CELLULAR AND HUMORAL RESPONSES TO INTRAUTERINE INOCULATION WITH VIABLE *STREPTOCOCCUS ZOOEPIDEMICUS* IN A GROUP OF MARES WITH VARYING RESISTANCE TO ENDOMETRITIS.

BY S.J. MUCHINA MUNYUA.

BVM (Nairobi, Kenya) BSc. Hons (Murdoch, W.A.).

Clinical Studies Department,

College of Agricultural and Veterinary Sciences,

University of Nairobi,

P.O. BOX 29053,

Nairobi, KENYA.

JULY, 1985

This thesis is submitted in fulfilment of the requirements for the degree of Master of Philosophy (MPhil) in Veterinary Theriogenology within Murdoch University.

DECLARATION

I DECLARE THAT, UNLESS OTHERWISE STATED, THIS THESIS IS MY OWN ACCOUNT OF MY RESEARCH AND CONTAINS AS ITS MAIN CONTENT WORK WHICH HAS NOT BEEN PREVIOUSLY SUBMITTED FOR A DEGREE AT ANY UNIVERSITY.

During the investigations described in this thesis the following procedures were perfo med by persons other than the author:-

1) Some routine bacteriology (Chapter 3);

2) Some immunoglobulin assays (Chapter 3);

3) Comassie Blue protein assay (Chapter 3);

4) Collection of some of the uterine biopsies and washings (Chapter 4);

5) Serotyping of Streptococci (Chapter 3).

S.J. MUCHIRA MURYUA

PETER E. WILLIAMSON (Principal supervisor)

JOHN PENHALE (Supervisor)

For every Cultural, Social, Political and Economic problem there is one solution that is very simple, straight forward, understandable, acceptable and wrong.

Ross, Mary and Phil Deschamp (Perth, 1984)

THIS THESIS IS DEDICATED TO MY MOTHER, HURO WA MUNYUA AND MY SONS SOLO AND FRED MUNYUA

30.4.85

ACKNOWLEDGEMENT

I wish to express my gratitude and appreciation to my principal supervisor, Dr. P. Williamson and acting supervisor Dr. J. Penhale (during Peter's absence) for their guidance, assistance, moral support, insights, patience, continued encouragement, understanding and constructive criticism during the course of this project. In addition I wish to thank Dr. C. Hawkins for his continued encouragement, insights and constructive criticism through out this study. As in any undertaking of this magnitude the efforts of many persons other than those of the supervisor(s) and the student make it possible for the task to be completed in time. To all these people I express my sincere and grateful appreciation.

I particularly wish to acknowledge the technical assistance of James Murray, Margaret Sharpe, Dr. Fred Sumbung, Barry Combs, David Brockway, John Morrel, Pamella Deery, Diane Leslie, Peter Hinchcliffe, Peter Fallon, Geoff Griffith and Murray Austin-Smith; to them I am grateful. I wish to express my sincere appreciation for the understanding, encouragement and moral support provided by my wife Mantema, son Solo, and my family in KENYA, throughout this study.

I also wish to express my appreciation, for the understanding and moral support provided by Lynn, Jenny and Nickie Williamson, Agnes, Marilyn, Stephen and Rono (Ronald) Sumbung, Karen, Kay and David Murray, Dr. Shannon Atkinson, Pat Clifford, Ray Moule, Prof. Ralph Swan, Dr. Kang Cheng Lun, Dr. Ating Tjondronegoro and Dr. Masu'd Hariadi.

I wish to thank Pat Clifford for typing the tables included in this thesis.

I wish to record my thanks to Australian Development Assistance Bureau for providing the fellowship, through the Kenyan Government, and the University of Nairobi for granting me the study leave. I particularly wish to thank Mr. K. Chambers (Supervisor ADAB, Perth). Dr. J. Kiptoon (Chairman Clinical Studies Department, Kabete), Prof. G. Mugera (Dean, Faculty of Veterinary Medicine, Kabete) and Prof. J.M. Mungai (Vice Chancellor, University of Nairobi, Nairobi) for making it all possible.

The data that is presented in this thesis was obtained in the course of a study on the immunology of the equine uterus financed by the Australian Equine Research Foundation and Murdoch University to whom we are grateful.

SUMMARY OF THESIS

This thesis records in part the search for a technique to identify mares with lowered resistance to Streptococcal endometritis. It also presents the findings of studies on the pathogenesis of equine Streptococcal endometritis, including the factors influencing the elimination of the experimentally infused Streptococcus zooepidemicus.

Acute endometritis was induced in 32 mares, which were classified as either resistant (21) or susceptible (11) to endometritis depending on their breeding histories and the degree and extent of endometrial pathology. Infection was induced by intrauterine inoculation of 10^9 viable S. zooepidemicus suspended in 2 mls of sterile isotonic mannitol solution. Three control mares were infused with 10 mls of sterile isotonic mannitol. Serial uterine swabs, washings and biopsies and blood samples were obtained before (0 hrs), and at fixed time intervals after intrauterine infusion of the Streptococci inoculum (2, 3, 4, 6, 9, 12 and 15 hrs and 1, 2, 4, 6, 10, 14 and 28 days).

The findings of physical examination and the results obtained from the serial samples were used to assess the degree, extent and duration of the inflammatory reaction and immune response, endometrial damage and the persistence of the infused Streptococci in the uterus.

The major findings of the present study were:-

1) A predominantly neutrophilic response, which did not differ significantly between resistant and susceptible mares was observed within 2 hours of challenge. Neutrophil function tests indicated that there were no significant differences in the chemotactic, phagocytic and intracellular killing ability of both circulating and

uterine neutrophils obtained from resistant and susceptible mares. Uterine washings obtained before and after challenge from both resistant and susceptible mares did not enhance phagocytosis.

2) An influx of serum proteins including immunoglobulin A, G, G(T) and traces of M, which reached a peak between 6 and 24 hours of challenge, was observed in all challenged and control mares. IgA and IgG concentrations in uterine washings obtained from the challenged mares, were significantly higher than those measured in uterine washings obtained from control mares. This finding and the observation that persisting infection resulted in continuing high levels of IgA indicated that the IgA was produced in response to the infused Streptococci. There was, however, no significant difference between the total protein concentrations in uterine washings and fluid obtained from resistant and susceptible mares. The concentrations of ceruloplasmin and alfa 2 - globulins, both documented modulators of inflammation, reached their highest levels between 6 and 24 hours after challenge. In contrast the concentrations of trypsin inhibitors, (also documented modulators of inflammation), remained unchanged throughout the observation period.

3) The stage of the oestrous cycle at challenge was determined using plasma progesterone levels (measured by the enzyme immunoassay (EIA)), clinical and physical examination findings and histological findings in uterine biosies obtained 10 days after challenge. Data available from the present study indicated that the stage of the oestrous cycle at challenge did not influence the response to Streptococcal challenge or the persistence of infection.

4) Light and electron microscopic studies showed that the degree and extent of cellular infiltration into the endometrium and the extent of uterine luminal epithelial damage after challenge was similar in both resistant and susceptible mares.

From the data obtained in the present investigations it was concluded that repeated uterine infections, in mares with lowered resistance to endometritis (susceptible), occured in the presence of IgA and that although these mares were capable of subsequently mounting a local immune response that was comparable to that mounted by the resistant mares, they were still unable to eliminate the infused Streptococci. This inability to resolve the experimentally induced endometritis was, however, not due to gross disorders in neutrophil function, lack of immunoglobulins or natural modulators of inflammation and neither was it dependent on the stage of the oestrous cycle at challenge.

SCIENTIFIC COMMUNICATIONS

1) Papers presented at conferences of learned Societies.

a) Munyua, S., Williamson, P., Penhale, J. and Murray, J. (1984). Acute cellular and humoral responses to equine Streptococcal endometritis. Proc. of A.S.R.B., Melbourne August 27-31, Page 60.

b) Williamson, P., Penhale, J., Munyua, S., and Murray, J. (1984). Acute reaction of the mare's uterus to bacterial infection. Proc. 10th Intern. Cong. Reprod . & AI., Illinois, June 10-14.

c) Munyua, S.J.M. (1984)
Postgraduate seminar (Murdoch University, School of Veterinary Studies). Cellular and Humoral responses to intrauterine inoculation with Beta haemolytic Streptococci in a group of mares with differing resistance to endometritis. School of Veterinary Studies, Postgraduate seminars, 13.11.84.

ABBREVIATIONS USED.

BSA .....Bovine serum albumin

C .....Degrees centigrade

cm .....Centimetre

EDTA .....di-sodium ethylenediamine tetra-acetate.

Fig. .....Figure

g .....Force of gravity

g .....Gram

hr(s) .....hour(s)

i.u or IU .....International units

kg .....Kilogram

L or l .....Litre

Log .....Logarithm

M .....Molar

mm .....Millimetre

mM .....Millimolar

ml .....Millilitre

No. or # .....Number

nl .....Natural log.

O .....bacteriology ....no growth

cytology.....no inflammatory cells observed.

IgA, IgG, IgG(T) and IgM .....no reaction

Ceruloplasmin and Trypsin inhibitors.... no activity.

Progesterone.....no detectable.

P .....Probability of outcome being due to chance.

S.E.M. .....Standard error of the mean.

t .....t statistics

u or U .....Units

µg .....microgram

µl .....microlitre

v/v .....volume to volume

w/v .....weight to volume

% .....Percent

= .....Equal to

+ .....Plus

+ .....Present (positive)

- .....Minus

- .....Absent (negative)

> .....Greater than

< .....Less than

TABLE OF CONTENTS

PART I

CHAPTER 1

REVIEW OF THE LITERATURE.

1.1) FUNCTIONAL PHYSIOLOGY AND PATHOLOGY OF THE NON-PREGNANT EQUINE UTERUS.

1.2) BACTERIAL INVASION OF MUCOSAL SURFACES

1.3) UTERINE INFECTION.

1.3.1) Acute endometritis

1.3.2) Chronic endometritis

1.3.3.) Senile endometritis.

1.4) UTERINE DEFENCE MECHANISMS

1.4.1) Anatomical barriers

1.4.2) Cellular and humoral responses to infection

1.4.3) The neutrophil - its role in host defence

1.5) FUNCTIONAL DISORDERS OF PHAGOCYTES

1.5.1) Disorders of chemotaxis

1.5.2) Disorders of opsonization

1.5.3) Disorders of phagocytosis

1.5.4) Disorders of intracellular killing

1.6) LEUCOCYTE FUNCTION TESTS

1.6.1) Evaluation of chemotactic ability

1.6.2) Evaluation of Phagocytic ability

1.6.2) Evaluating the bactericidal activity of neutrophils

CHAPTER 2.

MATERIALS AND METHODS (GENERAL)

2.1) ANIMAL SELECTION.

2.2) ROUTINE BACTERIOLOGY AND CYTOLOGY

2.3) PREPARATION OF BACTERIAL SUSPENSIONS

2.4) UTERINE FLUSHING PROCEDURE

CHAPTER 3.

CELLULAR AND HUMORAL RESPONSES TO EXPERIMENTALLY INDUCED ENDOMETRITIS IN MARES RESISTANT AND SUSCEPTIBLE TO ENDOMETRITIS

CHAPTER 4.

LIGHT AND ELECTRON MICROSCOPIC STUDIES OF THE TISSUE AND CELLULAR REACTION TO UTERINE INOCULATION WITH β HAEMOLYTIC STREPTOCOCCI, IN A GROUP OF MARES OF DIFFERING RESISTANCE TO ENDOMETRITIS

PART II

CHAPTER 5.

CHEMOTACTIC, PHAGOCYTIC AND INTRACELLULAR KILLING ABILITY OF CIRCULATING AND UTERINE NEUTROPHILS OBTAINED FROM MARES KNOWN TO BE EITHER SUSCEPTIBLE OR RESISTANT TO ENDOMETRITIS

5.1) INTRODUCTION

5.2) AIMS

5.3) MATERIALS AND METHODS

5.4) RESULTS

5.5) DISCUSSION

CHAPTER 6

NON SPECIFIC FACTORS AFFECTING THE RESOLUTION OF EQUINE ENDOMETRITIS IN A GROUP OF MARES WITH DIFFERING RESISTANCE TO BACTERIAL ENDOMETRITIS

6.1) INTRODUCTION

6.2) AIMS

6.3) MATERIALS AND METHODS

6.4) RESULTS

6.5) DISCUSSION

CHAPTER 7

CONCLUSION

FUTURE RESEARCH POSSIBILITIES.

INTRODUCTION

It has long been recognized that horses bred under intensive systems of management suffer disappointingly low fertility. Worldwide Surveys of fertility suggest that the percentage of mares producing live foals lies between 50% - 80%, varying between different populations of horses, management systems and with statistical methods used to analyse data (Rossdale and Ricketts, 1980). In most instances this infertility has been ascribed to poor management, the artificially imposed breeding season, stallion factors, hormonal and nutritional imbalances and pathology of the reproductive tract (Roberts, 1971; Rossdale and Ricketts, 1980).

Clinical observations in the mare indicate that the uterus is grossly contaminated with bacteria during coitus and parturition (Bain, 1966; Peterson, McFeely and David, 1969). Dimmock (1925) and Hughes, Loy, Asbury and Burd (1966) reported that they were able to recover bacteria from the genital tract in 80-90% of mares, 24-72 hours post coitus and 1-30 days post partum. Hughes and Loy, (1969; 1975) and Peterson et al., (1969) observed that the "normal mares" eliminated the contaminating bacteria in a short period of time and concurrently developed a transient inflammatory response in the endometrium. "Abnormal mares", on the other hand, were unable to eliminate the bacteria as rapidly and hence the inflammatory response in their endometrium persisted for longer periods.

In uterus of most mares the transient inflammation of the reproductive tract, initiated by bacterial contamination at service or parturition, is often aggrevated by the continual cycle of natural cover, infection, pregnancy and uterine involution. This cycle has been shown to result in repeated damage and progressive deterioration in the

intergrity of the uterus (Hughes and Loy, 1969; 1975; Kenney, 1978; and Ricketts, 1978).

In the first section of this review the general features of development, anatomy and physiology of the equine uterus will be described. And as the establishment and subsequent persistence of bacterial infection in the uterus is most likely preceeded by the adhesion of the invading bacteria to the luminal epithelial cells, this phenomenon is discussed in detail. Thereafter, the functional physiology and pathology of neutrophils and production of immunoglobulins and their role in the defense of the uterus will be dealt with.

CHAPTER 1.

PAGES

CHAPTER 1.

LITERATURE REVIEW

1.1) FUNCTIONAL PHYSIOLOGY AND PATHOLOGY OF THE NON PREGNANT EQUINE UTERUS

The tubular portion of the mares' reproductive tract including the fallopian tubes, uterus, cervix and vagina arises from the primitive mullerian duct system, while the vulva develops from the urogenital sinus (Roberts, 1971).

The uterine wall has three distinct layers: (1) the serous membrane which consists of an extension of the peritoneum (perimetrium), (2) the myometrium consisting of circular and longitudinal smooth muscles which are subject to considerable hypertrophy. (3) the endometrium, a highly vascularized structure, consisting of the epithelial lining of the uterine lumen and a glandular layer (Hafez, 1980; McDonald, 1980). The glandular layer is divided into stratum compactum, an area of glands and dense connective tissue, and stratum spongiosum, an area of glands and loose connective tissue (Kenney, 1978). Changes in the myometrium and the endometrium (oedema, atrophy, hypertrophy or vascularity) are cyclic, responding to varying levels of oestrogen and progesterone throughout the oestrous cycle and pregnancy (Hafez, 1980; McDonald, 1980).

The increase in vascularity and blood supply to the uterus during proestrus and oestrus is associated with an increase in the number of leucocytes in the blood vessels with or without infiltration into endometrial tissue. The extent and degree of this leucocytic infiltration into the uterine lumen and endometrial tissue, however,

varies with species (Knudsen, 1964; Kenney, 1978).

