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The Pathogenesis of Equine Endometritis Due to Streptococcus Zooepidemicus.

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**THE PATHOGENESIS OF EQUINE ENDOMETRITIS DUE TO
*STREPTOCOCCUS ZOOEPIDEMICUS***

A Dissertation

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy**

in

**The Interdepartmental Program in
Veterinary Medical Sciences through
the Department of Veterinary Microbiology and Parasitology**

by

**Robert Crawford Causey
B.S., University of Minnesota, 1985
D.V.M., University of Minnesota, 1989
December 1995**

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To Daddy
from
Robert and Charles

ACKNOWLEDGMENTS

It is impossible to acknowledge every one who contributed to this work, but to some a very special debt is owed. My major professor, Dr. William J. Todd, provided unwavering support and trust during my Ph.D. program. His understanding of Educating someone, instead of merely training them, is a priceless gift. Second, Dr. Dale Paccamonti, my clinical mentor, taught me by what he said, but especially by his example. He maintained a welcome for me in the clinics throughout this project, and always encouraged me to take vitamin C for my colds. Perhaps I should have.

My Mother and Father, who raised me, then paid for most of my education, and encouraged me to become a veterinarian when it seemed impossible, I can never repay. My Father, to whom this dissertation is dedicated, passed away a few months before its completion. But even now, his interest in my childish efforts seems as strong as ever.

The greatest reward of living in Baton Rouge has been to meet Dr. Rosanne F. Hirsch. It suffices to say that our relationship has been, and continues to be, at times unprofessional.

Finally, perhaps most important of all, I must acknowledge the horses with whom I worked. Standing next to a horse, one stands next to man's closest companion in war and peace

throughout the ages. When we work with horses, or any animal, our humanity always hangs in the balance.

PREFACE

This dissertation has its roots in personal experience. Personal experience is not always highly rated in science; casual observations may lead to mistaken conclusions. However, to fully understand the choice of project, literature, and experiments performed, one needs to appreciate the experiences I had during a 3 year residency in theriogenology (1989-1992) and how they led to a question in class, a hypothesis, and then to a Ph.D. program.

Theriogenology involves the veterinary aspects of animal reproduction, i.e., fertility, obstetrics and diseases of the reproductive system. It includes all animals, but especially deals with animals closest to man. During the residency most of my contact was with cattle and horses. Over those 3 years, bacterial infection of the uterus, and its impact on fertility, was the most frequent and frustrating problem that I encountered. An infected animal cannot become pregnant; pregnancy is either delayed until the infection is resolved, or prevented altogether. In cattle this directly translates to major economic losses in beef or milk produced each year. In horses, particularly Thoroughbreds, delayed pregnancy leads to late-born foals being at a disadvantage when racing against older competitors. Worse still, the cost of a valuable breeding animal may not be recovered if she produces no offspring. As a theriogenology resident, therefore, my main purpose in life was to make sure animals became pregnant,

quickly. My main obstacle in achieving this goal was uterine infection. It was particularly frustrating in horses, where uterine infection is most commonly manifested in a disease known as equine endometritis.

When I started the residency an extensive literature on equine endometritis already existed focusing on the phagocytosis and killing of invading bacteria by uterine neutrophils. The gram-positive organism, *Streptococcus zooepidemicus*, is the bacterium most commonly isolated from equine uterine infections. The studies recorded in the literature had focussed on the phagocytosis of this organism by uterine neutrophils. Indeed, phagocytosis of invading organisms was considered to be the major line of uterine defense in the horse. However, researchers were not fully satisfied with all the data obtained. Comparing horses that were resistant and susceptible to infection did not prove susceptibility to infection was due to defects in uterine phagocytosis; uterine infections were still a problem, especially in older horses.

Also at this time (January 1990), Dr. William J. Todd was lecturing in a class on Pathogenic Mechanisms of Bacteria. During a lecture he drew a picture of the human pathogen *Streptococcus pyogenes*, covered with a surface of what he described as "fluff" known as M protein. The importance of M protein was that organisms carrying it resisted phagocytosis by neutrophils and thereby evaded host defenses. At the end of the class, I asked if S

zooepidemicus also carried M protein. Dr. Todd, as I recollect, confessed that he had never heard of *S zooepidemicus* and referred the question to Dr. Hollis Cox. Dr. Cox explained that although *S zooepidemicus* was virtually ubiquitous as a common commensal of the respiratory and reproductive tracts, he was unaware of any studies to suggest that it carried M protein, but felt that it probably had not been looked into. This was in contrast to *Streptococcus equi*, which was well known as an important equine pathogen, and which did carry an "M protein."

Dr. Todd suggested that he could obtain anti M-protein monoclonal antibodies from Dr. Vincent Fischetti of the Rockefeller University. With Dr. Paccamonti, my clinical mentor, we proposed to collect clinical isolates of *S zooepidemicus*, and test them for the presence of M protein. With the assistance of Langston Hull, then a high school honors student, we obtained the following results using Fischetti's monoclonal's against our equine uterine streptococcal isolates. Of 11 isolates tested, 2 had bound the monoclonal. To the question "Does *Streptococcus zooepidemicus* have an M protein?" the answer had come back "Yes and No."

I now entered a Ph.D. program in the Department of Veterinary Microbiology and Parasitology and needed a dissertation topic. I wanted to quickly end the M protein question, and move onto a full-time Ph.D. project. The quickest way to achieve this, I thought, was to ask the question "Does

Streptococcus zooepidemicus resist phagocytosis?" If so, I could believe the monoclonal results, if not I could dismiss the results as spurious cross reactions. Growth of the organism in fresh blood was the classic demonstration of antiphagocytic properties of *S pyogenes* and could be performed quickly and easily. I tested 3 isolates, and one of the isolates which had bound Fischetti's monoclonal grew rapidly in fresh horse blood. After much doubt, and exploring worthy projects in other laboratories, the "quick M protein project" which I had been pushing aside became a Ph.D. project with Dr. Todd as my major professor.

Years later I have not regretted the decision. To document the antiphagocytic nature of *S zooepidemicus*, study its role in equine uterine infections, and demonstrate an M protein on the surface of the organism has been a rewarding and is a continuing challenge. It has given me a project directly applicable to my clinical discipline and I have had the luck to humanely test laboratory findings in the live animal. It has revealed the unexpected treasure of a literature which stretches back more than a hundred years, introducing me to the names of Oliver Wendell Holmes, Ignatz Semmelweiss, and Rebecca Lancefield. It has allowed me to meet and, in a small way, collaborate with such prominent contemporary researchers as John Timoney, Vincent Fischetti and Susan Hollinghead. It has taken me to conclusions that I simply had not anticipated. It has been, in short, everything I believe a Ph.D. project should be.

The body of the text is in the journal style of the American Veterinary Medical Association, with a few exceptions, such as the reference citations which are by author. I hope that chapters of this dissertation, published in the veterinary literature, will prove to have been steps in the right direction towards understanding equine endometritis.

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ABSTRACT

The ability of uterine isolates of *Streptococcus zooepidemicus* to resist phagocytosis was explored by the organism's ability to grow in equine fresh blood. Although isolates grew in fresh blood from many horses, any given isolate was killed by blood from certain horses. Killing required leukocytes, heat labile and heat stable components of plasma and appeared to follow phagocytosis of the organism by neutrophils. This led to the working hypothesis that killing was mediated by phagocytosis requiring complement and isolate-specific antibody to variable surface antigens. Variation in killing between horses was attributed to differences in circulating isolate-specific antibody. This is a situation analogous to requirements for phagocytosis of the human pathogenic M protein-positive group A streptococci, and supports existing evidence that uterine isolates of *S zooepidemicus* also carry M protein. However, in contrast to human systemic infections with the group A streptococci, killing ability in fresh blood was not closely related to resistance to equine endometritis. Horses in whose blood a given isolate grew were able to eliminate intrauterine inocula of that isolate. Similarly, horses in whose blood the isolate was killed were not necessarily resistant to intrauterine inoculation. Other sources of variation between horses and between isolates appeared to be the major determinants of disease. It was concluded that other mechanisms

of resistance, in addition to phagocytosis, were responsible for clearance of streptococci from the uterus. Physical clearance of carbon powder from the equine uterus was enhanced in horses which displayed mucus production at the endometrial surface. In addition, mucus appeared to block the binding of carbon to the endometrial surface. Since the equine endometrium is ciliated, it was suggested that mucociliary clearance may also contribute to removal of streptococci from the equine uterus.

INTRODUCTION

Equine endometritis, inflammation of the equine endometrium, is a major cause of infertility in horses. The most common cause of this disease is bacterial infection of the uterus, which was described in the veterinary literature almost 70 years ago. Estimates have suggested the condition leads to heavy economic losses to horse breeders and horse exporting countries. Consequently, much effort has been spent to prevent or cure the condition in afflicted horses.

Our current knowledge of the pathogenesis of equine endometritis suggests that the mare herself is the cause of the infection. Organisms that cause equine endometritis are usually harmless commensals of the cervix and vagina; *Streptococcus zooepidemicus* is the organism most commonly isolated from infected mares. These bacteria are frequently introduced into the uterus as part of the normal reproductive cycle of the mare, usually at foaling and breeding. In normal horses they are quickly eliminated from the uterus by the horse's natural defenses. However, in certain horses these defenses fail and prolonged or persistent infection of the uterus follows, leading to infertility as long as inflammation persists. These so called "susceptible mares" tend to be older animals, frequently with poor conformation of the perineum (anus and vulva), and large uteri

which tend to accumulate fluid. The infected mare usually shows no sign of systemic illness.

Because bacterial inoculation into the uterus leads to intense infiltration of neutrophils into the uterine lumen, phagocytosis of bacteria has also been thought to play a major role in uterine defenses. Deficiencies in the phagocytosis of bacteria have been proposed to explain the underlying weakness of the susceptible mare. Consequently the phagocytosis of *S zooepidemicus* by equine neutrophils has been an intensely researched area. Attempts have been made, with mixed success, to enhance the opsonic properties of uterine fluid by infusion of equine plasma into the uterus. However, it is still not clear if failure of phagocytosis underlies susceptibility to endometritis.

Phagocytosis of the human pathogen *Streptococcus pyogenes* has also been the subject of intense medical research for many years. *Streptococcus pyogenes* carries a fibrillar surface protein, known as M protein, which confers resistance to phagocytosis. The protein is highly variable, and was originally used as the basis for serotyping the many different strains of *S pyogenes*. To date, more than 80 serotypes exist. An important feature of streptococci which carry M protein (M+ streptococci) is that they can only be phagocytized in the presence of antibody specific for the variable region of the protein. Antibody which is specific for one serotype is only phagocytic for that serotype.

Limited evidence exists to suggest that *S zooepidemicus* also carries an M-like protein. This evidence comes from antigenic and genetic studies, and clinical similarities in diseases caused in man by *S pyogenes* and *S zooepidemicus*. However, it is not clear if the M-like protein of *S zooepidemicus* is a true functional homolog of the M protein of *S pyogenes*, nor have antiphagocytic properties of equine uterine isolates of *S zooepidemicus* been documented. It would therefore increase our knowledge of equine endometritis to know the answers to the following questions:

(1) Are uterine isolates of *S zooepidemicus* antiphagocytic?

(2) Do these isolates carry an M protein homologous to the M protein of *S pyogenes* ?

(3) What role, if any, do antiphagocytic properties play in uterine infection?

This dissertation aims to increase our understanding of equine endometritis by addressing these questions. The literature review focuses on equine endometritis, streptococcal M protein, and the existing evidence for an M protein on *S zooepidemicus*. The experimental work is presented in chapters written as scientific papers for publication in the veterinary literature. The conclusion indicates further work which may provide valuable results in the future.

CHAPTER 1 LITERATURE REVIEW

Two lines of independent scientific inquiry will be described, namely equine endometritis and streptococcal M protein. Most of our knowledge of equine endometritis comes from studies by veterinary clinicians. Streptococcal M protein, however, has been the concern of human bacteriologists. Researchers in the two fields have occasionally crossed paths, but mostly they have remained separate. This review, will summarize current knowledge in each field, and then explore the possibility that *Streptococcus zooepidemicus*, the most common bacterium isolated from equine endometritis, also carries streptococcal M protein.

1.1. PATHOPHYSIOLOGY OF EQUINE ENDOMETRITIS

Because of the importance of horse-breeding in veterinary medicine, the literature on equine endometritis is extensive. This, however, is in contrast to our understanding of the disease. Classic studies of the 1920's by Dimock, Edwards provided a solid basis of understanding, but more recent researchers have often been frustrated. Recognizing that this is a complex literature, it is worth emphasizing the essential points in advance. .

Much infertility in mares is due to uterine infection by *S zooepidemicus*, a normal inhabitant of the equine vestibule, vulva and clitoris. The infertility is due to embryonic death caused by inflammation of the endometrium. In addition, horses susceptible

to endometritis frequently have damaged endometria. Although the endometrium is a mucosal immune surface, it undergoes a typical acute inflammatory reaction when irritated, characterized by an inflow of neutrophils and serum proteins. However, failure of neutrophil migration, chemotaxis, phagocytosis, or diminished opsonic activity of uterine fluid do not appear to underlie susceptibility to equine endometritis. Accumulation of fluid, and failure to mechanically evacuate the uterus are currently considered the primary defects of susceptible mares. Fluid accumulation may promote uterine infections by causing a poor uterine environment for phagocytosis.

1.1.1 HISTORY, INCIDENCE AND ORGANISMS. Early scientific description of endometritis is remarkable for its accuracy and thoroughness. It defines the extent of the problem in the horse population, identifies causative agents of disease, describes the lesions, and explains the opportunistic nature of the infection. Subsequent surveys largely confirm this early work.

In 1928 Dimock and Edwards published a 6 year survey of approximately 4000 mares from central Kentucky breeding farms (Dimock and Edwards 1928). Of these mares 1,606, approximately 40%, were not pregnant at the end of at least one breeding season (barren). By uterine culture, using sterile technique, they subdivided the barren mares into non-infected and infected groups. Infected horses accounted for 36.5% of all the barren mares. Detailed bacteriological and pathological examinations of these

infected mares led to a conceptual framework of equine endometritis which has survived essentially intact for almost 70 years.

Bacteriological examination revealed that two organisms accounted for more than 75% of all infections, namely *Streptococcus genitalium* at 66%, and the encapsulated coliform *Encapsulatus genitalium* at 10% (Dimock and Edwards 1928). The remainder of the infections were due to "miscellaneous organisms." Although neither one of the foregoing names can be found in modern bacteriological texts, a thorough biochemical description of the organisms allows us to identify *Streptococcus genitalium* as *Streptococcus equi* subsp. *zooepidemicus* commonly referred to as *Streptococcus zooepidemicus* (Farrow and Collins 1984; Rotta 1984), and *Encapsulatus genitalium* as an unusually homogeneous collection of isolates probably belonging to *Klebsiella pneumoniae* subsp. *pneumoniae* (Brenner 1984).

The pathological picture of infection by either organism was essentially the same (Dimock and Edwards 1928). Clinically, a mucopurulent discharge was frequently detectable at the vulval lips or on vaginal speculum examination. If the uterine wall remained contracted the exudate was easily expelled, but if flaccid, exudate tended to accumulate in the uterus. Horses with perineal injuries incurred at parturition were more likely to be infected. Gross pathological lesions included suppurative endometritis, metritis, pyometra (due to cervical adhesions)

cervicitis and vaginitis. Salpingitis (inflammation of the oviduct) was rare but usually due to ascending infection. The endometrium was frequently hemorrhagic. Chronically, the endometrial folds would disappear, and the mucosal epithelium replaced by cicatricial tissue (circumferential scar tissue). Histologic sections revealed epithelial sloughing, degeneration of the uterine glands and their replacement by connective tissue. Round cell (mononuclear) infiltration extended throughout the endometrium and sometimes into the muscular coats. Fibrotic changes could also extend from endometrium to myometrium, with bundles of uterine wall musculature replaced by connective tissue. The serosal surface of the uterus, however, was unaffected. Although a definitive diagnosis required isolation of the organism, infection with *K pneumoniae* was marked by a thicker and more tenacious exudate.

It was also demonstrated that *S zooepidemicus* was a normal commensal of the vestibule and vagina, frequently multiplying in the uterus following a normal parturition but usually cleared by the foal heat (9 days *post partum*). *Streptococcus zooepidemicus* was shown to share culture characteristics with *S pyogenes*, and to be highly virulent for rabbits. Infection of the uterus with *S zooepidemicus* predisposed animals to infection with *K pneumoniae*. Stallions could transmit *K pneumoniae* from one mare to another. In later reports Dimock pointed out the possible connection between streptococcal

infection of the mare and neonatal septicemia in foals (Dimock 1935; Dimock, Edwards and Bruner 1947).

Dimock thus recognized the essentially opportunistic nature of streptococcal uterine infection: all mares harbored the organism; it gained entrance to the uterus at foaling and breeding; it was usually cleared by natural defenses; failure to clear the organism led to endometritis (Dimock 1935). However, he evidently did not explore why one mare might fail, and another succeed, in clearing streptococci from the uterus.

Dimock and Edwards' estimate of the overall impact of uterine infection on equine fertility remain accurate in many parts of the world. The percentage of barren mares in the brood mare population has been subsequently estimated from 30% to 50%, whereas for wild ponies it has been estimated at only 5% (Bain 1966; Collins 1964; Day 1939; Laing and Leech 1975; Millar and Francis 1974; Sullivan et al. 1975). Thus the domesticated mare appears to have dramatically lowered fertility compared to her wild counterpart (Day 1939). Such reproductive wastage has enormous economic impact, especially in horse exporting countries (Elliot, Callaghan and Smith 1971). Recent evidence, however, suggests that fertility in Thoroughbreds in the United Kingdom and Ireland has improved by 13% from 1970 to 1990, and the proportion of barren mares has almost halved (Ricketts and Young 1990). Uterine infection may account for 24% to 60% of barren mares (Bain 1966; Brooksby et al. 1965). *Streptococcus*

zooeidemicus is consistently the most commonly isolated organism from the uterus of mares accounting for approximately 50% of all positive cultures. *Escherichia coli* is the next most common at approximately 15% followed by *K pneumoniae* and *Pseudomonas aeruginosa*, each at less than 10% (Bain 1966; Brooksby et al. 1965; Elliot, Callaghan and Smith 1971; Shin et al. 1979).

Bacterial infection of the uterus is clearly linked to infertility, and particularly to infection with *S zooeidemicus*. In uterine cultures of mares cultured prior to breeding, barren mares yielded more positive cultures than foaling or maiden mares (Brooksby et al. 1965). The subsequent fertility reported for noninfected mares was greatly superior to the fertility of previously infected mares (Elliot, Callaghan and Smith 1971; Millar and Francis 1974). Previous infection with streptococci was particularly strongly associated with subsequent infertility; only 34% of mares infected with streptococci subsequently produced a live foal according to one estimate (Elliot, Callaghan and Smith 1971). In addition, previous scarring of vagina and cervix, incurred during parturition, predisposed animals to streptococcal infection (Elliot, Callaghan and Smith 1971).

Dimock's contention that *S zooeidemicus* was a commensal of the vulva and vestibule normally excluded from the uterus has been confirmed by subsequent research. Normal mares harbor potential uterine pathogens, including *S zooeidemicus*, in

the vestibule, vulva, and especially clitoris. These sites may provide a source of organisms that enter the uterus especially at foaling, breeding or during veterinary procedures. The anterior vagina, however, is essentially sterile in the normal mare, as is the uterus. However a few colonies of nonpathogenic organisms may be isolated from uterine cultures of normal mares (Hinrichs et al. 1989). Anaerobic bacteria, such as *Bacteroides fragilis*, also inhabit the external genital surfaces of mares, and may cause endometritis (Ricketts and Mackintosh 1987). Because of the possibility of culturing contaminants, diagnostic value of uterine culture is improved by concurrent use of uterine cytology (Wingfield-Digby and Ricketts 1982).

An important area where Dimock is contradicted today is his use of language. Dimock used the term "sterility" to describe reproductive failure in horses. Today, however, sterility is a term reserved for those animals which are permanently incapable of reproduction. Because barren mares may subsequently become pregnant, the term "subfertile" is preferred to describe decreased reproductive efficiency of these mares. Those mares whose subfertility results from a predisposition to bacterial infection of the uterus are termed "susceptible" mares; those who rapidly clear bacteria from the uterus are termed "resistant."

1.1.2. EMBRYONIC DEATH. The reproductive failure of subfertile mares is due, most often, to death of the developing embryo within 14 days of conception. Embryonic death may be

caused by primary defects in the embryo, and poor environment in the oviduct or uterus. Inflammation with fluid accumulation in the uterus is frequently observed clinically in subfertile mares. These factors appear to be age related, and probably contribute greatly to pregnancy loss as age of the mare increases.

Normal and subfertile mares appear to have similar fertilization rates, or they may be slightly reduced in the latter (Ball et al. 1986; Brinsko et al. 1994). However, embryo survival from days 2-14 post ovulation is consistently reduced in subfertile mares, probably due to a combination of primary embryonic defects and suboptimal environment of the oviduct and uterus (Ball et al. 1986; Ball et al. 1989; Brinsko et al. 1994). Because the embryo enters the uterus on day 5, the oviductal (day 0-5) or early uterine environment (day 5-7) could be responsible for early embryonic loss. Salpingitis is probably more prevalent in mares than Dimock's original report suggests, and may contribute to poor oviductal environment (Saltiel et al. 1986). In the absence of gross uterine pathology and acute uterine inflammation, embryos transferred to the uterus on days 7-8 survive equally well in normal or subfertile mares (Ball, Hillman and Woods 1987).

Acute uterine inflammation, however, may frequently be present in the subfertile mare under field conditions, and probably accounts for much embryo loss. Ultrasonographically, embryo loss after day 10 is accompanied by fluid accumulation and shortened

interestrus intervals strongly suggesting an inflammatory cause (Ginther 1985; Ginther et al. 1985a; Ginther et al. 1985b; Ginther and Pierson 1984; Woods et al. 1987). Uterine inflammation may lead to release of prostaglandins from the endometrium causing regression of the corpus luteum and loss of progesterone support for pregnancy (Neely et al. 1979). This hypothesis is supported by depressed progesterone levels, increased endometrial inflammation, and ultrasonic fluid accumulation at the time of embryo loss (Adams et al. 1987; Ginther et al. 1985b). Thus, uterine fluid accumulation in the uterus, particularly in diestrus, is strongly linked to early embryonic death and decreased pregnancy rates (Adams et al. 1987). The poor performance of older mares may be attributed, in part, to increased uterine inflammation and poor uterine tone, both of which can predispose to fluid accumulation in the uterus (Carnevale and Ginther 1992). Older mares also have a higher incidence of chronic, degenerative, endometrial disease (Kenney 1978; Ricketts and Alonso 1991).

1.1.3. THE ENDOMETRIUM. The endometrium of subfertile mares suffers damage at all scales of magnification which reduces its ability to support pregnancy. This damage has been observed by endoscopy, light microscopy and scanning electron microscopy.

The endometria of fertile, subfertile and pregnant mares have been compared using an endoscope inserted into the uterus via the cervix (Allen and Bracher 1992; Bracher and Allen 1992;

Bracher, Mathias and Allen 1992). The normal endometrium is thrown up into numerous longitudinal folds that are continuous with the folds of the cervix (Ginther 1992). The folds of the endometrium interdigitate so that the lumen of the normal uterus, as seen in cross section, consists of radially arranged capillary spaces between the longitudinal endometrial folds (Kenney 1978). In normal mares, the estrous endometrium is diffusely edematous, with occasional traces of clear luminal fluid. In diestrus, the endometrium appears "thinner" as the edema subsides. In subfertile mares the endometrial folds may suffer from uneven distribution, atrophy or scarring. Endometrial cysts and fluid accumulation are also common in subfertile mares. Occasionally, adhesions may span the uterine lumen. An interesting feature of the pregnant endometrium observed endoscopically is the visible mobility of the equine conceptus, which has been seen passing from one horn to the other, pushed along by peristaltic contractions of the myometrium (Allen and Bracher 1992).

The technique of endometrial biopsy has led to a thorough description of the cyclic changes and pathology of the endometrium at the light microscopic level (Kenney 1978; Kenney and Doig 1986; Ricketts and Alonso 1991). The endometrial surface is covered by a luminal epithelium, overlying loose connective tissue (lamina propria). Numerous uterine glands occupy the lamina propria, opening into the lumen of the uterus via narrow ducts. The luminal and glandular epithelia are continuous.

During estrus the lamina propria becomes edematous, increasing the distance between the glands. The cells of the luminal epithelium increase in height and neutrophils marginate in the endometrial capillaries. During diestrus the cells of the luminal epithelium decrease in height, the interstitial edema subsides, and the glandular density increases.

Pathological changes of the equine endometrium are usually either inflammatory (endometritis) or degenerative (endometrosis) (Allen 1993; Kenney 1978). In the acutely inflamed uterus, neutrophils migrate to the uterine lumen where they accumulate, but may be washed away during processing. Thus neutrophilic accumulations are rarely seen in endometrial biopsies, but are the predominant inflammatory cell type in cytology samples taken from the endometrial surface (Roszel and Freeman 1988). Chronic inflammatory infiltrates are commonly seen in the endometrium. They are usually lymphocytic, occurring under the luminal epithelium, around uterine glands, or around endometrial vessels and may be diffusely or focally distributed. Endometrosis is predominantly characterized by fibrosis around uterine glands. Such fibrosis may be a consequence of inflammation, or might be a response to cystic dilation of glands following blockage of the gland duct. Fibrosis may be distributed diffusely around glands throughout the endometrium, or focally around collections of dilated glands ("nests"). Severity of fibrosis may be objectively assessed by distribution (diffuse being more

detrimental than focal), numbers of layers of fibroblasts around affected glands, and the frequency of fibrotic nests in the lamina propria. A classification scheme (Kenney's Grades I, IIA, IIB, and III) has been developed combining assessments of endometritis and endometriosis from a single biopsy to assess a mare's ability to produce a live foal (Kenney 1978; Kenney and Doig 1986). Severe histopathologic changes indicate susceptibility to endometritis (Troedsson, deMoraes and Liu 1993). However, a history of recurrent endometritis is superior to biopsy as a predictor of susceptibility to endometritis (Williamson, Munyua and Penhale 1989). An additional feature of endometrial inflammation is the pattern of secretion of the luminal and glandular epithelia. Cells of the luminal epithelia normally secrete carboxylated mucins, and may increase secretion in response to endometrial inflammation. Endometrial glands, however, predominantly secrete glycogen (Freeman et al. 1990).

