig in group A (n=16) received $1.5 \times 10^{10}$ cfu of x4233 p.o., and pigs in groups B (n=16) and C (n=8) each received 10 ml sterile culture medium p.o. at 7 weeks of age. At 10 weeks of age, each pig in groups A and B received $1.4 \times 10^{10}$ cfu of x4347 p.o. Pigs in group C served as unexposed controls.

Clinical examination

Rectal temperatures were measured daily (0700-0900) for three consecutive days prior to exposure to strain 798, x4233, or x4347 to establish a baseline temperature for each pig. Temperatures were measured daily for 6 days after exposure to Salmonella. Pigs were observed daily for changes in appetite and demeanor and for signs of diarrhea.

Necropsy

Experiment 1. Two pigs from each of groups 1 and 2 and one pig from group 3 were necropsied 3, 7, 14, 21, and 28 days after exposure. The following tissues were collected: spleen, liver, ileocolic lymph nodes, terminal ileum (including the ileocecal junction), apex of cecum, mandibular lymph nodes, and palatine tonsils. The tonsils and ileum were cultured quantitatively; the remaining tissues were cultured qualitatively.

Experiment 2. Four pigs from each of groups A and B and two pigs from group C were necropsied 7, 14, 21, and 28 days after exposure to x4347 with the exception of two pigs from group B which became severely ill and were euthanized and necropsied on day 5 instead of day 7. Pigs necropsied on days 5 and 7 were grouped together for statistical analysis. The caudal ileum, including the ileocecal junction, was collected from all groups.
and cultured quantitatively (groups A and B) or qualitatively (group C) for *Salmonella*. The spleen, liver, apex of the cecum, and ileocolic lymph nodes were also collected from groups A and B for qualitative bacterial examination. The palatine tonsils were collected for qualitative examination from pigs in group A on days 21 and 28.

**Bacteriologic examination**

Tissues were cultured qualitatively for *S. typhimurium* by methods described previously (Wood et al., 1989) except for the following modifications: Tetracycline (25 µg/ml) or nalidixic acid (50 µg/ml) was added to differential media used to isolate χ4347 or χ4233, respectively, from mixed cultures. Quantitative analysis was performed using a most probable number (MPN) method as described previously (Wood and Rose, 1992) except that aliquots from the serial dilutions of mixed cultures were incubated in each of two sets of differential media containing either tetracycline or nalidixic acid as described above.

**Statistics**

Individual bacteriological counts were converted to log10 for statistical analysis. Mean logarithms of populations were analyzed by t-tests. Differences were considered significant at p=0.05.
RESULTS

Clinical response

In both experiments, pigs infected with χ4233 had a mild to moderate fever (peak mean=40.1°C) of approximately four days duration (Fig. 1). The rectal temperature response shown in Fig. 1 is based on data obtained during experiment 2; data from experiment 1 (results not shown) were similar. Occasional soft stools were noted. The appetite and demeanor of pigs infected with χ4233 were mildly depressed 24 hours after infection.

In experiment 1, clinical response to strain 798 was similar to that of χ4233. The febrile response was similar in magnitude and duration (results not shown). Occasional vomiting was noted.

Figure 1. Rectal temperature response of swine to exposure to a ΔcyaΔcrp mutant (χ4233) of S. typhimurium—experiment 2. Group A pigs were exposed to χ4233 on day -21. Group B pigs served as negative controls. There was a statistically significant difference (p<0.02) between groups on days -20 through -17 (first 4 days after receiving χ4233).
In experiment 2, pigs receiving \( \chi 4347 \) without previous exposure to \( \chi 4233 \) (group B) had a moderate to severe fever (Fig. 2), which peaked 2 days after infection (mean=40.5°C) and persisted throughout the monitored period (through 6 days after infection). The infected pigs were severely depressed and had diminished appetites for 2-3 days. Approximately 90% of the pigs experienced watery diarrhea. Rectal prolapses of varying degrees occurred in three pigs. One pig became dyspneic 7 days after infection; severe fibrinous pericarditis and peritonitis were present at necropsy.

Figure 2. Rectal temperature response of swine to challenge (day 0) with \textit{S. typhimurium} \( \chi 4347 \)--experiment 2. Group A pigs were exposed to a \( \Delta \text{cya}\Delta \text{crp} \) mutant (\( \chi 4233 \)) of \textit{S. typhimurium} 21 days prior to challenge. Group B pigs did not receive \( \chi 4233 \). Group C pigs received neither \( \chi 4233 \) nor \( \chi 4347 \) and served as unexposed controls. Group A temperatures were significantly increased over controls (\( p<0.05 \)) on day 1. Group B temperatures were significantly increased over controls (\( p<0.05 \)) on days 1-3 and 5-6.

Pigs challenged with \( \chi 4347 \) after prior exposure to \( \chi 4233 \) (group A) had a fever 24 hours after challenge, but rectal temperatures were statistically the same (\( p>0.05 \)) as controls by 48 hours (Figure 2). The magnitude of fever was significantly lower (\( p<0.05 \)) and of shorter duration than that of challenged pigs not previously exposed to \( \chi 4233 \).
Approximately 50% of the pigs in group A exhibited soft feces, but there was no liquid diarrhea. Appetites and demeanor, slightly depressed at 24 hours, returned to normal by 48 hours.

With the exception of the pig that developed fibrinous pericarditis and peritonitis, gross lesions of the organs examined at necropsy were limited to edematous lymph nodes, which were variably present in both groups.