The cow, ewe and the doe have high numbers of predominantly polymorphonuclear (PMN) leucocytes including neutrophils and few eosinophils in the endometrial stroma and uterine lumen during the follicular phase of the cycle (Finn and Porter, 1975; Dogameli, Aydin and Alancam, 1978). A similar influx of polymorphonuclear neutrophils (PMN) is seen in proestrous in the bitch and the queen (Shuttle, 1967; Prescott, 1972; Burke, 1975) and during the secretory phase just before and after mensturation in women (Novak and Woodruff, 1967; Finn and Porter, 1975). The mare, however, is unique in that despite the congestion and margination of blood vessels with PMN during the follicular phase there is rarely any leakage into the uterine lumen or stroma (Kenney, 1978; Munyua, 1982).

Evidence available to date indicates that in mammals secretory responses by neutrophils are critical for the initiation, development and regulation of acute inflammation. During the early phase of inflammation chemotactic factors promote the adhesion of leucocytes to vascular endothelium, the direct migration of the neutrophils and stimulate granule exocytosis (Becker and Showell, 1974; Wright and Gallin, 1979). In addition these chemotactic factors trigger off the oxidative metabolism at the plasma membrane (Lehmeyer, Synderman and Johnston, 1979).

1.2) BACTERIAL INVASION OF MUCOSAL SURFACES

The vulva, the posterior termination of the urogenital tract located just below the anus, provides a principal portal of entry for multitude of pathogens present in the environment.

Various species of bacteria are frequently isolated in samples obtained from the equine uterus (Shin et al., 1979; Rossdale and Ricketts, 1980). Whether the adherence of these bacteria to mucosal surfaces, including the uterine luminal epithelium, is critical for the establishment of an infection is yet to be elucidated (Waldman and Ganguly, 1976; Sugarman, 1980; Beachey, 1981).

Reputed mediators of attachment, including lipoteichoic acids (LTA) for Staphylococci (Aly, Shinefield, Litz and Maibach, 1980), and Streptococci (Beachey, 1976), and Type 1 and other pilli for gram negative bacilli (Woods, Straus, Johanson, Berry and Bass, 1980, Beachey, 1981), have been identified. However, the receptor sites on mammalian cells are not well characterized (Myhre and Kronvall, 1980; Beachey, 1981).

Waldman and Ganguly (1976), Sugarman (1980), and Beachey, (1981), suggested that a close association between the hosts' mucosal surfaces and the invading bacteria may be essential for the establishment of an infection. Weiss, (1972), Kozel, Reiss and Cherniak, (1980), and Tylewska, Hjersten and Wadstrom, (1981), showed that mammalian cells and micro-organisms display electrical potentials across their outer layers. These charges have been suggested as being involved in the interactions between organisms and receptor cells by simple attraction of the opposite charges and the expulsion of the like charges (Tylewska et al., 1981).

1.2.1. Factors affecting bacterial adherence.

Several factors have been shown to affect bacterial adherence to mucosal surfaces. Only the factors that could have a possible application to the uterus will be dealt with. The factors, most

likely to influence bacterial adherence in the uterus, include:-

1) Tissue tropism: Selectivity of adherence, the preference of particular bacteria for certain tissues over others, suggests that in many instances it may be a causative factor in colonization and infection. Frequently cited examples of tissue tropism is the apparent preference of Streptococci mutans for dental plaque (Gibbons and Houte, 1975; Mardh and Westrom, 1976; Gibbons, 1977; Sugarman, 1980), group A Streptococci, for the gastrointestinal tract, and N.gonorrhoeae for the urinary tract, in humans (Stotterman 1975), and diarrheagenic E. coli K88 infections in pigs (Jones and Rutter, 1974).

2) Ovarian hormones: In vitro adherence of Neisseria gonorrhoeae group B Streptococci, E. coli and Proteus mirabilis to isolated human genitourinary tract epithelial cells obtained from women have been reported to change with stages of the menstrual cycle of cell donors (Botta, 1979; Svanborg-Eden and Leffer, 1980). However, their results were inconsistent and other researchers failed to observe the reported cyclic changes in the adherence of these bacteria (Svanborg-Eden and Leffer, 1980; Schaeffer, Jones and Dun, 1981; Sobel, Schneider, Kaye and Levison, 1981). Sugarman and Epps (1982) showed that epithelial receptor cells, derived from the cervix (Hela), displayed oestrogen responsiveness and suggested that the modification of the outer Hela layers altered the subsequent bacterial attachment. Their data offered direct support for the hypothesis that hormone levels may influence bacterial attachment to genitourinary epithelial cells, thereby affecting colonization and infection in these and other areas (Holmes, Counts and Beaty, 1971; Baker, Goroff, Alpert, Crickett, Zinner, Evrard, Rosner and McCormick, 1977;

Botta, 1979; Forsin, Danielson and Falk, 1979; Schaeffer, Amundsen and Schmidst, 1979; Svanborg-Eden, Larsson and Lomberg, 1980). Sugarman and Epps (1982) proposed that hormonal changes may influence bacterial adherence by altering the epithelial cell number and morphology, modifying receptor site synthesis on epithelial cells, changing the surrounding pH or mucous composition. It is, however, apparent that all cells do not appear to respond similarly to hormone treatment.

3) Drainage: During good health, mucosal surfaces are constantly bathed with secretions laden with antibacterial agents including enzymes, antibodies and positively charged proteins which impede the attempts of pathogens to colonize the surface. In addition most unattached organisms are simply swept away mechanically. Thus, successful pathogens are those that are capable of not only penetrating these local defenses and attaching to the mucosa, but of eventually penetrating the "intact epithelial cell" barrier (Novak and Woodruff, 1967; Sugarman, 1980; Ofek and Beachey, 1980; Beachey, 1981). Cervical drainage in the mare has been implicated as one of the principal reasons why acute endometritis normally resolves (Hughes and Loy, 1969; 1975; Peterson et al., 1969).

4) Recurrent infections and the availability of binding sites: Fowler and Stammey (1977), Kallenius and Winberg (1978), and Svanborg-Eden and Jodal (1979), showed that periurethral epithelial cells of women who have experienced recurrent urinary tract infections bind a higher number of E. coli than do periurethral epithelial cells from females who are not prone to such infections. Sellwood et al. (1975), demonstrated that the gastrointestinal epithelial cells from piglets resistant to diarrhea

produced by *E. coli* K88 bound less organisms than the brush-borders of susceptible piglets. These workers concluded that the presence of specific receptors was critical for the establishment of these bacteria. They also suggested that the piglets which lacked receptor sites for bacterial attachment, for example *S. aureus* or *E. coli* K88 in the brush-borders of their gastrointestinal epithelial cells could be considered to be genetically immune.

Freter (1980), Sugarman (1980), and Beachey (1981) have, however, criticized some of the interpretations made from data obtained from *In vitro* systems designed to study bacterial adherence. They pointed out that researchers investigating bacterial adherence often overlook the complexities of the adherence phenomenon in the intact host, which include bacterial motility, chemotaxis, penetration of the mucous gel, adhesion to receptor cells, migration into deeper intervillous spaces, attachment to epithelial cells and/or elaboration of toxins. In addition Freter (1980), Sugarman (1980), and Beachey (1981), stated that some of the conclusions were based on data collected from small sample numbers hence it was not clear whether the reported selectivity reflected a true pathogenic mechanism in the etiology of colonization and infection. Sugarman, (1979; 1980), suggested that the observed specificity could have been reflecting the different handling of bacteria, a coincidence and/or the fact that adherence may in turn, be dependent upon another pathogenic (virulence) mechanism. Support is lent to their view by experimental evidence refuting the importance of bacterial adherence as a pathogenic mechanism (Selinger and Reed, 1977). These investigators, studied the *Streptococcus pneumoniae* attachment to oral mucosal cells and reported that increased capsule size, which has for long been presumed to be

an indicator of virulence, was associated with decreased adherence and that adherence of the serotype which affected humans was much lower than for other gram-negative organism. The technical arguments for and against bacterial adherence as a pathologic mechanism are beyond the scope of this review, hence interested readers are refered to the articles by Bjorksten and Kaijser, (1978), Svanborg-Eden and Hansson, (1978), Silverblatt and Ofek, (1978a; b), Sugarman, (1980) and Beachey,(1981).

1.3) UTERINE INFECTION

Inflammation represents a dynamic state, consisting of several interdependent reactions involving leucocytes, platelets, blood and lymph vessels and tissues. These reactions are incited in the host by chemical, physical and/or infectious irritants (Volanakis, Freeman and Stroud, 1975). Uterine infection in the mare is often a sequel to trauma of the reproductive tract; dystocia, foetal maceration and emphysema, retained after birth, abortions and poor management of mares at a stud (Roberts, 1971; Hughes and Loy, 1969; 1975; Rossdale and Ricketts, 1980).

Clinically, uterine infections are generally localized except in cases of severe puerperal or septic endometritis where systemic signs are evident. In cases of parametritis and perimetritis or uterine abscessation which results in peritonitis, the signs of the peritonitis mask those of endometritis (Nieberle and Cohrs, 1966; Novak and Woodruff, 1967; Roberts, 1971; Smith et al., 1972; Rossdale and Ricketts, 1980).

Most organisms isolated in cases of endometritis in the mare are opportunistic and are remarkably similar to those isolated in "normal" females of the same species (Hughes and Loy, 1969; Shin, Lein, Aronson and Nusbaunn, 1979; Munyua, 1982; Williamson et al., 1983). This is similar to the situation in women (Novak and Woodruff, 1967; Gibbs, O'Dell, McGregor, Schartz and Morton, 1975), the bitch (Osbalbson, 1978; Farstad, 1982), and the cow (Fukuyama, Kamimura, Itoh, Yoshida and Kudoh, 1978). Hughes and Loy (1969, 1975), reported that under certain conditions, most probably inherent to the particular female, pathogenecity of the same bacteria in the uterus varied. Endometritis, which is the inflammation of the endometrium, can be classified as either acute, chronic or senile, all three of which could be either mild, moderate or severe depending on the extent of stromal changes and intensity of cellular reaction in the endometrium and uterine lumen.

1.3.1. Acute endometritis.

1) Simple acute endometritis: Simple acute endometritis is characterized by intense PMN infiltration of the superficial layers of the uterine mucosa. PMN , which are seen migrating betweeen cells of the luminal epithelium, are sometimes retained in an amorphous layer over the surface of the epithelium.

2) Septic endometritis: Septic endometritis is characterized by severe leucocytic, predominantly PMN infiltration of the strata compactum and spongiosum with extensive inflammatory thrombotic process in vessels and interstitial haemorrhage. Marked stromal oedema, endometrial necrosis and ulcerations, perivascular cuffing, vascular obstruction and margination are frequent

findings. Perimetritis is a common sequel , with dissemination of the causative agent to localize in other tissues and organs.

3) Puerperal endometritis: Uterine infection during the puerperal period, especially in the cow is usually very severe with parametritis and perimetritis often being the sequel . Unless treatment is instituted early septic emboli cause multiple abscesses with high mortality (Novak and Woodruff, 1967). Histological findings in puerperal endometritis are similar to those in septic endometritis except in cases of parametritis and perimetritis where the myometrium and the uterine serosa are affected.

1.3.2 Chronic Endometritis.

The presence of plasma cells and degenerative changes are diagnostic of chronic endometritis, in both the mare and woman (Sen and Fox, 1967; Novak and Woodruff, 1967; Kenney, 1978).

1) Chronic endometritis: Chronic infiltrative endometritis is often a sequel to acute endometritis and is characterized by periglandular and perivascular aggregation of mononuclear cells and PMN . There is, in addition, a consistent PMN and mononuclear cell infiltration into the luminal epithelial cells. Persisting infection results in a change in the predominant cell type from PMN to mononuclear cells, thus resulting in chronic endometritis.

2) Chronic degenerative endometritis: In the mare, chronic degenerative endometritis is characterized by the presence of varying degree of glandular and stromal fibrosis. The glandular degenerative changes often observed are of 2 basic appearances:-

a) the glandular nests, where an area of glandular tissue becomes well circumscribed, often surrounded by a variable thickness of fibrous tissue lamellae. The luminal epithelium of the circumscribed glands appears low cuboidal and inactive and in most cases their histochemical staining affinity is low.

b) Glandular cysts, where the glandular lumen is significantly enlarged and surrounded by a variable thickness of fibrous tissue lamellae. In some cases these cystic endometrial glands appear full of insipissated amorphous secretions. Associated with cystic glands are lymphatic lacunae, which are pools of fluid in the lymphatic drainage system. In the bitch and queen chronic degenerative endometritis is often associated with prolonged oestrogenic stimulation, as is in cases of ovarian cysts (Roberts, 1971) with leucocytic infiltration of the uterine glands which then become cystic (Nieberle and Cohrs, 1966). Occasionally, however, spontaneous chronic degenerative endometritis in the bitch and the queen does occur after 7 years of age (DeCoster, Dieterren and Jose, 1979). In the human cystic glands and cystic hyperplasia are usually associated with persistent follicles and/or intensive oestrogen therapy during menopause (Ricketts, 1975).

3) Purulent chronic endometritis (Pyometra): Pyometra is most common in the bitch and the cow (Roberts, 1971; Colby, 1975). Hughes et al., (1979), proposed that pyometra in the mare development due to lowered endometrial resistance to infection and uterine adhesions. In the bitch pyometra may be secondary to hyperoestrogenism that is as a result of cystic ovaries and/ or ovarian tumours. In the bitch, as in most other species however, high pro-

gesterone levels in the presence of an infection are the most common cause of pyometra (Bloom, 1953; 1954; Roberts, 1971). Histologically, pyometra is characterized by an intense plasma cell infiltration into the endometrium, including the endometrial glands, with extensive sloughing of the endometrium and occassionally the stromal tissue. During healing some of the sloughed areas are replaced by granulation tissue and glands are absent over wide areas (Nieberle and Cohrs, 1966). In the woman there is disruption of the menstrual cycle but the tissue changes are usually less marked due to denudation of the uterine wall every 20-22 days (Novak and Woodruff, 1967).

1.3.3 Senile Endometritis.

1) Senile endometritis is a postmenopausal condition in women, which is often superimposed on postmenopausal atrophy. Senile endometritis is characterized by a thin endometrium which is heavily infiltrated by round and plasma cells. The resulting tissue damage from senile endometritis has been shown to be the cause of postmenopausal bleeding (Novak and Woodruff, 1967).

1.4) UTERINE DEFENCE MECHANISMS

1.4.1) Anatomical barriers

Anatomical barriers in the uterus include the intact mucous membranes, the cervix and vulval lips, with the cervix closing during the luteal phase when the uterus is supposedly most vulnerable to infection (Hughes and Loy, 1969; 1975). In addition the mucous gel, epithelial cell desquamation and uterine secretions impede attempts of pathogens

to colonize the surface, while unattached pathogens are mechanically swept away by cervical drainage. This cervical drainage, in the mare (Hughes and Loy, 1969; 1975; Woolcock, 1980), the doe and the cow (Black, Uberg, Kidder, McNutt and Casida, 1953; Winter, Broome, McNutt and Casida, 1960) and menstruation and cervical drainage in women (Novak and Woodruff, 1967) are among factors responsible for the tendency for endometritis to resolve.

1.4.2 Cellular and humoral response to uterine infection.

In the mare the immediate response to experimental infection with uterine pathogens is an early (within 6 hours) intense and predomenantly PMN infiltration into the endometrium and uterine lumen. By 72 hours post inoculation there is a decrease in the number of viable bacteria and the appearance of mononuclear cells in the uterine lumen and the endometrium (Hughes and Loy, 1969; 1975; Peterson et al., 1969). These workers observed that in cases where infection persisted, mononuclear cell infiltration into the endometrium and uterine lumen predominated within 7 days. Hughes and Loy (1969; 1975) and Peterson et al., (1969) proposed that neutrophils increased in numbers in mares that were capable of resisting infection whereas susceptible mares showed a poorer response. Asbury, Schultz, Klesius, Foster and Washburn (1982) and Munyua, Williamson, Penhale and Murray (1984), however, showed that there were no significant differences between the numbers of neutrophils observed in uterine washings of susceptible and resistant mares following experimental infection with B haemolytic Streptococci. In addition Asbury et al., (1982) reported that neutrophils harvested from resistant and susceptible mares showed similar chemotactic and phagocytic activities, but suggested that susceptible mares were deficient in opsonins. These investigators,

however, did not report on the possible effects of dilution on opsonic activity of the uterine washings or the presence and the absolute values of the various possible opsonins.