The studies of Dimock and Edwards reported much of the foregoing gross and microscopic pathology, but electron microscopy has subsequently revealed anatomy and pathology beyond their reach. Cyclic changes have been described at the ultrastructural level and surface pathology correlated with Kenney's grading system (Ferreira-Dias, Nequin and King 1994b; Samuel et al. 1979). The endometrium is further folded into small ridges and troughs. Two cell types are visible at the endometrial surface, the ciliated cells, each with a large tuft of several long

cilia, and microvillous non-ciliated cells. The small openings of endometrial glands can be seen clearly, frequently with a ring of cilia around them, opening into the endometrial lumen. The non-ciliated cells appear to be secretory, although blebbing may be an artifact of delayed fixation (Ferreira-Dias, Nequin and King 1994b; Samuel et al. 1979). The non-ciliated cells probably represent carboxylated mucin secreting cells seen by light microscopy (Freeman et al. 1990). During estrus the secretory cells increase in number, followed by a relative decrease during diestrus. On the other hand, the ciliated cells are more numerous during diestrus.

As one proceeds from normal to severely injured endometria, according to Kenney's system, parallel ultrastructural changes can be seen on the endometrial surface. There is a loss of ciliated cells in the diseased endometrium and thinning of the microvilli on the secretory cells. The secretory cells give a flat, polygonally tiled floor appearance in the healthy endometrium (grade I). However, epithelial cells of severely degenerated endometria (grade III) may sometimes swell, becoming rounded, and give the endometrium an appearance like a cobble-stone street.

Among the domestic species, the equine endometrium is unique in its rapid rate of involution following parturition. Histologically, involution is well advanced by day 4 *post partum*, and virtually complete by the foal heat on day 9. This rapid rate of involution may be explained by the rapid lysis and ingestion of the microcotyledons, and the clean break made at the placento-

utero interface (Gygax, Ganjam and Kenney 1979; Stevens et al. 1979). Thus, at normal parturition, the equine endometrium suffers minimal loss of epithelial tissue. However, following abortion, delivery of stillborn or dysmature foals, and manual removal of a retained placenta, the endometrium may suffer more extensive damage (Stevens et al. 1979).

1.1.4. MUCOSAL IMMUNITY. The equine endometrium is believed to be a mucosal immune system. This view is supported by immunoglobulin content of uterine fluid, immunohistochemical studies of the endometrium, and the endometrial response to local vaccination. However, most available information to date suggests that a primary defect in mucosal immunity does not underlie susceptibility to equine endometritis.

The immunoglobulins in equine uterine fluid appear to be actively transported from the serum or produced by plasma cells in the endometrium (Kenney and Khaleel 1975; Mitchell et al. 1982; Waelchli and Winder 1987; Watson and Stokes 1988b; Widders et al. 1984; Widders et al. 1985a). The low albumin to immunoglobulin ratio of uterine fluid suggests that minimal transudation of serum into uterine fluid occurs, unless the uterus is acutely inflamed (Pycock and Allen 1990; Widders et al. 1985b; Williamson et al. 1987). Like other mucosal immune systems, the endometrium preferentially secretes IgA. The ratio of IgA to the other immunoglobulins is increased 10-fold in uterine fluid compared to plasma, although IgG still predominates in uterine

fluid (Mitchell et al. 1982; Widders et al. 1984). In addition, the secretory component of IgA is found in the uterine epithelium (Widders et al. 1985b). Transport of immunoglobulins across the uterine epithelium may be both intercellular and intracellular (Waelchli and Winder 1987; Widders et al. 1985a).

Cells producing IgA, IgG, and IgM can be seen in the endometrium, and their numbers may increase in persistently infected horses (Kenney and Khaleel 1975; Mitchell et al. 1982; Waelchli and Winder 1987; Waelchli and Winder 1991; Widders et al. 1985a). An additional study noted that endometritic mares sometimes had markedly more plasma cells in their endometria than normal mares, but the association did not achieve statistical significance (Watson and Stokes 1988b). Numbers of immunoglobulin producing cells in the endometrium do not vary with the reproductive cycle (Waelchli and Winder 1987; Widders et al. 1985b). However, cells of the endometrium bearing MHC Class II antigens (macrophages, lymphocytes, monocytes, dendritic cells, epithelial cells and endothelial cells), especially those close to the uterine lumen, increase MHC Class II expression during estrus. In addition, horses with endometritis have greatly increased MHC class II expression and T lymphocyte numbers in the endometrium (Watson and Dixon 1993).

A mucosal immune response follows intrauterine immunization. Increased levels of IgA and IgG appear in uterine fluid some of which may be specific for *S zooepidemicus* antigens

(LeBlanc et al. 1991; Widders et al. 1985b; Widders, Warner and Huntington 1995). Levels of IgA and IgG are consistently higher in uterine fluid of susceptible mares than in resistant mares (Asbury et al. 1980; LeBlanc et al. 1991; Mitchell et al. 1982; Widders et al. 1984; Williamson et al. 1983), which probably reflects increased numbers of plasma cells and lymphocytes in the endometria of these horses. However, local vaccination only appears to be protective in resistant horses (Widders, Warner and Huntington 1995), and suggests that an unidentified component of uterine defenses fails in susceptible mares.

1.1.5. THE ACUTE UTERINE RESPONSE. The equine uterus undergoes an acute non-specific reaction to local irritation. Following a uterine insult there is a rapid influx of neutrophils, fluid, serum proteins and inflammatory mediators into the lumen. Although protective, the response may be accompanied by lysis of the corpus luteum (luteolysis). Many aspects of the response appear similar in resistant and susceptible mares. When the acute uterine response is induced by intrauterine inoculation of streptococci, numbers of bacteria recovered from the uterus decrease precipitously in both resistant and susceptible mares. However, after about 12 hours, bacterial numbers rebound in susceptible mares, suggesting a deficiency in local uterine defenses.

Stimuli which induce an acute uterine response do so by irritation of either the uterus or cervix. Such a response may

follow clinical endometritis, uterine massage, cervical dilation, uterine biopsy, or intrauterine infusions of saline, bacteria, streptococcal free filtrate, and oyster glycogen (Couto and Hughes 1985; Hughes and Loy 1969; Hurtgen and Ganjam 1979; Hurtgen and Whitmore 1978; Neely et al. 1979; Peterson, McFeely and David 1969; Pycock and Allen 1990; Watson, Stokes and Bourne 1988a; Watson et al. 1987a; Williamson et al. 1987). Saline infusions alone may not invoke the response, unless large volume are infused or infusion is accompanied by uterine massage (Strzeminski and Kenney 1984; Williamson et al. 1987). The more irritating an infusion, the more intense the response (Watson et al. 1987a). Extracellular products of *S. zooepidemicus* may provoke additional irritation (Couto and Hughes 1985). Oyster glycogen is a convenient way to induce an intense response experimentally without using bacterial antigens (Watson, Stokes and Bourne 1988a; Watson et al. 1987a).

The irritation appears to trigger an acute inflammatory cascade (Higgins and Lees 1984; Pycock and Allen 1990; Watson et al. 1987a). The response may be detected as an inflow of uterine fluid, uterine edema, increased uterine blood flow and cervical enlargement. Changes begin as early as 30 minutes following stimulation, peak in about 4-6 hours, may subside within 12 hours or persist for longer periods (e.g., 72 hours) depending on the outcome (Hughes and Loy 1969; Peterson, McFeely and David 1969; Pycock and Allen 1990; Williamson et al. 1987). During the initial

period inflammatory mediators such as prostaglandin (PG) F₂ alpha and its metabolite PGFM, PGE₂ and leukotriene B₄ peak in uterine fluid within 6 hours, subside by about 12 hours, and then may peak a second time 1 to 2 days later (Neely et al. 1979; Pycock and Allen 1990; Watson, Stokes and Bourne 1988a; Watson et al. 1987a). Serum proteins in uterine fluid also peak within 6 hours, but decline more gradually over 24 - 48 hours. Interestingly, levels of IgA appear to decrease slightly over the initial phase, possibly due to serum dilution, but then achieve maximal levels in 24-48 hours. Neutrophils are also rapidly recruited to the uterus, peaking in about 6 hours, and may remain elevated for several days depending on the nature of the irritation (Williamson et al. 1987). Because prostaglandin levels also become elevated in serum, the acute uterine response is commonly followed by luteolysis and shortened interestrus intervals (Hurtgen and Ganjam 1979; Hurtgen and Whitmore 1978; Neely et al. 1979).

In general, the acute uterine response starts similarly in both resistant and susceptible mares. Neutrophils, serum proteins, and inflammatory mediators increase, while bacterial numbers (if bacteria are used as the stimulus) peak quickly, then decrease precipitously during the first 12 hours of the response (Peterson, McFeely and David 1969; Watson, Stokes and Bourne 1988a; Watson et al. 1987a; Williamson et al. 1987). After the initial phase, a defining difference then emerges between resistant and

susceptible mares inoculated with *S zooepidemicus*. Resistant animals maintain the decrease in bacterial numbers, and soon eliminate bacteria from the uterus. Susceptible mares, however, suffer a rebound in bacterial numbers after the initial phase, and take longer, perhaps multiple estrous cycles, to eliminate the organism (Hughes and Loy 1969; Peterson, McFeely and David 1969; Williamson et al. 1987; Williamson et al. 1984). Exceptions to this pattern include susceptible mares with severely degenerated endometria which have a markedly reduced acute uterine response, possibly due to failure of the injured endometrium to secrete prostaglandins (Hughes and Loy 1969; Neely et al. 1979). Under normal circumstances, however, susceptible mares release slightly elevated levels of prostaglandins in uterine fluid compared to resistant mares (Watson et al. 1987a). Susceptible mares also accumulate more fluid in the uterus post inoculation, and clinically may display reduced drainage of uterine contents through the cervix (Hughes and Loy 1969; Troedsson and Liu 1992). This leads to an accumulation of uterine fluid which adversely affects the uterine environment (Troedsson and Liu 1992; Williamson et al. 1987).

In his initial study of the acute uterine response, Peterson did not detect a difference between phagocytic ability of uterine neutrophils in resistant and susceptible mares. Both groups of animals appeared to phagocytize inoculated streptococci during the acute uterine response. He argued that an event other than

phagocytosis accounted for the difference between resistant and susceptible mares, such as non-cellular defenses or the ability to kill successfully phagocytized organisms (Peterson, McFeely and David 1969). However, because of the intensely neutrophilic nature of the acute uterine response, neutrophil migration and phagocytosis were subsequently investigated extensively as potential causes of susceptibility to endometritis.

1.1.6. NEUTROPHIL MIGRATION. The influx of neutrophils into the uterus may be caused, in part, by an accumulation of chemoattractants in uterine fluid. Stage of cycle does not affect migration towards uterine fluid, however estrogen supplementation of a horse enhances migration towards that horse's serum *in vitro*. Migration is similarly enhanced towards uterine fluid from infected horses, and some chemoattractants in uterine fluid appear to be products of arachidonic acid metabolism. It is not clear if uterine neutrophils from susceptible mares have reduced migratory ability in comparison with resistant mares; differences have been detected in diestrus, but not estrus. If differences exist, they may be attributable to fluid accumulation in susceptible mares. No difference can be confirmed in migratory ability of peripheral blood neutrophils between resistant and susceptible mares.

Chemoattractants in uterine fluid are detected by inducing neutrophils to migrate through millipore filters towards uterine washings; the depth to which neutrophils penetrate the filter is

used as a measure of chemoattractiveness (Pycock and Allen 1988b; Strzemienski, Do and Kenney 1984; Strzemienski and Kenney 1984). An alternative method is to measure the distance neutrophils migrate over a flat surface underneath a bed of agarose (Lees, Dawson and Sedgwick 1986; Watson, Stokes and Bourne 1987c). Random migration of neutrophils may also be measured under agarose (Watson, Stokes and Bourne 1987c). Both serum and uterine fluid appear to possess chemoattractive properties (Strzemienski, Do and Kenney 1984). Serum from estrus or estrogen supplemented mares stimulates neutrophil migration more than serum from diestrus or progesterone supplemented mares. (Blue et al. 1984; Strzemienski, Do and Kenney 1984; Watson, Stokes and Bourne 1987b). Although this difference may not be carried into uterine fluid of cycling mares (Blue et al. 1984; Strzemienski, Do and Kenney 1984), it may be seen in uterine fluid of ovariectomized horses supplemented with estrogen *versus* progesterone (Watson 1988a).

The chemoattractiveness of uterine fluid appears to be enhanced during uterine infection (Blue et al. 1984). This may account for reported increases in uterine neutrophil migration from progesterone treated mares (Watson 1988a). During acute uterine inflammation induced by *S zooepidemicus*, uterine fluid greatly increases in chemoattractiveness within 30 minutes, and remains high for 240 minutes. Heating this fluid to 56 C for 30 minutes partially reduces neutrophil migration, suggesting that

complement proteins play a chemotactic role during acute uterine inflammation (Pycock and Allen 1988b). In addition, *S zooepidemicus* itself is not inherently chemotactic, thus the influx of neutrophils into the uterus probably involves activation of chemotactic substances produced by the horse (Pycock and Allen 1988a). Metabolites of arachidonic acid including leukotriene B₄ and prostaglandin E₂ are released into the uterus during acute uterine inflammation, and are chemotactic for neutrophils *in vitro* depending on concentration (Lees, Dawson and Sedgwick 1986; Watson, Stokes and Bourne 1987c). Release of arachidonic acid metabolites from the endometrium has been demonstrated *in vitro* (Watson 1989).

Depending on the stage of the estrous cycle, uterine neutrophils from susceptible mares may lose migratory ability faster than resistant mares, but the issue is controversial. During an acute uterine response to streptococci in diestrus, migratory ability and elasticity of uterine neutrophils from susceptible mares is greatly reduced compared to resistant mares at 12 hours after inoculation (Liu et al. 1985). After 12 hours, neutrophils from resistant mares also lose migratory ability. However, perhaps as a result of continued inflammation, there may be a second influx of neutrophils into the uterus of susceptible mares. Consequently, uterine neutrophils in susceptible mares recover migratory ability at 15 hours (Liu et al. 1986). This may be related to multiple releases of PGF_{2a} following acute uterine

inflammation during diestrus (Neely et al. 1979) However, following an acute uterine response in estrus, susceptible mares may have superior chemotaxis of uterine neutrophils compared to resistant mares (Troedsson, Liu and Thurmond 1993a). The reason for this discrepancy in stage of cycle is unclear. The poorer migratory ability of uterine neutrophils from susceptible mares, if real, could be attributed to poor uterine environment, a possible consequence of uterine fluid accumulation (Liu et al. 1986; Troedsson and Liu 1992).

A difference between resistant and susceptible mares cannot be confirmed in peripheral blood neutrophils (Liu et al. 1986; Liu et al. 1985). Significant differences which have been reported are in conflict (Troedsson, Liu and Thurmond 1993a; Watson, Stokes and Bourne 1987a).

1.1.7. KILLING, PHAGOCYTOSIS AND OPSONIZATION. Killing of invading streptococci has been thought to play a key role in equine uterine defenses. Consistent bactericidal activity is not demonstrable in the cell free supernatant of uterine fluid. However, neutrophils in uterine fluid are phagocytic, to a degree. The stage of the estrous cycle may influence phagocytosis, being enhanced by estrogen and depressed by progesterone. Metabolites of arachidonic acid may also influence phagocytosis. Although uterine neutrophils from susceptible mares sometimes have reduced phagocytic ability, the defect is not consistently demonstrated. No difference exist in macrophage function between

resistant and susceptible mares. In both resistant and susceptible mares, uterine fluid is poorly opsonic for *S zooepidemicus* compared to serum, probably due to the break down of complement proteins in uterine fluid. Overall, no consistent difference in opsonic activity of uterine fluid has been detected between resistant and susceptible mares.

It has been suggested that the cell free supernatant of uterine fluid contains antibacterial components. The survival of streptococci in diestral cell free uterine flushings appears to be markedly diminished in comparison to survival in Hank's balanced salt solution with 1% gelatin (HBSSG). Heat treatment for 30 minutes at 56 C fails to reduce antibacterial activity, but succeeds at 80 C, suggesting that complement is not involved (Strzemienski, Do and Kenney 1984). However, an independent attempt to repeat this finding, comparing uterine fluid from ovariectomized mares treated with progesterone and estrogen, has not been successful. Instead, uterine fluid may in fact support higher bacterial numbers than HBSSG, regardless of stage of cycle (Johnson, Oxender and Berkhoff 1994).

However, ovarian steroids consistently alter the phagocytic response of neutrophils in uterine fluid. In uterine fluid collected 48 hours after streptococcal inoculation, chemiluminescent or bactericidal measurements of phagocytosis are consistently greater in ovariectomized estrogen supplemented mares than unsupplemented controls. Furthermore, supplementation with

progesterone actively depressed phagocytosis relative to controls (Ganjam et al. 1980; Ganjam et al. 1982; Washburn et al. 1982; Watson et al. 1987b). Progesterone treated mares may be heavily infected at 48 hours post inoculation, whereas estrogen treated horses clear infection (Washburn et al. 1982). After 10 days of progesterone treatment, phagocytic and streptocidal ability of progesterone treated mares may recover, but high numbers of bacteria can nevertheless persist in the uterus (Washburn et al. 1982). Similarly, 24 to 144 hours following infusion of oyster glycogen, phagocytosis of *S zooepidemicus* is depressed in uterine fluid from progesterone treated mares (Watson et al. 1987b). Progesterone supplemented horses may remain infected for weeks (Ganjam et al. 1980).

Of the metabolites of arachidonic acid appearing in uterine fluid, Leukotriene B₄ promotes phagocytosis and intracellular killing *in vitro*. Prostaglandin F_{2a} enhances only intracellular killing and PGE₂ has no effect on either (Watson 1988b).

A single study has attempted to investigate macrophage function between resistant and susceptible mares (Watson and Stokes 1988a). Rate of clearance of intravenously injected ¹²⁵I labelled polyvinyl pyrrolidone, an *in vivo* index of macrophage function, suggests that no difference exists between resistant and susceptible mares with regards to macrophage function. A trend towards lowered macrophage function was detected in estrus, although it did not achieve statistical significance.

A defect in the phagocytic ability of uterine neutrophils may occur in mares susceptible to endometritis, depending on stage of cycle and time of collection, but the issue is controversial (Asbury and Hansen 1987; Cheung et al. 1985). When uterine neutrophils are collected 12 hours following streptococcal infusion into the uterus during diestrus, susceptible mares have greatly depressed phagocytic ability to *Candida albicans* blastopores than resistant mares (Cheung et al. 1985). This parallels decreased migratory ability of uterine neutrophils of susceptible mares at 12 hours (Liu et al. 1986).

During estrus, no consistent difference appears between uterine neutrophils from resistant and susceptible mares in phagocytic capacity. In an acute uterine response, uterine neutrophils collected at 4 hours appear to be equally phagocytic in resistant and susceptible mares (Asbury and Hansen 1987). However, through 36 hours post inoculation, uterine neutrophils from susceptible mares may be more phagocytic than those from resistant mares (Troedsson, Liu and Thurmond 1993a) During anestrus, collection at 18 hours may detect a deficiency in susceptible mares (Watson, Stokes and Bourne 1987a). Comparisons of phagocytic ability of uterine neutrophils between resistant and susceptible mares may be complicated by arrival of fresh neutrophils in susceptible mares due to persisting inflammation (Liu et al. 1986). If a defect in phagocytic ability exists in uterine neutrophils of susceptible mares, it is probably

secondary to a poor uterine environment, which may be attributed to aged accumulations of uterine fluid in susceptible mares (Troedsson and Liu 1992).

The opsonic ability of uterine fluid is poor compared to serum, and probably a result of destruction of complement in uterine fluid. (Asbury, Gorman and Foster 1984; Asbury et al. 1982; Blue et al. 1982; LeBlanc et al. 1991; Troedsson, Liu and Thurmond 1993a). However, the alternative pathway of complement activation is not capable of opsonizing *S. zooepidemicus* in serum, suggesting that the classical (antibody mediated) pathway of complement activation is responsible (Asbury, Gorman and Foster 1984). In contrast to serum, opsonic activity of uterine fluid during normal estrus or diestrus is negligible. However, during an acute uterine response in estrus, serum opsonins are released into uterine fluid providing a mild increase in opsonic activity (Brown, Hansen and Asbury 1985). This increase is short lived, being undetectable at the following estrus. The increase in opsonic activity is heat-stable, suggesting antibody but not complement is responsible for the limited opsonizing ability of uterine fluid (Brown, Hansen and Asbury 1985). Fractionation of opsonins by ammonium sulfate precipitation and column chromatography also points to IgG as a major opsonin in uterine fluid (Hansen and Asbury 1987). Complement, however, is rapidly destroyed in uterine fluid (Asbury, Gorman and Foster 1984). Occasionally, significant

complement activity may appear in uterine fluid, but this is inconsistent, and may be caused by uterine hemorrhage during collection (Brown, Hansen and Asbury 1985; Watson, Stokes and Bourne 1987a). Therapeutic benefits of intrauterine infusions of plasma or serum to improve opsonic activity of uterine fluid, though initially promising, have not been consistent (Adams and Ginther 1989; Asbury 1984; Colbern et al. 1987b; Watson and Stokes 1988c).

Susceptibility to uterine infection cannot yet be associated with deficient opsonins or opsonic activity of uterine fluid. Following streptococcal inoculation, uterine fluid from horses which become persistently infected is as opsonic as fluid from horses which subsequently clear infection (Brown, Hansen and Asbury 1985). Resistant and susceptible mares do not differ significantly in the opsonic capacity of non-inflammatory uterine washings, though susceptible mares yield significantly more anti-streptococcal antibody (LeBlanc et al. 1991). Following an acute uterine response to streptococcal infusion, susceptible and resistant mares have similar intrauterine immunoglobulin and complement levels. However, at 36 hours, significant reductions may be seen in IgG and complement concentrations in susceptible mare uteri, possibly due to continued consumption of opsonins by multiplying bacteria (Troedsson, Liu and Thurmond 1993b). The opsonic activity of uterine fluid within 24 hours following an acute uterine response may be significantly higher in susceptible

mares (Hansen and Asbury 1987; Watson, Stokes and Bourne 1987a). Susceptible mares may be more likely to have active complement components in inflammatory uterine fluid (Watson, Stokes and Bourne 1987a). In contrast, sometimes a decrease in opsonic ability of uterine fluid from susceptible mares may be detected, probably attributable to poor uterine environment from fluid accumulation (Troedsson and Liu 1992; Troedsson, Liu and Thurmond 1993a). Decreased opsonic activity of endometrial culture supernatant from susceptible mares has been demonstrated (Watson and Stokes 1990). Overall, most evidence suggests that a significant defect in the opsonic activity of uterine fluid does not underlie susceptibility to equine endometritis. When a deficiency does exist, it is probably secondary to accumulation of uterine fluid.

1.1.8. PHYSICAL CLEARANCE. Physical removal of fluid and particles from the uterus plays a major role in uterine defense. Progesterone induced susceptibility to endometritis is associated with impaired uterine clearance. In addition, susceptible mares cyclically accumulate uterine fluid, and have delayed clearance following inoculation of materials infused into the uterus. Impaired uterine clearance has been detected radiographically in susceptible mares. The pathogenesis of fluid accumulation in susceptible mares may result, in part, from decreased myometrial contractions. Oxytocin, by promoting myometrial contractions, can

result in effective fluid removal in susceptible mares, and may be useful in treatment of endometritis.

Uterine infection may be associated with failure to clear both fluid and particles from the equine uterus. An innovative inoculum, consisting of three different components suspended in saline, has been used to demonstrate the importance of physical clearance in uterine defenses (Evans et al. 1986; Evans et al. 1987). The inoculum consists of *S zooepidemicus* (an antigenic material cleared either by phagocytosis or mechanical clearance), charcoal (a non-antigenic material cleared by phagocytosis and mechanical clearance), and ^{51}Cr labelled 15 μm microspheres (a non-antigenic material cleared only by mechanical clearance). Following inoculation, the uterus is flushed with saline containing traces of ^{125}I labelled human serum albumin. Analyzing the collected flush makes it possible to determine how much fluid, bacteria, leukocytes, charcoal, and microspheres remain in the uterus. In acyclic maiden mares, progesterone treatment reduces physical clearance of inoculated markers compared to estrogen or no treatment. Failure to eliminate bacteria and leukocytes from the uterus parallels the reduced uterine clearance of fluid and particles (Evans et al. 1986). When treated with estrogen, susceptible mares have reduced physical clearance compared to resistant mares. However this difference disappears with progesterone treatment when all horses suffer impaired mechanical clearance and increased infection (Evans et al. 1987).

Although questions about the relative importance of physical clearance and killing have been raised (LeBlanc, Asbury and Lyle 1989), additional studies confirm that susceptibility to endometritis is associated with accumulations of fluid and delayed physical clearance of fluid and particles (Allen and Boyd 1990; Allen and Pycock 1988; Troedsson and Liu 1991; Troedsson and Liu 1992). Clinically, delayed clearance of fluid has been confirmed in susceptible mares scintigraphically by measuring clearance of an intrauterine infusion of Technetium 99m-albumin colloid (LeBlanc et al. 1994).