Colonization

Experiment 1. The ΔcyADcrp mutant (χ4233) of *S. typhimurium* colonized all of the tissues examined as did the parent strain (798). χ4233 and strain 798 persisted qualitatively in the liver and spleen for comparable periods. *Salmonella* was recovered from both organs on days 3 and 7 but not from either organ on days 14, 21, or 28. χ4233 was cleared from the ileocolic lymph nodes more quickly than was strain 798; on day 28, *Salmonella* was not recovered from the ileocolic lymph nodes of pigs infected with χ4233. The caudal ileum, cecum, mandibular lymph nodes, and palatine tonsils were

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<td></td>
<td>798</td>
<td>2/2</td>
</tr>
<tr>
<td>Ileocolic lymph nodes</td>
<td>χ4233</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>798</td>
<td>2/2</td>
</tr>
<tr>
<td>Cecum</td>
<td>χ4233</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>798</td>
<td>2/2</td>
</tr>
<tr>
<td>Mandibular lymph nodes</td>
<td>χ4233</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>798</td>
<td>2/2</td>
</tr>
</tbody>
</table>

^aNumber of culture-positive pigs/total pigs necropsied
culture-positive in all pigs at all necropsies (Table 1). Mean logarithms of populations of *S. typhimurium* in the terminal ileum ranged from 3.0 to 4.16 in pigs infected with the ΔcyAΔcrp mutant (x4233) and from 5.51 to 7.41 in pigs infected with the parental wild-type (798). The differences, shown in Fig. 3, were 100- to 1000-fold at all necropsies and were statistically significant (p<0.05) on days 3, 14, and 21. There were no significant differences between *Salmonella* populations in the palatine tonsil of pigs infected with either strain 798 or x4233 (Fig. 4). Mean logarithms of tonsil populations ranged from 4.16 to 6.40.

![Image](image.png)

**Figure 3.** *S. typhimurium* populations per gram of tissue in the caudal ileum of swine exposed on day 0 to either strain 798 (wild-type) or its ΔcyAΔcrp mutant (x4233)—experiment 1. Differences between groups were statistically significant (p<0.05) on days 3, 14, and 21. Populations were determined by a most probable number method.

**Experiment 2.** Mean logarithms of populations of x4347 in the terminal ileum of pigs previously exposed to x4233 (group A) ranged from 2.10 to 5.14. In pigs not previously exposed to x4233 (group B), mean logarithms of populations of x4347 in the terminal ileum ranged from 6.02 to 7.23. The mean differences, shown in Fig. 5, were
significant (p<0.05) on days 7, 21, and 28. The population difference on day 14 was also substantial (p=0.08). The difference between the ileal populations in the two groups of pigs examined became larger over the course of the study. The mean populations in both groups decreased over time, but the overall decrease in x4233-exposed pigs was greater than in pigs not exposed to x4233 (net log decrease 3.0 and 0.7, respectively).

![Graph](image)

Figure 4. Populations of *S. typhimurium* per gram of tissue in the tonsils of swine exposed on day 0 to either strain 798 (wild-type) or its ΔcyADcrp mutant (x4233)—experiment 1. There were no statistically significant differences (p>0.05) between groups. Populations were determined by a most probable number method.

Recovery of x4347 from tissues other than the ileum is summarized in Table 2. x4347 was not recovered from the livers or spleens of pigs in group A at any necropsy. The livers of pigs from group B were culture-negative by day 14 and the spleens were culture-negative by day 21. The ileocolic lymph nodes from pigs in group A were culture-
Figure 5. Populations of wild-type *S. typhimurium* (*x*4347) per gram of tissue in the caudal ileum of swine—experiment 2. All pigs were challenged with *x*4347 on day 0. Pigs in group A were exposed to a ΔcyΔcpr mutant of *S. typhimurium* (*x*4233) 21 days prior to challenge. Differences were statistically significant on days 7, 21, and 28 (*p* < 0.05). Populations were determined by a most probable number method.

Negative on day 21, but 50% of the ileocolic lymph nodes from group B still contained *x*4347 on day 28. With the exception of one pig from group A on day 28, the cecum remained colonized in both groups throughout the study. The palatine tonsil, which was retrieved only from pigs in group A on days 21 and 28, was colonized in all but one pig, which was necropsied on day 28.

Populations of *x*4233 in the terminal ileum of pigs in group A after challenge with *x*4347 were very low or undetectable. The mean populations, shown in Fig. 6, ranged from <1 to 100 organisms/g. *x*4233 could not be recovered at necropsy from the ileum of 50% of the post-challenge pigs in group A. The mean population of *x*4233 rose throughout the course of experiment 2. At no time, however, did the population of *x*4233 in challenged pigs become as high as that in the unchallenged pigs. Also, the incidence of
Table 2. Recovery of wild-type *Salmonella typhimurium* from internal organs of challenged pigs—experiment 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group</th>
<th>Days Post Exposure</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Spleen</td>
<td>A</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>2/4</td>
<td>1/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Liver</td>
<td>A</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Ileocolic lymph nodes</td>
<td>A</td>
<td>4/4</td>
<td>3/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Cecum</td>
<td>A</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
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<td>B</td>
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<td>4/4</td>
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<tr>
<td></td>
<td>B</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
</tbody>
</table>

*Pigs in group A were exposed to χ4233 (ΔcyaΔcrp mutant) 21 days prior to challenge.*

*bNumber of culture-positive pigs/total pigs necropsied.*

*cPigs in group B were not previously exposed to χ4233.*

N/D = not done

Cecal colonization by χ4233 immediately after wild-type challenge was lower than would have been expected in challenged pigs based on data from experiment 1 (Tables 1 and 2).