The hallmarks of the secretory immune system are the local synthesis of antibody (of which IgA usually predominates), and a distinctive mechanism of transport of polymeric IgA, in conjunction with the secretory component, across the secretory epithelium. The existence of a local or regional immunity that is not dependent on serum has been known for sometime (Waldman and Ganguly, 1976). It is now accepted that immunoglobulin A (IgA) which represents a relatively small fraction of the serum immunoglobulins, is the predominant species in the mucosae and glands of the digestive tract, udder, respiratory tract and the uterus (Vearman and Ferin, 1974; Asbury et al., 1980; Mitchel et al., 1983; Williamson et al., 1983). The humoral immune response of mucosal surfaces of the reproductive tract has recently been reviewed (Vearman and Ferin, 1974; Tomasi, 1980; Winter, 1982).

Most recent investigations suggest that most mammalian uteri, including that of the mare, are capable of mounting a local immunological response including the production of specific antibodies (Liu, Mitchell, Perryman and Stewart, 1981). Where such a system for local production of immunoglobulins is not available, as is the case in some young women, a secretory component possibly compensates by preferential secretion (extraction) or diffusion of polymeric IgA from serum (Rebello et al., 1975; Kelly and Fox, 1979).

Kenney and Khaleel, (1975) reported the presence of IgA and IgG positive lymphoid cells in equine endometrial biopsies. Kenney and Khaleel, (1975), Asbury, Halliwell, Foster and Longino, (1980) and

Williamson et al., (1983; 1984) reported the presence of IgA, IgG and IgG(T) in uterine washings obtained from uninfected mares. Asbury et al., (1980), Blue, Brady, Davidson and Kenney, (1982), Williamson et al., (1983; 1984), and Munyua et al., (1984) observed that natural and experimental uterine infections in the mare are associated with increased concentrations of immunoglobulins A, G and G(T) in uterine washings obtained from normal (resistant) mares and in mares with postulated lowered resistance (susceptible) to bacterial endometritis. Asbury et al., (1980) observed that normal (resistant) mares had lower total immunoglobulin levels than mares with postulated lowered resistance (susceptible) to streptococcal endometritis.

1.4.3. The effects of ovarian hormones on susceptibility to endometritis.

The results obtained by Asbury et al., (1980) indicated that the immunoglobulin content may be influenced by ovarian hormones. These investigators showed that young nulliparous mares had detectable IgA levels during dioestrus and IgM during both dioestrus and oestrus while older, chronically infected mares had both IgA and IgM in oestrus and dioestrus.

There are distinct species differences in the variation of chemical and cellular compositon of uterine secretions under the influence of ovarian hormones (Heap and Lamming, 1960; Kenney, 1978; Munyua, 1982). In ruminants and rabbits the uterus appears to be much more susceptible to bacterial infections during the luteal phase than during the oestrogen influenced follicular phase (Rowson, Lamming and Fry, 1953; Broome and Lamming, 1959; Broome, Winter, McNutt and Casida, 1960; Hawke, Turner and Sykes, 1960; Lamming and Haynes, 1964). In these

species, ovarian hormones seem to exert a profound influence on bacterial resistance via several mechanisms including alterations in cervical drainage, (Black, Simon, Kidder and Witback, 1954), influencing leucocyte response and modulating the presence or absence of a non-cellular bactericidal factors (Hawke, 1958; Winter, Broome, McNutt and Casida, 1960) and cellular infiltration into the endometrium (Kiubar, 1981). It was initially assumed, particularly by extrapolation from bovine data, that the mares' uterus is also resistant during oestrus and susceptible during the luteal phase (Roberts, 1971). However, subsequent investigations have presented conflicting results.

Hughes and Loy, (1969; 1975), Blue et al., (1982) Williamson et al., (1984) and Munyua et al., (1984), using intact cycling mares, showed that the clinical response to uterine infection in the mare was similar in extent and duration, during oestrus and dioestrus. In contrast Ganjam et al., (1980; 1982) and Washburn et al., (1982) using ovariectomized mares treated with oestrogen or progesterone to simulate their levels in circulation during the follicular or luteal phases, concluded that ovarian hormones influenced leucocytic response and neutrophil function during uterine infection. The discrepancies in observations reported by the two groups is difficult to explain other than propose that they could have been due to the different experimental designs used to study the pathogenesis of equine endometritis. Despite these differences, however, both groups agree that the influx of neutrophil into the uterus immediately following bacterial invasion plays a central role in the resolution of endometritis.

1.4.4. The neutrophil - its role in host defence.

The phagocytic system consists of circulating neutrophils and eosinophils (PMN), basophils, mononuclear cells (MM) and sessile macrophages. Neutrophils, normally released as mature cells from the bone marrow, have remarkable capabilities for chemotaxis (directional movement), phagocytosis (ingestion), oxidative metabolism and intracellular killing. In contrast mononuclear phagocytes, which are released as immature cells (blood monocytes), migrate into tissues where they develop into histiocytes or macrophages (Quie et al., 1979).

Neutrophils develop from the undifferentiated haemopoietic stem cells in the bone marrow. The human neutrophil, which has been extensively studied, is known to contain primary and secondary granules. The primary granules contain acidic hydrolases including acid phosphatase (AP), neutral proteases including collagenase, cationic bactericidal proteins, glucoronidase (B-glu), myeloperoxidase (MPO), lysozyme and acid mucopolysaccharide. The secondary granules contain lysozyme, lactoferrin, cobalophilin (Vitamin B12 binding protein), collagenase and acid proteins (Valentine et al., 1951; Bainton et al., 1966; Bagglioni et al., 1970; Bagglioni, 1972; Klebannoff and Clark, 1978; Wright, 1982). A number of biologically active granule components which still await further characterization have been described (Wright and Gallin, 1977; Wright and Greenwald, 1979; Wright, 1982).

The loss of granulation(s) from the neutrophils engaged in an inflammatory response or during phagocytosis is both an intracellular (Hirsch and Cohn, 1960) and extracellular phenomena. The extent of extracellular release during phagocytosis is dependent on the degree of phagocytic challenge (e.g. the number of particles: neutrophils),

the type of particles ingested and the particular granule component under study (Henson, 1971a, b; Wright and Malawista, 1972).

Experiments by Hoffstein et al., (1974), showed that although degranulation is associated with phagocytosis, it is not dependent upon it, and that degranulation occurs at the plasma membrane, whether the membrane is internalized in forming a phagocytic vacuole or remains at the cell surface.

The relevance of extracellular degranulation to the pathology of inflammation was clearly supported by the identification of diverse proteolytic enzymes in the neutrophil granules which could digest tissue substrates (Janoff, 1972; 1975; Wright, 1982) and the observation that certain anti-inflammatory drugs, including colchicine and corticosteroids, inhibited degranulation and the extracellular release of granule constituents (Wright and Malawista, 1973; Hawkins, 1974; Goldstein, 1976). Wright, (1982), suggested that during their functional life, neutrophils may encounter a variety of stimuli which can induce extracellular degranulation, some of which (stimuli) appear to promote a selective exocytosis of the secondary granules and others the combined exocytosis of both the primary and secondary granules. Wright (1982) further suggested that when the neutrophils encounter humoral stimuli such as chemotactic factors, become adherent to the microvascular endothelium and emigrate into the extravascular spaces, secondary granule exocytosis is significantly increased. He concluded that when neutrophils encounter phagocytic stimuli (opsonized microorganisms, phagocytosable tissue, cellular debris), at the site of inflammation significant secretion of the primary granule components can occur, enhanced further by toxic factors which ultimately lead to neutrophil lysis and death.

1.4.5. Consequences of secretion for the host defence.

There has been considerable investigation of the extracellular release of proteases and lysosomal constituents from primary granules by neutrophils, because of their relevance the tissue damage that characterizes inflammatory disease processes (Review - Henson, 1972; Weissmann, Zurier and Hoffstens, 1973; Weissmann, Smolen and Korchark, 1980; Wright, 1982). It is now evident that neutrophil secretory responses involving secondary granule exocytosis and the release of metabolites generated at the plasma membrane, may be just as important to the physiology of acute inflammation. Secretory products may: (i) deliver antimicrobial activity to the extracellular millue, (ii) amplify, facilitate and control the various features of the inflammatory responses, (iii) influence the function of the inflammatory monocytes and lymphocytes and (iv) regulate myelopoiesis (Wright, 1982).

1.4.5.1. Extracellular antimicrobial effects.

Wright, (1982), proposed that the day-to-day functions of neutrophils are to contribute to the integral barriers and to constantly restore sterility in mucosal and submucosal tissues when invaded by small, insignificant numbers of micro-organisms that have circumvented these barriers. Large numbersof neutrophils are continually delivered to the mucosal surfaces of the body, particularly in the gastrointestinal tract where diverse micro-organisms reside and proliferate as "normal flora" (Klinkhamer, 1963; Teir, Rytomaa, Cedaburg and Kiviniemi, 1963). Wright (1982) proposed that this constant supply of neutrophils to such sites clearly constitutes an important part of the barrier against microbial invasion, for when the production and turnover of the

neutrophils ceases, overwhelming sepsis with organisms ensues. Thus, in the gastrointestinal tract and possibly the uterus, the neutrophils do not represent a latent system of phagocytes that becomes active only with an acute infection. In this state, however, secretion of primary granule constituents would not appear to occur to an extent to cause tissue damage. A substantial secretion of secondary granule components may occur in the course of normal neutrophil turnover in the absence of phagocytic stimuli (Wright, 1982) for the secondary granule exocytosis appears to be an intrinsic feature of neutrophil adherence, response to chemotactic factors and emigration into extracellular spaces (Wright and Gallin, 1979).

Lysozyme (muramidase) (Flemming and Alison, 1922), lactopherin (Iron binding protein) (Kirkpatrick, Green, Rich and Schade, 1971; Reiter, Brook and Steel, 1975) and cobalophilin (Gullberg, 1974) are contained in the secondary granules and these components have a direct and synergistic antimicrobial activity (Wright, 1982). In addition non-phagocytic stimuli that induce secondary granular exocytosis coincidentally stimulate an oxidative metabolic response at the neutrophil surface, resulting in the extracellular release of hydrogen peroxide (H_2O_2) and oxygen derivatives such as superoxide. These oxygen metabolites may provide short term antimicrobial activity in the extracellular millieu particularly when combined with exogenous peroxidases in the environment (Klebanoff and Clark, 1978; Klebanoff, 1982). Furthermore, oxygen metabolites have been clearly implicated as mediators of the extracellular cytotoxic effects of neutrophils, particularly when combined with extracellular peroxidase and halide (Clark and Klebanoff, 1977; Klebanoff, 1982).

Pathologic conditions in which there is a large scale invasion of tissues by micro-organisms may provide a sufficient stimulus for the extracellular release of primary granule constituents and exteriorization of antimicrobial activities that are normally, confined to phagocytic vacuoles. This sort of secretory response, however, albeit of probable importance in eliminating infection, is at the expense of normal tissues (which are destroyed) in the extracellular environment (Weissmann, Zurier, Band and Hoffstein, 1972).

1.4.5.2 Amplification and modulation of the inflammatory responses.

During the early phase of inflammation, chemotactic factors promote direct migration of the neutrophils and stimulate granule exocytosis (Becker and Showell, 1974; Wright and Gallin, 1979) and in addition, these factors, trigger off the oxidative metabolism at the plasma membrane (Lehmeyer, Synderman and Johnston, 1979). Secondary granules exocytosis and the consequent delivery of acidic proteins to the cell surface has been implicated in the greatly increased cell adhesiveness that results from exposure to chemotactic factors (Bockenstedt and Goetzl, 1980). Neutrophil adhesiveness to vascular endothelium is also promoted by products of membrane lipid turnover and metabolism in stimulated neutrophils (Stenson and Parker, 1979; 1980). These changes in the adhesiveness appear to be critical in the irreversible adherence of circulating neutrophils to capillary endothelium which initiates the accumulation of these cells at the site of inflammation. Secretory events also appear to be involved in loosening the endothelial cell junctions and in splitting the basement membrane, thereby creating gaps in the capillary vessel walls through which the neutrophils can emigrate into the tissues. Neutrophils

have been shown to loosen the intercellular and surface attachments of endothelial cells and fibroblasts in culture, through a combined effect of secreted oxygen metabolites and proteases, derived most likely from the primary granules (Harlan, Killen, Harker and Striker, 1980). Secreted collagenase is stored in both primary and secondary granules and may facilitate the penetration of neutrophils through the microvascular basement membrane (Gadeck, Fells, Wright and Crystal, 1980). The secretion also appears to provide a mechanism by which neutrophils initially engaged at an inflammatory site may amplify the inflammatory response and thus accelerate the accumulation of additional neutrophils. This amplification effect may be mediated both by the generation of the vasoactive mediators that enhance local blood flow and by the generation of chemotactic factors. Activated neutrophils have been shown to release various biochemically active proteins and proteases including:-

1) those that act on the peptides with bradykinin-like activity, (leucokinionogen), in serum (Movat, Habal and Macmorine, 1976).

2) Plasminogen activator, which acts on plasminogen to produce plasmin which acts on kininogen to release kinin (Miller,Webster and Melmon, 1975).

3) granule derived mediators, that cause degranulation of the tissue mast cells with a consequent histamine release from cells (Kelly, Martin and White, 1971).

4) cationic proteins, that include the release of the vasoactive amines from platelets (Hallgren and Venge, 1976).

5) a product of the secondary granules, that generates the chemotactic fragments of C5 through the activation of the intact complement system in serum and proteases (primary granules) that can generate chemotactic molecules through the direct cleavage of complement components (Wright and Gallin, 1975).

6) and prostaglandins (Zurier, 1976).

Although secretory events may serve to amplify the response of the neutrophils to inflammatory stimuli they also provide for negative feedback regulation of inflammation. Proteases derived from the primary granules, extracellular H_2O_2 and myeloperoxidase have been shown to inactivate chemotactic factors (Wright and Gallin, 1977; Brozna, Senior, Krentzer and Ward, 1977; Clark and Klebanoff, 1979) and to degrade vasoactive kinins (Granelli-piperino, Vassali and Reich, 1977).

Plasma protease inhibitors such as alpha 1-antitrypsin or alpha 2-macroglobulin defend the tissues against the escape of neutral proteases. In addition antioxidants or free radical scavengers, such as ceruloplasmin, a copper containing protein, and superoxide dimutase are generated as acute phase reactants, and they dampen the response of the host to products secreted by the neutrophils (Goldstein, Kaplan, Edelson and Weissman, 1979; Weissman, Korchak, Perez, Smolen, Goldstein and Hoffstein, 1979).

Predictably these complex cellular and humoral immunological interactions and the resulting initiation, modulation, amplification and/or inhibition of inflammation, has led to the establishment of numerous assays for the study of neutrophil function. Equally predictable is the intensity of the claims and counter claims of

superiority of a particular assay (Miller, 1976; Fuenfer, Scott and Pold, 1976). Through this controversy, three facts have emerged:-

1) that there are no "standard assays" for either chemotaxis, phagocytosis or intracellular killing ability. This is basically due to multiple variables that are difficult to standardize and the relatively short period of functional integrity of neutrophils.

2) that the effects of the complex sets of interactions between the phagocytes and humoral components in most, if not all, functions on particular assay systems are yet to be ellucidated let alone agreed upon.

3) that the method(s) of interpreting the data obtained from the currently available techniques are not always readily acceptable, especially in view of the fact that conditions of In vitro phagocyte assays differ substantially from what is believed to be the actual In vitro conditions under which neutrophils function. This situation is aggravated by the scarcity of information on the interactions and the relative importance of individual components of inflammatory response (Miller, 1976; Fuenfer et al., 1976).

It is, therefore, often difficult to ascribe to a particular In vitro defect as the cause of recurrent infections or impaired immunity in a given subject (or patient). This is particularly true in those situations in which combined defects involving chemotaxis, phagocytosis and intracellular killing ability are demonstrated (Quie, 1979; Wright, 1982).

1.5) FUNCTIONAL DISORDERS OF PHAGOCYTES.