Myometrial contractions also play an important part in effective physical clearance. Myometrial contractions during estrus are more intense, more frequent, and of shorter duration than in diestrus (Troedsson et al. 1993). Following intrauterine inoculation of streptococci, susceptible mares have reductions in myometrial contraction that become most apparent 10-20 hours following inoculation (Troedsson et al. 1993). Interestingly, this time frame coincides with the rise in bacterial numbers in susceptible mares (Williamson et al. 1987). The ability of oxytocin to induce susceptible mares to eliminate accumulated uterine fluid may prove to be a valuable and simple treatment for some cases of endometritis (Allen 1991; LeBlanc et al. 1994).

1.1.9. A MODEL OF ENDOMETRITIS. Long term progesterone treatment has been used to develop a model of endometritis in normal horses to evaluate pathogenesis and uterine therapy. Such

a model has not been successful in evaluating different therapeutic regimens. Nevertheless, it has helped to reveal important differences between resistant and susceptible mares.

Progesterone treatment reliably induces endometritis in ovariectomized horses, and is superior to dexamethasone for this purpose. Progesterone treated horses inoculated with *S zooepidemicus* are unable to eliminate infection (Colbern et al. 1987a). The evaluation of intrauterine therapy using this model is difficult. Intrauterine plasma is not of therapeutic benefit using this model (Colbern et al. 1987b), but the benefit of plasma is controversial based on other studies (Adams and Ginther 1989; Asbury 1984). A progesterone based model of endometritis favors growth of a variety of bacteria, yet experimentally inoculated organisms may not become established. In addition, prolonged antibiotic administration in this model may lead to fungal endometritis, and therapeutic benefits of antibiotics may not be consistent (Hinrichs, Spensley and McDonough 1992). Because it does not include events occurring during a complete estrous cycle, this model of endometritis does not truly mimic the susceptible mare (Allen 1993). However, progesterone administration by others has helped to reveal the importance of physical clearance in uterine defense, and led to detection of deficiencies in physical clearance in susceptible mares (Evans et al. 1986; Evans et al. 1987). Thus the model, with due regard to its limitations, has yielded valuable information.

1.1.10. BACTERIAL PATHOGENESIS. It is surprising that the etiologic agents of endometritis have been so neglected, given the powerful tools currently available for their study. Bacteria have been implicated in luteolytic prostaglandin release, inflammation, and adherence to the endometrium. Some bacteria are clearly more virulent than others, and interactions between bacteria may contribute to pathogenesis.

Intravenous administration of *Salmonella typhimurium* endotoxin leads to a luteolytic release of prostaglandins (Daels et al. 1988; Daels et al. 1987) The resulting decline in progesterone can terminate pregnancy, although progestogen supplementation can prevent pregnancy loss (Daels et al. 1991b). Early administration of flunixin meglumine may prevent endotoxin mediated prostaglandin release (Daels et al. 1991a).

Streptococcus zooepidemicus may produce substances which accentuate the acute inflammatory reaction of the mare's uterus. Intrauterine infusion of cell free filtrates of media in which streptococci have been cultured produce a more intensely suppurative reaction than media alone (Couto and Hughes 1985). Adhesion is an important means by which bacteria become established at mucous membranes (Beachey 1981). *S zooepidemicus* adheres to equine endometrial tissue *in vitro*, whereas *S pneumoniae* does not. In addition, streptococcal adherence is greater to endometrium of biopsy grade III during estrus than to endometrium of grades I or II (Ferreira-Dias, Nequin

and King 1994a). *Streptococcus zooepidemicus* adherence is greater to endometrium samples from ovariectomized mares treated with estrogen than with progesterone (Watson, Stokes and Bourne 1988b). Ultrastructural studies of *Taylorella equigenitalis*, the etiologic agent of contagious equine metritis, reveal a variety of surface structures which may play a role in bacterial adherence or resistance to phagocytosis (Hitchcock et al. 1985). Following intrauterine inoculation, *Taylorella equigenitalis* persists longer in the equine uterus than *Pseudomonas aeruginosa* (Strzemienski et al. 1984).

Uterine infection with *S zooepidemicus* may predispose to uterine infection with *K pneumoniae*. Inoculation of *K pneumoniae* alone consistently fails to establish infection unless inoculated in conjunction with killed *S zooepidemicus*. A basis for this interaction may be the presence of streptococcal immunoglobulin-binding Fc receptors on the surface of *S zooepidemicus*. Antibody-receptor complexes activate the complement cascade and may deplete available opsonins (Lyle et al. 1991). It is interesting to note that Dimock and Edwards also reported that *S zooepidemicus* predisposed mares to infection with *K pneumoniae* (Dimock and Edwards 1928).

1.2 STREPTOCOCCAL M PROTEIN

The dawn of medical science dimly illuminated streptococcal disease 200 years ago; as the light grew stronger, knowledge multiplied. Consequently, the history of streptococcal

disease, and streptococcal M protein, is virtually a history of medical science. It began with an inability to explain the exact cause of disease. But with stubborn insistence on epidemiological interrelationships, it led to the discovery of etiological agents as the germ-theory of disease was gaining acceptance. Discovery of the antiphagocytic properties of streptococci brought a broader appreciation of bacterial pathogenesis. Breakthroughs in serotyping and recognition of streptocidal activity of blood accompanied the growth of immunology. More recently, the chemical, genetic and molecular aspects of pathogenesis and epidemiology have accompanied the development of molecular biology. To fully understand M protein, one needs to understand all this, which ordinarily would be impossible. However, one person established a simple framework upon which the old knowledge rests and where new knowledge still finds room. Her name was Rebecca Lancefield.

1.2.1. EPIDEMIOLOGY AND HUMAN DISEASE. To appreciate Rebecca Lancefield, one must first understand the importance of epidemiology in streptococcal disease prevention. Even before streptococci were known as human pathogens, diseases which we now know were caused by streptococci were controllable by accurately identifying the risk factor involved. But the challenge in those days was to convince others of the association. The epidemiological truth did not always prevail against widespread and entrenched ignorance. Two early examples, puerperal fever

and epidemic sore throat, revealed the importance of identifying how disease is contracted and spread. In the case of puerperal fever it was to identify a particular obstetrician whom the disease followed like a shadow. In the case of epidemic sore throat it was to identify a dairy distributing contaminated milk.

Throughout the last century, before streptococci had been identified as pathogens, physicians in Europe and the United States suspected the contagious nature of puerperal fever, an acute, suppurative, and frequently fatal infection of women in child birth. Ignatz Semmelweis, a young obstetrical assistant in Vienna, suspecting the contagious nature of puerperal fever, conducted one of the earliest successful clinical trials to test his theory. He saved many lives in the process. In 1845, two obstetrical hospitals existed in Vienna, one for obstetrical teaching, the other where midwives were primarily responsible for deliveries. Death rates due to puerperal fever in the first clinic were 4 times the death rates in the second. Semmelweis proposed that the high mortality of the first clinic was due to students performing *post mortem* examinations on cases of puerperal fever, and then carrying toxic material directly to patients in the wards. By insisting that students wash their hands in "chloride of lime" following *post mortem* examinations, he dramatically reduced the death rate of the first clinic to the level of the second clinic. However, he was denounced by the medical authorities of the day, and received little recognition in his

lifetime. The hand washing practice was discontinued by his superiors, and he was removed from his position. He himself died of the disease, from an infected cut obtained during *post mortem* examination of a puerperal fever victim (Semmelweis 1975).

In the United States, the Boston physician and novelist, Oliver Wendell Holmes, met similar opposition. Women frequently gave birth at home, attended by a visiting doctor or midwife. Holmes gathered medical records as far back as eighteenth century Britain to document, beyond reasonable doubt, a horrifying pattern of disease. Certain practitioners would suddenly be cursed with a series of frequently fatal cases of puerperal sickness in patients under their care. The doctor would rush from delivery to delivery, without washing his hands or changing his clothes, and in rapid succession his patients would contract the disease. In addition Holmes noted that a Scotsman, Dr. Gordon of Aberdeen, announced as early as 1795 that it was possible to predict which woman would become afflicted, after hearing which midwife attended the delivery. Holmes particularly noted the association of disease outbreaks following a physician performing an autopsy on cases of puerperal fever, or after treating patients with erysipelas (a septicemic streptococcal disease with severe skin lesions). He even records a physician who attended an autopsy of a case of puerperal fever, and then carried the pelvic viscera of the dead woman in his pocket to a class. He attended another woman in the evening without having changed his clothes. That woman was

the first of a series he assisted who subsequently died. Because puerperal fever occurred sporadically in unattended deliveries, occurring on the average 3 times in every 1000 births, Holmes argued that a succession of 2 cases was highly unlikely due to chance, whereas the chance occurrence of a series of 3 cases was virtually impossible.

Many opposed his views bitterly, for obvious reasons. Countless physicians had encountered such series in their own practices, and winced to acknowledge that they were directly responsible for so many unnecessary deaths. Because of the wide scope of his documentation, probably assisted by his rhetorical and literary ability, he prevailed. His essay, *The Contagiousness of Puerperal Fever*, first published in 1843, is one of the earliest contributions to medical research by an American physician (Holmes 1892).

In December 1911, Chicago suffered a severe and widespread epidemic of sore throat and fever, afflicting over 10,000 people, for which no explanation could be made at the height of the epidemic. The severity of the outbreak was matched in only one other community in the state, the nearby town of Batavia, Illinois. An earlier outbreak in Boston had suggested a causal link between milk supply and epidemics of sore throat. Two physicians chose to investigate the possibility that the Chicago outbreak was a milk-borne disease (Capps and Miller 1912). It quickly became clear that a disproportionate number of cases were associated with

consumption of milk from one dairy (dairy X), which also supplied Batavia. Of all the cases of sore throat, 87% occurred in drinkers of milk from dairy X. Furthermore, the incidence of sore throat was 14 times higher in drinkers of milk from dairy X than from any where else. Cases of bovine mastitis had occurred on the farms supplying dairy X, simultaneously with cases of sore throats in milkers on those farms. But the most significant finding lay in the milk plant. Failures in achieving adequate pasteurization temperature were consistently recorded on days which immediately preceded the greatest numbers of new cases.

However, bacteriologic investigations of the outbreak ran into difficulties. A girl on one of the farms had suffered from sore throat, with arthritic complications, for many weeks but had not drunk any milk from the cows at the dairy. One cow on the same farm had mastitis. Similar organisms were cultured from the girl, the cow, and the throats of afflicted milk drinkers in Chicago. It was proposed that the same organism caused all these infections, and was named *Streptococcus epidemicus* (Davis 1912). It was asserted, based largely on colony morphology that *S epidemicus* was distinct from the other streptococci isolated from the udder of cows, and distinct from other cases of septic streptococcal disease in humans, such as scarlet fever, erysipelas and streptococcal sore throat unassociated with milk. However, other epidemics of milk borne streptococcal disease were often associated with erysipelas and scarlet fever. Consequently the

unique identity of *S epidemicus* was questioned (Williams et al. 1932). Attempts were made to classify other streptococci as distinct organisms linked to specific diseases, such as *S scarlatinae* from cases of scarlet fever (Sherman 1937). But distinguishing between the isolates could not be performed satisfactorily. There was no simple way to distinguish the many hemolytic streptococci which could be cultured from humans, nor clearly distinguish between human and animal strains. Investigators were severely hampered by an inability to confidently identify organisms, lacking any subdivisions by which streptococci from diverse sources could be grouped (Dochez, Avery and Lancefield 1919; Sherman 1937).

1.2.2. LANCEFIELD'S CLASSIFICATION OF STREPTOCOCCI: THE "M" AND "C" SUBSTANCES. Lancefield solved the problems of streptococcal classification in a single stroke. She first developed a system by which the many streptococci of human origin could be distinguished and this led directly to a broader classification scheme by which streptococci of human origin could be distinguished from a variety of animal sources. Now epidemiologists could pursue the origin and spread of a streptococcal epidemic with greater confidence, and more rapidly control an outbreak.

The acid extract of Lancefield, a modified procedure of Porges, released a substance from the cell wall which varied across the range of human streptococci, but was identical within

isolates of the same type. Different isolates could be identified by serologically recognizing this "type-specific substance". Lancefield had previously typed a small collection of human streptococci by agglutination and mouse protection tests (Dochez, Avery and Lancefield 1919). She then digested these streptococci in a mixture of sodium chloride and dilute hydrochloric acid, releasing cell wall carbohydrate and proteins into solution. The supernatant was divided into an alcoholic precipitate (protein) and a supernatant that resisted alcoholic precipitation (carbohydrate). The protein precipitate was redissolved in saline and then added to serum from rabbits immunized with the typed streptococci. Extracts homologous to the type used to immunize the rabbit tended to precipitate in the rabbit serum, whereas heterologous extracts did not. However the pattern was not consistent; sometimes heterologous extracts also precipitated. However, by absorbing out cross-reacting antibodies from serum with a packed column of heterologous streptococci, Lancefield was able to generate a bank of type-specific sera which only precipitated extracts of homologous isolates. These sera could be used to type field isolates collected during an epidemiologic investigation (Lancefield 1928a). To give due credit, Hitchcock had suggested precipitin reactions as a basis for streptococcal grouping some years earlier (Hitchcock 1924).

Although type-specific antigens of other organisms were often carbohydrate, Lancefield demonstrated that the type-

specific substance of her human streptococcal isolates, designated substance "M", was a protein. She showed that the substance was precipitated by common protein precipitants (alcohol, acetic acid, picric acid), contained a high nitrogen content, was destroyed by removal of amino groups, and was readily digested by trypsin and pepsin (Lancefield 1928b). Furthermore, the M substance appeared to be associated with virulent organisms and "matt" colonies, whereas organisms which lacked M protein were avirulent with "glossy" colonies (Todd and Lancefield 1928).

The protein fraction of the acid extract contained other substances precipitated with the M protein. These included the "R" antigen, which resisted tryptic digestion, lacked association with virulence, and was confined to only a few streptococcal types (Lancefield and Perlmann 1952). A "T" antigen present on both matt and glossy streptococci did not precipitate in homologous serum, but caused type specific-agglutination of glossy variants (Lancefield 1940). The non-specific components of the acid extract, which necessitated the absorption of the typing sera, were apparently nucleoproteins common to most streptococci (Lancefield 1928b).

Then Lancefield leaped over the divide between human and veterinary medicine. She proceeded to serologically differentiate streptococci of humans from streptococci of animals using the carbohydrate fraction of the acid extract (Lancefield 1933). This

component, designated substance "C" had been found on all the hemolytic streptococci isolated from human diseases, and was believed to be common to all hemolytic streptococci (Lancefield 1928c). However, when Lancefield analysed streptococci from animals, she saw that additional "C" substances existed. The milk-borne epidemics of septic sore throat had spurred an effort to distinguish between different groups of streptococci: those originating from human sepsis (group A), bovine mastitis (group B), diverse animal infections (group C), cheese (group D), and non-pathogens of milk (group E). Other workers had already tentatively assembled streptococci into these 5 groups based on a multitude of biochemical tests. Lancefield showed that the "C" substance was distinct in each of these 5 groups. This "group-specific substance" in the acid extract was precipitated in sera of rabbits previously immunized with representative strains of each group (Lancefield 1933). Using the grouping sera, one could distinguish the etiologic agents of sore throat from those of bovine mastitis or from non-pathogenic streptococci. Thus epidemiologists were given the tools to investigate and control streptococcal disease in the human population (Brown 1937).

1.2.3. GROWTH IN FRESH BLOOD AND RESISTANCE TO PHAGOCYTOSIS. Long before Lancefield, it was recognized that phagocytosis by neutrophils was the primary defense of animals against systemic streptococcal disease. Phagocytic killing was conveniently demonstrated by killing of streptococci in fresh

blood of immune individuals, and confirmed by microscopic examination of inoculated blood smears. However, group A streptococci multiplied rapidly in the blood of non-immune individuals. Two group A streptococcal components could explain this resistance to phagocytosis: M protein and the hyaluronic acid capsule.

A century ago, pioneers in cellular immunity, from the laboratory of Metchnikoff, revealed fundamental differences in the killing of gram negative *versus* gram positive organisms. The gram negative *Vibrio cholerae* could be directly killed in immune serum (Bordet 1895). However, streptococci were unharmed in serum of immune animals; in fact, they could multiply abundantly. Nevertheless, passive immunization of a rabbit with immune serum was highly protective against streptococcal infection. By examination of peritoneal exudates following intraperitoneal inoculation, Bordet concluded that clearance of streptococci required phagocytosis by neutrophils (Bordet 1897). Similarly, subcutaneous injection of streptococci into a horse led to local edema; macrophages then became packed with streptococci, but subsequently ruptured. Only following an influx of neutrophils (microphages) did the tide turn in favor of the host (Salimbeni 1898). As Metchnikoff said: "These microphages seize the free streptococci that have struggled so victoriously against the macrophages." The phagocytosis of the invading streptococci by

neutrophils was the critical event in clearing systemic streptococcal infection (Metchnikoff 1907).

As knowledge expanded, streptococci revealed a capability to resist phagocytosis. Bordet noted that following injection into the peritoneal cavity, streptococci developed a halo which apparently prevented their being phagocytized by neutrophils. Development of the halo was critical in determining the fate of the injected rabbit. If all streptococci could be phagocytized before they had time to develop the halo, the rabbit lived. However, if a few streptococci had enough time to develop the halo, they multiplied extracellularly despite the presence of many neutrophils, and the rabbit quickly died. At the time of death, encapsulated cocci would be found multiplying unchecked in the rabbit's circulation (Bordet 1897).

The battle between the streptococci and the neutrophils was also studied in tubes of fresh blood. In 1918, it had been demonstrated that streptococci on an agar plate were killed by neutrophils, but not serum (Wright, Fleming and Colebrook 1918). However, successful demonstration of killing in a tube required complete rotation of the blood tube end-over-end. In standing blood the cellular components settled to the bottom of the tube where streptococci were killed, but streptococcal multiplication continued unchecked in the supernatant serum. After a 3 hour incubation, blood lost its ability to kill streptococci (Todd 1927b). Microscopic examination of smears of such blood, where

streptococcal numbers were rapidly falling, revealed intense phagocytosis by neutrophils (Lyons and Ward 1935; Seastone 1934). However, in blood of immunologically naive individuals, virulent streptococci replicated rapidly (Todd 1927c), and microscopic examination subsequently confirmed their resistance to phagocytosis (Ward and Lyons 1935). The ability to replicate in blood and resist phagocytosis appeared to be a special adaptation of virulent streptococci which could be lost after extensive passage of streptococci on artificial media (Foley, Smith and Wood 1959; Todd 1927c). Fresh blood subsequently became a standard medium in which to study group A streptococcal phagocytosis (Kass and Seastone 1944; Morris and Seastone 1955; Rothbard 1945a; Rothbard 1948; Rothbard and Watson 1948; Seastone 1934; Wiley and Wilson 1956).

The connection between M protein, resistance to phagocytosis and virulence is central to M protein's fame. Todd originally showed that streptococci lapsed into avirulent forms following culture on artificial media, and lost the ability to grow in fresh blood (Todd 1927c). The colony morphology of the virulent and avirulent forms, respectively designated matt and glossy, correlated with M protein production, M protein being more abundant in the matt forms (Todd and Lancefield 1928). Others independently confirmed the association between Todd's colony descriptions, virulence and resistance to phagocytosis. It should be noted, however, that some matt strains which were virulent for

humans were avirulent for mice; thus M protein was not sufficient for virulence in all species (Hare 1931; Todd and Lancefield 1928; Ward and Lyons 1935). The ability to grow in fresh blood was directly linked to M protein production; strains that did not produce M protein were unable to grow in fresh blood (Rothbard 1945a). During the course of streptococcal infection in humans, streptococci isolated at weekly intervals gradually lost M protein, and this loss was associated with gradual loss of resistance to phagocytosis in blood of naive-individuals. Interestingly, these streptococci recovered production of their original M protein following mouse passage (Rothbard and Watson 1948).

The hyaluronic acid capsule of streptococci was also associated with resistance to phagocytosis. Following Bordet's observation of an antiphagocytic halo around streptococci, presence of a capsule was confirmed in young streptococcal cultures (Seastone 1934). It was conveniently demonstrated by inoculating non-immune human blood with streptococci, and then examining a Wright's stained smear after a 1.5 hour incubation. The capsule appeared as a granular border surrounding the streptococci (Seastone 1934). Chemical analysis of the capsule revealed a polysaccharide consisting of N-acetylglucosamine and glucuronic acid units, a polymer now known as hyaluronic acid. Unlike the polysaccharide capsule of the pneumococcus, the hyaluronic acid capsule was neither antigenic nor type-specific, probably because hyaluronic acid is a common component of many

mammalian tissues (Hirst 1941; Kendall, Heidelberger and Dawson 1937). Streptococci whose capsules were removed by age (Seastone 1934), heating (Seastone 1934), or digested with hyaluronidase (Kass and Seastone 1944; Rothbard 1948) were more easily phagocytized in fresh blood.

What, then, were the relative contributions of M protein, and hyaluronic acid in resistance to phagocytosis? Because M protein could be atraumatically removed from streptococci by digestion with trypsin (Lancefield 1943) and the capsule by hyaluronidase (Hirst 1941), tools necessary to answer the question were available. A consistent picture emerged in which loss of either M protein or the capsule rendered the streptococcus susceptible to phagocytosis, but the combined loss of both was the most debilitating treatment. It was difficult to assign a greater importance to either component across all serotypes, and the combined antiphagocytic action of both could not be ruled out (Foley and Wood 1959; Hirsch and Church 1960; Morris and Seastone 1955). Because encapsulated streptococci could be killed in immune blood, it was suggested that the M protein extended far into the capsule, even to the capsule's outer edge. Release of M protein following hyaluronidase treatment, coupled with absorption of type specific antibodies by encapsulated streptococci, supported this suspicion (Morris and Seastone 1955). It thus appeared that the capsule and M protein might structurally cooperate in conferring resistance to phagocytosis. However, a

mechanism of such cooperation, if it exists, has yet to be described.

1.2.4. KILLING IN FRESH BLOOD AND TYPE-SPECIFIC ANTIBODY.

Although group A streptococci multiplied in the blood of non-immune individuals, blood would subsequently develop dramatic killing ability following streptococcal infection. However, killing was type-specific; only the strain isolated from the infection was killed. It became clear that type-specific antibodies to the type-specific M protein were an important component of immunity against streptococcal infection, and could be measured by the bactericidal activity of fresh blood. So great was the specificity of killing in fresh blood that Lancefield chose the bactericidal test to M type streptococci that had cross reacting R antigens in precipitin tests. The bactericidal test thus became the standard assay of type-specific antibody, and the most rigorous method of M typing group A streptococci.

Following streptococcal infection, blood develops intense bactericidal activity that is type-specific for the infecting strain. Todd demonstrated that serum from a patient with puerperal sepsis, when added to blood of a normal human, invoked intense killing of the infecting strain. However, interpretation of his early demonstration is difficult for many reasons. He lacked preinfection sera; he did not demonstrate growth of the isolate in normal blood; he failed to detect increased phagocytic activity; sera was obtained during acute sepsis, too soon for the

development of type-specific antibodies from the current infection. But he showed that the derived glossy variant was killed *less* in the presence of the patient's serum than the original virulent organism. This suggests that he was detecting anti-M protein antibodies that found targets on the virulent strain that were absent on the glossy variant (Todd 1927a). Some years later, Seastone, using his own blood to demonstrate antiphagocytic properties of the streptococcal hyaluronic acid capsule, accidentally infected himself with the isolate he was studying. Fortunately he survived to document an intense increase in the type-specific bactericidal activity of his blood to the isolate (Seastone 1934). More extensive studies by Lyons and Ward showed that protection against peritoneal challenge could be achieved in mice by passive immunization. However, resistance was strictly type-specific, requiring sera from a rabbit immunized with the homologous strain. Resistance correlated with increased phagocytosis of peritoneal neutrophils (Lyons and Ward 1935). He and his coworkers showed, by absorption experiments, that the type-specific opsonization was probably directed against M protein. Interestingly, one of them also experienced streptococcal pharyngitis with the isolate they were studying, and similarly experienced an increase in type-specific bactericidal activity in blood. They showed that infants lacked bactericidal activity to most streptococcal isolates. However, adults possessed the ability to kill particular isolates, although

each adult killed different isolates, presumably as a result of different individual exposure over the years. Each isolate thus exhibited a different profile of killing in a panel of 5 adult blood donors (Lyons and Ward 1935). Hirst and Lancefield then showed that preparations of M protein could be used to actively and passively immunize mice against streptococcal challenge. The immunity was essentially type-specific in passively transferred antibody. Active immunization was also predominantly type-specific, although an element of cross protection could be observed (Hirst and Lancefield 1939).

Because of its probable importance in immunity, much effort was therefore spent to develop techniques to detect type-specific antibody in people; ultimately, the bactericidal test became accepted as the most reliable assay. Measurement of anti-M protein antibodies in people was attempted by a variety of other methods, but none was altogether satisfactory. Rothbard summarized the shortcomings as follows: agglutination reactions were non-specific; precipitin tests resulted in cross reactions, and absorption with heterologous antisera eliminated type-specific precipitation; mouse protection tests were accurate but could only be applied to mouse virulent strains, and required large volumes of high titer sera; direct measurement of opsonization could be difficult to interpret (Rothbard 1945a). To gather serologic evidence for type-specific immunity, Kuttner recorded the development of bactericidal activity of fresh blood in a

children's institution where periodic epidemics of streptococcal pharyngitis occurred (Kuttner and Lenert 1944). From her results it was clear that blood became bacteriostatic for the homologous strain post infection, whereas a heterologous strain would multiply. However, only bacteriostatic (not bactericidal) activity could be demonstrated against the homologous strains. This may have been due to her failure to rotate the tubes end-over-end, which Todd had emphasized was necessary for effective killing (Kuttner and Lenert 1944; Todd 1927b). However, Rothbard, who rotated the tubes as he was supposed to, added serum from convalescing patients to blood of infants in which the streptococci normally multiplied (indirect bactericidal test). He demonstrated that serum from patients initially lacked type-specific antibody, but that strictly type-specific killing could be demonstrated some weeks following infection. The type-specific nature of the killing was confirmed by absorbing out the type-specific antibodies with killed homologous, but not heterologous, streptococci. In addition, absorption by M groups, and not T (agglutination) groups, specifically eliminated bactericidal activity, confirming that M protein was the target of type-specific antibody. However, because strains lacking M protein could not grow in fresh infant's blood, the assay was only applicable to detection of antibodies to strains carrying M protein (Rothbard 1945a). More detailed study revealed that killing required leukocytes, complement, and a heat stable component of

plasma. Calcium binding anticoagulants disrupted phagocytosis whereas killing proceeded in the presence of heparin. Human blood cells were necessary to detect antibodies in human sera; cells of other animal species were ineffective with human sera (Rothbard 1945b).