The liver and ileocolic lymph nodes were cleared of χ4233 in all pigs from group A at necropsy. All spleens were culture negative for χ4233 except for that of one pig necropsied on day 28. χ4233 was recovered from the cecum of 1 of 4 pigs at each necropsy. The palatine tonsils contained χ4233 in 2 of 2 pigs on day 21 and 3 of 4 pigs on day 28.

The palatine tonsils were not originally one of the samples to be harvested at necropsy during experiment 2. Because of the extremely low populations of χ4233 recovered from χ4233-exposed pigs on days 7 and 14, though, the tonsils were collected in subsequent
necropsies in an effort to characterize more accurately the status of $\chi_{4233}$ in these animals. In several pigs, the tonsils were the only tissues cultured from which $\chi_{4233}$ was recovered.

There was only one $\chi_{4233}$-exposed pig in the study from which $\chi_{4233}$ could not be recovered from any tissue examined. Rectal samples taken shortly after exposure to $\chi_{4233}$ confirmed that this pig was indeed infected and was shedding $\chi_{4233}$ in its feces at that time. Interestingly, this pig exhibited the largest population of $\chi_{4347}$ in its ileum of any of the $\chi_{4233}$-exposed pigs necropsied on that day (day 28), but, in general, there was no relationship between populations of $\chi_{4233}$ and $\chi_{4347}$ in individual pigs. There were no pigs identified which had cleared both $\chi_{4233}$ and $\chi_{4347}$.

*Salmonella* was not recovered from any of the unexposed control pigs in either experiment.

![Graph showing populations of bacteria](image)

Figure 6. Populations of a ΔcyaΔcrp mutant ($\chi_{4233}$) of *S. typhimurium* per gram of tissue in the caudal ileum of infected swine. All pigs received $\chi_{4233}$ on day 0. Challenged pigs received wild-type *S. typhimurium* on day 21. Unchallenged pigs did not receive wild-type *S. typhimurium*. Populations were determined by a most probable number method. In the group of challenged pigs, 2 of 4 pigs were culture negative on day 28, and 3 of 4 pigs were culture negative on each of days 35 and 49. Populations of the parental wild-type ($\chi_{4347}$) in challenged pigs are shown for comparison.
DISCUSSION

A ΔcyaΔcrp mutant of *S. typhimurium* strain 798 was able to colonize swine in a manner similar to parental wild-type with respect to tissue distribution but colonized the ileum in reduced numbers. Oral exposure of swine to this ΔcyaΔcrp mutant resulted in a reduction in the severity of clinical signs and a reduction in the colonization of internal organs associated with subsequent parental wild-type challenge.

Contrary to the avirulent behavior of *S. typhimurium* ΔcyaΔcrp mutants in mice, the ΔcyaΔcrp mutant of *S. typhimurium* strain 798 was mildly virulent in swine, consistently inducing a mild fever and variably formed feces. It was not entirely unexpected that the ΔcyaΔcrp mutant behaved differently in swine than it did in mice. Wild-type *S. typhimurium* also elicits clinically distinct syndromes in these two species. *S. typhimurium* in mice causes septicemic disease with high mortality (Hsu, 1989); in pigs the same organism typically causes localized enterocolitis (Wilcock, 1986).

The clinical signs associated with wild-type *S. typhimurium* infection in experiment 2 were consistent with previous reports (Wilcock, 1986; Wood et al., 1989). Washed bacterial cells were used for oral exposure to strain 798 in experiment 1, whereas the complete culture of χ4347 (a tetracycline-resistant derivative of strain 798) was used in experiment 2. The complete culture was selected for experiment 2 in order to maximize the effect of wild-type challenge. The absence of factors present in the culture supernatant may have contributed to the diminished severity of clinical signs observed with strain 798 in experiment 1.

The ΔcyaΔcrp mutant (χ4233) colonized the liver, spleen, and ileocolic (mesenteric) lymph nodes of swine as did the parental wild-type, but it was cleared from the lymph nodes more quickly than wild-type *S. typhimurium*. This is consistent with data reported
by Curtiss and Kelly (1987) who showed that ΔcyaΔcrp mutants exhibited a diminished ability to reach or persist in the spleen and mesenteric lymph nodes of mice. Because quantitative examination of these organs was not performed in our study, it is not known whether x4233 colonized these porcine organs in reduced numbers.

x4233 colonized the caudal ileum of swine at lower populations than did strain 798, but the mean population of x4233 was fairly stable throughout the study. Thus it appears that even though x4233 existed in the ileum in lower numbers, it was not cleared more readily from the ileum than was strain 798 during the first four weeks after infection. The populations of strain 798, in the range of 10^5 to 10^7 organisms per gram of ileum, were consistent with previous quantitative studies of wild-type infections in swine (Wood and Rose, 1992) and indicated that colonization by strain 798 in experiment 1 occurred as expected despite the relative lack of associated clinical signs.

There are several possible explanations for the reduced colonization and/or persistence of the S. typhimurium ΔcyaΔcrp mutant in porcine internal organs. Many metabolic functions are altered by deletions in the adenylate cyclase and cAMP receptor protein genes. Among these is the synthesis of flagella (Curtiss and Kelly, 1987). Flagella may have a role in the growth and survival of Salmonella in extraintestinal organs of mice (Finlay and Falkow, 1988). They may enhance resistance to macrophage bactericidal activity or they may enhance intracellular bacterial multiplication. The ΔcyaΔcrp mutation also affects synthesis of certain outer membrane proteins (Curtiss and Kelly, 1987). A 59-kD outer membrane protein of S. typhimurium plays a role in resistance to oxidative killing by human neutrophils (Stinavage et al., 1990). Cyclic AMP is necessary for the activation of numerous bacterial operons involved in catabolic processes, including those involved in the utilization of certain carbohydrates as nutritional substrates (Lewin, 1990,
Depression of cAMP levels, therefore, may affect the ability of the bacteria to survive under diverse nutritional conditions.