Efficient chemotaxis, phagocytosis and the subsequent intracellular killing have been shown to be important factors in resistance to disease and often play a decisive role in the delicate balance between host and pathogenic bacteria. Patients with gross functional disorders of the phagocyte system suffer from frequent infections which are often prolonged, inspite of appropriate antibacterial therapy and recurrent lesions are the rule (Quie et al., 1979). The virulence of some of the pathogens is related in part to their ability to resist ingestion by phagocytes whereas that of others is related to their ability to resist intracellular killing once ingested (Wood, 1951; Cleat and Coid, 1981; 1982). Effective phagocytosis and intracellular killing in the early course of bacterial invasion may limit the spread of infection. However, ineffective phagocytosis and/or killing may lead to uncontrolled bacterial multiplication and overwhelming infection (Quie et al., 1979).

The sequence of events leading to intracellular killing of bacteria by phagocytes may be conveniently divided into four distinct but inter-related phases including, i) Directional movement - chemotaxis, ii) Preparing for uptake - opsonization, iii) ingestion - phagocytosis and iv) intracellular killing (Winklestein, 1973; Fuenfer et al., 1976).

1.5.1 Disorders of chemotaxis.

Chemotaxis is the directional movement of leucocytes (WBC) towards chemoattractants, resulting in the accumulation of WBC at the foci

of inflammation. Chemotactic factors, $\overline{C567}$, C3a and C5a (products of the serum complement cascade, activated by Antigen-antibody complexes) and PMN granules and extracts (chemotactic in presence of serum) have so far been identified (Hersh and Bodey, 1960; Baehner, 1974). However, the trigger which initiates chemotaxis has yet to be ellucidated (Baehner, 1974; Callin and Quie, 1978; Quie et al., 1979).

Boxer, (1974); and Boxer et al., (1977) incriminated defective microtubular function as the cause of the impaired chemotaxis and degranulation observed in peripheral neutrophils obtained from patients with Chediak Higashi Syndrome. No cases of impaired chemotaxis per se have been reported. Most reported cases of impaired chemotaxis were observed to occur in the presence of other conditions including neutropeania and recurrent infections (Miller et al., 1971), reduced gammaglobulins in circulation (Steerman et al., 1971), reduced neutrophil metabolism and intracellular killing ability, (Sigh et al., 1972), Chediak-Higashi syndrome (Blume and Wolf, 1974; Clark and Kimbal, 1974), and in malnutrition (kwashiokor) (Schoffer and Douglas, 1976). Hence it is likely that impaired chemotaxis is a secondary condition.

1.5.2 Disorders of Opsonization.

The process of opsonization involves serum factors which alter the physiochemical surface of the antigen (including bacteria), to enhance phagocytosis. These factors, opsonins, may either be heat labile or stable, including specific antibodies and serum factors distinct from both complement and antibody (Winklestein, 1973;

Barrett, 1976). In most situations opsonization requires the participation of both immunoglobulin G and M antibodies, the complement system or the properdin system (alternative pathway), before the attachment of the opsonized bacteria onto specific receptors on both the PMN and monocytes (Smith and Wood, 1969; Alper et al., 1972).

Quie, et al., (1968) and Messner and Jelinek, (1970), showed that the $F(ab)^2$ portion of the antibody combines with the surface antigen of the bacteria and the Fc portion of the antibody is then free to attach on to specific receptor sites on the surface of the phagocyte. Alper et al., (1972) and Baehner, (1974), reported that, unlike the complement system, the alternative pathway involving the properdin system does not depend upon specific antibodies for its action and it is likely that it plays a role in the humoral defense during the early preantibody stages of infection. If the infection persists, immunoglobulins G and M (IgG & IgM respectively) specific antibodies would be generated and activated the complement system. These workers suggested that the phase of infection following an immune response may not require complement due to super-saturating amounts of specific antibodies. This suggestion has since been substantiated by Cleat and Coid, (1981; 1982), who demonstrated that effective phagocytosis of group B Streptococci bacteria was a non-specific event requiring only non specific antibody or complement. They suggested, however, that neutrophil bactericidal activity was mediated by the pathogenecity of the infecting organism. These workers further observed that Streptococci of low pathogenicity were killed by neutrophils alone, although greater numbers were killed in the presence of specific antibody or complement. Furthermore the alternative pathway of the complement appeared to play a

significant role in the killing of these bacteria. In contrast, highly pathogenic Streptococci were killed only in the presence of specific antibody and neither of the complement pathways appeared to play a role in the process. Moreover, addition of specific antibody to neutrophils which had already ingested highly pathogenic organisms immediately initiated the killing process, suggesting an important non-opsonic role for the specific antibody, perhaps activation of Fc receptors on the neutrophil surface.

Profound deficiency in some components of the complement system, especially C3 and C5, may produce life long infection (Alper et al., 1970; Jacobs and Miller, 1972). Deficiency of specific antibodies such as opsonins are found in patients with defects of humoral immunity (Janeway, 1968). The sera of children with sickle cell disease, who are at risk of developing life threatening pneumoccocal septicemia and meningitis have been shown to be markedly deficient in the heat labile opsonins for pneumococcus (Winklestein, 1968; Barrett-connor, 1971).

1.5.3 Disorders of phagocytosis.

Direct observations of phagocytosis, the uptake of opsonized particles/ micro-organisms, shows that the outer membrane in contact with the phagocytosed particle is internalized. In the process of internalization considerable changes occur in lipid metabolism (Baehner, 1974). Baehner, (1974), suggested that the stability of the phagocytic vesicle that is created was possibly related to the high concentration of fatty acids in the cell membranes. The fact that defective phagocytosis is almost always associated with abnormal chemotaxis, which in turn is often related to the abnormalities in the humoral

factors, suggests a close relationship of these functions (Baehner, 1974, Callin and Quie, 1978, Quie et al., 1979).

1.5.4 Intracellular killing disorders.

Intracellular killing of organisms following phagocytosis depends on degranulation of cytoplasmic granules into the phagocytic vacuole stimulation of the oxidative metabolism within the phagocyte, and death and digestion of the ingested bacteria within the phagocytic vacuole (Baehner, 1974). When phagocytosis occurs rapid degranulation takes place, facilitated by microtubules. The cytoplasmic granules fuse with the phagocytic vacuole and discharge hydrolases and bactericidal proteins (Malawista and Vinblastine, 1971; Bainton, 1973). In vitro studies have shown that an increase in oxygen (O_2) consumption, H_2O_2 production and glucose oxidation occurs soon after addition of bacteria or particles to a phagocyte suspension (Baehner, 1974). In addition to the enzymatic system a variety of microbiocidal agents and systems are known to exist in the PMN, including localized areas of acidosis and leukin (Skarnes and Watson, 1956; Mandel, 1970), phagocytin (Hirsch, 1956), cationic protein, (Zeiyer and Spitznagel, 1969), bacteriostatic and hydrolytic factors, (Baehner, 1974) and the hydrogen peroxide-myeloperoxidase halide system (Klebanoff, 1967; 1968; 1970).

Genetic studies of disease conditions characterized by defective intracellular killing ability of neutrophils suggest an autosomal recessive inheritance (Baehner and Nathan, 1967; Holmes et al., 1967; Klebanoff, 1970; Salmon et al., 1970; Clark and Kimbal, 1971; Klebanoff and Hannon, 1972). Several other disease conditions have

also been cited as causing intracellular killing defects, including severe thermal injury (Alexander and Wilson, 1970), some severe infections (Park et al., 1968; McCal et al., 1971) and malnutrition (Kwashiokor) (Salvaraj and Bhat, 1982).

The major difficulty encountered in studying the molecular basis of phagocytic disorders is their infrequency of occurence (Miller, 1976; Fuenfer et al., 1976; Baehner, 1974; Quie et al., 1979), along with the complex inter-relationship between cellular and humoral function and hence defects (Miller, 1976; Fuenfer et al., 1976; Callin and Quie, 1978; Quie et al., 1979). The lack of animal models with natural deficiencies (Rausch and Moore, 1975) and the lack of standard, specific, sensitive and readily reproducible assay procedures (Miller, 1976; Fuenfer et al., 1976) compound these problems. The discussion below summarizes the methodologic limitations of currently available neutrophil function assay systems. The term "neutrophil function" will be used to encompass cellular adhesiveness to vessel walls, emigration and chemotaxis to the site of infection, ingestion (phagocytosis) and ultimately intracellular killing of the organisms.

1.6) LEUCOCYTE FUNCTION TESTS

A number of invitro neutrophil function tests are available but their accuracy in indicating the true In vitro defects is questionable. The following review evaluates the methods and procedures for the invitro and invivo tests currently in use for the evaluation of neutrophil function.

1.6.1. Evaluation of chemotactic ability of neutrophils

Chemotaxis, the directed movement of cells towards or away from a substance in a concentration gradient, is exhibited by polymorphonuclear leucocytes (PMN) to several substances including zymosan activated whole serum.

1.6.1.1. Migration through filters.

Chemotaxis was initially observed *In vitro* when leucocytes were placed in a capillary tube, between a slide and a cover slip or on a cover slip incubated in a humid chambers (Nelson, Quie and Simmons, 1975). In 1962 Boyden (Quoted by Nelson *et al.*, 1975) described an assay for the measurement of chemotaxis using filters. There are many modifications of the Boyden assay, but all employ the same general principle. In the Boyden method the cells under study are placed in the upper portion of the Boyden chamber and separated from the chemotactically active source in the bottom chamber by a filter. After incubation at $37^{o}C$ the filter is removed, cleared in a series of concentrated alcohols and stained to microscopically assess the number of cells that have migrated through it. To determine the chemotactic index, the value obtained using a buffer is subtracted from that obtained using the chemoattractant(s).

The advantages of measuring the chemotactic ability of neutrophils with the Boyden chambers are its rapidity and economy of its performance. The technique is also reproducible and easy to set up in standard laboratory. The results obtained, however, are affected by factors such as the concentration of the chemoattractant, type and quality of the filter, cell adhesion to the filter material, and tortuosity and

size of the pore channels. In addition the Boyden procedure requires large numbers of test cells (Nelson, Quie and Simmons, 1975; Miller, 1976).

1.6.1.2 Migration through agarose

Chemotactic assay using the "migration through agarose" method is dependent upon gel concentration and incubation conditions (Nelson et al., 1975). In a standard assay, a set of wells is punched into agarose gel in a 10 x 35 mm plates. The middle wells in the test plates are filled with the chemoattractant or buffer (control). After incubation at 37°C in a humid chamber for 1 hr, the remaining wells are filled with test cells and incubated for a further 2-3 hrs. At the end of the incubation period the cells are fixed in methanol-formalin fixative before being stained with Wright's stain and examined under a light microscope. Chemotaxis is then evaluated by calculating the migration index, which is the ratio of the distance migrated towards the attractant (directional migration) to that migrated towards the buffer (random migration).

The advantages of the chemotaxis under agarose method are the rapidity and economy of its performance and the small amounts of blood required. The results obtained, however, are affected by factors such as the concentration and type of agarose, the concentration of the albumin used to make up the gels and concentration of the chemoattractant (Nelson et al., 1975; Miller, 1976).

1.6.2. Evaluation of the phagocytic ability of neutrophils

Phagocytosis is the uptake or ingestion of particles by a phagocyte.

Methods for the study of this process, phagocytosis, are many and varied but there is no agreement as to whether the methods available accurately measure the phagocytic process (MIller, 1976).

1.6.2.1. Ingested particle count.

Many particles and agents have been utilized in assays that directly quantitate uptake of particles. Briefly, leucocytes are incubated with the opsonized particles at 37°C in humid chambers for a 30-60 minutes, before they are pelleted and smears made. Phagocytosis is quantitated directly using a light microscope.

The advantages of the ingested particle count procedure for assessing the phagocytic ability of the neutrophils include the fact that the method is relatively easy to establish and cheap to perform, and is easily reproduced. The procedure is, however, cumbersome to perform and relatively imprecise (Miller, 1976).

1.6.3 Evaluation of Intracellular Killing ability of Neutrophils

1.6.3.1. Nitrobule Tetrazolium (NBT) test.

The NBT test, an In vitro assay for granulocyte function, was developed by Park, Fikrig and Smith, (1968). This method involves incubation of leucocytes and NBT, in the presence or absence of a stimulant, and then examining smears prepared from the incubated cell suspension under oil immersion for reduced dye. The reduced dye appears as deep blue black formazan particles in the neutrophils. Baehner and Nathan, (1968) and Park et al., (1968) originally proposed the NBT test as a test of lysosomal, function to differentiate between bacterial and non-bacterial diseases.

This original test system was based on the assumption that hydrogen peroxide production was linked to the oxidation of NADH to NAD+ and consequently the amount of NBT reduction would reflect the intracellular levels of hydrogen peroxide, a potent bactericidal agent. It has since been established that the reduction of the yellowish NBT to formazan requires adequate levels of the flavo-enzyme Nicotinamide adenine dinucleotide (NADH) oxidase, which catalyses the oxidation of NADH to NAD+. However, the exact sequence of events leading to the reduction of NBT is yet to be ellucidated.

In the original test system neutrophils capable of reducing NBT were said to be NBT-positive while those that did not were NBT-negative. On this basis the mean proportion of NBT positive neutrophils in humans, was found to be 8.5% in healthy controls, between 5.8 and 9.5% in patients with non-bacterial illness and 29 to 47% in patients with bacterial infection (Fuenfer et al., 1976). Park and Good, (1970) devised a "stimulated NBT test" in which bacterial endotoxin was used to detect some disease states which show low or normal NBT test values even when an active bacterial infection is present. This stimulation may be accomplished by incorporation of a large range of substances, including:- bacterial culture filtrates (Matula and Paterson, 1971; Freeman and King, 1972), Latex particles (Hicks and Bennett, 1971; Wegner and Bole, 1973), zymosan and glass contact (Gifford and Malawista, 1972), high concentrations of heparin (Bjorkstein, 1974; Rothwell and Doumas, 1975) or bacterial extract (Sigma tech. bull. No. 840, 1978) into the blood-NBT incubation mixture. In vitro stimulation of blood from normal individuals results in a marked increase in the percentage of formazan containing neutrophils. Neutrophils of patients with cellular, humoral or granulocyte metabolic defects fail to exhibit

a positive response even when stimulated (Baehner and Nathan, 1968; Gifford and Malawista, 1972; Ochs and Igo, 1973; Belcher and Czarnetziki, 1973). Numerous papers have appeared (Review - Segal, 1974; Fuenfer et al., 1976) either disputing or confirming the original claims by Baehner and Nathan, (1968) and Park et al., (1968) concerning the clinical (diagnostic) value of the NBT test. Segal (1974), concluded that the NBT test was of little value in the diagnosis of pyogenic infection due to the high false negatives and positives. In contrast Fuenfer et al., (1975) concluded that the NBT test was valuable in detection of recurrent suppurative infection in patients especially susceptible to infection (Kaunder, 1968; Nathan, Baehner and Weaver, 1970; Holmes, 1974), those undergoing immunosuppressive therapy and those who have sustained major injuries. Fuenfer et al., (1975), however, conceded that technical difficulties and the occurence of false negative (Park and Good, 1970; Chretien and Garagusi, 1971; Matula and Paterson, 1971) and false positive (Giron, 1971; Chan and Todd, 1972; Feigin, Shackleford and Choi, 1973) results in a wide variety of clinical situations. The induction of positive NBT test by viruses, parasites and fungi also detracted from its reliability.

The conflicting results from different laboratories may, however, arise from the use of poorly standardized modifications of the original test. Thus, despite its inherent weaknesses the NBT test may still be the most rapid, inexpensive and non-hazardous neutrophil function test available.

1.6.3.2 Direct measurement of intracellular killing ability of neutrophils.

The direct invitro assay of the bactericidal capacity of PMNs was

initially devised by Maaloe (1946) and later modified by Quie, (1967) and Alexander, Windhorst and Good, (1968).