The usefulness of the bactericidal test exceeded the mouse protection test. The mouse protection test was hitherto the most reliable way to demonstrate type-specific antibody, but was severely limited by requiring mouse virulent strains. Because many mouse avirulent streptococci grew rapidly in fresh human blood, the bactericidal test was well suited to these strains. In addition, with established sera of high type-specific antibody titer, bactericidal tests agreed well with mouse protection tests (Maxted 1956). Because of its high specificity, Lancefield herself used the bactericidal test to differentiate streptococci which had a cross reacting R antigen in precipitin tests. She concluded that the bactericidal test distinguished streptococci with a high degree of accuracy, and was superior to mouse protection tests in measuring type-specific antibody (Lancefield 1957).

1.2.5. THE STUDY OF M PROTEIN BY MODERN TECHNOLOGY.

Medical science has analyzed M protein with all the tools of modern technology; the streptococcal surface has been described by electron microscopy; the type 6 M protein has been mapped by X-ray diffraction; a molecular basis for M protein's resistance to phagocytosis has been proposed; M protein molecules have been

probed with monoclonal antibodies; M protein genes have been cloned, sequenced, and mutated. Today, the earlier contributions of Lancefield and her contemporaries are all but forgotten. But the solid foundation laid by Lancefield's generation made the successes of modern technology possible.

The early digestion and absorption studies suggest that M protein is located on the surface of the streptococcus, extending far into the capsule. Electron microscopy reveals that group A streptococci are indeed covered by a fuzzy coat of fimbriae. With confidence one can assert that M protein comprises part of this coat: M protein positive (M+) strains possess the layer, whereas variants of these strains that have become M protein negative (M-) do not; the layer disappears following trypsin digestion; ferritin labelled type-specific antibodies bind to targets in the fuzzy layer on homologous streptococci (Swanson, Hsu and Gotschlich 1969). The 3 dimensional structure of the phage-lysin extracted type 6 M protein reveals a dimer in an alpha-helical coiled-coil arrangement. The isolated fimbriae are 50 nm in length. Fragments of the protein extracted by pepsin or detergent are substantially shorter (Phillips et al. 1981).

The ability of M protein to resist phagocytosis has received much attention; one mechanism of antiphagocytic activity appears to arise from interference with the alternate pathway of complement activation. Peptidoglycan of streptococcal cell walls is a potent activator of the alternate complement pathway

(Greenblatt, Boackle and Schwab 1978; Tauber et al. 1976; Tauber, Polley and Zabriskie 1976). This activation leads to the rapid phagocytosis of M- streptococci (Bisno 1979). However, in the absence of type-specific antibody, M+ streptococci activate complement to a much lesser degree and resist phagocytosis (Bisno 1979; Peterson et al. 1979). This resistance to complement and phagocytosis can be removed by tryptic or peptic digestion of M+ streptococci, i.e. by removing the M protein from the cell wall (Bisno 1979). Furthermore, C3 is deposited circumferentially on M- streptococci, but only in patches on M+ streptococci. This decreases the number of ligands on the M+ streptococci to which neutrophils bind (Jacks-Weiss, Kim and Cleary 1982). The impaired C3 deposition on streptococci appears to involve the same mechanism that prevents C3 deposition on host cells. M protein and M+ streptococci avidly bind complement control factor H, which is normally only bound by host tissues. Factor H leads to the dissociation of the C3b,Bb complex, which normally acts as a ligand for the neutrophil. C3b becomes accessible to inactivation by complement control factor I, leading to its degradation and dissociation from the streptococcal surface. The streptococcal surface now lacks receptors to which the neutrophil can bind, thus preventing phagocytosis (Horstmann et al. 1988). Binding of fibrinogen to the conserved region of M protein may also block complement deposition (Whitnack and Beachey 1982).

The resistance of M⁺ streptococci to phagocytosis is lost in the presence of type-specific antibody against M protein, but such type-specific protection is narrow because of many existing M types. When type-specific antibody is available, phagocytosis is rapid due to a circumferential deposition of C3 on the streptococcal cell wall. Type-specific antibody alone is not sufficient to phagocytize group A streptococci; the classical pathway of complement appears to be necessary (Jacks-Weiss, Kim and Cleary 1982). This confirms the early demonstration by Rothbard that opsonization of M⁺ streptococci requires complement and antibody (Rothbard 1945b). The variability of different types of M protein appears as great differences in size between M proteins of different types, and sometimes differences in strains of the same M type, but only from different outbreaks. M protein molecules from the same outbreak do not vary, nor does variation occur in strains passaged extensively. Thus size variation in M protein molecules is not rapidly evolving (Fischetti, Jones and Scott 1985).

Studies with monoclonal antibodies have revealed that the amino half of the M protein molecule, the half extending away from the streptococcus, varies greatly between M types. The carboxy half, extending towards the cell wall, is conserved between M types. Monoclonal antibodies to the conserved region react to several types of M protein (Jones et al. 1986; Jones et al. 1985; Manjula et al. 1986). However, only antibodies to the

variable amino terminal half are opsonic. Cross reactive antibodies directed against the carboxy half of the molecule are unable to opsonize streptococci (Fischetti and Windels 1988; Jones and Fischetti 1988). The exact reason for opsonic failure of antibodies to the conserved region is unclear. It may be related to this region's ability to bind complement control factor H, preventing the deposition of complement (Horstmann et al. 1988). In addition, fibrinogen binds to the conserved region of some M protein molecules, and may block antibody binding (Whitnack, Dale and Beachey 1984).

Molecular biology of group A streptococci has revealed not only variable genes coding for Lancefield's M substance, but also subfamilies of M protein genes. Cloning and expression of the type 6 streptococcal M protein in *Escherichia coli* produces a protein identical to the native M protein. Using the bactericidal test, type-specific antibodies are demonstrable in rabbits vaccinated with the expressed protein (Fischetti et al. 1984; Scott and Fischetti 1983). The C terminal sequence reveals a hydrophobic membrane anchor, followed by a proline rich region that spans the cell wall. The bulk of the molecule consist of 3 sections of repeating blocks of amino acid sequences. A 7 amino acid periodicity persists throughout, wherein the first and fourth amino acids are hydrophobic. Overall, the primary sequence is consistent with an alpha helical coiled-coil (Hollingshead, Fischetti and Scott 1986). Comparison of multiple M proteins

confirms that the carboxy third of the molecule is conserved, with 95% homology across M types. The amino half of the molecule is variable (Hollingshead, Fischetti and Scott 1987; Scott, Hollingshead and Fischetti 1986). Features of M protein genes are shared with other streptococcal surface proteins, including the seven amino acid periodicity and a highly conserved sequence (LSPTGE) close to the membrane anchor at the carboxy terminal (Fischetti, Pancholi and Schneewind 1991). In some cases, the M protein and Fc-receptors genes may have resulted from duplication of an ancestral gene (Heath and Cleary 1989). More recent study of many streptococcal M types shows M protein genes are grouped in clusters of 1, 2 or 3 genes downstream from a common promoter. An M protein gene may belong to 1 of 4 subfamilies. Subfamily 1 (SF-1), which includes M6, probably represents an allele that evolved directly from the ancestral M protein gene. Streptococci carrying SF-1 alleles are designated class I, and are more frequently associated with virulence (Hollingshead et al. 1993). Overall 9 different patterns of subfamily clusters have been demonstrated. These patterns may be the product of successive gene duplications, originating with duplication of the ancestral SF-1 gene (Hollingshead et al. 1994).

1.3. *STREPTOCOCCUS ZOOEPIDEMICUS* AND M PROTEIN.

We must now return to the horse, and consider similarities between group A and group C streptococci. *Streptococcus zooepidemicus* represents the bulk of isolates in Lancefield's

group C. Just as the group A streptococci are isolated from suppurative and septicemic conditions in humans, so *S zooepidemicus* is isolated from similar conditions in animals, especially horses. Since its inception, streptococcal isolates from the equine uterus have been recognized as important members of Lancefield's group C. Unlike the group A, however, isolates of *S zooepidemicus* are usually not primary pathogens, but commensals, frequently appearing as opportunistic invaders. Although *S zooepidemicus* rarely causes disease in humans, when it does the clinical picture is indistinguishable from group A streptococcal infection. One is therefore inevitably led to the question, does *S zooepidemicus* carry an M protein? Although at first glance it would seem an easy question to answer, it quickly becomes complicated. For the M protein of the group A streptococci is not a single protein, but a typing system. To identify M protein on the group C streptococci requires development of a similar typing system using acid extracts or bactericidal tests. A molecular approach to identification of M protein, outside the framework of such a system, is difficult.

Early in the 20th century, the horse was still an animal of war, and epidemics of equine respiratory disease could cripple army divisions relying on horses for cavalry or draught. Thus, unlike today, research on equine diseases enjoyed military financial support. In these early studies, streptococci were commonly isolated from cases of respiratory and septicemic

disease. However, while many cases of influenza terminated in severe, and sometimes fatal streptococcal pneumonia, streptococci could not transmit the condition. In addition, normal horses carried hemolytic streptococci in the nasal mucosa and throat. Thus streptococcal respiratory infections, it was argued, resulted from predisposing factors, such as viral infection, poor environment and stress (Jones 1919). Early on, the equine pathogen *S equi* was distinguished by its failure to ferment lactose, sorbitol and trehalose, and by its strict association with the disease known as equine strangles (Edwards 1933; Edwards 1934). The great majority of equine streptococcal isolates, including isolates from equine endometritis, fermented lactose and sorbitol, but not trehalose. Similarly, the great majority of pyogenic streptococci from other animals (excluding the group B streptococci of bovine mastitis) shared this fermentative pattern. Thus a wide variety of streptococcal infections in animals were attributable to the same organism, now known as *S zooepidemicus* (Farrow and Collins 1984; Rotta 1984). Because of its clinical similarities to the group A streptococcus it was regarded as the "animal *pyogenes* " (Sherman 1937). When Lancefield serologically defined the animal streptococci as group C, more than 90% of her isolates were *S zooepidemicus*, including 4 isolates from the equine uterus. When Edwards confirmed Lancefield's groups A and C, similarly more than 90% of group C cultures were *S zooepidemicus*. More than half of these were isolates from the

equine uterus, the same isolates he and Dimock had obtained from the survey of Kentucky breeding farms described at the beginning of this chapter. Thus Rebecca Lancefield and the pioneers of equine endometritis briefly crossed paths (Edwards 1933; Edwards 1934; Lancefield 1933).

Infection of humans by *S zooepidemicus* is extremely rare, but when it occurs a serious infection results which is clinically indistinguishable from group A streptococcal infection. Early work suggests that human disease due to *S zooepidemicus* never occurs (Brown 1937; Edwards 1934), but this is now known to be untrue. The most common source of human infection by *S zooepidemicus* is consumption of improperly pasteurized milk, resulting in milkborne sore throat. Sometimes acute glomerulonephritis, a classic group A streptococcal sequela, may follow. These outbreaks have been the result of failed pasteurization (Duca et al. 1969), or a general public knowingly consuming unpasteurized milk as a "health food" (Barnham et al. 1989; Barnham, Thornton and Lange 1983; Edwards and Roulson 1988). In other parts of the world, pork has been identified as a vehicle of human infection with *S zooepidemicus*. (Yuen et al. 1990). Close human contact with horses has been associated with pneumonic pleuritis due to *S zooepidemicus*, clinically indistinguishable from classic group A streptococcal pneumonia (Rose, Allen and Witte 1980).

The question "Do equine uterine isolates of *S zooepidemicus* carry M protein?" becomes a natural one to ask for several reasons: *S zooepidemicus* and the group A streptococci cause similar diseases within their hosts; extensive work in endometritis focusses on phagocytosis of *S zooepidemicus* by neutrophils; there is no serologic typing system in use to differentiate and epidemiologically track different strains of *S zooepidemicus*. Lancefield commented, in 1941, that preliminary studies suggested that a trypsin labile, type-specific substance existed among group C strains (Lancefield 1941). However, more recently, Moore and Bryans demonstrated a type-specific substance in equine isolates of *S zooepidemicus* which could be used as the basis of a typing system. Using acid extracts they repeated Lancefield's demonstration of immunoprecipitation using homologous, but not heterologous antisera. Of 155 cultures, 8 antisera successfully defined 52 isolates (Moore and Bryans 1969). Unfortunately their work, unlike Lancefield's, was an isolated demonstration. There were no mouse protection or bactericidal tests results to independently confirm their serotypes. Their typing sera were never widely used in subsequent epidemiologic investigations by others, and were subsequently discarded (Timoney et al. 1995). Nevertheless, their work comes tantalizingly close to demonstrating an M protein in equine isolates of *S zooepidemicus* in the spirit of Lancefield. It

is especially interesting that 29 of the isolates which they typed originated from the equine uterus.

Attempting to identify and clone an M-like protein of *S zooepidemicus*, without the frame work of well defined typing sera, is difficult. Mutanolysin digestion of *S zooepidemicus* releases a number of potentially M-like proteins, which differ substantially between isolates in molecular weight, suggesting great potential antigenic variability between strains of *S zooepidemicus* (Timoney and Mukhtar 1992). One of these proteins, when injected into guinea pigs, produces opsonic antisera to *S zooepidemicus* in a modified bactericidal test (Timoney and Mukhtar 1992). The same protein from a strain of *S zooepidemicus* (W60) was cloned in *E coli* and sequenced (Timoney et al. 1995). When mice were immunized with the *E coli* recombinant they were protected against the original *S zooepidemicus* strain, but not against *S equi*. This cloned M-like recombinant protein, now known as the *S zooepidemicus* protective protein (SzP), was presumptively identified in many of the Moore and Bryans strains by the polymerase chain reaction. However, the variation in SzP sequences between W60 and the Moore and Bryans isolates is confined to a small hypervariable region in the center of the molecule which does not extend throughout the N terminal half like the group A M proteins (Walker and Timoney 1994). The SzP lacks many other features normally associated with group A M proteins, such as repeating blocks of

amino acids and possesses a shorter signal sequence. However, like other streptococcal cell wall proteins, SzP contains a highly conserved carboxy terminal region, with a typical membrane anchor sequence and the highly conserved LPSTGE motif (Walker and Timoney 1994). It is not known if SzP is the type-specific protein identified by Moore and Bryans, since the typing sera were discarded. Proof that SzP is the type-specific antigen would require creation of a new set of typing sera (Timoney et al. 1995). In other words, accurate molecular definition of M protein genes in *S zooepidemicus* requires the framework of the typing system established by Lancefield in 1928.

1.4. SUMMARY: M PROTEIN AND EQUINE ENDOMETRITIS. Research on equine endometritis has focussed to a large extent on streptococcal phagocytosis by neutrophils. Streptococcal M protein is a well established antiphagocytic virulence factor of the group A streptococci, but its presence on *S zooepidemicus* is still open to question. Although it is difficult to demonstrate the presence of M protein on *S zooepidemicus*, antiphagocytic properties of M+ streptococci are easily demonstrated by their ability to grow in fresh blood. In addition, killing in fresh blood is a convenient method to demonstrate the presence of type-specific antibodies to M+ organisms and may be used as the basis for a simple typing system, a necessary prerequisite for subsequent demonstration of M protein. A simple way of attacking the M protein issue as it relates to equine endometritis is to explore the

ability of *S zooepidemicus* to grow in fresh equine blood. It combines the ability to demonstrate resistance to phagocytosis, type isolates, demonstrate anti-streptococcal antibody, and elucidate the requirements for successful streptococcal opsonization and phagocytosis. These issues are important to understand phagocytosis of *S zooepidemicus* and may in turn shed light on the pathogenesis of equine endometritis.

CHAPTER 2
ANTIPHAGOCYtic PROPERTIES OF *STREPTOCOCCUS ZOOEPIDEMICUS*
AND MECHANISMS OF KILLING IN FRESHLY OBTAINED BLOOD OF
HORSES*

The pathogenesis of endometritis, a major cause of infertility in brood mares, is incompletely understood although much progress has been made in recent years (Allen 1993; Varner and Blanchard 1990). An approach commonly followed has been to compare mares that are resistant with those that are susceptible to bacterial infection of the uterus (Asbury et al. 1980; Asbury et al. 1982; Cheung et al. 1985; Evans et al. 1987; LeBlanc et al. 1991; LeBlanc, Asbury and Lyle 1989; Troedsson and Liu 1991; Troedsson and Liu 1992; Watson and Stokes 1990; Williamson et al. 1983). *Streptococcus zooepidemicus* is the organism most commonly isolated from infected mares (Shin et al. 1979), and has been used extensively in experimental infections. Current knowledge of endometritis in mares identifies accumulations of poorly opsonic uterine fluid, particularly in older animals, as a major contributor to the disease (Allen 1993; Asbury et al. 1982; Cheung et al. 1985; LeBlanc, Asbury and Lyle 1989; Troedsson and Liu 1991). Although accumulation of fluid may result from impaired mechanical clearance or cervical dysfunction, the factors underlying opsonic failure are still unclear (LeBlanc et al.

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1991). This is largely attributable to rapid degradation of complement in uterine fluid (Asbury, Gorman and Foster 1984), making studies in this milieu difficult. This is further compounded by scarcity of published data on general requirements for phagocytosis of uterine isolates of *S zooepidemicus*. Experiments were therefore conducted in fresh heparinized equine blood to determine whether *S zooepidemicus* could resist phagocytosis and, if so, what were the components of an effective phagocytic response.

2.1. MATERIALS AND METHODS.

2.1.1. HORSES. Horse A was a healthy, adult, ovariectomized Quarter Horse mare housed separately from other horses. Horses B, C, D, E, and F were healthy brood mares of various ages and breeds selected arbitrarily from the veterinary school teaching herd.

2.1.2. COLLECTION OF ISOLATES. Isolates UF-1 (Dr. Michelle LeBlanc, University of Florida, Gainesville) and CA-1 (Dr. Irwin K. Liu, University of California, Davis) were obtained from ongoing research programs in equine endometritis at other institutions. Isolates H-1 and H-2 were obtained from uterine lavage fluid from clinical cases at the Louisiana State University Veterinary Teaching Hospital and Clinic. All other equine uterine isolates were obtained from clinical cases, using standard uterine culture techniques and a sterile guarded swab (Accu-med Corporation, Pleasantville, NY), and were isolated in the clinical microbiology section of the Louisiana Veterinary Medical Diagnostic Laboratory.

2.1.3. EXPERIMENT 1. The ability of *S zooepidemicus* to evade blood's natural bactericidal properties was assessed by its ability to grow in freshly obtained equine blood. Twenty-two animal streptococcal isolates, predominantly from the uterus of mares, were tested in a single semi-quantitative bactericidal test to determine whether they could grow in fresh heparinized blood from a single horse (horse A). Isolates were also tested for growth in the sugars lactose, maltose, sorbitol, and trehalose to confirm their identity.

2.1.4. EXPERIMENT 2. To document that growth in experiment 1 was not restricted to horse A, and to illustrate antigenic variation of the strains, isolates were tested for their ability to grow in blood from additional horses. Five isolates that were able to grow in blood from horse A were, therefore, tested for their ability to grow in blood from horses B, C, D, E, and F in semi-quantitative bactericidal tests. Sufficient blood was drawn from each horse to allow testing of all 5 isolates simultaneously, thus ensuring that all isolates were incubated in blood from a given horse. The test was repeated, using the same isolate and horse, within 3 hours of the first test, and consistency of results was assessed by comparison of the outcome of the first and second tests.

2.1.5. EXPERIMENT 3. The purpose of this experiment, performed using quantitative bactericidal tests, was to determine whether killing required leukocytes, and whether a specific signal

for killing resided in plasma. Isolate CA-1 and blood from horse C were used as a prototype model to study killing mechanisms. Killing of isolate CA-1 was confirmed by incubation in fresh blood from horse C. Isolate CA-1 was then incubated in cell-free plasma of horse C. In these 2 experiments, blood and plasma respectively of horse A were included as controls. Three tubes of blood from horse C were then centrifuged. The buffy coat was left intact in the first tube, partially removed in the second, and completely removed in the third. After resuspension of the blood, samples were taken from each tube for leukocyte count determination. The ability of isolate CA-1 to grow in blood from variable leukocyte counts was then tested. To study the role of plasma, blood from horse A was centrifuged and the plasma supernatant was replaced with 3 combinations of plasma from horses A and C (A/C = 5.0 ml/0 ml, 4.5 ml/0.5 ml, 0 ml/5.0 ml). The growth of CA-1 was then measured in this mixture of horse-A cells resuspended in combinations of horse-A and horse-C plasma.

2.1.6. EXPERIMENT 4. To investigate the potential roles of antibody and complement, the plasma supernatant of horse-A centrifuged blood was replaced with heat-treated horse-C plasma. Growth of CA-1 was measured in this mixture of heat-treated horse-C plasma and horse-A cells. Heat treatment (57 C for 30 minutes) destroys complement, but leaves antibody unaffected (Asbury, Gorman and Foster 1984; Hansen and Asbury 1987; LeBlanc et al. 1991). Horse-A cells resuspended in horse-A plasma

and unheated horse-C plasma were included as controls. The experiment was performed 3 times. The protocol was then adjusted to remove residual horse-A plasma as follows. Horse-A blood was centrifuged, and the supernatant was replaced with heat-treated horse-C plasma. After resuspension, the mixture was centrifuged a second time and the supernatant was removed. The plasmas representing treatment (heated horse-C plasma) and controls (unheated horse-A and unheated horse-C plasma) were then added as described previously. This adjustment was tested once at 3 streptococcal concentrations. Finally, having washed horse-A cells in heat-treated horse-C plasma according to our adjusted protocol, different ratios of heated horse-C plasma (HC) and fresh horse-A plasma (UA) were added (HC/UA = 5 ml/0 ml, 2.5 ml/2.5 ml, 0 ml/5 ml). The ability of isolate CA-1 to grow was measured after fresh plasma of horse A was returned to the system. This experiment was performed 3 times at 3 streptococcal concentrations. The experiment also was performed, using isolate H-2 and heated plasma from horse D. It was repeated, using a streptococcal isolate (T-5) from a recent case of endometritis, using heated plasma from the mare infected with that isolate (horse D).

2.1.7. EXPERIMENT 5. To determine the stability of expression of growth in fresh blood, growth was assessed before and after extensive passage of the isolates through artificial media. Isolates used in experiment 2 were passed through Todd-

Hewitt broth for 10 passages. They were then tested for their ability to grow in fresh blood from horse A.

2.1.8. COLLECTION AND HANDLING OF BLOOD. Blood was obtained by sterile jugular venipuncture into sterile vacuum tubes containing 143 U of lithium heparin (Vacutainer, Beckton Dickinson, Rutherford NJ). Collected blood was immediately brought to the laboratory, and maintained at 20 to 22 C and processed according to which experiment was being performed. Incubation of blood for growth tests was achieved within 2 hours of collection.

2.1.9. INOCULATION OF BLOOD. The blood was inoculated as follows. Each blood sample or processed blood mixture was added to a set of 8 sterile glass tubes in 1.5-ml aliquots. The first tube was inoculated with approximately 0.025 ml from a fresh blood stock of the organism. The remaining tubes were inoculated by the serial passage of approximately 0.025 ml from tube to tube, starting from the inoculated first tube, to provide a series of up to 8 tubes with variable streptococcal concentrations, labeled from 1 to 8 in order of increasing dilution. This usually provided a series of at least 3 tubes that would give convenient values for counting.

2.1.10. SEMI-QUANTITATIVE BACTERICIDAL TEST. This test was used in all experiments except experiment 3. Immediately after inoculation, a 0.05-ml aliquot was taken from the tubes and streaked over the entire half of a blood agar plate. The tubes were

then incubated for 3 hours at 37 C, being gently rotated end over end at approximately 8 RPM. After incubation, a 0.05-ml aliquot from each tube was streaked over the entire half of each plate opposite the streak from the same tube 3 hours earlier. After incubation of the blood plates at 37 C for 24 hours, the streaks at the beginning (0 hour) and end (3 hours) of incubation were then compared. When a streak yielded 99 colonies or fewer, the actual number of colonies was recorded. If 100 or more colonies were present, they were scored as follows: +++ = 100 or more separate individual colonies; ++++ = partly lawn and partly individual colonies; +++++ = continuous lawn of bacteria.

2.1.11. QUANTITATIVE BACTERICIDAL TEST. This protocol was followed in experiment 3 only. After inoculation of the tubes, a 0.1-ml aliquot was taken from each tube at 0 hour and placed in a sterile petri dish, into which was poured approximately 22 ml of fresh blood agar. The mixture was gently agitated to evenly disperse the inoculum. After the agar solidified, the plates were incubated at 37 C. The procedure was repeated in a second set of petri dishes after the tubes had been incubated at 37 C for 3 hours, being gently rotated end over end at approximately 8 RPM. The next day, colonies in the plates were counted, and the numbers from each tube at 0 hour and 3 hours were compared. Individual colonies were easily seen by their zone of complete hemolysis. Colonies were counted on a back-lighted colony counter (American Optical, Buffalo, NY).