Competition between $\chi^{4233}$ and $\chi^{4347}$ for attachment sites may have played a role in the decreased colonization of internal organs by $\chi^{4347}$ and the apparent reduction in $\chi^{4233}$ populations of $\chi^{4233}$-exposed pigs after wild-type challenge. However, it is unlikely that these events can be explained by competition alone. It is possible that $\chi^{4233}$-exposed pigs had enhanced phagocytic cell activity and enhanced cellular resistance to invasion by *S. typhimurium*.

Removal by phagocytic cells is considered to be a primary means of *Salmonella* clearance from infected animals (Guo et al., 1986). It has been shown that neutrophils from pigs exposed to *S. typhimurium* have increased bactericidal activity against *S. typhimurium* when compared to nonexposed controls (Coe et al., 1992). Killing was enhanced in all pigs by pre-incubation of bacteria with specific antibody and complement. In mice, the effectiveness of neutrophils against *S. typhimurium* is also increased by the presence of opsonizing antibodies (Baron and Proctor, 1984). Increased levels of anti-*Salmonella* antibodies may have been produced via an anamnestic response in $\chi^{4233}$-exposed pigs. $\chi^{4233}$-exposed pigs may also have had a larger number of activated neutrophils and macrophages. These phagocytes, which demonstrate increased antimicrobial activity, may be activated by a number of cytokines and products of inflammation (Roitt et al., 1989).

Several cytokines have been implicated in host resistance to *S. typhimurium*. The protective effect of interferon (IFN) against *Salmonella* infections in bovine (Peel et al., 1990), murine (Bukholm et al., 1984; Muotiala and Makela, 1990), and human cell (Bukholm and Degre, 1983) models has been reported. IFN appears to increase host cell resistance to invasion by *S. typhimurium* in mice and humans. IFN-γ, or immune
interferon, is produced by sensitized T-lymphocytes (Roitt et al., 1989) and may have been increased after challenge in χ4233-exposed pigs. Tumor necrosis factor (TNF), which is produced by multiple cell types during inflammation, has a protective effect against *S. typhimurium* in mice (Nakano et al., 1990) and is synergistic with IFN in its action. Interleukin-6, which is increased during the acute phase response associated with bacterial challenge, inhibits intracellular bacterial growth in infected macrophages (Flesch and Kaufmann, 1990). The actions of these cytokines in a porcine *Salmonella* model have not been reported. However, if the actions are similar, then IFN and TNF may have reduced the invasiveness of the parental challenge, and IL-6 may have played a role in the reduction of resident χ4233 populations.
REFERENCES CITED


PAPER III. EVALUATION OF FACTORS AFFECTING BACTERICIDAL ACTIVITY OF PORCINE NEUTROPHILS AGAINST *SALMONELLA TYPHIMURIUM*
Evaluation of Factors Affecting Bactericidal Activity of Porcine Neutrophils Against *Salmonella typhimurium*

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James A. Roth, DVM, PhD

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Salmonella typhimurium infections in swine are typically characterized by a persistent carrier state. Little is known about the mechanisms that allow S. typhimurium to persist in porcine tissues, but professional phagocytes, such as neutrophils and macrophages, are typically important for clearing bacteria from host tissues. In this study, we examined factors that may affect the bactericidal efficiency of porcine neutrophils against S. typhimurium. Initially, opsonized bacteria were more efficiently killed by porcine neutrophils than were nonopsonized counterparts, but the difference in efficiencies was usually decreased or lost by 4 hours. A phoP mutant of S. typhimurium was progressively more efficiently killed than its parent strain after 2 hours. There was little difference in neutrophil bactericidal efficiencies between a ΔcyaΔcrp mutant and its parent strain and between an ΔaroA mutant and its parent strain after 2 hours. Treatment of porcine neutrophils with recombinant human tumor necrosis factor alpha (rHu-TNFα) 2 hours prior to assay resulted in an initial decrease in bactericidal efficiency when compared to nontreated neutrophils, but by 4 hours, rHu-TNFα-treated neutrophils demonstrated a small, but statistically significant, enhancement of bactericidal efficiency over nontreated controls.
INTRODUCTION

Salmonella typhimurium infection in swine readily causes a persistent carrier state with variable fecal shedding (Wilcock, 1986; Wood et al., 1989). Carrier animals are a major reservoir of infection for S. typhimurium, and Salmonella from infected animals at slaughter can contaminate food intended for human consumption. Reduction and/or elimination of the carrier state of S. typhimurium in swine is therefore desirable, but very little is known about the host-bacteria interactions that influence the establishment and/or maintenance of the carrier state. In a previous study we showed that several porcine neutrophil functions are altered in Salmonella-infected pigs, including increased bactericidal activity against S. typhimurium (Coe et al., 1992a). In the present study, we examined specific host and bacterial factors that may influence porcine neutrophil bactericidal activity against S. typhimurium: specific bacterial mutations (ΔcyaΔcrp, phoP, and ΔaroA), opsonization of bacteria with antibody and complement, exposure time, and cytokine activation of neutrophils.
MATERIALS AND METHODS

Animals

Crossbred pigs, 3-6 months of age, were maintained as blood donors for this study. Blood was collected from the vena cava of randomly selected pigs into syringes containing 7.5% EDTA in 0.85% saline (1:30 v/v). Each assay variable was tested with neutrophils from each of 5-7 pigs.