Basically the method involves the incubation of fixed ratio of PMNs: bacteria with varying sources of opsonins and/or buffer. By utilizing isolated leucocytes in a system containing serum opsonins and a fixed ratio of leucocytes to bacteria, means are provided for accurate differentiation of disease-induced effects on phagocytic and intracellular killing aspects of neutrophil function. In the modified procedure of Alexander et al., (1968) two identical systems are established for the incubation of bacteria with PMNs. At predetermined intervals of incubation antibiotics are added to aliquots from one of the test mixtures to kill extracellular (free floating and those attached to leucocytes) bacteria. Then, using the pour plate technique, the total number (extracellular and intracellular) of viable bacteria may be discerned after predetermined incubation intervals (Verhoef, Peterson and Quie, 1977).

Modifications of the Maaloe (1946) and Alexander et al., (1968) techniques have been employed to study the effects of various glucocorticoids on neutrophil function (Yielding and Tomkins, 1959; Mandel, Rubin and Hook; 1970; Fuenfer et al., 1976).

The major advantage afforded by the direct measurement tests is that kinetics of opsonization, attachment and intracellular killing of the various bacteria can be studied in a more closely duplicated system than offered by the NBI test. However, besides being a long, expensive and tedious exercise, the results can also be affected by small changes in the neutrophil environment with respect to quality and quantity of nutrients, pH and temperature.

1.6.3.3. Inherent and luminol dependent chemiluminescence.

Chemiluminescence (CL) or the phenomena of PMNs emitting light during phagocytosis was originally described by Allen, Stjernholm and Steel (1972) and Stjernholm, Allen, Waring and Harris (1973). The emission of light has been related to increased glucose exidation via the hexose monophosphate shunt (Allen et al., 1972) and direct oxidation of the particle being phagocytosed, with the production of high energy compounds intimately involved with bactericidal activity of the phagocyte (Rosen and Klebanoff, 1976; Cheson, Christensen, Sperling, Kohler and Babior, 1976; Nelson, Herron, Schmidtke and Simmons; 1977).

The technique, chemiluminescence (CL); involves the incubation of PMNs, at 37°C, with opsonized particles, including live or heat inactivated bacteria, in the absence (inherent) or presence (luminol dependent) of luminol. Inherent and luminol dependent chemiluminescence have been used as assays for both opsonic activity (Stjernholm et al., 1973; Hemming, Hall, Rhodes, Shigeoka and Hill, 1976; Steven, Winston and Van dyke, 1978) and phagocytic activity (Stevens and Young, 1977; Stevens et al., 1978). The addition of 5 amino-2, 3-dihydro 1, 4 phthalazinedione (luminol) to a "phagocytic - chemiluminescence system" reduces the number of cells required to produce a given amount of light. Luminol, a cyclic hydrazide that can be digested to emit light, readily diffuses into granulocytes and reacts with the oxidizing products released during phagocytosis, which in turn leads to the formation of electrically excited aminophalate ion which releases light on returning to the ground state. This secondary chemiluminescence is the basis of the increased sensitivity of the lumino-dependent system (Van Dyke, Trush, Wilson,

Steely and Miles, 1977; Stevens et al., 1978).

Initially chemiluminescence was measured at ambient temperature, using a scintilation counter (Allen et al., 1972; 1973). Currently, however, various types of counters including an ATP spectrophotometer (Van Dyke et al., 1977; Hatch, Gardner and Menzel, 1978), prototype photometer (Anderson and Brendel, 1978) and "Easmon version" ATP spectrophotometer (Easmon, Cole, Williams and Hastings, 1980) have been designed or modified to overcome the high cost of installing a scintillation counter. Although less sensitive than the liquid scintillation counters, the various designs of the ATP spectrophotometers, photometers and luminometers can effectively measure and detect experimentally induced and clinical neutrophil functional defects using small numbers of PMNs (Easmon et al., 1980). Young and Stevens (1977) validated the chemiluminescence technique by comparing it to, invitro killing assays, post phagocytic oxygen consumption and morphological phagocytic indices. They concluded that of the then available techniques of assessing PMN function chemiluminescence was the most rapid and simple useful screening method to determine possible opsonic-phagocytic deficiences of PMN and serum.

The production and measurement of inherent and luminol dependent chemiluminescence is quenched by the presence of contaminating erythrocytes, composition of the media (especially the presence of phenol red, Hepes buffer or foetal calf serum), and pH below or above 8.5 (Nelson, Heron, Schmidthe and Simon, 1977; Anderson and Amirault, 1979; Easmon et al., 1980; Hastings et al., 1982). Addition of gelatin, protein solutions and amino acids to Hanks buffered salt solution also depresses chemiluminescence (Anderson and Amirault, 1979; Easmon et al., 1980). Nelson et al., (1977)

observed that addition of certain amino acids to Hanks buffered salt solution enhanced inherent chemiluminescence.

It is necessary that a highly uniform preparation of neutrophils and medium including buffers and protein additives be established to enable comparison of data to be made between laboratories.

1.6.3.4. Quantitative phagocytic assay by determination of the iodination

The iodination test, which has been used successfully in the study of the human and bovine neutrophils, is based on the determination of radiolabelled iodine bound to the PMN after incubation of the cells with pre-opsonized zymosan, NaI and Iodine 125 (1^{125}) (Klebanoff and Clark, 1977; Roth and Kaeberle, 1981).

The iodination test measures the ability of the polymorphonuclear cells to convert inorganic iodine to a trichloroacetic acid precipitable (protein bound) form. The iodine is covalently bound to a suitable acceptor molecule, such as the tyrosine residues of proteins via the actions of hydrogen peroxide and myeloperoxidase. This system, has been found to exhibit a marked toxicity towards bacteria, fungi and some viruses (Belding and Klebanoff, 1970; Simmons and Karnovsky, 1973).

1.6.3.5. Quantitative phagocytic assay using 3_H Hymidine labelled bacteria.

Miller and Beck, (1975) developed a rapid quantitative assay for studying the kinetics of PMN engulfment of 3_H-Thymidine - labelled Staphylococcus albus, with magnesium ions and bovine

serum albumin, without serum. The procedure involves the incubation of a fixed ratio of PMN and radiolabelled bacteria at 37C for 30 minutes. Phagocytosis is determined by assaying for leucocyte associated radioactivity after centrifugation and washing of the leucocytes. Phagocytosis is then arbitrarily expressed as the average counts per minute per 0.1 ml at 30 minutes minus the average counts per minute per 0.1 ml at zero time. These authors suggested that this technique was especially useful in circumstances where it was necessary to avoid the effects of serum bactericidins. Verhoef, Peterson and Quie, (1977) and Santivatr, Maheshwaran, Newman and Pomeroy, (1982) modified the technique to study the separate but inter-related process of bacteria opsonization, phagocytosis and intracellular killing of 3_H - Thymidine labelled Staphylococcus aureus by human PMN and chicken mononuclear cells respectively. In their studies, opsonization was studied by incubating bacteria with an opsonic source for varying durations and then adding leucocytes. By treatment of the samples with the muralytic enzyme, lysostaphin, the attachment and ingestion phases of phagocytosis could be separated. Sampling for colony forming units (CFU) permitted them to measure bactericidal ability. Using this modified technique differences in kinetics of Staphylococcal opsonization by normal and complement deficient sera were defined, opsonic influence on attachment and ingestion phase of phagocytosis were delineated and the influence of the different opsonins and leucocyte population on killing were determined. The main drawbacks of the 3_H Thymidine and Iodine 125 labelled bacteria techniques include; high background disturbance, leakage of radioactive label and extra expenses including high overhead costs (Sugarman, 1980).

CHAPTER 2

PAGES

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

This chapter contains techniques used throughout the experimental work recorded in this thesis. Sample collection, including uterine flushing, swabbing and collection of biopsies, and isolation and identification of bacteria were all performed using these standard laboratory procedures.

Procedures limited to particular experiments, such as quantitative and qualitative protein assays, scanning and transmission microscopic examination of uterine biopsies and neutrophil isolation and function tests are described in the appropriate chapter.

2.1) ANIMAL SELECTION

Mares selected for this project were refered by practicing Veterinarians (mares with a history of recurrent endometritis) or purchased through dealers (most of the resistant mares) and had well documented reproductive histories. Upon arrival they were weighed and drenched with Neguvon powder (800 gm per kg Trichlorophon - Bayer Aus. Ltd.) given orally at the rate of 2.5 g per 65 kg live weight (maximum dose 7 x 2.5 gm) and Thiabenzole (176gm per litre of Thiabendazole - Merck, Sharp and Dohme, (Aust) PTY Ltd, WA) given at the rate of 25 mls per 100 kg live weight. The mares were then identified by brand or marking, given a numbered collar and released onto irrigated pastures. Before the challenge experiments all selected mares were allowed an adjustment period of 4-6 weeks. At the end of the adjustment period the mares were weighed and

given a final clinical assessment, which included a rectal examination and uterine swabbing; for routine bacteriology and cytology; and biopsy.

2.2) ROUTINE BACTERIOLOGY AND CYTOLOGY

2.2.1 Routine bacteriology

Endometrial swabs for bacteriology were obtained and processed as described by Munyua, (1982), and Williamson et al., (1983).

To obtain a uterine sample for bacteriology, a guarded uterine swab was introduced into the uterus through the cervix. Upon entry into the uterus the swab, contained in the inner protective tubing, was firmly but gently pushed through the protective casing and rolled onto the uterine luminal epithelium for 15-20 seconds. The swab was subsequently withdrawn into the protective casing which was then withdrawn from the uterus and immediately placed into Stuarts' transport media and refrigerated. All swabs were streaked onto a selective media, McConkey's agar, and an enriched media, blood agar, within 1 hr of collection and incubated at 37C for 24 to 48 hrs.

Bacteriological examination of uterine washings was performed on each sample collected by spreading a loopful of the uterine washings on blood agar and McConkey's agar plates. These plates were then incubated at 37C for 24 to 48 hrs before they were read. The loops used in the present experiment held approximately 10µl of fluid.

Routine biochemical procedures were used for further identification of any bacteria isolated from the swabs and/or uterine washings (Deibel and Seeley, 1974). The results obtained in the present study were interpreted using criteria adopted by Shin et al., (1979). These

investigators suggested that a count of 20 or more pure colonies of any bacteria that is a documented as a potential uterine pathogen (Rossdale and Ricketts, 1980) was significant, and hence was recorded as positive bacteriology.

2.2.2. Uterine cytology

A second guarded swab was obtained from each mare at each sample collection. A smear for cytological examination was prepared by rolling the swab onto two microscope glass slides coated with 1% gelatin. The smears were then stained with Wrights' stain, using an automatic slide stainer (Miles Laboratory, Perth, WA). The slides were subsequently assessed as described by Pearman, (1972). The meander technique used for the examination of blood smears was adopted for scanning and examination of the uterine smears. In the present study the finding of 50 or more leucocytes per slide was considered to be indicative of infection and was recorded as positive cytology (Munyua, 1982). The presence of plasma cells or the predominance of mononuclear cells including lymphocytes was taken to be indicative of chronic inflammation while, karyorrhexis of neutrophils or other inflammatory cell types was taken to indicate a rapid cell death due to the presence of a toxin and/or a virulent organism (Pearman, 1972).

2.3) PREPARATION OF STREPTOCOCCAL AND STAPHYLOCOCCAL SUSPENSION

2.3.1 Preparation of Streptococcal inoculum for uterine challenge.

The test organism was a strain of S. zooepidemicus (Beta haemolytic

Streptococci) isolated from a mare with an active severe endometritis. Uterine inoculum was prepared from fresh blood agar cultures of this bacteria which were grown overnight in Todd-Hewitt broth, before being concentrated by centrifuging at 15,000g for 15 minutes. Two ml aliquots of sterile peptone water was heavily inoculated with a loopful of the pelleted β haemolytic streptococci and incubated for 1 hr at 37C. Portions of the inoculated peptone water (0.1ml) were then added to five 100ml lots of sterile peptone water and incubated overnight at 37C. A gram stain was made from each incubation to check for purity before the inoculi were combined and thoroughly mixed. A 5 ml aliquot was taken from the pooled inoculated peptone water and stored frozen for future reference and serotyping (see section 2.6). The remaining β haemolytic streptococci suspension was dispensed into centrifuge tubes for ultracentrifugation at 15,000 g for 15 minutes. After centrifugation the resulting pellets were washed twice in physiological saline, each time centrifuging at 15,000 g, before being suspended in physiological saline solution to give a concentration of x 10^9 colony forming units of β haemolytic streptococci per ml. Two ml aliquots of the Streptococcal suspension were then transported in glass bottles placed in containers with crushed ice, to the Veterinary School farm for use in the challenge experiments.

2.3.2 Preparation and quantitation of Staphylococcus aureus

Staphylococcus aureus suspension, used to assess the phagocytic index of harvested peripheral neutrophils, was prepared by overnight growth in nutrient broth at 37C and the bacteria were then pelleted by ultracentrifugation at 15,000 g for 15 minutes. After washing with physiological saline to an optical density of 0.22 at 525 nm in a

Bausch and Lomb spectronic 20 spectrophotometer (Townson and Mercer, WA.).

Quantitative cultures showed that the number of bacteria remained unchanged for at least 1 hr when placed in Hanks buffered salt solution (Hbss) or physiological saline without any leucocyte or serum. In addition it was confirmed that at the optical density used, 0.22 at 525nm, the Staphylococcal suspension contained about 2.5×10^8 colony forming units of the Staphylococci per ml (Alexander, Windhorst and Good, 1968).

Hbss, pH 7.0 - 7.4, used throughout this work was prepared by dissolving a satchet of pre-prepared Hanks buffer salts (Flow laboratories, WA.) in 1000 mls of sterile distilled water. Where Hbss with carbonate, pH 7.0 - 7.4, was required, it was prepared by adding 6.3 ml of 5.6% solution of $NaHCO_3$.

2.4 UTERINE FLUSHING PROCEDURE

Uterine washings were obtained using a sterile Foley catheter with an inflatable cuff (Zavy et al., 1978; Williamson et al., 1983).

With the hand in the vagina the catheter was guided into the uterus through the cervix and the cuff inflated with 50 ml of air after it had passed the cranial os of the cervix. Twenty ml of sterile isotonic mannitol solution (5.6%) was infused into the body of the uterus at 2, 3, 4, 6, 9, 12 and 15 hrs after challenge, while 50 ml of mannitol solution was infused at 0, 1, 2, 4, 10, 14 and 28 days after challenge. The uterus was then massaged gently for about 4 minutes before the fluid was allowed to flow from the uterus into sterile plastic containers. When sufficient fluid (20 ml) had been collected, the procedure was halted to minimize

the time and trauma of collection. Three ml of the washing from each mare, to be used for leucocyte enumeration were immediately cooled to 4C. The remainder of the washings was centrifuged at 2,000g, aliquoted into 5 ml lots and stored frozen at -20 or -70^{o}C until required.

Isotonic mannitol solution was selected for flushing because:-

a) Mannitol solution did not contain sodium or potassium, which were to be used in the estimation of uterine fluid volume,

b) Quantitative cultures showed that the genital strain of β haemolytic streptococci used in the present experiments tolerated suspension in isotonic saline and mannitol (5.6%) solution equally well, and better than they tolerated suspension in isotonic, sorbitol (0.308 M), sucrose, (0.308 M), inositol, (0.308 M), dulcitol, (0.308 M), glucose, (5.4%), dextrin, inulin, ficol and glycogen, and distilled water (Drs. P. Williamson and J. Penhale, Personal communication).

2.5) UTERINE BIOPSY COLLECTION AND EVALUATION

The technique of collection, processing and assessment of uterine biopsies was as described by Kenney (1978). Immediately following collection, the biopsies were carefully divided into 2 portions. The first portion of each biopsy obtained was placed in Bouins fixative, while the other was fixed in 10% formal saline. All biopsies were subsequently dehydrated in alcohols and embeded in paraffin wax for the production of routine histological sections. Sections of the formal saline fixed portions were stained with carbochromotrope - methylene green-pyronin, for the demonstration

of plasma cells and eosinophils. Duplicate sections were gram stained to check for the presence of bacteria. Sections from the Bouins fixed portions were routinely stained with hematoxylin eosin for histopathological examination.

The following grading criteria was used to arrive at a diagnosis (Kenney, 1978, Munyua, 1982, Williamson et al., 1983):-

1) the presence, type, number and distribution of inflammatory cells.

2) type and condition of the luminal epithelium.

3) density, amount and distribution of connective tissue especially in relation to the uterine glands.