2.1.12. MAINTENANCE AND STORAGE OF ISOLATES. Prior to experiment 1, all isolates were cultured on artificial media for the limited number of passages necessary to obtain a pure culture and make a frozen stock in skim milk. However, the isolates used in experiments 2-5 were subsequently maintained by growth in fresh blood from horse A as follows. Up to 8 tubes of blood from horse A were inoculated to variable dilutions similarly to the bactericidal tests. They were then incubated at 37 C, while being gently rotated end over end, for 24 hours. After incubation, tubes were generally either completely hemolyzed (port wine appearance) or unhemolyzed. In the hemolyzed tubes, bacteria had grown to high numbers, whereas in the unhemolyzed tubes, bacteria were absent. The second lowest dilution to be completely hemolyzed was used as a stock for experiments and continued propagation of the isolate. The isolate was stored in hemolyzed blood at 4 C for up to a week. Long-term storage was accomplished by freezing and storing freshly grown hemolyzed blood cultures at - 70 C.

2.1.13. BLOOD CENTRIFUGATION. All blood centrifugation was performed at 800 x g for 20 minutes.

2.1.14. LEUKOCYTE COUNTS. Leukocyte counting was performed, using a hemocytometer.

2.2. RESULTS.

2.2.1. EXPERIMENT 1. Of 22 isolates screened, 8 had the ability to grow in fresh blood from horse A. All of these 8 were

speciated as *S zooepidemicus* and came from the equine uterus, except 10323, which was originally isolated from an equine nasal swab specimen (Table 2.1).

Table 2.1-Bactericidal test results (+ or - growth) of 22 streptococcal isolates in blood from horse A (experiment 1)

Isolate	Identification*	Growth	Source
UF-1	<i>S zooepidemicus</i>	+	Equine uterus
CA-1	<i>S zooepidemicus</i>	+	Equine uterus
H-1	<i>S zooepidemicus</i>	+	Equine uterus
H-2	<i>S zooepidemicus</i>	+	Equine uterus
4589	<i>S zooepidemicus</i>	+	Equine uterus
10323	<i>S zooepidemicus</i>	+	Eq nasal swab
10258	<i>S equisimilis</i>	-	Equine uterus
10118	<i>S zooepidemicus</i>	-	Equine uterus
10137A	Uncertain	-	Equine uterus
7340	<i>S zooepidemicus</i>	+	Equine uterus
7106	<i>S equi</i>	-	Eq lymph node
6931	<i>S zooepidemicus</i>	-	Equine uterus
3069	<i>S zooepidemicus</i>	-	Equine uterus
30878	<i>S zooepidemicus</i>	-	Equine uterus
31377	<i>S zooepidemicus</i>	-	Equine uterus
31956	Uncertain	-	Canine Vagina
32174	<i>S zooepidemicus</i>	-	Equine uterus
9651	<i>S zooepidemicus</i>	-	Equine uterus
8167	<i>S zooepidemicus</i>	-	Equine uterus
6540	<i>S zooepidemicus</i>	-	Equine uterus
7939	<i>S zooepidemicus</i>	+	Equine uterus
9999	Uncertain	-	Equine uterus

* By growth in lactose, maltose, sorbitol, and trehalose
Eq = Equine

2.2.2. EXPERIMENT 2. All isolates grew in blood from some horses, but were killed in blood from other horses. However, the horses in whose blood an isolate could grow or was killed were different for each isolate, with the exceptions of H-1 and H-2. The first test results for all horse/isolate pairs agreed with the second test results, with the exceptions of isolate UF-1/ horse C and CA-1/ horse F (Table 2.2).

Table 2.2-Paired bactericidal tests, 3 hours apart, of 5 streptococcal isolates in blood from 6 horses (experiment 2)

Isolate	Horse											
	A		B		C		D		E		F	
UF-1	+	+	+	+	+	+/-	+	+	+	+	+/-	+/-
CA-1	+	+	-	-	-	-	+	+	+	+	-	+/-
10323	+	+	+	+	-	-	+	+	+	+	-	-
H-1	+	+	-	-	+	+	-	-	+	+	-	-
H-2	+	+	-	-	+	+	-	-	+	+	-	-

+ = streptococcal numbers consistently increase

- = streptococcal numbers consistently decrease

+/- = no consistent change in streptococcal numbers

2.2.3. EXPERIMENT 3. When incubated in fresh blood from horse A, numbers of CA-1 increased over the 3-hour incubation period, whereas they decreased in blood from horse C. Numbers of CA-1 increased in cell-free plasma of horses A and C (Table 2.3). As more leukocytes were removed from blood from horse C, numbers of CA-1 decreased less precipitously (Fig 2.1). As the

Table 2.3-Summary of bactericidal test results of isolate CA-1 after incubation in fresh blood and plasma of horses A and C (experiment 3)

Medium	Horse A	Horse C
Fresh blood	+	-
Plasma	+	+

+ = Streptococcal numbers consistently increase
 - = Streptococcal numbers consistently decrease

Table 2.4-Summary of bactericidal tests of isolates after incubation in blood from horse A, before and after 10 passages through artificial media (experiment 5)

Time	UF-1	CA-1	10323	H-1	H-2
Before	+	+	+	+	+
After	-	-	-	-	-

+ = Streptococcal numbers consistently increase
 - = Streptococcal numbers consistently decrease

Figure 2.1 (Overleaf)-Results of a 3-hour incubation of isolate CA-1 in blood from horse C (experiment 3), in which CA-1 is killed, at 3 WBC counts (top), and in blood from horse A (bottom), in which CA-1 grows, having replaced the plasma supernatant with different combinations of horse-A plasma (A) and horse-C plasma (C). Points above the dotted line represent growth; points below the dotted line represent killing.

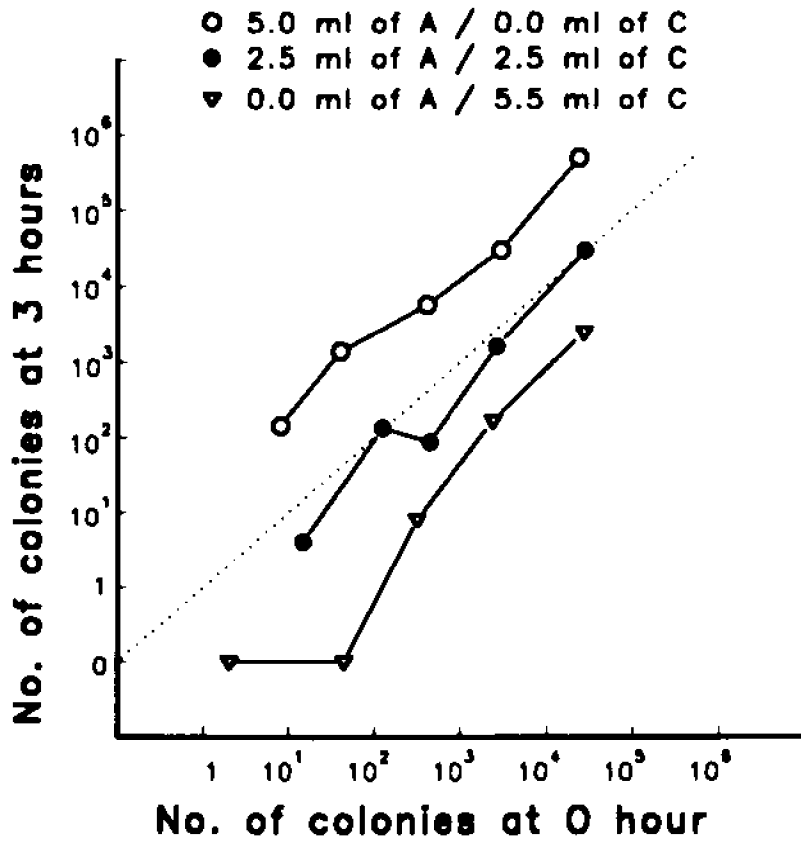
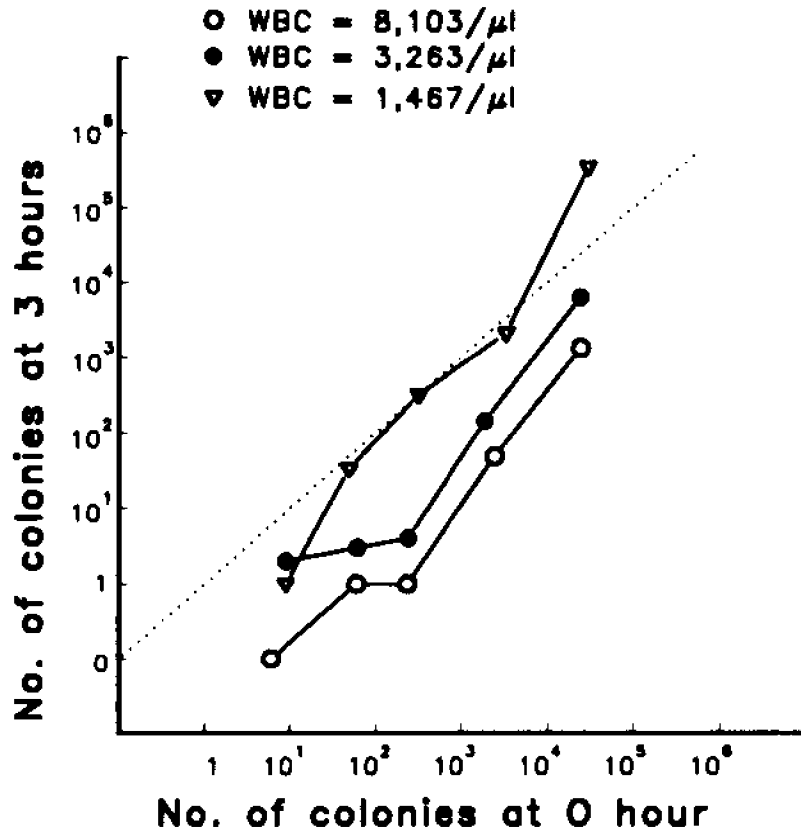


Figure 2.2 (Overleaf)-Results of a 3-hour incubation of isolate CA-1 in blood from horse A (experiment 4), having replaced the plasma supernatant with heated or unheated plasma of horse C (top). Results with unheated plasma of horse A are included as a control. Each point represents an average value from 3 runs. The experiment was then repeated for a single run after washing horse-A cells in heated horse-C plasma to remove residual traces of horse-A plasma (bottom). Points above the dotted line represent growth; points below the dotted line represent killing.

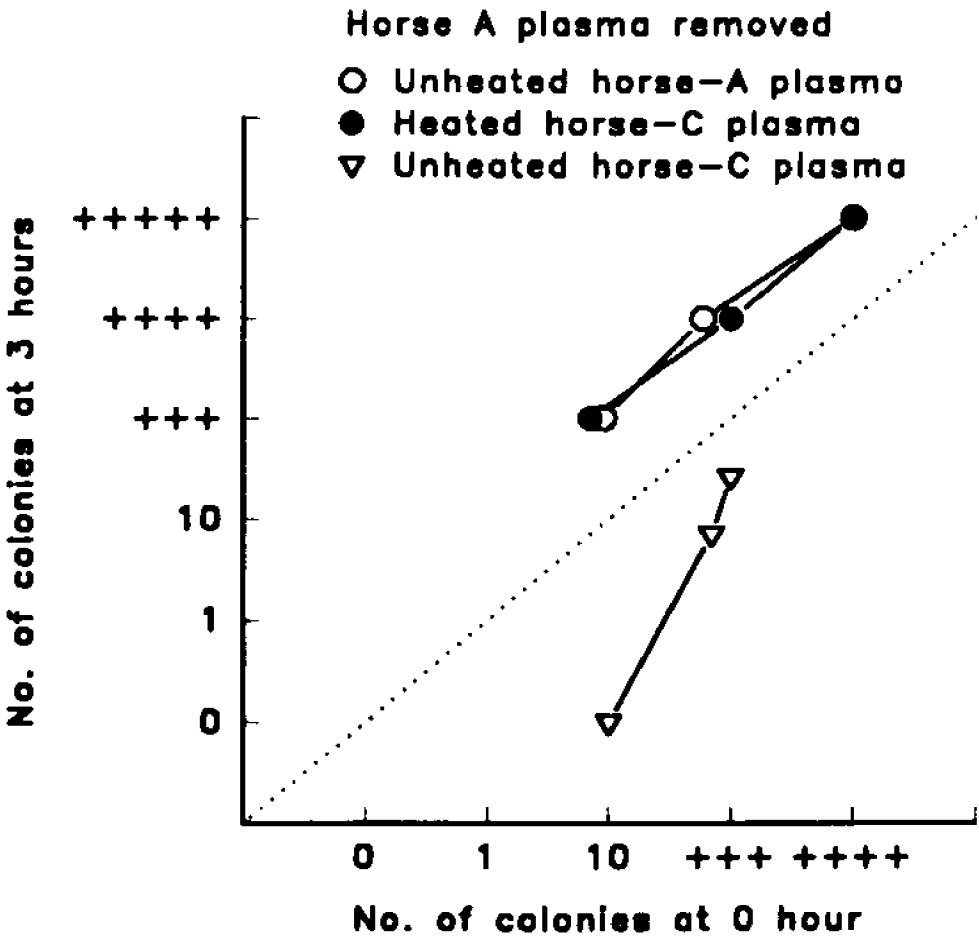
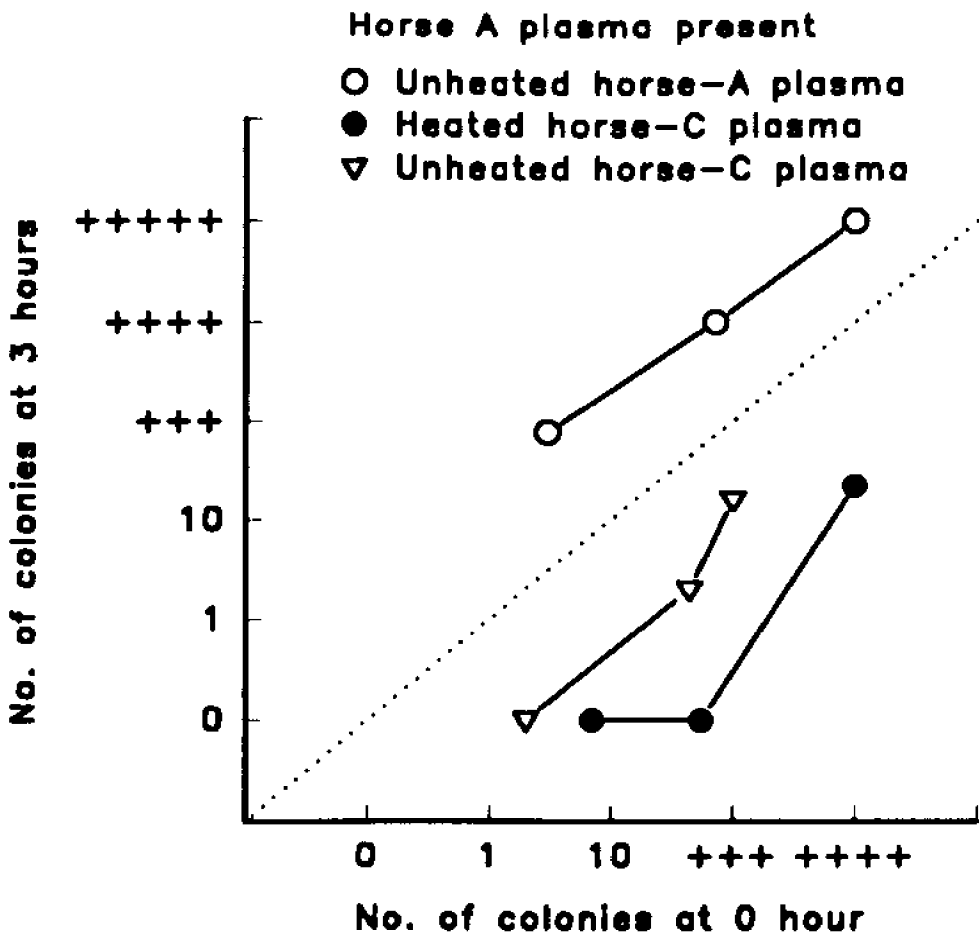
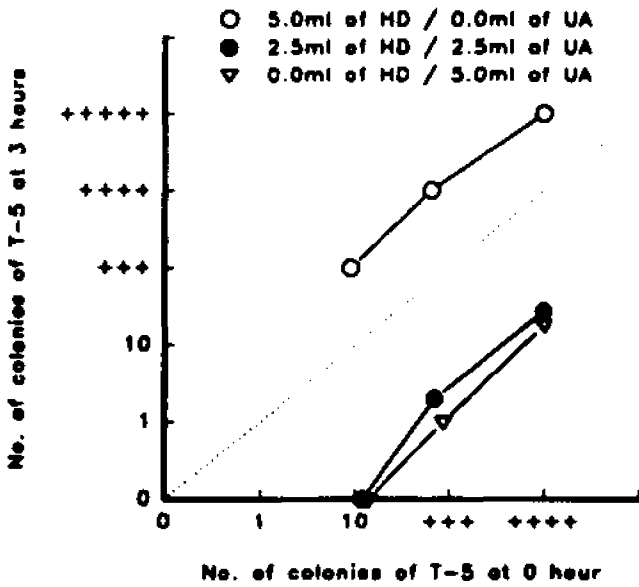
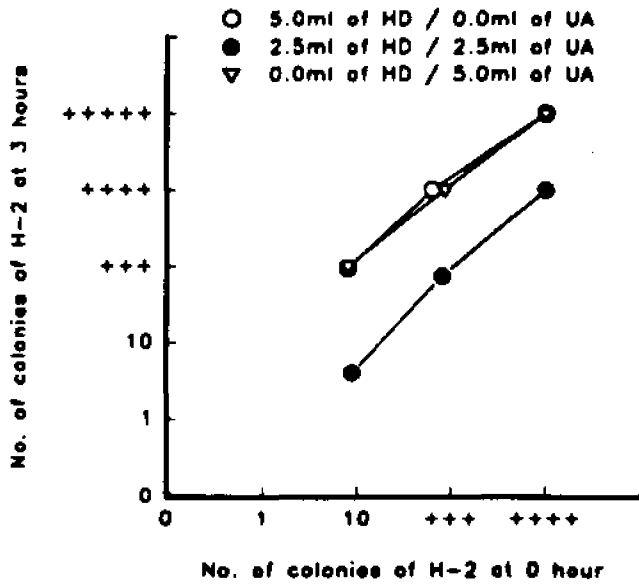
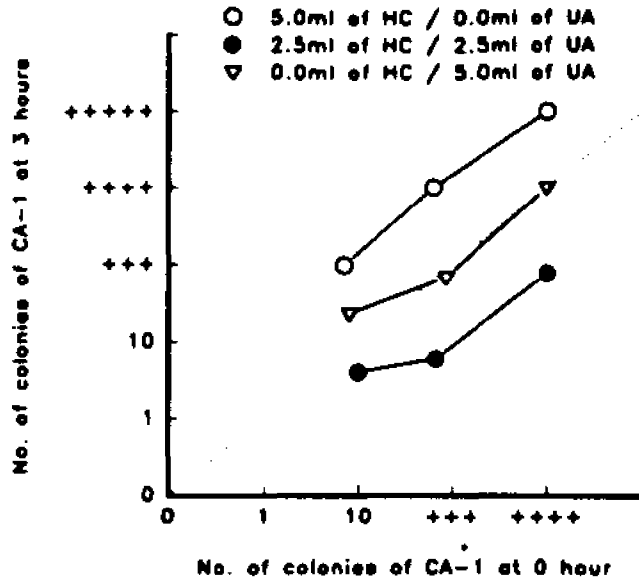


Figure 2.3 (Overleaf)-Results of a 3-hour incubation of isolate CA-1 in the cell fraction of horse A (experiment 4 continued), washed in heated horse-C plasma, and resuspended in a supernatant consisting of combinations of heated horse-C plasma (HC), and unheated horse-A plasma (UA; top). The experiment was repeated using isolates H-2 (middle) and T-5 (bottom) incubated in the cell fraction of horse A, washed in heated horse-D plasma (HD), and resuspended in a supernatant consisting of combinations of HD and UA. (Isolates H-2 and T-5 grew in blood from horse A, but were killed in blood from horse D). Each point represents an average value from 3 runs. Points above the dotted line represent growth; points below the dotted line represent killing.



amount of plasma from horse C added to horse-A blood cells increased, growth of CA-1 was less, and numbers decreased markedly in the tube with most horse-C plasma.

2.2.4. EXPERIMENT 4. Heat treatment of plasma from horse C did not diminish its ability to direct blood cells of horse A to kill CA-1 when traces of horse A-plasma were present. However, when all horse A-plasma was removed from the horse-A cell fraction, the cells, to which heat-treated plasma was added, no longer had killing ability (Fig 2.2). When fresh plasma from horse A was added back, killing resumed most strongly when approximately equal proportions of unheated horse-A plasma and heated horse-C plasma were added (Fig 2.3). The degree of killing was less when H-2 was tested in this system, using heated plasma from horse D. Nevertheless, growth was markedly reduced when approximately equal proportions of heated and unheated plasma were present. Killing of T-5 was marked when heated plasma of horse D, the horse from which the organism was isolated, was used in this system (isolate T-5 grew in fresh blood from horse A). In all these horse/isolate pairs, killing only occurred when unheated plasma of horse A was present.

2.2.5. EXPERIMENT 5. After 10 passages through Todd-Hewitt broth, the isolates had lost the ability to survive in blood from horse A (Table 2.4). Whether this loss was permanent or temporary has not yet been fully assessed, but limited attempts to induce

isolates that are killed to grow in fresh blood have not yet been successful (data not shown).

2.3. DISCUSSION

Experiments were conducted to document antiphagocytic properties of *S zooepidemicus*, and the requirements for successful killing of the organism in fresh equine blood. Experiment 1 indicated that at least 8 of 22 clinical streptococcal isolates were able to evade the natural bactericidal properties of fresh blood. This was confirmed in experiment 2, when 5 of these isolates were tested and grew in blood from other horses. Of the 14 isolates killed in blood from horse A, it is possible that some would have grown when tested in blood from other horses. However, the possibility also exists that some of these isolates lack the ability to grow in fresh blood altogether, or have lost the ability during isolation or passage on artificial media. Natural bactericidal properties of blood are believed to arise from activation of the alternate pathway of complement by recognition of foreign material, such as peptidoglycan (Bisno 1979; Greenblatt, Boackle and Schwab 1978; Tauber, Polley and Zabriskie 1976). This nonspecific recognition leads to deposition of opsonins, particularly C3B, on streptococcal cell walls, leading to phagocytosis. Growth of streptococci in fresh blood, therefore, suggests evasion of the alternate pathway of complement activation (Bisno 1979).

However, having documented growth in fresh blood from some horses, experiment 2 also indicated that the clinical isolates were killed in blood from other horses. Furthermore, blood from different horses killed different isolates, with the exception of isolates H-1 and H-2. These 2 isolates were obtained from 2 horses on the same farm during the same month, and probably represent the same strain. The consistency of the test and retest results (23 out of 25 agreeing pairs) indicates that the bactericidal tests were sufficiently specific to differentiate between strains. It was, therefore, hypothesized that the isolates carried variable surface epitopes, specific antibodies to which provoked an effective phagocytic response. These streptococci would be able to withstand nonspecific phagocytosis mediated by alternative pathway of complement activation, but would succumb to specific phagocytosis mediated by specific antibody to epitopes on the bacterial cell surface. *Streptococcus zooepidemicus* has been documented by others to have substantial antigenic and genetic variability (Moore and Bryans 1969; Skjold et al. 1987; Timoney et al. 1991).

The first step in testing this hypothesis was to determine whether killing required leukocytes, in addition to specific signals in plasma. The horse/isolate pair, horse C/isolate CA-1, was arbitrarily selected to study the killing process. In experiment 3, isolate CA-1 was rapidly killed in fresh blood from horse C, but grew in fresh plasma of horse C, and killing was less

as leukocytes were depleted from fresh blood from horse C. Therefore phagocytosis appeared to be a possible mode of killing the organism, as opposed to complement-mediated cell lysis. Examining the role of plasma, horse A's blood cells were induced to kill isolate CA-1 when resuspended in horse-C plasma. Plasma of horse C, thus, appeared to mediate killing of CA-1 by horse-A leukocytes and was, therefore, considered likely to carry specific opsonins for CA-1.

If this was the case, the question, therefore, arose whether these opsonins were antibodies or complement. In experiment 4, heat-treated plasma of horse C, in which complement was thermally destroyed, retained its ability to direct horse A's leukocytes to kill CA-1. Therefore, specific antibody to CA-1 probably directed the killing process. However, when cells of horse A were washed in heat treated plasma of horse C to remove all residual traces of horse-A plasma, killing did not occur, suggesting a role for complement, in addition to antibody, in killing the organism. Furthermore, when horse A's blood cells, similarly washed in heated horse-C plasma, were resuspended in 3 combinations of unheated horse-A plasma and heated horse-C plasma, killing returned as unheated horse-A plasma was added back to the system. Killing was maximal when unheated horse-A plasma and heated horse-C plasma were present in approximately equal quantities. Returning unheated plasma from horse A also was necessary to inhibit growth of isolate H-2, and to cause

killing of isolate T-5. Thus, although all 3 isolates grew rapidly when incubated in fresh blood from horse A, unheated plasma of horse A appeared necessary for killing in this system, supporting the hypothesis that killing requires complement.