Bacterial strains

The three parent-mutant pairs of *S. typhimurium* used are described in Table 1. Log-phase bacterial cultures were grown and harvested as described previously (Coe et al., 1992a). All of the mutant strains were derived directly from the designated parent strains.

Cytokine

Recombinant human tumor necrosis factor α (rHu-TNFα) was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Concentrated rHu-TNFα was diluted in M199 medium immediately prior to use.

Neutrophil isolation

Neutrophils were isolated from whole blood by a combination of hypotonic RBC lysis and ficoll-diatrizoate density centrifugation as described previously (Coe et al., 1992). The cell concentration was adjusted to 10^7 cells/ml in RPMI-1640 medium except where specifically indicated otherwise.

MTT bactericidal assay

The MTT bactericidal assay is a colorimetric assay that utilizes the reduction of yellow MTT (3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan by live bacteria as an indicator of the bactericidal activity of phagocytes (Stevens et al., 1991). The amount of formazan formed, which can be quantitated spectrophotometrically,
Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Mutation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>798</td>
<td>porcine isolate</td>
<td>N/A</td>
<td>Wood et al., 1989</td>
</tr>
<tr>
<td>x4233</td>
<td>798</td>
<td>deletion in genes encoding adenyl cyclase and cyclic AMP receptor protein</td>
<td>Coe and Wood, 1992</td>
</tr>
<tr>
<td>SR-11</td>
<td>murine isolate</td>
<td>N/A</td>
<td>Galan and Curtiss, 1989</td>
</tr>
<tr>
<td>x43687</td>
<td>SR-11</td>
<td>point mutation in gene regulating nonspecific phosphatase</td>
<td>Galan and Curtiss, 1989</td>
</tr>
<tr>
<td>UK-1</td>
<td>equine isolate</td>
<td>N/A</td>
<td>Roy Curtiss, III Washington Univ. (unpublished)</td>
</tr>
<tr>
<td>x4467</td>
<td>UK-1</td>
<td>deletion in genes encoding aromatic amine synthesis (auxotrophic for aromatic amino acids)</td>
<td>Roy Curtiss, III Washington Univ. (unpublished)</td>
</tr>
</tbody>
</table>

is proportional to the amount of live bacteria remaining after incubation with phagocytes and subsequent lysis of the phagocytes. The percentage of bacteria killed by a given phagocyte preparation is extrapolated from a standard curve generated from absorbance readings of bacteria incubated without neutrophils. In this study, the assay was performed as described previously (Coe et al., 1992) except that 10% porcine antiserum and 2.5% fresh normal porcine serum (complement source) were used to opsonize bacteria and the neutrophils were resuspended in appropriate culture medium (RPMI-1640 or M199) instead of phosphate-buffered saline. RPMI-1640 medium was used throughout all of the assays except those involving rHu-TNFα, in which M199 medium was used.
The neutrophil:bacteria ratio for the MTT assays was optimized by incubating aliquots of porcine neutrophils simultaneously for 90 minutes with each of three concentrations of bacteria to achieve neutrophil:bacteria ratios of 1:10, 1:25, and 1:40 (results not shown). The 1:40 ratio was selected for use in the study presented here because it resulted in a strong challenge to the neutrophils and generated adequate amounts of formazan necessary for assay sensitivity.

In assays testing the effect of exposure time, the aliquots of neutrophils were incubated with either opsonized or nonopsonized bacteria, and bactericidal activity was evaluated at 0.5, 1, 2, 3, and 4 hours. Replicate sets of assay plates were made and incubated at 37°C/5% CO₂, and one set was developed after incubating for each of the designated time intervals. To test the effect of rHu-TNFα on bactericidal activity, porcine neutrophils (5 x 10⁷ cells/ml) were incubated with rHu-TNFα (250 ng/ml) or M199 medium alone (nontreated controls) for two hours at 37°C/5% CO₂ prior to use in the MTT bactericidal assay. The neutrophils were further diluted 1:5 in M199 medium after incubation but before distribution into 96-well plates. Opsonized and nonopsonized bacteria were used, and the exposure times evaluated were the same as described above.

**Statistical analysis**

The mean absorbance for each sample was calculated from triplicate (cytokine assays) or quadruplicate (all other assays) samples of each neutrophil preparation. The percentage of bacteria killed for each sample was extrapolated from standard curves calculated from standard wells incubated on the same microtitration plate to eliminate error due to plate-to-plate variation. Direct data comparisons were made only among data generated on a single day to eliminate error due to day-to-day variation. Treatment differences were assessed by analyses of variance; differences were considered significant at p-values of 0.05 or less.
RESULTS

Effect of bacterial mutations

The phoP mutant (x3687) was less efficiently killed (based on percentage of bacteria killed) by porcine neutrophils than its parent strain, SR-11, shortly after exposure (0.5 and 1 hour), but by 3 and 4 hours, x3687 was significantly (p<0.05) more efficiently killed (figure 1). Overall, the ΔaroA mutant (x4467) was significantly less efficiently killed than its parental strain, UK-1, except at 4 hours when nonopsonized x4467 was significantly more efficiently killed than nonopsonized UK-1 and at 1 and 2 hours when there were no significant differences between the killing of opsonized UK-1 and opsonized x4467. Strain 798 was significantly more efficiently killed than its ΔcyaΔcrp mutant (x4233) at 0.5 and 1 hour. The differences were lost by 2 hours, but by 4 hours, opsonized x4233 was significantly more efficiently killed than opsonized 798.