4) the endometrial glands, were examined for size, activity, number and distribution. In addition it was determined whether the glands were clustered by connective tissue (nesting) or enlarged (cystic).

5) the condition of blood supply to the endometrium (congestion, haemorrhage and/or oedema).

2.6) SEROTYPING OF STREPTOCOCCI

Throughout this study randomly selected B haemolytic streptococci isolates were sent to the Commonwealth Scientific and Industrial Research Organization Laboratories (CSIRO, Perth, WA) at regular intervals for serotyping to establish whether the

isolates after challenge were the same serotype as those that were infused at challenge.

Results indicated that the isolated B haemolytic streptococci after challenge were the same serotype as those that were inoculated.

2.7. STATISTICAL ANALYSIS

The significance of differences between means was tested using analysis of variance and Tukey's honestly significant difference (hsd) procedure where appropriate (Snedecor and Cochrane, 1967). Where wide variations were observed the data was transformed to natural logarithms before statistical analysis was performed (Steel and Torrie, 1960, Snedecor and Cochrane, 1967).

The performance characteristics of biopsy findings and reproductive history as diagnostic tools for predicting resistance to uterine infection were described in terms of diagnosability, sensitivity and specificity. In the present study sensitivity was taken to be the probability of a diagnostic test correctly identifying as resistant (positive) those mares that were truly resistant (positive). And specificity was the probability of identifying as negative (susceptible) those mares that truly lacked the characteristic of interest (Shwabe, Riemann and Franti, 1977).

The sensitivity (Se) and Specificity (Sp) were calculated as shown below:-

Table 2.1
The performance characteristics of biopsy findings and reproductive history, described in terms of sensitivity and specificity.

		True state of nature (population).		
		+	-	
Test Result	+	a++	b+-	a+b
	-	c-+	d--	c+d
		a+c	b+d	N..= population size

Where

True state of the nature = Whether the mare was resistant (+) or susceptible (-) to uterine infection after challenge.

Test results = whether the mare was resistant (+) or susceptible (-) according to biopsy findings or reproductive history.

N.. = a+b+c+d = population size.

a/a+c = Sensitivity, b/b+d = Specificity,

a/a+b = diagnosability and d/c+d = the proportion of the tests that were truly negative.

b/a+b = the probability of a false positive (false resistant),

c/a+b = the probability of a false negative (false susceptible) and d/c+d = the proportion of test negatives (susceptible) that are really negative.

If Se + Sp was $\leq$ 1.0 then test was interpreted as selecting resistant and/or susceptible mares at random.

CHAPTER 3

THE CELLULAR AND HUMORAL RESPONSE OF MARES TO EXPERIMENTALLY INDUCED ENDOMETRITIS.

CHAPTER 3.

THE CELLULAR AND HUMORAL RESPONSE OF MARES TO EXPERIMENTALLY INDUCED ENDOMETRITIS

3.1 INTRODUCTION

At present the factor(s) preventing the establishment of bacterial infections in the mare's uterus are not well understood. It has, however, been suggested that the uterine defense has numerous components, including soluble and cellular factors (Hughes and Loy, 1969; 1975; Peterson et al., 1969; Asbury et al., 1982; Williamson et al., 1984; Munyua et al., 1984.) These investigators considered the initial cellular infiltration into the endomentrium and uterine lumen to be responsible for the early removal of invading bacteria in normal mares. Asbury et al., (1982) reported that there were no significant differences between mares susceptible or resistant to endometritis in the neutrophils in uterine washings after experimental infection or the phagocytic function of peripheral neutrophils.

The uterine fluid and washings obtained from both resistant and susceptible mares have been shown to contain uterine specific (Blue et al., 1982) and serum derived proteins, including immunoglobulins A and G, (Mitchel et al., 1982; Williamson et al., 1983; 1984; Munyua et al., 1984). In addition, these investigators reported that the protein content in uterine washings, including IgA and IgG, increased

in response to naturally occurring and experimentally induced uterine infection.

3.2 AIMS

The present studies were undertaken to develop a technique for identifying mares with lowered resistance to uterine infection (endometritis) and to study the pathogenesis of equine streptococcal endometritis in resistant and susceptible mares.

To attain these aims two related but separate experiments were designed.

3.3 MATERIALS AND METHODS

3.3.1 Classification of Mares for Experiments 1 and 2.

Thirty two mares with no clinical signs of endometritis were tentatively assigned to "resistant" or "susceptible" groups using 2 criteria.

1. Biopsies collected at least 7 days before the experiment were evaluated, and those mares with significant endometrial degeneration, (grades 2 and 3; Kenney, (1987)) were classified as susceptible to endometritis, whereas those mares with histologically normal endometria were classified as being resistant to endometritis.
2. Mares with a clinical history of recurrent uterine infection were classified as susceptible to endometritis, whereas those mares without a history of recurrent infection, and clean to swabbing at their initial clinical examination were considered resistant to endometritis.

The two methods of classifying the mares were subsequently tested by comparing the ability of the mares to eliminate infection when challenged by intrauterine innoculation with an active culture of β haemolytic streptococcus.

EXPERIMENT 1

The aims of Experiment 1 were:-

1) to monitor the persistance of β-haemolytic streptococci in the uterus of mares at different intervals after experimental infection,

2) to study the appearance of the various inflammatory cell types at specific intervals after intrauterine inoculation with β haemolytic streptococci (challenge),

3) to establish criteria for assessment and classification of mares as having either "resistant" or "susceptible" status,

4) to identify and measure the soluble components of the inflammatory response (total protein and immunoglobulin concentrations) in uterine washings following challenge with β haemolytic streptococci.

5) to examine uterine washings and fluids collected 6 hours after bacterial challenge (at the peak of the protein influx into the uterus), for leucocidal factors.

3.3.2 Induction of acute endometritis.

Acute edometritis was induced in 26 mares by intrauterine inoculation of at least 10^9 β haemolytic streptococci suspended in 2ml isotonic mannitol solution. Three control mares were infused with 10 ml of sterile isotonic mannitol. Uterine swabs for bacteriology and cytology, and uterine washings for leucocyte counts and neutrophil

function tests were obtained from the challenged and control mares, at fixed intervals after challenge 2, 4, 6, 12 and 15 hours, and 1, 2, 4, 10, 14, and 28 days (18 mares) and at 0, 3, 6, 9 and 12 hours and at 1, 4 and 10 days (8 mares)) to measure each mare's response to challenge.

3.3.3 Uterine bacteriological examination.

A guarded uterine swab was used to obtain uterine samples for bacteriology. Swabs were processed as previously described (see section 2.2.1).

3.3.4 Preparation and examination of uterine smears.

A second uterine swab from each mare was rolled onto two gelatin coated slides which were subsequently stained with Wright's stain. The technique applied in the examination and grading of the uterine cytological smears is as previously described (see section 2.2.2).

3.3.5 Collection of uterine washings.

Uterine washings were obtained as previously described (see section 2.4). Three ml of the washing from each mare, to be used for leucocyte enumeration and neutrophil function test, was immediately cooled to 4C. The rest of the washings were centrifuged at 2,000g, and the supernatant aliquoted into 5ml lots and stored frozen at -20 or -70c until required.

3.3.6 Enumeration of leucocytes in uterine washings.

Total leucocyte counts (total WBC) were performed on fresh washings using a Coulter electronic particle counter. In samples where erythrocytes were observed it was assumed that bleeding had occured during sample collection and a correction factor based on the approximate erythrocyte-leucocyte ratio (RBC:WBC) was used to estimate the number of leucocytes that had leaked into the uterus during sample collection (Coles, 1974). This estimated leucocyte count was subtracted from the total number of leucocytes (total WBC) to obtain the number of leucocytes present in the uterus apart from those contributed by the leakage. The proportion of the various leucocyte types was then estimated using a differential count (DF%) performed on concurrent uterine smears.

Total WBC count - Estimated WBC count = Actual WBC count.

Actual WBC X Differential count % (DF%) = Actual count of the various inflammatory cell types.

3.3.7 Quantitative protein assays in uterine washings and serum.

Total serum and uterine protein levels were determined by spectophotometry (Bradford, 1976; Spector, 1978). Serum or uterine washing samples (50ul), were diluted with 450ul of water and incubated with 5ml Comassie blue working solution at room temperature for 5 minutes. The stock solution for the Comassie blue protein assay was prepared by dissolving 100mg of Comassie brilliant blue G-250 (Kodak Ltd., WA) in 50ml 95% ethanol. To prepare a daily working solution

50ml Comassie reagent (0.2%) was mixed with 100ml phosphoric acid (85%) before being made up to a litre with distilled water. Optical density was measured on a spectrophotometer set at 595, within 1 hour /nm, of incubation. Absolute values were read from a standard curve prepared from serially diluted bovine serum albumin (BSA). The BSA standards were prepared from a stock solution (1mg/ml) serially diluted (5, 10, 15, 20, 25 and 50 ug/ml), while PBS buffer was used as blanks (0ug/ml).

3.3.8 Immunoglobulin assay in uterine washing using the single radial immunodiffusion method.

Single radial immunodiffusion is a procedure in which the serum or uterine washing sample being tested diffuses through an agarose gel containing specific antiserum against the given immunoglobulin (Mancini, Carbonara and Heremans, 1965).

The concentration of immunoglobulins, IgA, IgM, IgG and IgG(T) in uterine washings was determined by the single radial immunodiffusion method, using both commercial (Flow laboratories, W.A.) and laboratory prepared antisera (Courtesy of Prof. J. Penhale, Immunology section, Murdoch University). Where purified standard immunoglobulin preparation was available, immunoglobulin concentrations in samples were determined in absolute quantities. However, in those cases where purified standards were unavailable, samples were compared to a colostrum standard and recorded as a percentage of the colostrum (mg %) (Williamson et al., 1983).

3.3.9 Bactericidal activity of uterine washings obtained from mares.

Bactericidal activity of uterine washings was assessed using the same principle employed in routine assays for bacterial drug sensitivity (Buxton and Fraser, 1977). The genital strain of β haemolytic streptococci, used for the challenge experiments, was used as the test organism.

Fresh uterine washings (25µl), collected 6-12 hours after bacterial challenge, were dropped at regular intervals of approximately 2cm over the surface of blood agar plates which had been evenly inoculated with Beta - haemolytic streptococci 1-2 hours previously. The plates were subsequently incubated at 37c for 24 to 48 hours before being read. Bactericidal activity was then quantitated by assessing, both macroscopically and microscopically, the diameter of any clear zones immediately around the drop of uterine washing or fluid.

3.3.10 Leucocidal activity of uterine washings obtained from resistant and susceptible mares at 6 hours after challenge.

Uterine washings obtained from resistant and susceptible mares 6 hours after challenge, were examined for the presence of leucocidal factors using isolated peripheral neutrophils. In addition uterine fluid obtained at post mortem from two mares with severe chronic streptococcal endometritis, was included in the assays. Peripheral neutrophils were isolated as described elsewhere (see section 5.3.3.1).

To assess leucocidal activity of the uterine washings, pooled

peripheral neutrophils (100µl) were incubated at 37C with uterine washings (100µl), obtained at the peak of the "protein flush" (6 hours after challenge), uterine fluid (100µl) obtained from two mares with chronic streptococcal endometritis, or Hbss buffer (100µl), at 37c for 0, 1, 2, 3 and 4 hours. The number of viable neutrophils was then established by performing a viability count using the Trypan blue exclusion method at the one hourly intervals.

The Trypan blue exclusion test was accomplished by adding 4-5 drops of 1% Trypan blue dye to duplicate incubation tubes at the end of each hour and making two cytological smears after three months incubation. Viable (live) neutrophils were those that did not take up the dye after the 3 minutes incubation with the dye. The results were then expressed as a percentage (Rabinowitz, 1964; Majeski and Alexander, 1976).

% viable neutrophils = X / N x 100

where

X = the number of neutrophils that did not take up the dye after 3 minutes incubation.

N = the total number of neutrophils counted per slide (200).

EXPERIMENT 2

The aim of Experiment 2 was to identify the main components of the protein influx into the uterus during the acute phase of uterine infection. In this experiment 6 mares were challenged by infusion with 10^9 viable β haemolytic streptococci and serial uterine samples were obtained after challenge without flushing the uterus with sterile

isotonic mannitol. Thus the diluting effect of the mannitol was avoided, as was distortion of any inflammatory reaction caused by periodic flushing in Experiment 1.

3.3.11 Induction of acute uterine infection and collection of samples.

Acute endometritis was induced by inoculation of 10^9 β haemolytic streptococci in 10ml mannitol into the uterus (Section 3.3.2). Serial uterine swabs for bacteriology (see section 2.2.1) and cytology (see section 2.2.2), and uterine fluid for qualitative and quantitative protein assays (see section 3.3.7) were obtained at 3, 6, 9, 12 and 15 hours and 1, 4, and 10 days after challenge, without flushing the uterus.

In addition to the uterine fluids collected following experimental uterine infection, fluid was collected at post mortem from two mares with chronic streptococcal endometritis. These samples were included in the qualitative and quantitative protein assays for comparison with those uterine fluids obtained during the acute phase of induced infection.

3.3.12 Electrophoretic analysis of uterine washings and serum.

Electrophoretic analysis was performed on the undiluted uterine samples using a Gelman semi microelectrophoresis chamber and Gelman sepraphore (Clifford Instruments Inc. 1969).

The Gelman super sepraphore (Cellulose acetate) strip was soaked in Gelman high resolution buffer for 10 minutes before 10μl of sample was

loaded with a standard applicator. After 25 minutes of electrophoresis the acetate strips were stained with Ponceux S for 10 minutes and the background decolourized in 3 changes of 5% acetic acid in 10 minutes. The strips were then oven dried for one hour before scanning. The total protein values obtained using the Comassie blue assay (see section 3.4.6) were used to calibrate the strip scanner.

All buffers were prepared according to Clifford instruments instruction manual (1969). The Gelman high resolution buffer consisted of 5.778g of trimethamine (Tris), 2.466g of barbital and 9.756g of sodium barbital made up to 800ml with distilled water.

3.4 RESULTS

A total of 35 mares were used in Experiments 1 and 2. All experimental mares, 32 challenged and 3 unchallenged controls, showed a clinical response to intrauterine infusion of β haemolytic streptococci or sterile isotonic mannitol. This reaction was characterized by a copious mucopurulent discharge from the vulva within 24 hours of inoculation.

3.4.1 Relationship between history, biopsy findings and challenge results:

Light microscopic examination of uterine biopsies obtained before challenge from mares used in experiments 1 and 2 revealed that of the 32 mares, 21 had no significant degenerative changes in their uterine biopsies while 11 showed severe, diffuse, chronic degenerative and inflammatory endometrial changes.

Eleven (11/32) of the mares used in Experiments 1 and 2 had a history of recurrent uterine infections. Five of these 11 mares had uterine biopsies that were classified as normal. Twenty one (21/32) of the mares had no history of recurrent uterine infections. Sixteen of the biopsies obtained from these 21 mares were classified as normal since they showed little or no pathological changes (Table 3.1).

Fifteen of the 21 mares that were challenged in Experiment 1 eliminated the inoculated β haemolytic streptococci in around 4 days. These mares were classified as resistant. In contrast, the mares

still infected after 10 days were designated as susceptible to uterine infection (6 mares). Five of the mares designated as having lowered uterine resistance to infection (susceptible) on the basis of their challenge results had biopsies that were classified as normal and had no history of recurrent endometritis (Table 3.1).

The accuracy of biopsy findings or reproductive history in predicting the mares that were resistant or susceptible to uterine infection were described in terms of sensitivity (Se) and specificity (Sp) (Shwabe, et al., 1977).

Uterine biopsy results correctly predicted the challenge results in 21/32 mares, while reproductive history correctly predicted the ability of 31/32 mares to eliminate infections (Experiments 1 and 2).

Assuming that the challenge results were the true indicators of susceptibility, reproductive history was observed to be highly sensitive (Se = 0.92) in picking both resistant and susceptible mares. Biopsy findings were moderatley accurate (Se = 0.58) in classifying mares into either resistant or susceptible status (Table 3.2).