A possible explanation for variation in the degree of killing observed in these 3 horse/isolate pairs is variation in antibody titer in the heated plasma. The highest antibody titer would be in heated plasma of horse D against isolate T-5, followed by horse-C plasma against isolate CA-1, and finally horse-D plasma against isolate H-2. This trend can be most easily appreciated by studying the degree of killing when heated plasma (0 ml of HC or HD) was not added as supernatant. These tubes all contained small quantities of their respective heated plasma because this was the medium in which the blood cells of horse A had last been washed. Thus, this small quantity presumably mediated killing when it was present in highest titer (HD/T-5), prevented overall growth at an intermediate titer (HC/CA-1), and was unable to prevent growth at the lowest titer (HD/H-2). More killing might have been observed for isolate H-2 with heated plasma of horse D if a higher ratio of HD to UA had been tested. As a whole, these data are consistent with the hypothesis that killing requires a heat-labile component of plasma, probably complement, in addition to a heat stable component, probably antibody.

The documentation, in experiment 5, that isolates lost the ability to survive in fresh blood after passage in artificial media

suggests that growth in fresh blood is a special ability that can be lost. Because promoting growth capacity by repeated passage in fresh blood from isolates that cannot grow in fresh blood has not been successful, it is possible that the ability to grow in fresh blood may result from an evolved adaptation requiring substantial genetic information.

Of interest in this regard is the possible role of an antiphagocytic capsule. Colony morphology and negative staining, using india ink before and after passage, has not revealed any obvious differences between isolates that can and cannot grow in fresh blood (data not shown). Thus, presence of a large capsule inhibiting phagocytosis is doubtful. However, the possibility of a smaller capsule cannot yet be ruled out, nor can the possibility of a larger capsule on other isolates yet to be examined. Such a capsule might have a role in resistance to phagocytosis, and as a target for opsonic antibody. Whether a capsule exists on these strains, and has an antiphagocytic or immunogenic role will require further study.

These results may provide insight into elimination of *S zooepidemicus* from the uterus of mares. These results suggest that some isolates of *S zooepidemicus* are inherently antiphagocytic and that killing requires specific antibody to surface structures. Cross protection does not occur, because these structures presumably vary from isolate to isolate. When appropriate specific antibody is present, killing appears to be

mediated by leukocytes and complement. The components of the effective killing response, which we have thus suggested, namely, leukocytes, specific antibody, and complement, can be detected in equine uterine fluid. However, our results suggest that horses deficient in any one of these components might have difficulty in eliminating some isolates of *S. zooepidemicus* from the uterus, assuming that phagocytosis of the organism is necessary for its elimination. Although functional leukocytes reliably appear in the infected uterus (Asbury et al. 1982), availability of the other 2 components is, perhaps, less consistent. Specific antibody might be absent early in infection unless previous exposure to the infecting strain had already occurred. Complement, on the other hand, is rapidly degraded in uterine fluid (Asbury, Gorman and Foster 1984). In bactericidal tests, equine blood apparently loses its killing ability after about 6 hours. This might explain why accumulation of uterine fluid is associated with susceptibility to endometritis (LeBlanc, Asbury and Lyle 1989; LeBlanc et al. 1994). Mares that accumulate uterine fluid will have "older" fluid on average, and therefore, lower active complement values, than do mares that do not accumulate fluid. Hence, accumulated uterine fluid might lack opsonic activity (LeBlanc et al. 1991). Despite the data reported here, however, the effective elimination of streptococci from the equine uterus probably also involves local factors not present in fresh equine blood.

Interestingly, these results provide data to support the hypothesis that isolates of *S zooepidemicus* carry M proteins. The behavior of our isolates is strikingly similar to that of the M protein-positive (M+) group A streptococci, the causative agents of streptococcal pharyngitis, rheumatic fever, and acute glomerulonephritis in humans (Lancefield 1957). M Protein is a highly variable surface protein of group A streptococci (Fischetti 1989; Fox 1974; Lancefield 1928a; Lancefield 1962). Streptococci that are M+ are able to resist phagocytosis mediated by the alternative pathway of complement activation (Bisno 1979; Horstmann et al. 1988; Jacks-Weiss, Kim and Cleary 1982), and can only be phagocytized in the presence of type-specific antibody to the specific M protein carried by each M serotype. This is indicated by the ability of M+ streptococci to grow in fresh blood, unless specific anti-M protein serum is added (Lancefield 1957). Evidence already exists suggesting that M protein is carried by *S zooepidemicus* (Moore and Bryans 1969; Timoney et al. 1991), and is expressed by clinical isolates from horses with endometritis (Appendix 1).

CHAPTER 3
PHAGOCYtic KILLING OF *STREPTOCOCCUS ZOOEPIDEMICUS* BY
NEUTROPHILS IN FRESH EQUINE BLOOD

It was previously shown that fresh blood from horses varied in ability to kill specific uterine isolates of *Streptococcus zooepidemicus*. Killing appeared to require leukocytes, heat-stable and heat-labile components of plasma. This led to the current working hypothesis that uterine isolates of *S zooepidemicus* display variable surface antigens, and are killed by phagocytosis requiring neutrophils, isolate-specific antibody and complement. Variation in killing ability of fresh blood would be due to variations in isolate-specific antibody between horses. This pattern of growth and killing of *S zooepidemicus* in fresh equine blood is remarkably similar to behavior of M+ group A streptococci in fresh human blood (Lancefield 1957; Lyons and Ward 1935; Rothbard 1945b; Todd 1927c).

To provide additional support for this hypothesis a comparison was made of phagocytosis of the same isolate in blood from 2 horses by light microscopy. Intracellular killing was estimated by fluorescent microscopy using acridine-orange to assess viability of phagocytized streptococci. It had been shown previously that in blood from one of the horses the isolate multiplied, but in blood from the other horse the isolate was killed.

3.1. MATERIALS AND METHODS.

3.1.1. ISOLATE. A single uterine isolate of *S zooepidemicus*, (SZ), which had been isolated from a case of equine endometritis, was used. This was isolate H-2 from the previous chapter. The isolate was maintained and passaged as previously described.

3.1.2. HORSES. Two adult horses of light breed were used, horse A and horse B. In blood from horse A the isolate was known to multiply, whereas in blood from horse B the isolate was known to be killed.

3.1.3. EXPERIMENTAL OVERVIEW. Identical volumes of the same streptococcal culture were simultaneously inoculated into tubes of fresh blood from each horse. Buffy coat slides were made at 15 minutes and 60 minutes, stained with acridine orange or Giemsa stain, and examined by fluorescent or light microscopy. Microscopic evaluations of killing (percent of intracellular streptococci killed) and phagocytosis (number of streptococci per neutrophil) were made. Number of colony forming units in each of the inoculated bloods was determined at 15 and 60 minutes. Complete white blood cell counts, including differentials, were made on the collected blood from each horse. The experiment was performed on 3 separate occasions, and the combined results statistically analysed.

3.1.4. PREPARATION OF INOCULUM. A drop of a hemolyzed blood culture of SZ was evenly dispersed over the entire surface of a blood agar plate, and the plate incubated overnight at 37 C.

The following day the culture was visually examined to confirm pure and homogenous growth of beta hemolytic streptococci. Streptococci were harvested by rolling a sterile cotton swab over the entire surface of the plate, and then twisting the tip of the swab back and forth in 1.5 ml of sterile saline to release the streptococci. The resulting streptococcal suspension was used to inoculate blood from both horses.

3.1.5. COLLECTION AND INOCULATION OF BLOOD. Jugular venipuncture of horse A and B was performed as previously described. Each horse was sampled within 5 minutes of the other. For each horse, 3 ml aliquots of blood were placed into 3 sterile glass tubes. The first tube (tube 1) was inoculated with 0.5 ml of the freshly prepared streptococcal suspension. The second tube (tube 2) was inoculated by addition of 0.5 ml from freshly inoculated tube 1. The third tube (tube 3) remained uninoculated as a control.

3.1.6. PREPARATION OF BUFFY COAT SLIDES Immediately following inoculation, 1.5 ml samples from tube 2 and 3 were transferred to smaller tubes and centrifuged at 800 g for 15 minutes prior to buffy coat removal. The original tubes 2 and 3, each with 1.5 ml of inoculated blood remaining, were then incubated for 45 minutes at 37 C, being gently rotated at 8 RPM end over end. Following incubation the contents of both tubes were then transferred to smaller tubes and likewise centrifuged at 800 g prior to removal of the buffy coat. Buffy coats were aspirated

with a pasteur pipette and added to a tube containing a single drop of 0.14 mg/ml acridine orange (Sigma Chemical Co, St Louis, MO), also dispensed through a pasteur pipette. After 5 minutes, 4 smears were made of each buffy coat aspirate, 2 for examination by fluorescent microscopy, and 2 for Geimsa staining (Leukostat, Fischer Scientific, Orangeburg, NY) and examination by conventional light microscopy. Because of elapsed time in centrifugation and staining, during which phagocytosis and killing may continue, preparations made prior to incubation were recorded as taken at 15 minutes post inoculation, and those following incubation taken at 60 minutes post inoculation.

3.1.7. DETERMINATION OF COLONY FORMING UNITS. Numbers of streptococcal colony forming units in tube 2 at 15 minutes and 60 minutes were estimated as follows. A 0.1 ml aliquot was serially transferred along 6 tubes, each containing 0.9 ml of sterile phosphate buffered saline. From the last 3 dilutions (10^{-4} , 10^{-5} , 10^{-6}) a 0.1 ml aliquot was spread evenly over a blood agar plate and incubated at 37 C for 24 hours, after which all colonies on the plates were counted. The streptococcal concentration was estimated as the average yielded by the 10^{-4} and 10^{-5} dilutions.

3.1.8. WHITE BLOOD CELL COUNTS AND DIFFERENTIALS. Total white blood cell counts were determined on an automated cell counter (Baker 9000, Baker Instruments Corporation, Allentown, PA) and differential counts were performed manually. The number

of neutrophils or polymorphonuclear leukocytes (PMN) was calculated per μ l of blood.

3.1.9. EVALUATION OF PHAGOCYTOSIS. Giemsa stained smears were labelled, randomized and examined blindly, including uninoculated slides. The entire feathered edge was examined, or 100 PMN, whichever ever came first. Following evaluation the slides were numbered, and only after all slides had been evaluated was the origin of each slide revealed. A phagocytosis index was computed as follows:

$$\text{Phagocytosis index} = \frac{\text{number of PMN associated streptococci}}{\text{total number of PMN examined}}$$

Preliminary studies suggested that the most significant variable affecting phagocytosis index is the total number of streptococci per PMN in the blood. Consequently, a corrected phagocytosis index was computed adjusting for the colony counts and PMN counts as follows:

$$\text{Corrected phagocytosis index} = \text{Phagocytosis index} \times \frac{\text{PMN}}{\text{streptococcal CFU}}$$

Errors in identification of streptococci in Giemsa stained slides were estimated by recording the % of uninoculated slides in which streptococci were observed.

3.1.10. EVALUATION OF INTRACELLULAR KILLING. Acridine orange stained slides were labelled, randomized and examined blindly, including uninoculated slides. Slides were numbered

following examination, and only after all slides had been evaluated was the origin of each slide revealed. The feathered edge of the smear was systematically examined for ingested bacteria. Slides were examined under epi-fluorescence, with mercury arc source of illumination, an excitation filter of 490 nm, and a barrier filter of 510 nm. Green streptococci were counted as live; yellow or orange streptococci were counted as dead. Either the entire feathered edge of each slide was examined, or 100 PMN evaluated, whichever came first. A killing index for each slide was calculated as follows:

$$\text{Killing index} = \frac{\text{Number of dead intracellular streptococci}}{\text{Total number of intracellular streptococci}}$$

An estimate of error in identification of streptococci in acridine orange stained slides was determined by recording the % of uninoculated slides in which live or dead streptococci were observed.

3.1.11. STATISTICAL ANALYSIS. Data was analysed by analysis of variance using the General Linear Models Procedure (GLM) of statistical analysis software (SAS Institute Inc., Cary, NC).

The killing index, which is not normally distributed, underwent transformation prior to analysis as follows:

$$\text{Transformed killing index} = 2 \times \arcsin (\text{killing index})^{1/2}$$

The effect of horse, time and the horse-time interaction was determined on the following dependent variables: transformed killing index, phagocytic index, and corrected phagocytic index.

3.2. RESULTS

3.2.1. PHAGOCYTTIC ABILITY. The mean phagocytic indices of horses A and B were 2.84 and 10.85, a highly significant difference ($P < 0.0001$). Similarly, the corrected phagocytic indices of horses A and B were 0.26 and 4.26 respectively, also significantly different to a high degree ($P = 0.0003$). Phagocytic indices increased significantly with time ($P < 0.0001$) as did corrected phagocytic indices ($P = 0.0037$). Although no significant horse by time interaction could be observed in the uncorrected phagocytic index ($P = 0.1778$), a significant interaction was detected in the corrected phagocytic index ($P = 0.0094$) in which phagocytosis appeared to increase more rapidly in horse B than horse A over time.

No streptococci were identified in any of the Giemsa stained smears from uninoculated blood.

3.2.2. INTRACELLULAR KILLING. Intracellular killing, measured by the transformed killing index, was not significantly different between horses, being 1.08 for horse A and 1.23 for horse B ($P = 0.4826$). However, a highly significant increase in killing was observed over time ($P < 0.0001$). A significant horse by time interaction was observed ($P = 0.015$), in which killing was

Table 3.1-Comparison of phagocytic and intracellular killing ability of horse A versus horse B blood against the same uterine isolate of *Streptococcus zooepidemicus*

	Phagocytic Index	Corrected Phagocytic Index	Transformed Killing Index
Horse A	2.84 (0.67)	0.26 (0.65)	1.08 (0.14)
Horse B	10.85 (0.67)	4.26 (0.65)	1.23 (0.14)
P value	0.0001	0.0003	0.4826

Standard error of the mean in parentheses

Table 3.2-Mean streptococcal colony forming units (CFU) in fresh blood from horse A and horse B before and after a 45 minute incubation

	CFU per microlitre of blood	
	Before	After
Horse A	39,000	71,000
Horse B	47,000	16,000

less in horse A at 15 minutes than in horse B, but at 60 minutes horse A exceeded horse B.

In 24 acridine orange stained uninoculated slides, only 1 slide was recorded as having streptococci, giving an estimated 0.04 error rate in streptococcal identification. In this uninoculated slide a small number of green objects were mistakenly identified as live streptococci; no mistaken identifications of dead streptococci occurred in this or any other uninoculated slide.

3.2.3 STREPTOCOCCAL NUMBERS. In horse A streptococcal numbers increased in all 3 experimental runs with an average 1.8 fold increase in streptococcal numbers over a 45 minute period. In horse B streptococcal numbers fell in all 3 experimental runs with an average 2.7 fold reduction in streptococcal numbers over the same period.

3.2.4. NUMBERS OF NEUTROPHILS. In the 3 successive runs, horse A blood yielded PMN counts of 5100, 4920, and 3800 PMN/ul. Horse B yielded 4800, 6560, and 4600 PMN/ul.

3.2.3. DISCUSSION.

Overall, these results support the hypothesis that differences in the ability of horse blood to kill *S zooepidemicus* may be attributable to differences in phagocytosis. Significant differences were demonstrated in the ability of blood from different horses to phagocytize the same isolate of *S zooepidemicus*. Intracellular killing of phagocytized streptococci

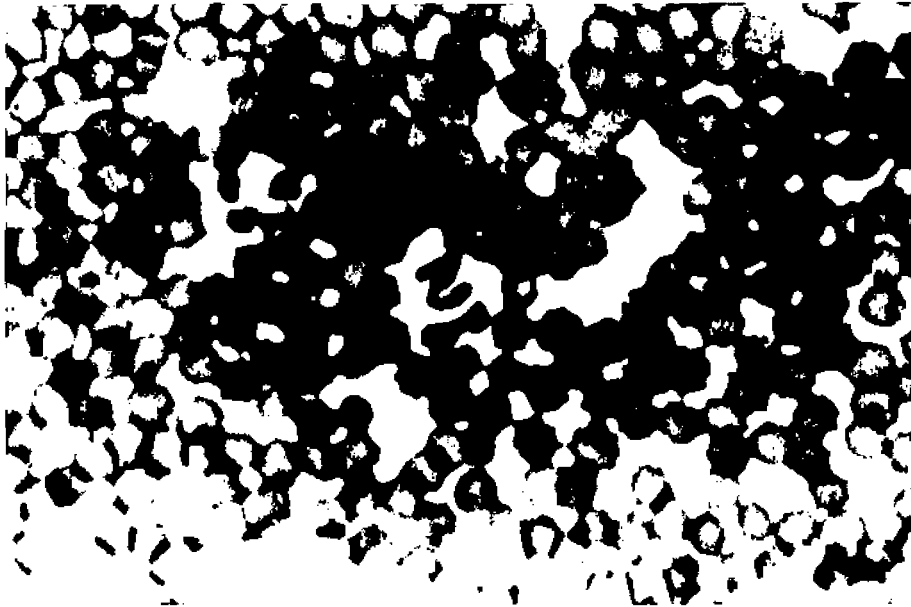


Figure 3.1-Horse A buffy coat at 15 minutes with minimal phagocytosis. Giemsa stain, 1000 X.

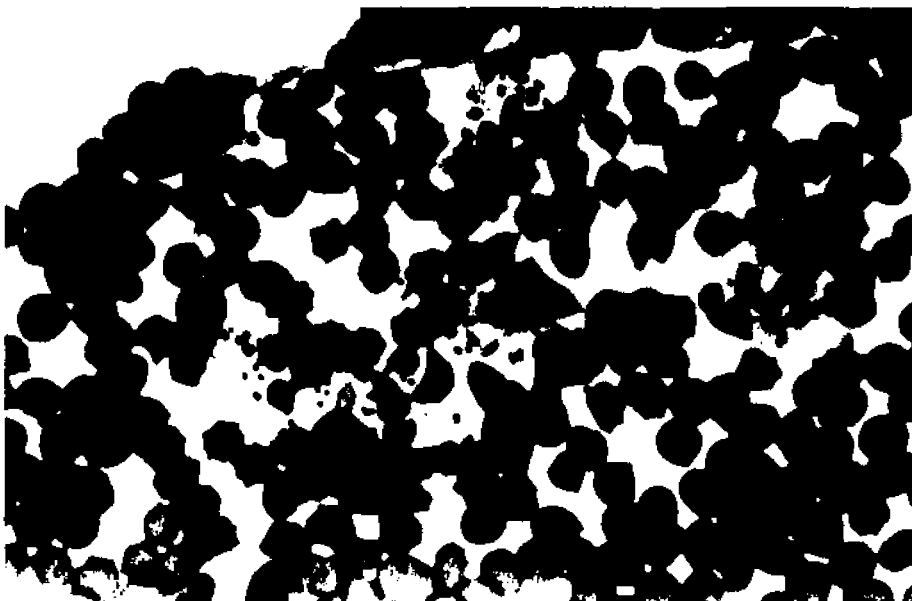
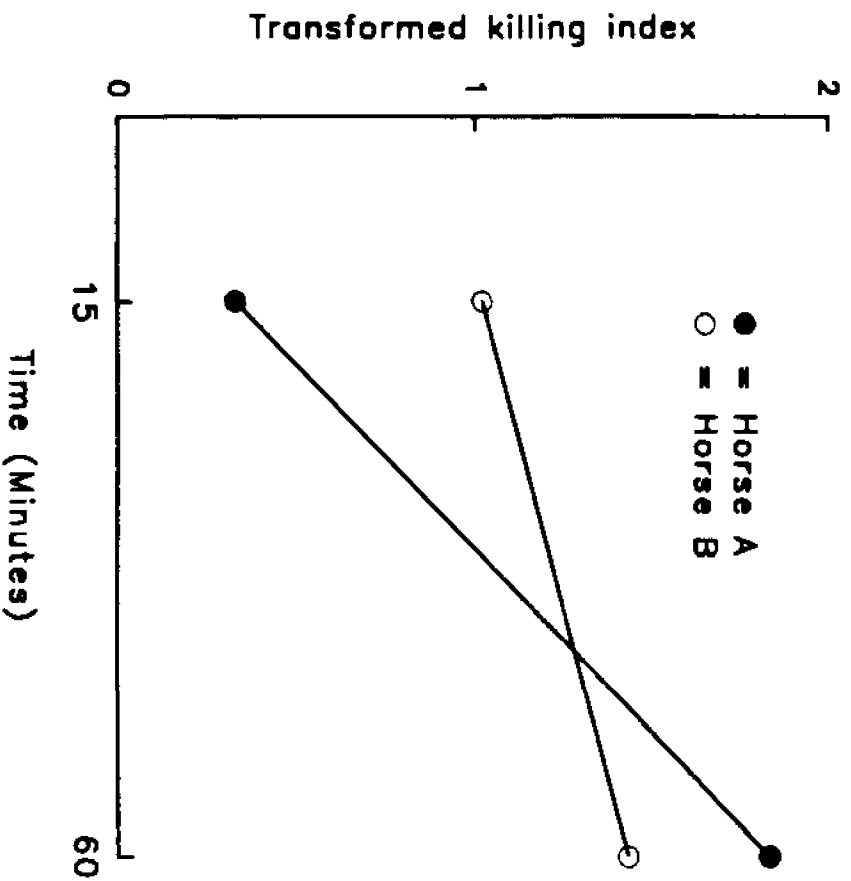
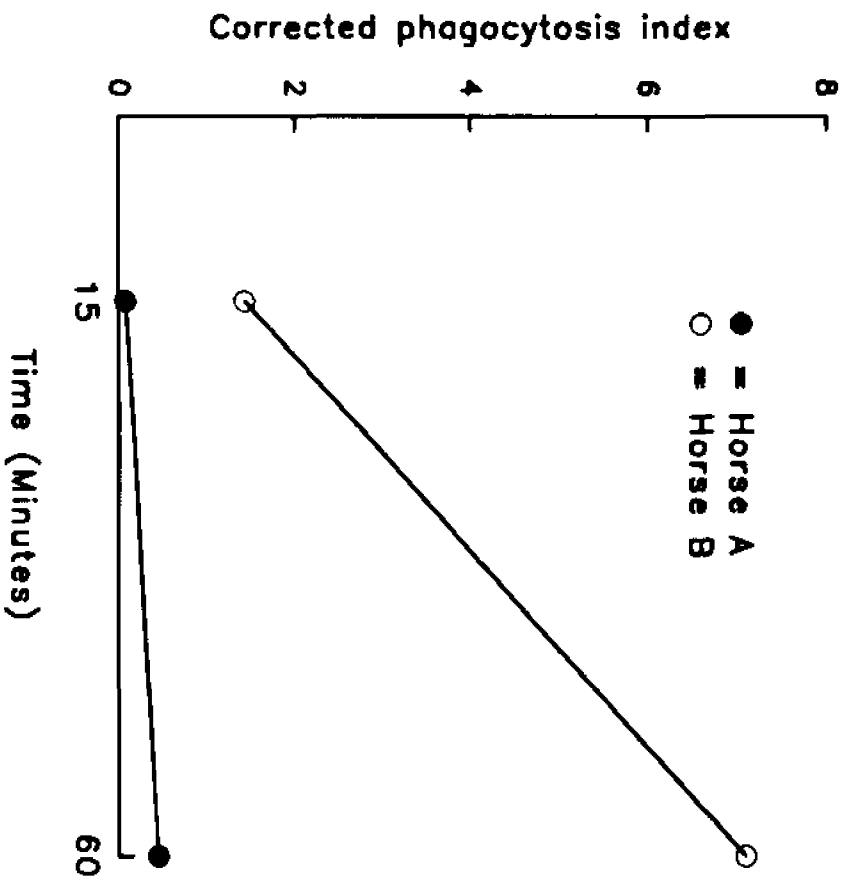


Figure 3.2-Horse B buffy coat at 15 minutes with aggressive phagocytosis. Giemsa stain, 1000 X.

Figure 3.3 (Overleaf)- Changes in corrected phagocytic index and transformed killing index over time in horses A and B.



did not differ between horses, but the degree of killing and phagocytosis increased with time in both horses. Significant horse by time interactions might be explainable by different rates of phagocytosis.

Over the course of the incubation, streptococcal numbers increased in horse A and decreased in horse B, paralleling differences in phagocytic ability between the horses. There was a 4.8 fold difference, in the colony forming units between the 2 horses over the incubation period, with a variation of 30% observed between runs. The 3.8 fold difference in the phagocytic index between the 2 horses falls within this range. Thus the relative differences between the horses in streptococcal colony counts and phagocytosis were similar. When phagocytosis was adjusted for streptococcal numbers and PMN counts, the corrected phagocytic index suggested a 13 fold superiority for phagocytosis for horse B. Thus differences in phagocytosis, based on these results, are of sufficient magnitude to account for the differences in the streptocidal ability of blood from the 2 horses. Due to the results of our previous study these differences are unlikely to be due to inherent defects of horse A's neutrophils, but may result from differences in heat-stable factors in plasma.

Neither horse appeared to be superior in the ability to kill phagocytized streptococci. In acridine orange stained slides, no difference could be detected between horse A and horse B in the proportion of dead to live bacteria. In fact, the killing indices for

both horses were remarkably similar (0.34 for horse A and 0.37 for horse B). Acridine orange has been used by others to determine viability and killing of phagocytized bacteria in PMNs (Cheung et al. 1985; Horn, Hansmann and Federlin 1985; Pantazis and Kniker 1979). At the concentration of acridine orange used, only intracellular (not extracellular) bacteria were stained through trapping of the dye within the PMNs; intracellular granules of eosinophils stained green.

The significant horse by time interaction in the transformed killing index may result from a greater increase in killing over time in horse A than horse B. This arises from significantly lower levels of killing of streptococci in horse A at 15 minutes than horse B. This may be because slower phagocytosis of streptococci in horse A results in recently ingested streptococci that have not yet been killed. More aggressive phagocytosis of horse B results in earlier phagocytosis of streptococci and a greater proportion of killed streptococci at 15 minutes. As time elapses, the streptococci ingested by horse A have had more time to be ingested and the number of killed streptococci proportionately increases relative to horse B. At 60 minutes, the killing index of horse A exceeded horse B, although the magnitude had not reached statistical significance ($P = 0.1680$).