Effect of opsonization

Bacteria opsonized with antibody and complement were usually killed more efficiently than their nonopsonized counterparts (figure 2). With strain 798 and its ΔcyaΔcrp mutant (x4233), the advantage of bacterial opsonization on neutrophil bactericidal efficiency was significant (p<0.05) for the first 3 hours after contact between neutrophils and bacteria. However, by 4 hours after exposure, the percentage of opsonized bacteria killed was not significantly different from that of nonopsonized controls. Similar trends were observed with strain UK-1 and its ΔaroA mutant (x4467), but the differences between opsonized and nonopsonized bacteria were greatly reduced by 3 hours. With strain SR-11 and its phoP mutant (x3687), significant enhancement of bactericidal efficiency due to opsonization was
Fig. 1. Effect of opsonization and incubation interval on bactericidal efficiency of porcine neutrophils against *S. typhimurium* as measured by MTT reduction. Neutrophils were incubated with either a parent strain of *S. typhimurium* or its specific mutant (1:40 ratio) for the time period shown. Opsonized bacteria were pre-incubated with specific antibody and complement for 30 minutes prior to incubation with neutrophils. A) Parent strain 798 and its ΔcyΔcrp mutant, χ4233; B) Parent strain SR-11 and its phoP mutant, χ3687; C) Parent strain UK-1 and its ΔaroA mutant, χ4467.
not evident with χ3687 until 2 hours, but the enhancement remained significant through 3 and 4 hours. Opsonized SR-11 was more efficiently killed than nonopsonized SR-11 throughout the first 4 hours.

**Effect of exposure time**

With all but one bacterial preparation (nonopsonized SR11), there was an overall increase in the percentage of bacteria killed over the 4-hour time period monitored (figure 1).

**Effect of neutrophil treatment with rHu-TNFα**

Porcine neutrophils treated with rHu-TNFα demonstrated reduced bactericidal efficiency at early times after exposure to bacteria (0.5 and 1 hour). By 2-3 hours, the bactericidal inhibition was reduced, and by 4 hours, rHu-TNFα-treated neutrophils demonstrated enhanced bactericidal efficiency over nontreated controls. This trend was evident for all bacterial preparations tested (figure 2). The differences were most pronounced with strain UK-1 and χ4467 (ΔaroA mutant); the treatment difference was significant (p<0.03) at all time points except 3 hours. With strain 798 and χ4233 (ΔcyaΔcrp mutant) and with strain SR11 and χ3687 (phoP mutant), the differences were significant at 0.5 and 4 hours (798/χ4233; p<0.03) and 2 and 4 hours (SR11/χ3687; p<0.05).
Fig. 2. Effect of recombinant human tumor necrosis factor alpha (rHu-TNFα) on the bactericidal efficiency of porcine neutrophils against *S. typhimurium* as measured by MTT reduction. Neutrophils were pre-incubated in either rHu-TNFα—enriched or unenriched culture medium for 2 hours. Opsonized bacteria were pre-incubated for 30 minutes with specific antibody and complement. Neutrophils were then incubated with either a parent strain of *S. typhimurium* or its specific mutant for the time period shown. Differences between the percentage of bacteria killed by TNF-treated and sham-treated neutrophils are shown for each parent:mutant pair of *S. typhimurium*. A) Parent strain 798 and its ΔcyaΔcrp mutant, χ4233; B) Parent strain SR-11 and its phoP mutant, χ3687; C) Parent strain UK-1 and its ΔaroA mutant, χ4467.
DISCUSSION

The bactericidal actions of macrophages and neutrophils are important for the clearance of *Salmonella* from animal hosts. Although most research has focused on the role of macrophages in *Salmonella* clearance, the neutrophil is an important mediator of defense, especially in the early stages of infection. Neutrophil infiltrates have been identified in intestinal lesions of pigs exposed to *S. typhimurium* (Reed et al., 1986). It has also been shown that neutrophils can ingest and kill *S. typhimurium* (Takeuchi, 1967).

Bactericidal efficiency, measured in this study as the percentage of bacteria killed when compared to bacteria incubated in the absence of neutrophils, is influenced by the dynamic interaction of neutrophil bactericidal activity and bacterial growth. No attempt was made to compare absolute numbers of bacteria killed by porcine neutrophils in this study because the growth characteristics of the bacterial preparations may have been altered by the presence of neutrophils. Also, no comparisons were made between bactericidal efficiencies measured on different days. Occasionally, the calculated percentage of bacteria killed was a negative number and, in one instance, the percentage of bacteria killed when compared to bacteria incubated without neutrophils decreased over time. It is possible in these cases that neutrophil-derived factors enhanced bacterial growth of *S. typhimurium* at a rate greater than that at which the bactericidal activity of the neutrophil could destroy *S. typhimurium*. It has been shown that certain cytokines, such as IL-2 and GM-CSF, can enhance the growth of *E. coli* (Denis et al., 1991). Even though it is doubtful that significant amounts of IL-2 or GM-CSF were present in our neutrophil-bacteria cultures, it is possible that other inflammatory factors, secreted by neutrophils as by-products of plasma membrane lipid metabolism, enhanced the growth of virulent *S. typhimurium*. 
Of the specific bacterial mutations that we tested (ΔcyΔcrp, phoP, ΔaroA), the difference in bactericidal efficiency between parent and mutant bacteria was most pronounced with the phoP mutant, which was more efficiently killed than strain SR-11. It has been shown that the phoP gene of *Salmonella typhimurium* is involved in the regulation of genes responsible for virulence and survival in murine macrophages, specifically in resistance to defensins (Groisman and Saier, 1990; reviewed by Miller, 1991). Defensins are microbicidal cationic proteins contained in secondary granules of neutrophils and macrophages that facilitate oxygen-independent microbicidal activity in these cells (Lehrer et al., 1990). The finding that the phoP mutant exhibited increased sensitivity to the bactericidal activity of porcine neutrophils correlates well with a previous study (Coe et al., 1992a), in which it was shown that porcine neutrophil bactericidal activity was increased against *S. typhimurium* even in the face of depressed oxidative neutrophil metabolism and suggested that oxygen-independent killing mechanisms of neutrophils may play an important role in the killing of *Salmonella typhimurium* by porcine neutrophils.