The proportion of false resistant (positive) and false susceptible (negative) were much higher for biopsy findings than for reproductive history (Table 3.2). The proportion of susceptible mares (negatives) that were truly susceptible (negative) was higher when classification was based on reproductive history (1.0) than on biopsy findings (0.7) (Table 3.2).

Reproductive history

*R Mares without a history of recurrent uterine infections

**S Mares with a history of recurrent uterine infections

Biopsy Results

R^1 Uterine biopsies showed little or no degenerative changes (normal endometrium)

S^2 Moderate to severe diffuse chronic degenerative changes observed in uterine biopsies obtained before challenge

Challenge Results

R^a Mares that eliminated the infused β haemolytic Streptococci by around the 4th day of challenge

S^b Mares that needed more than 10 days to eliminate infused bacteria

Table 3.1 The criteria used to tentatively (reproductive history and biopsy and finally (challenge results) classify mares into either resistant or susceptible status

Mare No	Tentative Selection Criteria		Challenge Results
	Reproductive History	Biopsy Result	
67	R*	R[1]	R[a]
153	R	R	R
155	R	R	R
160	R	R	R
72	R	R	R
35	S**	R	S[b]
55	S	R	S
141	R	S[2]	R
39	R	R	R
71	R	S	R
68	R	R	R
142	S	S	S
144	S	S	S
145	S	S	S
130	S	S	S
173	R	R	R
172	R	S	R
73	R	S	R
54	R	R	R
53	R	R	R
56	S	R	S
170	R	S	R
48	R	R	R
186	S	R	S
201	R	R	R
202	R	R	S
203	R	R	R
204	S	S	S
108	S	S	S
207	S	S	S
183	S	R	S
93	R	R	R

Table 3.2 The Performance characteristics of reproductive history and biopsy findings expressed in terms of sensitivity (Se), specificity (Sp), diagnosability and probability (P) of false positive and false negative, assuming that response to challenge indicates true resistance or susceptibility to infection.

	The test results	
	Biopsy	History
Sensitivity (Se)	0.58	0.92
Specificity (Sp)	0.70	1.0
Predictive Value (Diagnosability)	0.54	1.0
Proportion of test negatives (susceptible) truly susceptible	0.7	1.0

3.4.2 Bacteriological examination of uterine swabs and washings.

The mean numbers of β haemolytic streptococci recovered from uterine washings of mares classified as resistant or susceptible in Experiment 1 are plotted in Figure 3.1. There was a sharp decline in the number of β haemolytic streptococci isolated from uterine washings obtained from both resistant and susceptible mares within 6 hours of challenge. This initial decline was followed by a rapid rise in the number of β haemolytic streptococci recovered from the uterine washings obtained from both resistant and susceptible mares at 9-15 hours after challenge. A second sharp decline in the number of β haemolytic streptococci recovered was observed in uterine washings obtained from both resistant and susceptible mares between 15 and 24 hours after challenge. This second decline was followed by re-emergence of the β haemolytic streptococi in washings at 24 to 48 hours after challenge. Significantly higher numbers of bacteria were recovered from susceptible mares during this period. Bacteria generally persisted in susceptible mares for greater than 10 days, while they were eliminated from uteri of the resistant mares by the 4th day. No bacteria were grown from washings from the control mares throughout the sampling period.

In Experiment 2, serial bacterial swabs were obtained from challenged mares, without the infusion of washing fluid. The results of this experiment confirmed the bacterial isolation patterns for resistant and susceptible mares seen in washings from Experiment 1 mares following challenge (Figure 3.2). Both resistant and susceptible mares eliminated about 30% of the infused B haemolytic streptococci by 12-24 hours after challenge. At 10 days after challenge, however,

Figure 3.1 The mean number of Streptococci colony forming units/10µl recovered from uteri of resistant (-●-●-) and susceptible (-o-o-) mares following intrauterine inoculation with Streptococci. Control mares remained uninfected (Exp. I).

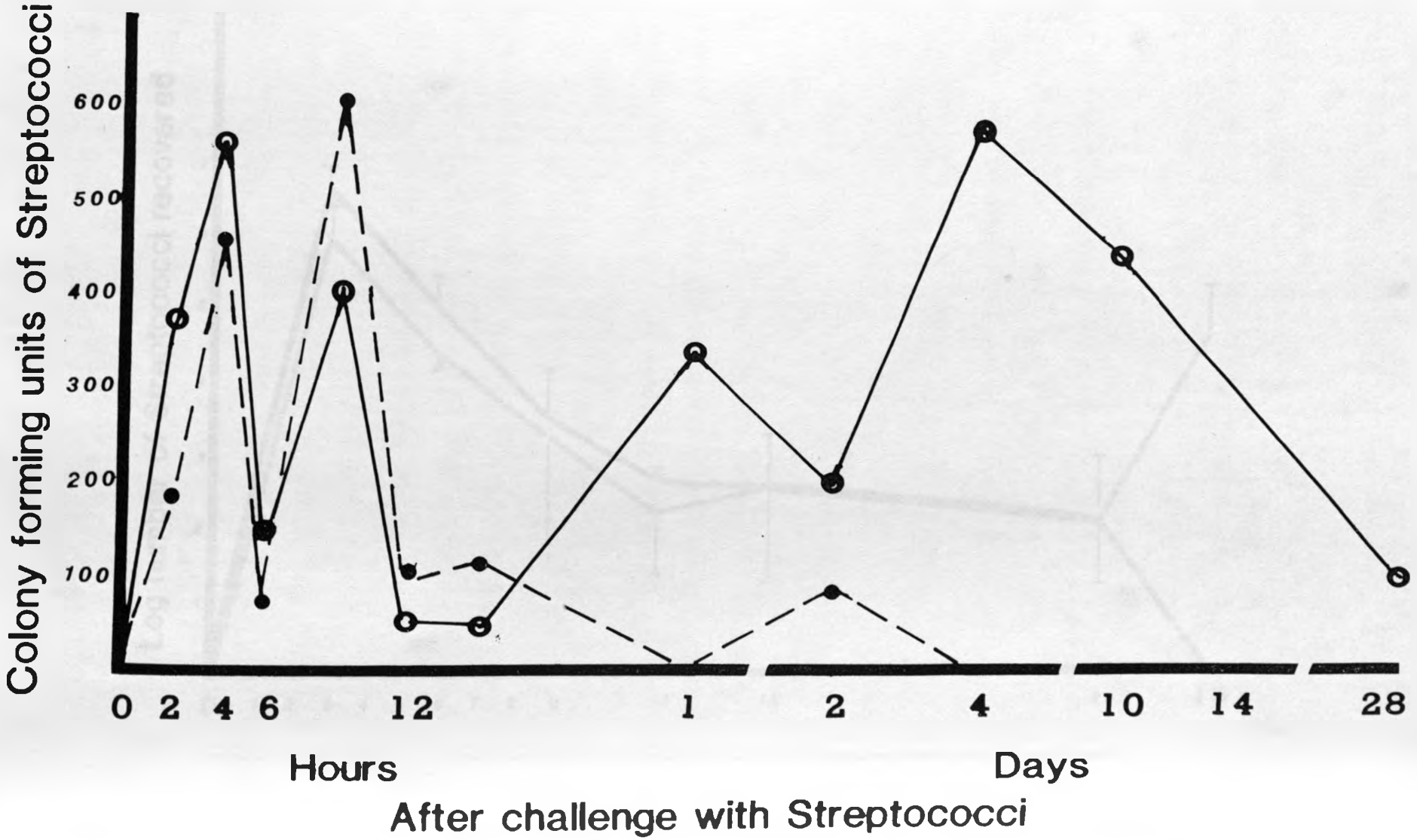
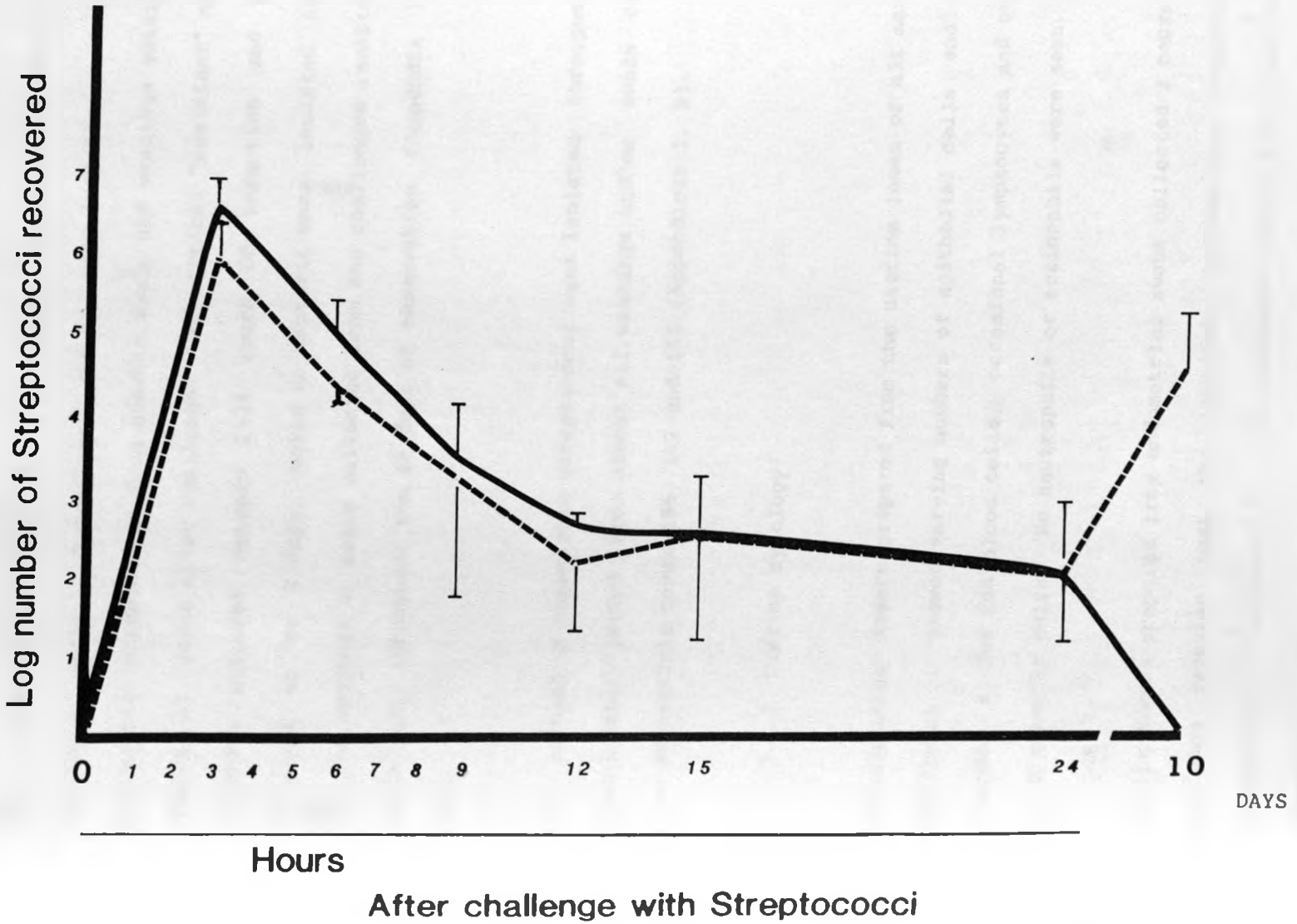


Figure 3.2 The mean number of Streptococci /ml recovered from uteri of resistant (——) and susceptible (----) mares following challenge with Streptococci without flushing the uterus.



only the mares with lowered resistance still had β haemolytic streptococci in their uterine fluids.

The dynamics of bacterial infection differed markedly between individual mares. Appendices 1 and 5, show isolation patterns of β haemolytic streptococci from uterine swabs and washings obtained from individual mares after challenge. The typical "resistant" mare had negative cultures between 2-24 hours of infection and positive cultures up to 4 days, while no bacteria were isolated from the uterine washings or swabs collected from one challenged resistant mare (mare 68) throughout the 28 days of observation (Appendix 1, page 221).

The infused β haemolytic streptococci were isolated throughout the experimental period from almost all washings and/or swabs obtained from susceptible mares 186, 142 and 145 (Appendices 1, 5).

3.4.3 Uterine cytology

Pre-challenge smears prepared from the uterine lumen of all mares in Experiment 1 showed varying numbers of epithelial cells and mucous strands, a few red blood cells, occasional lymphocytes and oval and round granular cells. No neutrophils or eosinophils were seen.

Uterine smears prepared from endometrial swabs collected 2 hours after challenge revealed that the immediate inflammatory response was predominantly neutrophilic. This cellular reaction was similar in smears obtained from resistant mares and susceptible mares (Appendices 1, 5). The cellular response subsided in most mares classified as

"resistant" within 4 days and persisted in "susceptible" mares for a minimum of 10 days (Appendix 1). Lymphocytes rarely exceeded 8% on differential count except in "susceptible" mares 35 (day 2, 24%), 55 (24 hours, 16%) and 186 (day 10, 15%; day 14, 75%) (Appendices 1, 5). Eosinophils, which rarely exceeded 5%, were readily observed in most uterine smears prepared 4 hours after challenge. Eosinophils persisted in uterine smears from the mares that remained infected. Phagocytosed bacteria and cellular karyorrhexis were observed in uterine smears made from swabs collected at 4, 6, 24 and 48 hours after challenge.

In Experiment 2 serial uterine samples, including uterine fluid and endometrial swabs were obtained after challenge without flushing. The inflammatory response, as observed on uterine cytological smears, was similar to that observed in the previously challenged group of mares (Experiment 1) except that since this group was sampled for cytology earlier 1 hour after challenge), an earlier cellular reaction was detected. Mares were classified as resistant (2/6) in this experiment, if they had eliminated the inoculated bacteria by the 4th day after challenge, while those designated as susceptible (4/6) remained infected for more than 10 days (Appendix 1). Eosinophils, which rarely exceeded 5% of the total leucocytes counted, were observed in uterine smears prepared 4-12 hours after challenge. Cellular karyorrhexis and phagocytosed bacteria were observed in smears prepared from swabs obtained at 4-48 hours after challenge. A neutrophilic response was observed in smears made from the control mares, for the first 48 hours of the observation period (Appendix 1).

3.4.4 Leucocyte concentration in uterine washings obtained in Experiment 1.

The acute phase of the cellular reaction in mares classified as either resistant or susceptible to uterine infection was similar. The mean leucocyte concentrations and cellular reaction patterns for the resistant and susceptible mares are shown (Figure 3.3, Appendices 1, 3, 5). No significant difference (N.S.) was observed in leucocyte numbers in uterine washings obtained from the "susceptible" and "resistant" mares after challenge. The unchallenged control mares showed a milder, predominantly neutrophilic response within 2 hours (Figure 3.3, Appendices 1, 3). Following the initial leucocyte peak a secondary increase in neutrophil numbers, which coincided with the re-emergence of the β haemolytic streptococci, was observed in uterine washings samples obtained from most of the challenged mares (Figures 3.1, 3.3, Appendices 1,4). Leucocyte numbers declined in "susceptible" mares in uterine samples obtained 10 days after challenge despite the persisting, active infection (Figures 3.3. Appendices 1,4,5).