In conclusion, it appears that the differences in killing of *S zooepidemicus* in horse blood is a result of different rates of phagocytosis. In view of the results of previous work, a

reasonable explanation for different rates in the phagocytic ability between the 2 horses would be differences in isolate-specific antibody. This is consistent with the current working hypothesis that uterine isolates of *S zooepidemicus* carry variable surface antigens, antibodies to which lead to effective opsonization and phagocytosis.

CHAPTER 4
KILLING ABILITY OF PERIPHERAL BLOOD DOES NOT DETERMINE
RESISTANCE TO *STREPTOCOCCUS ZOOEPIDEMICUS* INFECTION OF
THE EQUINE ENDOMETRIUM

Phagocytic killing of invading streptococci by neutrophils is considered to be a major component of equine uterine defenses (Asbury, Gorman and Foster 1984; Asbury et al. 1982; Cheung et al. 1985; Peterson, McFeely and David 1969; Washburn et al. 1982). Following intrauterine inoculation of irritants or streptococci, there is a rapid increase in neutrophils, total protein, serum immunoglobulins and prostaglandin E₂ (PGE₂) in uterine fluid (Pycock and Allen 1990; Watson et al. 1987a; Williamson et al. 1987). This response appears to be typical of acute inflammation in other sites in the horse wherein serum proteins and neutrophils are rapidly recruited to the site of inflammation from peripheral blood (Higgins and Lees 1984). Although this response strongly indicates that phagocytosis is an important component of uterine defense, it is uncertain if impaired phagocytosis is an underlying cause of susceptibility to endometritis (Hansen and Asbury 1987; LeBlanc et al. 1991).

It was previously shown that horses consistently differed in their ability to kill specific uterine isolates of *S. zooepidemicus* in peripheral blood. Some isolates were able to grow rapidly in blood of some horses, but were killed in blood of other horses. Killing was shown to require white blood cells,

plus heat-labile and heat-stable components of plasma, suggesting that phagocytosis, complement and isolate-specific antibody were required for killing. The variation in killing between horses was tentatively attributed to variation in isolate-specific antibody in peripheral blood between horses. *Streptococcus zooepidemicus* has been shown by others to display considerable genetic and antigenic variation between isolates (Moore and Bryans 1969; Skjold et al. 1987; Timoney et al. 1991). The bactericidal activity of blood, or direct measurements of phagocytosis, are frequently used to demonstrate opsonizing anti-streptococcal antibodies in serum (Fischetti et al. 1984; Lancefield 1957; Timoney et al. 1991; Timoney et al. 1995).

It is hypothesized that if phagocytic killing by blood-derived neutrophils and serum opsonins is an important component of uterine defense, isolate-specific killing of streptococci in fresh blood would be manifested as enhanced clearance of that isolate following intrauterine inoculation. Horses were therefore inoculated intrauterinely to determine if a relationship existed between the bactericidal activity of fresh blood and the ability to clear intrauterine inocula of *S. zooepidemicus*.

4.1. MATERIALS AND METHODS.

4.1.1. OVERVIEW OF EXPERIMENTAL INOCULATIONS. Four horses received 3 sets of intrauterine inoculations, with a different uterine isolate of *S. zooepidemicus* each time. Horses were first inoculated with isolate SZ-1, then SZ-2, and finally

SZ-3. After each inoculation horses underwent uterine culture and cytology at 48 hours post inoculation (PI) and at the first estrus PI, and were monitored for development of uterine fluid and vaginal discharge. Each horse received each of the 3 isolates, except for horse D who was only inoculated with SZ-2 and SZ-3. The ability of each isolate to grow in the blood of the horses was determined prior to inoculation, and during the weeks following inoculation.

4.1.2. HORSES. Four light breed mares were selected for inoculation from the veterinary teaching herd. The following selection criteria were used: no detectable abnormalities of the reproductive tract on palpation *per rectum*, ultrasonography or vaginal speculum examination; freedom from pre-existing uterine infection based on uterine culture and cytology; convenient profiles of killing and non-killing in peripheral blood of available uterine isolates.

4.1.3. ISOLATES. Three uterine isolates of *S. zooepidemicus* (SZ-1, SZ-2, SZ-3), all of which had previously demonstrated the ability to grow in blood of at least 1 horse, were used in this study. Isolate SZ-1 was originally obtained from another institution conducting research on equine endometritis (Dr Michelle LeBlanc, University of Florida, Gainesville). Isolate SZ-1 had been passaged for more than 20 passages in fresh blood before inoculation. Isolate SZ-2 was obtained by uterine culture using a sterile guarded swab from a horse with clinical endometritis in

the veterinary teaching herd (horse D in this study). Isolate SZ-2 had been passaged 3 times in fresh blood. Isolate SZ-3 was obtained from uterine lavage fluid from a clinical case of endometritis presented to the veterinary teaching hospital. Isolate SZ-3 had been passaged 4 times in fresh blood.

Isolates SZ-A, SZ-C and SZ-D were recovered from horses A, C, and D respectively at the first estrus post SZ-3 inoculation. These isolates were subsequently tested for their ability to grow in blood of 5 horses selected at random from the veterinary teaching herd. Isolates SZ-2 and SZ-3 were tested in blood of the same 5 horses at the same time for comparison (Table 4.3). Previous results (Chapter 2, Table 2.2) had indicated that bactericidal tests in a similar panel of horses might be able to identify and distinguish between isolates. The purpose of studying isolates SZ-A, SZ-C, and SZ-D, in this way, was to determine if these isolates were the same as the isolate originally inoculated at the previous estrus (SZ-3).

4.1.4. PREPARATION OF HORSES FOR INOCULATION Horses were teased, palpated and examined by rectal ultrasonography 3 or more times per week to assess stage of the estrous cycle and follicular development. Negative uterine cultures were obtained at the onset of each estrus in which inoculation took place. Bactericidal tests were repeated prior to each inoculation to confirm killing or non-killing of the isolate in blood of the horse to be intrauterinely inoculated.

Inoculations occurred on successive estrus periods, or with an intervening estrus between inoculations if horses were still infected at the first estrus PI. Following inoculation horses returned to the first estrus PI naturally. The following cycle was sometimes shortened by intramuscular injection of 10 mg of prostaglandin F2 alpha (Lutalyse, The Upjohn Company, Kalamazoo, MI) to synchronize horses for subsequent inoculations.

4.1.5. PREPARATION AND DELIVERY OF INOCULUM. Organisms were grown in an overnight culture in 1.5 ml of freshly obtained blood of the horse to be infected. In cases where the horse killed the organism the buffy coat was removed from the blood prior to inoculation of the blood. Serial inoculation of blood was performed as previously described, with minor modifications. Briefly, aliquots of 1.5 ml of fresh heparinized blood from the horse to be infected were added to 8 sterile tubes. The first tube was inoculated with 0.025 ml of a hemolyzed blood culture of the organism. A volume of 0.025 ml was then withdrawn from the first tube and the same volume serially transferred from tube to tube, along the remaining 7 tubes. After incubation at 35 C for 24 hours, the tubes being gently rotated end over end at approximately 8 RPM, the tube with the most dilute inoculum (last tube of the inoculated series to show hemolysis) was used as a source of organisms for infection. Based on colony counts of serial dilutions, such a tube has a streptococcal concentration of approximately 5×10^7 colony forming units (CFU) per ml. This

tube of hemolyzed blood was resuspended in 30 ml of sterile saline, and used for inoculation.

Horses were inoculated, in estrus, in the presence of a preovulatory dominant follicle (> 35 mm in diameter), followed by administration of 2500 IU of human chorionic gonadotrophin IV (HCG, Follutein, Solway Animal Health, Inc, Mendota Heights, MN). The inoculum was delivered by direct infusion into the uterus via the cervix using a sterile infusion pipette. Prior to inoculation, the vulva was cleaned using repeated applications of povidone scrub and clean water from a bucket containing a new plastic liner for each horse.

4.1.6. ADDITIONAL MONITORING POST INOCULATION. Horses were monitored daily for up to 5 days PI by ultrasound and vaginal speculum examination to detect time of ovulation, uterine fluid, and cervical discharge. Bactericidal tests were performed periodically (every 7 to 10 days) for up to a month PI to determine if a change in bactericidal test results occurred following inoculation of a given isolate.

4.1.7. CULTURE AND CYTOLOGY. Samples for uterine culture and cytology were obtained using a sterile guarded swab (Accumed Corporation, Pleasantville, NY) introduced into the uterus via the cervix with a sterile sleeved arm placed in the vagina to provide digital guidance of the swab through the cervix. The swab was advanced out of its plastic guard and exposed directly to the uterine lumen for 30 seconds. It was then withdrawn into its

plastic guard, and the hollow end of the guard tip was pushed back and forth over the uterine surface to obtain endometrial cells and exudate for cytology. The entire culturette was then completely withdrawn from the horse, and the exterior of the guard wiped with a clean piece of tissue paper. Uterine cells and exudate were then dislodged from the guard interior onto a glass slide and gently spread using the guard tip. The culturette was then taken to the laboratory.

For cytology, smears were stained using a 3-step modified Giemsa stain (Leukostat Fischer Scientific, Orangeburg, NY). The number of neutrophils in the smears was estimated as the percent of neutrophils in the total population of cells observed. They were scored as follows. - = no neutrophils observed; + = neutrophils observed, but at a proportion of 10% or less; ++ = 10% - 75% neutrophils; +++ = greater than 75% neutrophils.

The culturette guard was opened using sterile technique, and the swab rolled over one half of a blood agar plate. The opposite half of the plate was streaked for isolation of individual colonies. Beta hemolytic streptococci (BHS) were scored as follows: - = no growth; + = less than 10 colonies (light growth); ++ = 10-99 colonies (moderate growth); +++ = 100 or more colonies (heavy growth).

4.1.8. BACTERICIDAL TESTS. Bactericidal tests were performed as previously described. Briefly, 5 tubes containing 1.5 ml of freshly obtained heparinized blood of the horse to be tested

were serially inoculated by adding 0.025 ml of a hemolyzed fresh blood culture into the first tube, followed by transfer of 0.025 ml serially between the remaining tubes. This provided a series of tubes of decreasing streptococcal concentration. A volume of 0.05 ml was drawn from each of the last 3 tubes of the inoculation series and was streaked evenly over half of a blood agar plate, a different plate for each of the 3 tubes. The inoculated tubes were then incubated for 3 hours, rotated gently end over end at approximately 8 RPM, at 37 C. After incubation, a 0.05 ml aliquot was drawn from each tube, and streaked evenly over the unstreaked half of the plate containing the streak from the same tube before incubation. The 3 plates were incubated for 24 hours and growth or killing in a tube was assessed by comparing the number of colonies in the streak made before incubation with the number of colonies in the streak made after incubation.

4.1.9. ENDOMETRIAL BIOPSY. Samples of endometrium were taken for biopsy from all horses prior to the study and after all inoculations had been completed.

4.2. RESULTS.

Overall, there was little correlation between bactericidal activity of blood and resistance or susceptibility to endometritis (Table 4.1). In the first set of inoculations, horses had no bactericidal activity against SZ-1 in their blood prior to inoculation, but 2 out of 3 horses had almost eliminated the organism by 48 hours, and all 3 horses had cleared infection by

the next estrus PI. In the second set of inoculations, all horses had marked bactericidal activity against SZ-2 in blood prior to inoculation, but horse C remained infected at the next estrus PI. However, in the third set of inoculations some evidence of correlation between bactericidal activity and resistance to endometritis could be seen. Of the 4 horses inoculated with SZ-3, the only horse with bactericidal activity in her blood, horse D, had noticeable reduction in bacterial numbers at 48 hours PI. An isolate was recovered at the next estrus PI from this horse. However, it had a bactericidal test profile identical to SZ-2. Development of bactericidal activity in blood to the inoculated isolate was observed in 3 cases approximately 2 weeks following inoculation. All of these horses were marked by heavy growth of the isolate in uterine culture at 48 hours PI.

Detailed descriptions of the 3 sets of inoculations in each horse are as follows.

4.2.1. INOCULATION WITH SZ-1. Horses A, B, and C were inoculated with SZ-1 at a dose of 5×10^7 CFU. The isolate grew in blood of all 3 horses prior to inoculation.

Blood of horse A acquired the ability to kill isolate SZ-1 following inoculation. Horse A developed uterine fluid and cervical discharge. At 48 hours PI uterine culture of horse A yielded heavy growth of BHS, but less than 10% neutrophils. At the first estrus PI uterine culture and cytology of horse A were negative.

Table 4.1-Bactericidal tests and uterine culture results following intrauterine inoculation of horses A, B, C, and D with isolates SZ-1, SZ-2, and SZ-3

Isolate	Horse	BT Before	Uterine culture		BT After
			48 hours	First estrus	
SZ-1	A	+	+++	-	-*
SZ-1	B	+	-	-	+
SZ-1	C	+	+	-	+
SZ-2	A	-	+	-	-
SZ-2	B	-	+	-	-
SZ-2	C	-	+++	+++	-
SZ-2	D	-	-	-	-
SZ-3	A	+	+++	++	+
SZ-3	B	+	+++	-	-*
SZ-3	C	+	+++	++	-*
SZ-3	D	-	++	+++	-

BT= Bactericidal test result: += growth; -= killing

*Developed bactericidal activity in blood post inoculation

Before= Before inoculation; After= After inoculation

See text for explanation of uterine culture results

Blood of horse B did not acquire the ability to kill isolate SZ-1. A trace of uterine fluid was observed in the uterus of horse B but no cervical discharge was observed on vaginal speculum examination. At 48 hours PI uterine culture and cytology of horse B were negative. At the first estrus PI uterine culture and cytology of horse B were negative.

Blood of horse C did not acquire the ability to kill isolate SZ-1. Horse C did not develop uterine fluid or cervical discharge. At 48 hours PI, uterine culture of horse C yielded only 2 colonies

of BHS, and uterine cytology was negative. At the first estrus PI uterine culture and cytology of horse C were negative.

4.2.2. INOCULATION WITH SZ-2. Horses A, B, C and D were inoculated with isolate SZ-2 at a dose of 5×10^7 CFU. The isolate was killed in the blood of all 4 horses prior to inoculation. Blood of the 4 horses retained the ability to kill SZ-2 after inoculation. The 4 horses developed uterine fluid and cervical discharge.

At 48 hours PI uterine culture of horse A yielded fewer than 10 colonies of BHS, and uterine cytology yielded 10-75% neutrophils. At the first estrus PI uterine culture and cytology of horse A were negative.

At 48 hours PI uterine culture of horse B yielded fewer than 10 colonies of BHS, and uterine cytology was negative. At the first estrus PI, uterine culture and cytology of horse B were negative.

At 48 hours PI uterine culture of horse C yielded heavy growth of BHS and uterine cytology yielded greater than 75% neutrophils. At the first estrus PI, uterine culture of horse C yielded heavy growth of BHS and uterine cytology yielded less than 10% neutrophils.

At 48 hours PI, uterine culture of horse D was negative, and uterine cytology yielded less than 10% neutrophils. At the first estrus PI uterine culture and cytology of horse D were negative.

4.2.3. INOCULATION WITH SZ-3. Horses A, B, C, and D were inoculated with isolate SZ-3 at a dose of 5×10^7 CFU. Prior to

inoculation the isolate grew in blood of horses A, B and C but was killed in blood of horse D.

Table 4.2-Comparison of endometrial inflammation parameters (uterine cytology, uterine fluid or cervical discharge) observed in horses A, B, C, and D following intrauterine inoculation with isolates SZ-1, SZ-2 and SZ-3

Isolate	Horse	Fluid or discharge*	Uterine Cytology	
			48 Hours	First Estrus
SZ-1	A	+	+	-
SZ-1	B	+/-	-	-
SZ-1	C	-	-	-
SZ-2	A	+	++	-
SZ-2	B	+	-	-
SZ-2	C	+	+++	+
SZ-2	D	+	+	-
SZ-3	A	+	+++	+
SZ-3	B	+	+++	-
SZ-3	C	+	+++	-
SZ-3	D	+	+	+++

* + = Uterine fluid and discharge; - = No uterine fluid or discharge

+/- = Trace of uterine fluid but no discharge

See text for explanation of uterine cytology results

Blood of horse A did not change its ability to kill isolate SZ-3. Horse A developed uterine fluid and cervical discharge. At 48 hours PI uterine culture of horse A yielded heavy growth of BHS and uterine cytology yielded greater than 75% neutrophils. At the first estrus PI, uterine culture of horse A yielded moderate growth of BHS (isolate SZ-A), and less than 10% neutrophils.

Blood of horse B acquired the ability to kill isolate SZ-3 following inoculation. Horse B developed uterine fluid and cervical discharge. At 48 hours PI uterine culture of horse B yielded heavy growth of BHS and uterine cytology yielded greater than 75% neutrophils. At the first estrus following inoculation (the diestrus period was only 9 days) uterine culture and cytology of horse B were negative.

Blood of horse C acquired the ability to kill isolate SZ-3 following inoculation. Horse C developed uterine fluid and cervical discharge. At 48 hours PI uterine culture of horse C yielded heavy growth of BHS and uterine cytology greater than 75% neutrophils. At the first estrus PI uterine culture yielded moderate growth of BHS (isolate SZ-C) but uterine cytology was negative.

Blood of horse D, which killed the isolate prior to inoculation, continued to kill isolate SZ-3 following inoculation. Horse D developed uterine fluid and cervical discharge. At 48 hours PI uterine culture yielded moderate growth (32 colonies) of BHS and uterine cytology yielded less than 10% neutrophils. At the first estrus PI uterine culture yielded heavy growth of BHS (isolate SZ-D) and greater than 75% neutrophils.

When growth of the 3 isolates SZ-A, SZ-C, and SZ-D were compared to isolates SZ-2 and SZ-3 in blood of 5 randomly selected horses, SZ-A and SZ-C had the same bactericidal test results as SZ-3, but isolate SZ-D had the same results as SZ-2 (Table 4.3).

Table 4.3-Bactericidal tests of isolates SZ-2 and SZ-3 compared with isolates (SZ-A, SZ-C, SZ-D), recovered at first estrus following SZ-3 inoculation.

Horse*	SZ-2	SZ-3	SZ-A	SZ-C	SZ-D
M	-	+	+	+	-
N	-	+	+	+	-
O	+/-	+	+	+	+/-
P	-	-	-	-	-
Q	-	-	-	-	-

*Horses selected at random from veterinary teaching herd

+ = streptococcal numbers consistently increase

- = streptococcal numbers consistently decrease

+/- = no consistent change in streptococcal numbers

4.2.4. ENDOMETRIAL BIOPSIES. Results of endometrial biopsies using the modified grading system of Kenney (Kenney and Doig 1986) were as follows for each horse (before inoculations/after inoculations): horse A-IIB/IIB; horse B-III/III; horse C-IIA/IIB; horse D-IIB/III. Horses which dropped biopsy grades over the experimental period did so due to increased mononuclear infiltration.

4.3. DISCUSSION.

Overall, these data suggest that resistance of the equine uterus to streptococcal infection is not heavily dependent on circulating humoral responses, as measured by the bactericidal activity of freshly obtained equine blood. Horses in whose blood the organism grows appear to be capable of achieving effective

clearance of the organism. Similarly, horses in whose blood the organism is killed may be susceptible to infection. Nevertheless, some of the results suggest that killing in peripheral blood may signify some additional protection to infection that is of assistance in rapid clearance of the organism immediately following inoculation. Significant determinants of resistance to infection, which apparently outweighed the bactericidal tests, may reside in differences between horses and, interestingly, differences between isolates.

Several cases can be seen where the organism grew in fresh blood of the horse but was nevertheless effectively cleared from the uterus. Isolate SZ-1 grew in blood of all horses at the time of inoculation, yet was cleared in all 3 inoculations by the first estrus PI, and almost cleared by 48 hours PI in 2 of the horses. Clearance did not depend on killing activity of fresh blood since 2 of the 3 inoculations in which SZ-1 was cleared were not accompanied by a development of blood bactericidal activity. Horse A did not develop killing ability in blood to isolate SZ-3, but yielded negative uterine culture and cytology (data not shown) at a subsequent estrus, without therapeutic intervention.

Examples also exist where isolates were killed in blood but protection against uterine infection was not evident. Horse C carried isolate SZ-2 to the first estrus PI, despite killing the organism in her blood. In addition, development of bactericidal activity was detected in horse C to isolate SZ-3 12 days PI, but

the organism was nevertheless isolated from her uterus at the subsequent estrus (19 days PI).

However, some evidence suggests that killing of the organism may provide some additional protection against infection, particularly in the immediate post inoculation period during an acute inflammatory response. The primary evidence for this comes from the uterine culture at 48 hours PI in the four horses infected with SZ-3. All horses had high numbers of intrauterine bacteria at 48 hours PI, except horse D, in which markedly fewer colonies were detected at this time and which was the only horse to kill the organism in fresh blood prior to inoculation. Studying the first estrus PI this pattern appears to be sustained. In horse D, although heavy growth of streptococci were isolated from the uterus at the subsequent estrus (isolate SZ-D), this isolate did not coincide with isolate SZ-3 in bactericidal tests. Interestingly, it coincided with isolate SZ-2, an isolate originally isolated from horse D. However, horses A and C yielded isolates SZ-A and SZ-C, both of which coincided with SZ-3 in bactericidal tests (Table 4.3). It is possible that horse A carried uterine infection with SZ-3 to the first estrus PI, but that horse D may have retained isolate SZ-2 from the previous inoculation. Alternatively, SZ-2 may have been a natural inhabitant of the vestibule, vulva or clitoris in horse D, and reentered the uterus during the procedures in this study. Because uterine cytology of

horse C was negative, SZ-C may have originated from an extrauterine source

Although only 4 horses were used in this study, horse-to-horse variation was minimized by repeating inoculations within horses. A significant source of random error might lie in different durations between inoculation and the end of the estrus period when uterine defenses are thought to lose some effectiveness as progesterone levels increase. This was minimized by administration of HCG in the presence of a preovulatory follicle immediately post inoculation to induce ovulation.

Why does such a weak relationship exist between killing in peripheral blood and the ability to clear an intrauterine inoculum of *S zooepidemicus* ? The most likely reason for this is that there are sources of variation between horses and between isolates which override the events measured by the bactericidal test. The sources of variation between horses have been extensively investigated in the context of explaining why some mares appear to be resistant to endometritis, and others are susceptible. This variation was most evident in horse C, who appeared to be more susceptible to infection than the other 3 horses, irrespective of the bactericidal test. Previous inoculations in this horse had confirmed her increased susceptibility to endometritis. Currently, an age related decline in the mechanical clearance of fluid and particles appears to be the most significant source of such variation between mares (Evans et al. 1987; LeBlanc, Asbury and

Lyle 1989; LeBlanc et al. 1994; Troedsson and Liu 1991; Troedsson and Liu 1992). Bactericidal and opsonic activity of equine uterine fluid have also been investigated as sources of variation in uterine resistance between mares (Asbury, Gorman and Foster 1984; Asbury et al. 1982; Cheung et al. 1985), but appear, overall, to be of less significance than mechanical clearance (Allen 1993; Hansen and Asbury 1987; LeBlanc et al. 1991). The data contained in this paper would also suggest a secondary role for opsonic activity of uterine fluid. Opsonins and leukocytes in the acutely inflamed uterus are largely derived from circulating blood (Pycock and Allen 1990; Watson et al. 1987a; Williamson et al. 1987), but these data show that the bactericidal activity of blood does not tightly correlate with resistance to infection. Additionally, in 5 clinical cases of endometritis (data not shown), presented to the Louisiana State University teaching hospital, in which heavy growth of *S. zooepidemicus* was cultured from the equine uterus, strong bactericidal activity was detected to the cultured isolate in fresh blood despite ongoing infection.

However these data also confirm that, in many cases, the ability of the equine uterus to clear bacteria from the endometrial surface is impressive (Hughes and Loy 1969). Before each inoculation, negative uterine cultures and cytologies were obtained from each horse. Following inoculation, horses yielded negative uterine cultures and cytologies without therapeutic intervention.

If uterine isolates of *S. zooepidemicus* can resist phagocytosis but, as in the case of isolate SZ-1, are effectively cleared from the uterus, it would appear that mechanisms other than phagocytosis are responsible for their clearance. Physical clearance by myometrial contractions, while necessary, would alone probably not be sufficient to achieve complete uterine bacteriologic clearance. Fluid having been removed, the mucosal surface itself would still need to be cleared of potentially adherent streptococci (Ferreira-Dias, Nequin and King 1994a) and inflammatory debris. This would suggest that bacteria are killed at the endometrial surface, or they are physically cleared from the endometrium by local micro-anatomic mechanisms or that both processes act in concert. Blocking of bacterial adherence, coupled with mucous clearance mechanisms, are common themes in resistance to infection at mucous membranes (Sleigh, Blake and Liron 1988). The equine endometrium is variably ciliated (Ferreira-Dias, Nequin and King 1994b; Samuel et al. 1979), and mucus production is demonstrable at the endometrial surface of the estrous mare (Freeman et al. 1990). This provides an anatomic basis for mucociliary clearance, although such clearance has yet to be demonstrated in the equine uterus. In addition, extensive evidence already exists to suggest that the equine uterus is a typical mucosal immune surface (Kenney and Khaleel 1975; Mitchell et al. 1982; Widders et al. 1984; Widders et al. 1985a; Widders et al. 1985b). In this study, only 3 seroconversions were

documented following intrauterine inoculation, as measured by a change in bactericidal tests in peripheral blood, also suggesting that the endometrial mucosal surface is somewhat independent of the systemic immune system. Because these seroconversions occurred in cases where high streptococcal growth was observed, one might argue, cautiously, that mucosal resistance temporarily had been breached, and that some systemic exposure to the organism had occurred. This might have been facilitated during endometrial scraping for cytology in the presence of high streptococci at the endometrial surface. A complete understanding of how bacteriologic clearance of the uterus is achieved in the resistant mare will require further study.