AroA mutants are aromatic-dependent auxotrophs, requiring p-aminobenzoic acid (PABA) for growth (Hoiseth and Stocker, 1981). They are less virulent because host cells lack PABA, which limits their growth, but they are not necessarily less resistant to bactericidal activity of neutrophils. The data from this study showed that, in general, the opsonized ΔaroA mutant (x4467) was less efficiently killed than the opsonized parent strain, UK-1 (figure 1); there was little difference between the nonopsonized parent and mutant.

*Salmonella typhimurium* ΔcyΔcrp mutants contain deletions in the genes encoding adenyl cyclase and cyclic AMP receptor protein. Cyclic AMP, produced via the catalytic action of adenyl cyclase, is involved in the regulation of many genes, including those responsible for glucose utilization, amino acid metabolism, synthesis of flagella and pili,
and the synthesis of certain outer membrane proteins (Curtiss and Kelly, 1987). It appears from our study that none of these alterations decreases resistance to the bactericidal activity of porcine neutrophils up to 4 hours after exposure.

Increased neutrophil bactericidal efficiency against strains of *S. typhimurium* in vitro does not necessarily correlate with reduced bacterial virulence and/or persistence in vivo. Both strain SR-11 and its phoP mutant, χ3687, cause very mild enteritis in pigs, with variably formed feces for a few days after exposure. Three days after exposure, *S. typhimurium* was recovered from the tonsils, ileum, and associated lymph nodes of pigs exposed either to strain SR-11 or χ3687, but only strain SR-11 was recovered from the liver and spleen (unpublished data). Strain χ3687 was less invasive; this correlates well with our observation that χ3687 has increased susceptibility to bactericidal activity by porcine neutrophils. Strain 798 can cause severe enterocolitis in pigs, but χ4233 typically elicits a much milder enteritis. Both strains colonized similar internal organs, but χ4233 colonized the ileum in much lower numbers than did strain 798 (Coe and Wood, 1992). χ4233 was less virulent than strain 798 in vivo, but this could not have been predicted based on the relative bactericidal efficiency of porcine neutrophils against χ4233 when compared to its parent strain.

The increased bactericidal efficiency of porcine neutrophils against opsonized bacteria when compared to nonopsonized bacteria was not unexpected as it is well established that opsonization with specific antibody and complement aggregates bacteria into more easily phagocytosed particles and promotes uptake through specific Fc and C3b receptors on phagocyte membranes (Roitt, 1989, p. 1.6). Complement, especially when coupled with specific antibody, has a variable amount of direct bactericidal activity on Gram negative bacteria, such as *Salmonella* spp. (Roitt et al., 1989, p. 13.12), but this factor was accounted for in the assay design used in this study. All bacterial preparations were pre-
opsonized with antibody and complement prior to the introduction of neutrophils. Therefore, starting bacterial viability should have been similar between wells receiving neutrophils and wells without neutrophils. Baron and Proctor (1984) demonstrated that specific antibody enhanced murine neutrophil bactericidal activity against *S. typhimurium*; our study is consistent with those data. The advantage of opsonization was often diminished or lost by 4 hours. Perhaps by this time phagocytic uptake of bacteria by the neutrophils was maximal and opsonization no longer conferred a selective advantage. The plates in this study were not shaken after the initial mixing of neutrophils and bacteria. Settling of the plate contents over time might have increased the physical contact between bacteria and neutrophils and enhanced phagocytic uptake of bacteria.

Tumor necrosis factor is one of the first cytokines released by cells in response to injurious stimuli (reviewed by Fong and Lowry, 1990). Neutrophils are the first phagocytes to arrive at inflammatory foci, thus it is possible that TNF modulation of neutrophil function plays a role in early resistance to *S. typhimurium*. Ideally, TNF modulation of porcine neutrophil bactericidal efficiency should be studied using porcine-origin TNF, but porcine cytokines are not commercially available. Recombinant human TNFα (rHu-TNF), however, is commercially available and is active on porcine neutrophils (Coe et al., 1992b). It also does not affect the growth of *S. typhimurium* (Nakano et al., 1990). In a previous study, we showed that treatment of porcine neutrophils with 250 ng/ml rHu-TNF caused a significant reduction in neutrophil random migration and iodination and a near significant (*p*=0.08) reduction in antibody-dependent cell-mediated cytotoxicity. In the present study, treatment with rHu-TNF inhibited bactericidal efficiency of porcine neutrophils shortly after exposure to *S. typhimurium* but enhanced bactericidal efficiency 4 hours after exposure. These findings are consistent with those of Nakano et al. (1990), who showed that tumor necrosis factor had a protective effect in
mice and caused an enhancement of bactericidal capacity if administered 6 hours before challenge with *S. typhimurium*. The protective effect was not evident if TNF was given less than 6 hours before challenge. In our study, neutrophils were pre-incubated with rHu-TNF for 2 hours. Enhanced bactericidal activity was evident by 4 hours. This corresponds well with the time required for enhanced bactericidal capacity to develop in mice. The enhancement we observed at 4 hours, though statistically significant, was numerically small; whether the enhancement detected at 4 hours would increase with additional time cannot be predicted from this data.

Porcine neutrophil bactericidal efficiency against *S. typhimurium* is influenced by bacterial mutations in the phoP gene, bacterial opsonization, incubation interval, and neutrophil stimulation by rHu-TNFα. Bactericidal activity is incomplete even when these variables are optimized, and this probably plays a role in the development and maintenance of the carrier state of *S. typhimurium* in swine. Strategies aimed at increasing the bactericidal efficiency of porcine phagocytes against *S. typhimurium* might be useful in reducing the incidence of the carrier state and/or magnitude of bacterial shedding from carrier pigs.
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GENERAL SUMMARY

In order to develop strategies to reduce or eliminate the carrier state of *Salmonella typhimurium* in swine, we must first understand the host-bacterial interactions that occur after exposure of swine to *S. typhimurium*. In this dissertation, the results of two studies were presented that dealt with interactions of *S. typhimurium* and porcine neutrophils in vivo and in vitro. The effects of exogenous and endogenous biological response modifiers on these neutrophil-bacteria interactions were evaluated. The results of a third study described the alterations in the colonization and persistence of wild-type *S. typhimurium* in swine after exposure to a less virulent *Salmonella* mutant.

Porcine neutrophil function was altered in swine exposed to *S. typhimurium* when compared to *Salmonella*-free controls. Oxidative metabolism, as measured by cytochrome C reduction and iodination, was depressed 1-2 days after exposure. Motility, as measured by random migration and chemotaxis, and antibody-dependent cell-mediated cytotoxicity (ADCC) of neutrophils from exposed swine were also depressed. Neutrophil bactericidal activity against *S. typhimurium* from exposed pigs was enhanced throughout the 14-day monitoring period. Neutrophil function except for bactericidal activity normalized 3-4 days after exposure to *S. typhimurium*, but neutrophil iodination and ADCC were depressed again 7-14 days after exposure. Enhanced bactericidal activity in the presence of depressed oxidative metabolism suggests that oxidative microbicidal mechanisms are not the limiting factor for the destruction of *S. typhimurium* by porcine neutrophils. The data from this study also suggest that alterations in neutrophil function may be biphasic, similar to the biphasic clinical response that is often seen in *S. typhimurium*-infected swine.

Neutrophils from pigs treated with bacillus Calmette-Guerin (BCG) and not exposed to *Salmonella* demonstrated reduced cytochrome C reduction and random migration 3-4 days
after treatment, but treatment of swine with BCG did not reverse Salmonella-induced alterations in porcine neutrophil function. It also did not alter the colonization and persistence of S. typhimurium in porcine internal organs. Thus it appears that BCG treatment, as reported here, is not effective as a biological response modifier to alter the pathogenesis of S. typhimurium in swine.

Porcine neutrophil bactericidal efficiency against S. typhimurium was affected by bacterial opsonization, incubation interval, bacterial mutations, and by pre-treatment with the biological response modifier (cytokine), recombinant human tumor necrosis factor-alpha. Porcine neutrophils were usually more efficient at killing S. typhimurium opsonized with antibody and complement than nonopsonized counterparts, especially when the neutrophils and bacteria were incubated together 0.5-2 hours before assay. The advantage of opsonization was often decreased or lost by 3-4 hours. A phoP mutant of S. typhimurium was more efficiently killed than the parental strain, suggesting that the phoP gene product plays a role in bacterial resistance to killing by porcine neutrophils. Other bacterial mutations (i.e., ΔcyADcrp, ΔaroA) did not appear to confer increased sensitivity to porcine neutrophil bactericidal activity during the time period evaluated (up to 4 hours co-incubation).

Treatment of porcine neutrophils with recombinant human tumor necrosis factor-alpha (TNFα) caused a decrease in bactericidal efficiency when compared to nontreated controls after 0.5-1 hour of exposure of bacteria to neutrophils. The bactericidal inhibition was reversed within 2-3 hours, and by 4 hours, the bactericidal efficiency of TNFα-treated neutrophils was enhanced compared to controls. The differences in bactericidal efficiency due to TNFα treatment, although significant, were often small.

Exposure of swine to a ΔcyADcrp mutant of S. typhimurium (x4233) altered the subsequent colonization of swine by the fully virulent parental wild-type strain (798). Oral
exposure to χ4233 caused a mild, transient fever and soft feces in swine. Exposure to strain 798 typically caused a prolonged, often biphasic, febrile response and liquid diarrhea. However, the clinical response of swine to oral challenge with strain 798 was much milder in pigs previously exposed to χ4233. The populations of wild-type *S. typhimurium* in the ileum of χ4233-exposed pigs were 100- to 10,000-fold lower than those in pigs not exposed to χ4233, and the liver, spleen, and ileocolic lymph nodes were cleared of wild-type *S. typhimurium* more quickly after challenge in χ4233-exposed pigs.

Exposure to χ4233, as reported in this study, would not be an acceptable method to reduce carrier populations of *S. typhimurium* in swine because initial exposure to χ4233 caused transient, albeit mild, clinical illness. However, from this data, it appears that exposure to a suitable avirulent mutant of *S. typhimurium* might be useful to reduce carrier populations of virulent *S. typhimurium* in swine subsequently exposed to wild-type strains.

The studies reported in this dissertation demonstrate that exposure to *S. typhimurium* alters porcine neutrophil function; these functional alterations may play a role in the establishment and maintenance of the carrier state in swine. Treatment with biological response modifiers and pre-exposure to a *S. typhimurium* mutant were evaluated as potential methods to reduce either the incidence of the carrier state or the magnitude of *S. typhimurium* populations in internal organs of carrier swine. Additional knowledge of *S. typhimurium* interactions in swine is needed before effective strategies for reducing or eliminating the carrier state can be developed.
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