Turning from variation between horses to variation between isolates, these data show that isolates of *S. zooepidemicus* might vary in their ability to produce disease, unrelated to their ability to resist phagocytosis. Disease, in equine endometritis, can be clinically detected as inflammation of the endometrial surface (Asbury and Lyle 1993; Roszel and Freeman 1988). In this study the presence of uterine fluid, cervical discharge, and uterine cytology were the parameters used to assess endometrial inflammation (Table 4.3). Overall, isolate SZ-1 appeared to induce only mild clinical signs. Comparing SZ-1 with SZ-3, isolate SZ-3 appears to induce markedly more disease than SZ-1. Originally a clinical isolate from a horse with equine endometritis, isolate SZ-1 had a history of extensive passage in freshly obtained blood

(more than 20 passages) in this study before being used for intrauterine inoculations. In contrast, isolates SZ-2 and SZ-3 had less than 4 passages, but these latter 2 isolates produced consistent disease in the post inoculation period. It is possible, though not certain, that the ability to produce disease was attenuated in SZ-1 by extensive passage in fresh blood. This suggests loss of virulence factors other than those conferring resistance to phagocytosis. Candidates for such factors might include products inducing a hyperinflammatory response, or enhancing streptococcal adherence to the endometrium (Ferreira-Dias, Nequin and King 1994a). Effects of repeated inoculation might also account for the increased disease observed as one proceeds from SZ-1 to SZ-2 to SZ-3. If so, this represents an additional source of variation which outweighs the bactericidal test. Nevertheless, according to a previous study, multiple repeated intrauterine inoculations in resistant horses do not lead to increased susceptibility to intrauterine infections (Hughes and Loy 1969).

In conclusion, resistance to mucosal infection of the equine endometrium by *S. zooepidemicus* is not highly correlated with the bactericidal activity of fresh blood, although some effect apparently may be detected in the acutely inflamed uterus. These results may not apply to systemic streptococcal infections. The descriptive nature of this study does not allow firm inferences to be drawn, but suggests that mucosal *versus* systemic resistance

and virulence differences between bacteria are more important determinants of disease.

CHAPTER 5 THE POSSIBLE ROLE OF MUCUS IN PHYSICAL CLEARANCE AT THE EQUINE ENDOMETRIUM

It was previously shown that resistance to equine endometritis is independent of killing in fresh equine blood. Horses in whose blood uterine isolates of *S zooepidemicus* multiply are sometimes able to clear infection within 48 hours. Similarly, killing in fresh blood is not sufficient to protect a susceptible mare from endometrial infection. Overall this suggests a more minor role, than previously thought, for phagocytosis in clearance of *S zooepidemicus* from the equine endometrium. This is supported by the poor opsonic activity of uterine fluid in resistant and susceptible mares in comparison to serum (Asbury, Gorman and Foster 1984; Brown, Hansen and Asbury 1985; LeBlanc et al. 1991).

In contrast, resistance to equine endometritis is strongly associated with physical clearance of fluid and particles from the uterus (Evans et al. 1987; Troedsson and Liu 1991; Troedsson and Liu 1992). Defects in clearance may be due, in part, to defective myometrial contractions (Troedsson et al. 1993; Troedsson et al. 1993). Delayed uterine clearance may predispose to endometritis, in part, by impairment of neutrophil function or opsonic activity due to retention of aged uterine fluid (Troedsson and Liu 1992; Troedsson, Liu and Thurmond 1993b).

Mucus is an important defense component at many mucosal surfaces. It both blocks attachment of micro-organisms to the epithelium, and promotes their physical clearance (Sleigh, Blake and Liron 1988). The equine endometrium is characterized by both mucus production (Freeman et al. 1990), and ciliary activity (Ferreira-Dias, Nequin and King 1994b; Samuel et al. 1979). However, the role of mucociliary clearance from the equine uterus has not been suggested or studied, and may account for much of the resistance to equine endometritis by its contribution to physical clearance. Charcoal has been used to assess physical clearance from the equine uterus (Evans et al. 1986; Evans et al. 1987). A descriptive study was therefore performed to investigate the interaction of carbon powder, a substance similar to charcoal, and mucus at the endometrial surface.

5.1. MATERIALS AND METHODS.

5.1.1. EXPERIMENTAL OVERVIEW. Four horses received a single intrauterine infusion of carbon powder (American Norit Company, Atlanta, GA) suspended in saline during estrus. At 4 hours and 48 hours post infusion horses were examined by vaginal speculum to assess carbon evacuation, and samples were taken for endometrial biopsy and cytology. All samples were stained for mucus production.

5.1.2. HORSES. Four adult mares of light breed with normal reproductive organs based on palpation, ultrasound, and vaginal

speculum examination were used. Horses are identified as W, X, Y and Z.

5.1.3. PREPARATION OF HORSES FOR INFUSION. Follicular activity was monitored by teasing, rectal palpation and ultrasonography 3 times a week. Horses were infused during estrus in the presence of a dominant follicle.

5.1.4. INFUSION. A carbon suspension was prepared by suspending 10 ml of carbon powder in 60 ml sterile saline. After briefly shaking the suspension to disperse the carbon, 50 ml were infused into the uterus via a sterile fusette. Horses were then placed in a stall until the first sampling at 4 hours, after which they were turned out to pasture.

5.1.5. VAGINAL SPECULUM EXAMINATION. Vaginal speculum examination was performed at 4 hours and 48 hours post infusion using a cardboard tube mare speculum. The vaginal mucosa and cervix were examined for presence of carbon, indicating its elimination from the uterus.

5.1.6. ENDOMETRIAL CYTOLOGY AND BIOPSY. Samples for endometrial cytology were obtained at 4 hours and 48 hours post infusion of carbon. Uterine exudate was smeared onto a slide, and immediately fixed in 95% ethanol. Samples for endometrial biopsy were obtained by introduction of an alligator type mare uterine biopsy punch into the uterus via the cervix, massaging a dorsal endometrial fold into the jaws of the punch *per rectum*, closure of the jaws, and removal of a small piece of endometrial tissue

(approximately 1.00 X 0.5 X 0.25 cm). Samples were fixed in zinc formalin solution. Slides for both cytology and biopsy were stained for mucus with Best's mucicarmine. Mucus stains pink, chromatin black, and protein yellow with this stain.

5.2. RESULTS.

Overall, the results divided the 4 horses into 2 pairs. One pair (horses X and Y), had minimal endometrial mucus production visible on endometrial biopsy, marked adherence of carbon to the endometrium, and delayed uterine clearance of carbon. In the other pair (horses W and Z) extensive mucus production was evident on endometrial biopsy. No carbon was observed adhering to the endometrium, and uterine clearance of carbon appeared to be efficient.

In endometrial biopsies 4 hours post infusion, carbon was seen adhering to the endometria lacking epithelial mucus production of horses X and Y. The epithelium appeared to be partially disrupted where carbon was bound. Vaginal speculum examination did not reveal carbon in the vagina of these 2 horses at 4 hours post infusion, indicating retention of carbon within the uterus. At 48 hours, endometrial biopsy revealed continued absence of mucus production in horse X, and very sparse production in horse Y. No carbon was observed in these 48 hours biopsies, however, and the epithelium appeared intact in both horses. Horse X was notable in having accumulated carbon on the floor of her uterus at 48 hours, an unexpected finding detected by

uterine ultrasound. Her uterine cytology was also remarkable in revealing a thick yellow staining exudate.

Table 5.1-Summary of results obtained at 4 hours and 48 hours following intrauterine infusion of carbon in 4 horses

	Horse			
	W	X	Y	Z
4 HOURS				
Carbon in vagina	+	-	-	+
Adherence to luminal epithelium	-	+	+	-
Mucus production	+	-	-	+
48 HOURS				
Mucus production	+	-	+/-	+

Carbon in vagina determined by vaginal speculum examination

Adherence to uterine luminal epithelium and mucus production determined from endometrial biopsy

In contrast, endometrial biopsies which displayed mucus production at 4 hours, in horses W and Z, had no adherence of carbon to the luminal epithelium. These 2 horses also had carbon in the vagina at 4 hours, indicating effective clearance of carbon from the uterus. In biopsies at 48 hours, extensive mucus production was again observed in the endometrium of both horses. At this time no carbon was present, and the epithelium appeared



Figure 5.1-Horse W 4 hours post carbon infusion. No carbon seen adhering to an endometrium with mucus production. 400X.



Figure 5.2-Horse Y 4 hours post carbon infusion. Carbon adhering to an endometrium without mucus production. 400X.

intact. Horse W was notable in having a uterine cytology at 48 hours with numerous neutrophils. Many of these neutrophils appeared to be completely covered by a thin coat of mucus.

In contrast to the biopsy, no interaction between mucus and carbon could be detected on uterine cytology. With the exception of the neutrophilic cytology of horse W, mucus did not appear to stain well in uterine cytologies. Carbon was evident in uterine cytologies in all mares at 4 hours, and greatly reduced or absent at 48 hours.

Results are summarized in table 5.1.

5.3. DISCUSSION.

Mucus protects mucosal surfaces by inhibiting bacterial adherence and providing a cleansing flow perpendicular to, and parallel to, the epithelium (Sleigh, Blake and Liron 1988). Mucus production at the equine endometrium was originally demonstrated by Freeman (Freeman et al. 1990), who demonstrated its production at the luminal surface, but its apparent absence from the uterine glands. Samuel, and more recently Ferreira-Dias, have demonstrated the presence of cilia in normal equine endometria using scanning electron microscopy (Ferreira-Dias, Nequin and King 1994b; Samuel et al. 1979). Thus anatomical requirements for mucociliary clearance appear to exist at the endometrium of the mare. This possibility is particularly compelling given the importance of physical clearance in equine uterine defenses (Evans et al. 1986; Evans et

al. 1987; Troedsson and Liu 1991; Troedsson and Liu 1992; Troedsson et al. 1993; Troedsson et al. 1993).

Based on the results of this small study, adherence, and clearance of carbon may be closely related to endometrial mucus production. Carbon adhered to endometria only in the absence of mucus, specifically to endometria lacking mucus production, or to any cut surface on an endometrial biopsy. The 2 horses which produced mucus at the endometrial surface appeared to have superior physical clearance than the 2 horses which lacked mucus production, based on arrival of carbon in the vagina at 4 hours.

The differences in mucus production between the 2 pairs of horses appeared to be consistent, being evident at 4 hours and 48 hours. However, whether this is due to a true difference between horses, slightly different stages of the cycle or some other reason is unclear.

The observation that mucus coats neutrophils was unexpected. Whether this coating is of functional significance is unknown. However, it seems plausible that it might come between opsonins and their receptors on the neutrophil. This might partially explain the poor opsonic activity of uterine fluid in comparison to serum (Asbury, Gorman and Foster 1984; LeBlanc et al. 1991).

Results of such a small study must be interpreted with caution. Current knowledge of endometrial anatomy and endometritis is consistent with mucociliary clearance at the

equine endometrium. Although such clearance has not been previously proposed, these limited results provide support for such clearance.

CONCLUSION

This dissertation has attempted to shed some light on the pathogenesis of equine endometritis due to *Streptococcus zooepidemicus*. In the introduction 3 questions were posed, to which we now can offer some answers.

The first question was to ask if equine uterine isolates of *Streptococcus zooepidemicus* are antiphagocytic. Growth in fresh blood is the classic demonstration of resistance to phagocytosis of the group A streptococci, and *S zooepidemicus* appears to behave in horse blood exactly as antiphagocytic group A streptococci behave in human blood. Thus it appears to be beyond reasonable doubt that at least some isolates of *S zooepidemicus* are antiphagocytic.

The second question was to determine if uterine isolates of *S zooepidemicus* carry a homolog of the group A streptococcal M protein. This question, so simple to ask, becomes a challenge to definitively answer. Lancefield's M protein is not defined as a single entity, but as a typing system of many proteins all differing from each other. Thus, definitive proof of its presence on *S zooepidemicus* would require 2 sequential steps: first, the development of a typing system that mimics Lancefield's; second confirmatory biochemical and genetic analysis of the typing antigen. This first stage was essentially completed for *S zooepidemicus* by Moore and Bryans, but loss of the typing sera

has prevented completion of the second stage by others (Moore and Bryans 1969; Timoney et al. 1995). But because many other distinctive features of M+ streptococci are shared by *S zooepidemicus*, the argument for its existence on the latter is reasonably strong. That evidence now includes the following. First, *S zooepidemicus* infection in humans is clinically indistinguishable from group A streptococcal infections. Second, growth and killing of uterine isolates of *S zooepidemicus* in fresh equine blood closely mimics behavior of M+ streptococci in human blood. This includes the ability to differentiate streptococci by the bactericidal test, and the requirements for successful phagocytosis. This behavior is directly attributable to M protein in the group A streptococci, suggesting a similar explanation for *S zooepidemicus*. Third, electron microscopy of *S zooepidemicus* reveals an M protein-like fuzzy coat that is partially lost from isolates that can no longer grow in fresh blood (appendix 1). Fourth, some uterine isolates of *S zooepidemicus* possess antigens which bind anti-M protein monoclonal antibodies (appendix 2). Fifth, DNA of equine uterine isolates of *S zooepidemicus* is amplified by PCR primers specific for M protein subfamily 1 genes (appendix 3). However, none of this constitutes proof of its existence on *S zooepidemicus*.

The third question was to investigate if these antiphagocytic properties of *S zooepidemicus* were of importance in equine endometritis. This was approached by using the variation

in killing ability of blood of different horses to compare resistance to uterine inoculation. The results showed that killing activity of equine fresh blood did not strongly correlate with resistance to equine endometritis. This is in marked contrast to the situation in human streptococcal disease where phagocytosis of the organism, measured by killing in fresh blood, plays a major role in protection. Uterine defenses of a different nature appear to be involved in the horse.

If uterine phagocytosis is so impaired, how can horses effectively eliminate streptococci from the endometrial surface? Physical clearance is of major importance, but can myometrial contractions alone rid the endometrial surface of adherent bacteria? To shed light on this question, the interaction of mucus and carbon powder at the endometrial surface was studied, and the limited data suggest that extensive mucus secretion prevents carbon adherence to the endometrium. In addition, cilia at the mucosal surface may aid mucus flow as in the respiratory tract. Thus uterine resistance to streptococcal infection may be partly due to mucociliary clearance.

Gross anatomy of the uterus appears well adapted to support mucociliary action. Endometrial folding eliminates large fluid spaces in the uterus, bringing opposite walls together, and decreasing the depth of fluid over the endometrial surface, improving ciliary performance (Kenney 1978). Further more, the endometrial folds of the uterus are continuous with the folds of

the cervix, suggesting continuous tracks along which material can be propelled out of the uterus (Ginther 1992). In addition, many reported deficiencies of the susceptible mare may be directly related to mucociliary clearance. These include accumulation of fluid (Allen and Pycock 1988; Evans et al. 1987; LeBlanc et al. 1994; Troedsson and Liu 1992); loss of cilia (Ferreira-Dias, Nequin and King 1994b); loss of endometrial folds (Bracher, Mathias and Allen 1992); defective physical clearance of particles (Evans et al. 1987; Troedsson and Liu 1991). Thus, attempts to better understand equine endometritis might be well spent focussing on the role of mucus and cilia in uterine defense and disease.

In closing, I would like to emphasize the importance of keeping up to date with the out of date literature, in fact the more out of date the better. Almost a century ago, Metchnikoff said the following in reference to resistance at mucosal surfaces: "Nature does not make use of antiseptics to protect the skin and the mucous membrane. The fluids which moisten the surface of the mouth and of other mucous membranes are not microbicidal, or are so to a very slight degree, and then rather of an exceptional nature. Nature rids the mucous membranes and the skin of a large number of micro-organisms, eliminating them by epithelial desquamation, and expelling them along with fluid secretions and excretions. Nature, like the doctors of the present day who replace antisepsis of the mouth, intestine, and other organs by washing

with pure physiological saline solution, has chosen this mechanical method." (Metchnikoff 1907) Two things make this quote particularly relevant to equine endometritis. First, uterine lavage with physiological saline solution is today one of the few effective treatments for equine endometritis (Troedsson, Scott and Liu 1995). Secondly, part of Metchnikoff's argument is based on work from one of his own students, Cahanesco, who investigated the bactericidal properties of mucous secretions of the mare genital tract. Cahanesco could not demonstrate any bactericidal properties in mucus collected from the equine genital tract, despite plenty of neutrophils in the collected secretions (Cahanesco 1901). It is, therefore, rather perplexing to discover that the major conclusions of this dissertation were predicted almost 100 years ago.

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APPENDIX 1

Twelve uterine isolates of *Streptococcus zooepidemicus* were digested by phage lysin and immunoblotted with various anti-M protein monoclonal and polyclonal sera. Two isolates reacted with an anti-M6 monoclonal antibody. These 2, and an additional isolate, reacted with anti M6 polyclonal sera. All isolates reacted with polyclonal antisera against SzP, a cloned M-like protein of *S zooepidemicus*. Figure A.1 (overleaf) shows an immuno blot of one of the isolates (UF-1), which reacted with all 3 antisera. These results provide presumptive evidence for M protein on uterine isolates of *S zooepidemicus*.

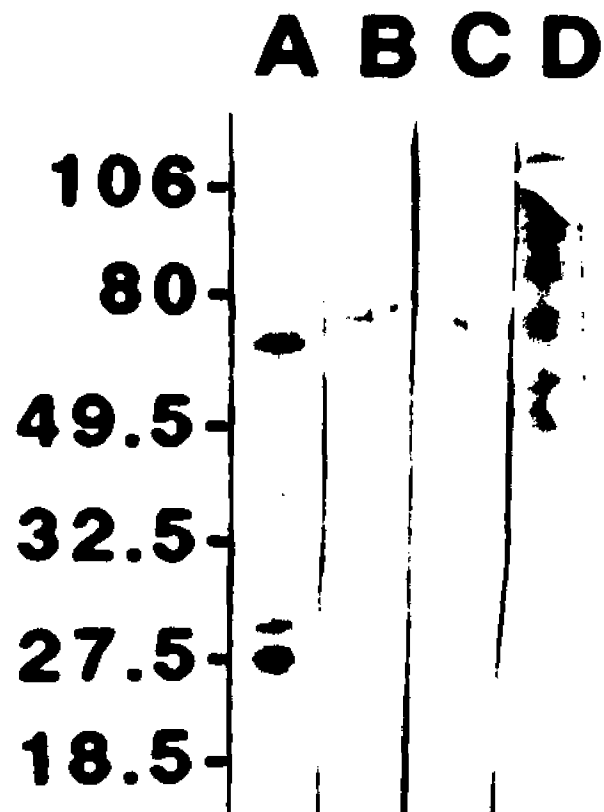


Figure A.1-Mutanolysin digest of *Streptococcus zooepidemicus*, isolate UF-1. immunoblotted with the following antisera: Lane A, total protein stain with india ink; lane B monoclonal antibody against type 6 M protein (Vincent Fischetti); lane C, polyclonal antibody against type 6 M protein (Vincent Fischetti); lane D, polyclonal antibody against M-like proteins of *Streptococcus zooepidemicus* (John Timoney).

APPENDIX 2

Figure A.2-Results of screening uterine isolates of *Streptococcus zooepidemicus* for the presence of group A streptococcal M protein subfamily 1 (SF-1) genes. (group A, type 6 streptococcal M protein is a member of subfamily 1.). Isolates screened were UF-1, 10323, H-1 and H-2. Variants of these Isolates which had lost the ability to grow in fresh blood were also screened. All isolates possessed an SF-1 like gene, however, isolates which had lost the ability to grow in fresh blood appeared to have genetic alterations in the promoter region.



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May 5, 1995

Robert C. Causey
Dept. of Veterinary Microbiology and Parasitology
Louisiana State University
Baton Rouge, LA 70820

Dear Dr. Causey,

I have finished the initial search for genetic evidence of emm and emm-like genes in your strains of *S. zooepidemicus* and am happy to report that there is an SF1-like gene. The *mga* gene is not like those normally found in front of SF1 genes, rather, it is more like the *mga* found in front of pattern 5 (SF4-SF2-SF3) strains, when it is present. There is also evidence for an *ScpA*-like gene in some strains but I haven't managed to get the whole gene out (yet!). Please see the enclosure for a quick summary to date.

Susan will be available after May 9th and please feel free to call me at any time with questions. I'm most easily reached between the hours of 7 a.m. and 3 p.m. at (205)934-5512. If you have any interest in sequencing the PCR products we would be happy to forward them to you.

Sincerely,

A handwritten signature in black ink, appearing to read 'Terri Readdy', written in a cursive style.

Terri Readdy

APPENDIX 3

Uterine isolates of *Streptococcus zooepidemicus* were examined electron microscopically. Isolates were prefixed in lysine, stained with alcian blue and uranyl acetate. Figures A.3.1 and A.3.2. (overleaf) show that *S zooepidemicus* possess a fuzzy coat, which may be partially lost in isolates which can no longer grow in fresh blood. However, as seen from the figure, isolates which do not grow in blood may retain some surface fimbriae, in contrast to published electron micrographs of M- group A streptococci which are usually non-fimbriated.

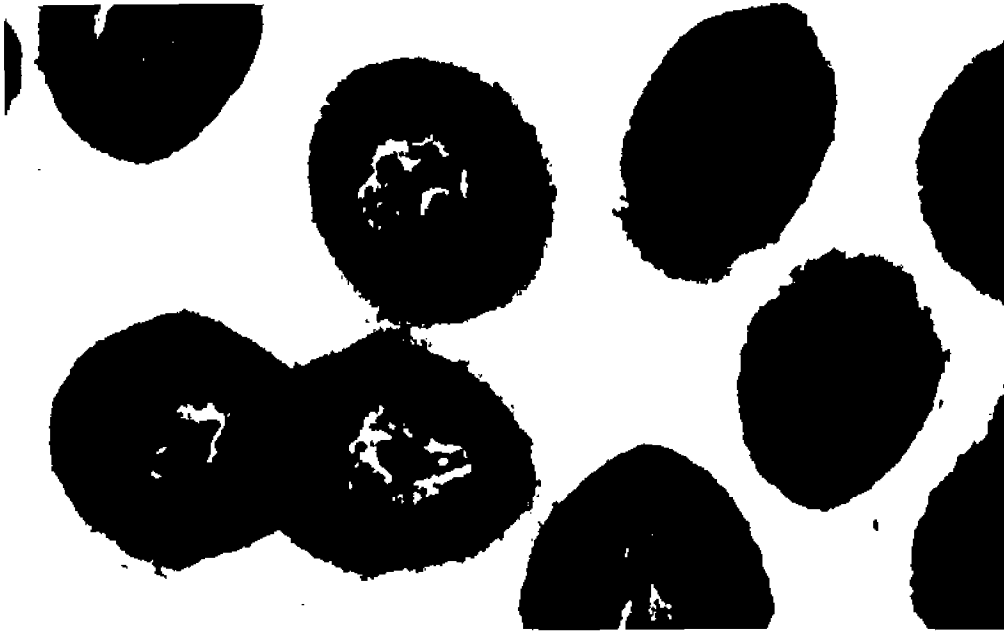


Figure A.3.1-*Streptococcus zooepidemicus* isolate H-2 prior to passage on artificial media and able to grow in fresh equine blood. 61200 X.



Figure A.3.2-Isolate H-2 following extensive passage on artificial media and unable to grow in fresh equine blood. 61200 X.

APPENDIX 4

1 00-92-225 10-26-95
faxed 10-26-95

TO 91788925:3230043325

001 *[Handwritten initials]*

To: Dr. Kurt Matushak
American Veterinary Medical Assoc.
1931 N. Meacham Rd., Suite 100
Schaumburg, IL 60173-4260

Post-it Fax Note	7871	Date	10-26-95	pages	1
To	Robert Causey	From	10-26-95		
	JVM	Co	AVMA		
	346-3312		925-329		
	346-5715		925-329		

RE: Antiphagocytic properties of uterine isolates of *Streptococcus zooepidemicus* and mechanisms of killing in freshly obtained blood of horses. R.C. Causey, D.L. Paccaroni, W.J. Todd, Am.J. Vet. Res., Vol. 56, No. 3, March 1995, pages 321-328.

Dear Mr. Matushak:

I am applying for permission to include the above article as a chapter in my Ph.D. dissertation. I intend to include the text of the article, figures, and tables, with a few formatting changes to satisfy Graduate School requirements.

If you could fax the letter of permission to the following number I would greatly appreciate it.

FAX: 504-346-5715

Yours sincerely,
Robert Causey

Dept. of Veterinary Microbiology and Parasitology
School of Veterinary Medicine
Louisiana State University
Baton Rouge, LA 70803
Tel: 504-346-3313

October 26, 1995

Permission granted, provided that the American Journal of Veterinary Research is clearly credited as the source.

[Handwritten Signature]
Jouis E. Audin, MS, DVM
Editor-in-Chief

VITA

Robert Causey was born in Harrogate, Yorkshire, in 1961. His parents moved to the United States in 1979, and he followed two years later. After obtaining his B.S. in 1985, and D.V.M. in 1989, both from the University of Minnesota, he came to Louisiana State University as a resident in Theriogenology. This experience provided him with the interest to pursue a Ph.D. program in the Department of Veterinary Microbiology and Parasitology under the direction of Dr. William J. Todd. Robert plays classical guitar, and currently takes guitar lessons from Professor Elias Barreiro at Tulane University.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Robert Crawford Causey

Major Field: Veterinary Medical Sciences

Title of Dissertation: The Pathogenesis of Equine Endometritis due to Streptococcus zooepidemicus

Approved:

William J. Todd
Major Professor and Chairman

John M. Schick
Dean of the Graduate School

EXAMINING COMMITTEE:

John K. Schultz

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Jack Rasmussen

Ronald F. Thorne

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Date of Examination:

10/20/95