3.4.5 Quantitative protein measurements in uterine washing and serum obtained from mares used in Experiment 1.

Uterine washings and serum samples were subjected to both quantitative and qualitative measurements of proteins. The protein concentration in the pre-challenge uterine washings obtained from mares designated as susceptible to uterine infection were similar to those in the washings from the resistant (0.45 ± 0.18 mg/ml cf. 0.50 ± 0.30 mg/ml, N.S.) and the control mares (0.35 ± 0.10 mg/ml, N.S.). Both the

Figure3.3 The mean leucocyte concentration/ml uterine washing obtained from challenged, resistant (———), susceptible (----) and unchallenged control (-o-o-) mares

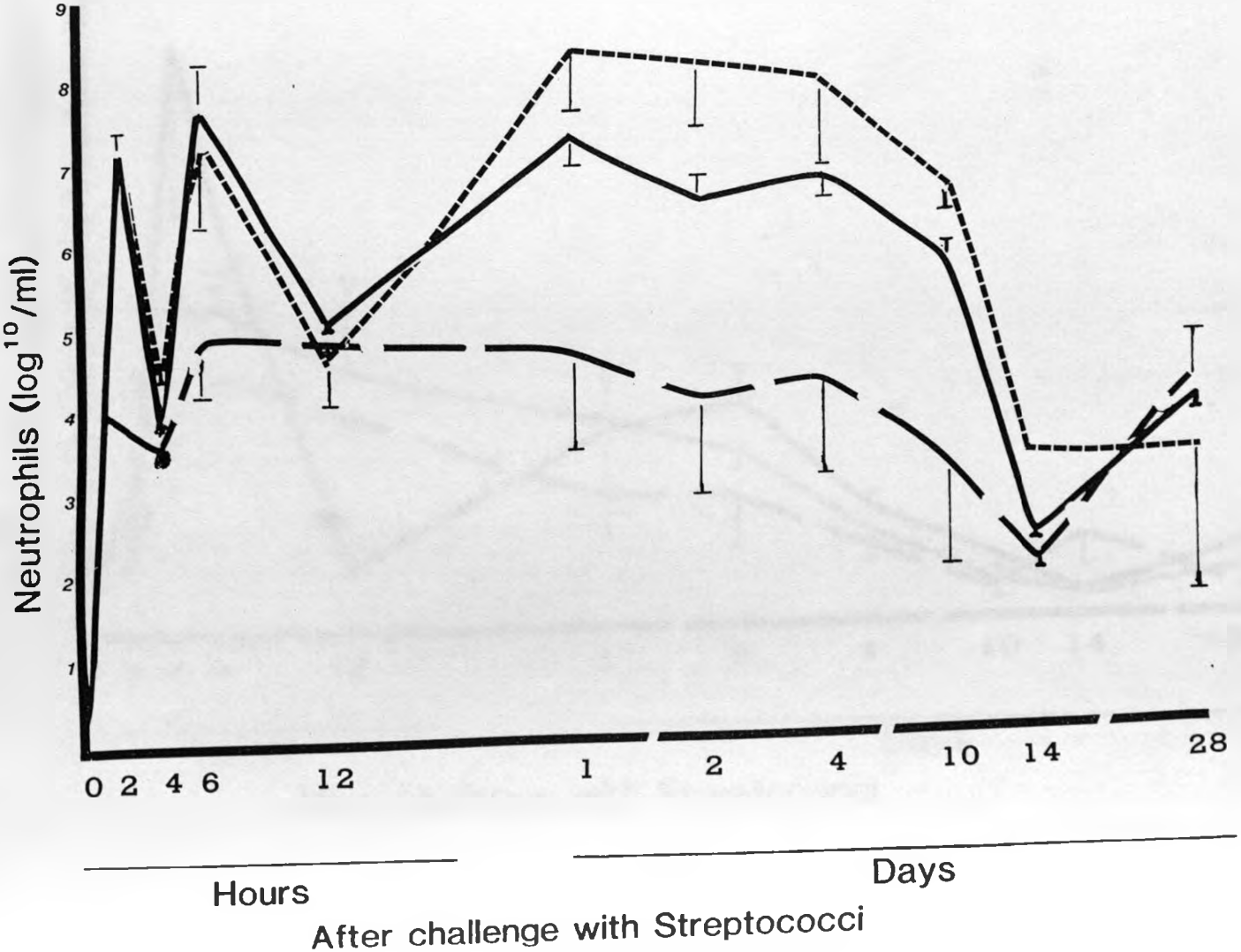


Figure 3.4 The mean protein concentration (mg/ml) in uterine washings obtained from challenged, resistant (————), susceptible (-----) and unchallenged, control mares (-o-o-)

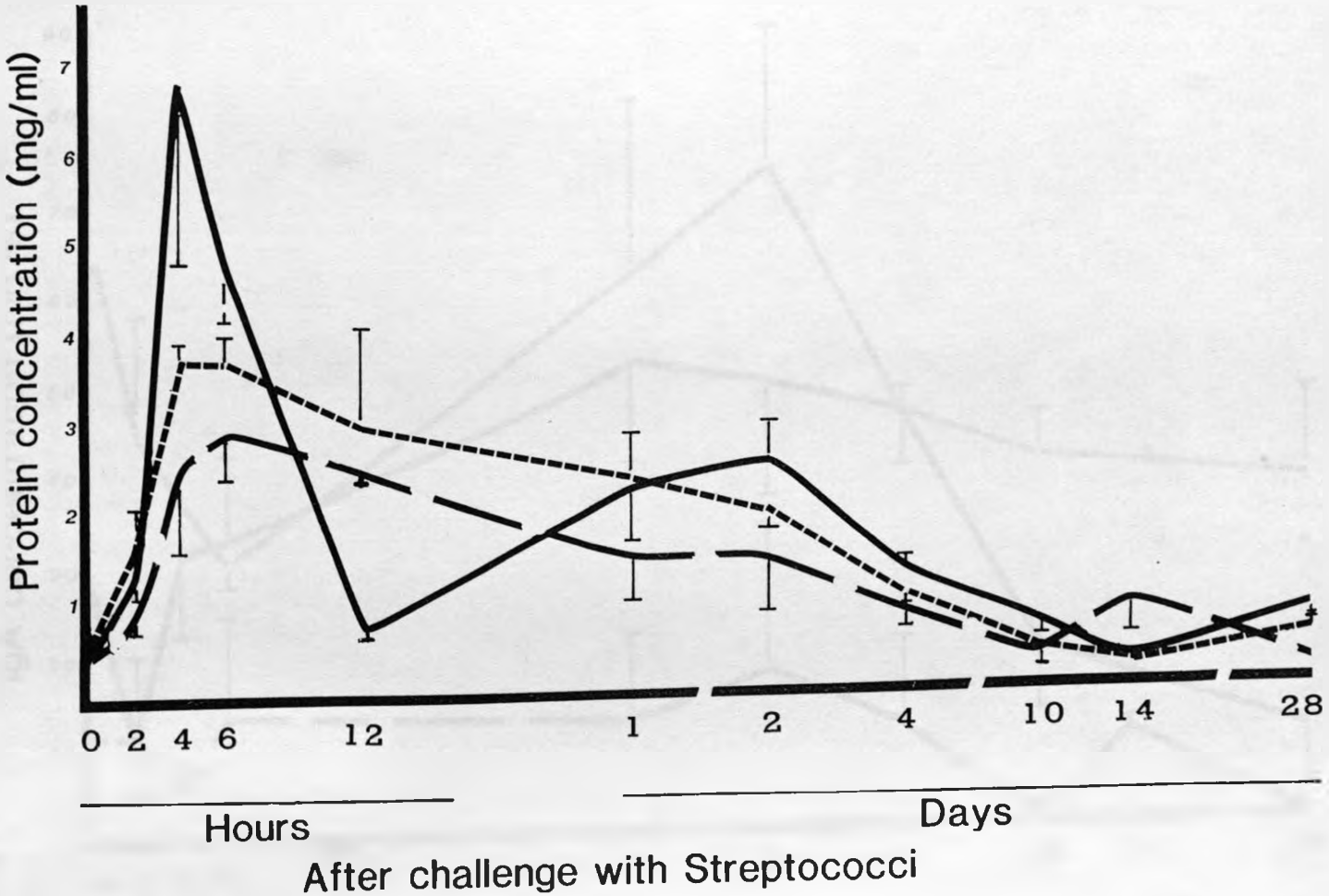


Figure 3.5 The mean IgA (mg%) concentration in uterine washings obtained from challenged resistant (——) and susceptible (----) and unchallenged control (-o-o-) mares.

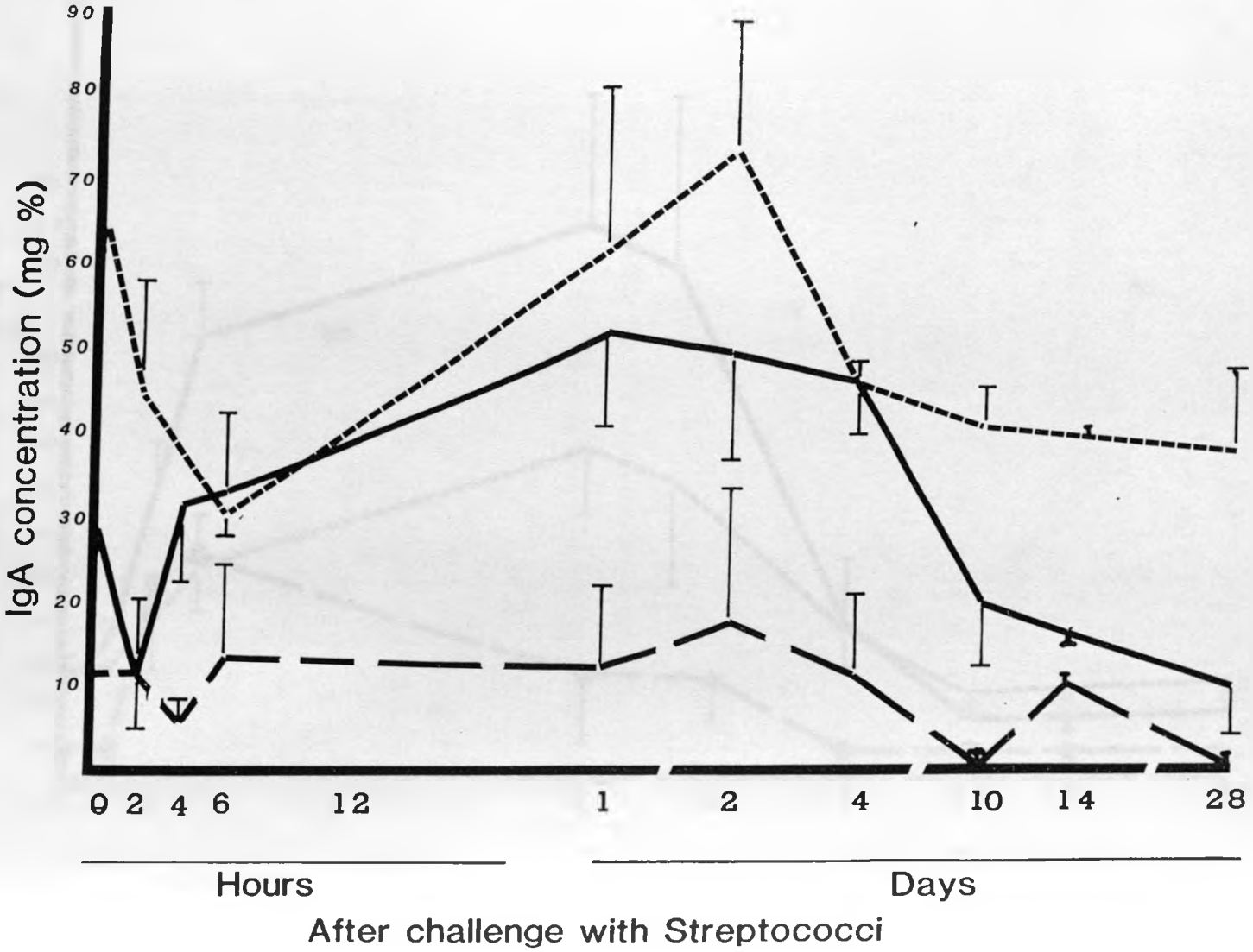
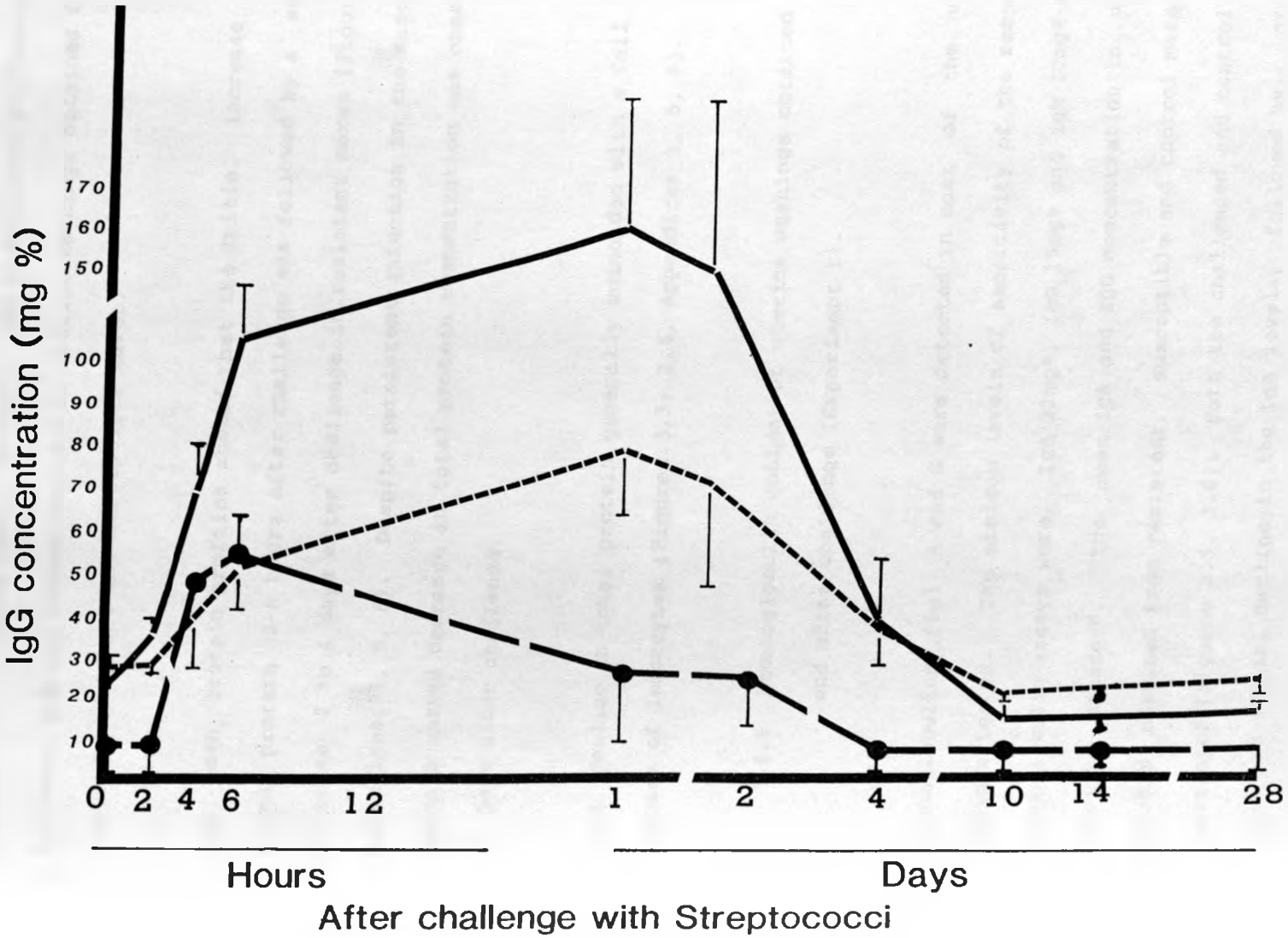


Figure3.6 The mean IgG concentration (mg%) in uterine washings obtained from resistant (——), susceptible (---) and control mares (-●-●-) following intrauterine inoculation with Streptococci



control and challenged mares subsequently showed a marked increase in protein concentration within 6 hours of challenge (figure 3.4, Appendix 5). There were, however, no significant differences (N.S) between protein concentrations in uterine washings obtained from the control, resistant and susceptible mares.

The mean protein profiles showed that the initial increase in the total protein 3-6 hours after challenge was followed by a secondary increase 2 to 4 days after challenge in resistant mares (Figure 3.4, Appendices, 1, 5, 6). Despite persistent infection in the susceptible mares a marked decrease in total protein concentration was observed by 10 days after challenge.

This decline in total protein generally coincided with a fall in the numbers of leucocytes (Figures 3.3; 3.4, Appendices 1, 5, 6).

3.4.6 Immunoglobulin content of uterine washings obtained before and after challenge (Experiment 1).

Immunoglobulins (Ig) A and G were detected in most of the uterine washings tested. The minimum levels of sensitivity of the respective immunoglobulin assays were, IgA 22mg%, IgG 15mg% and IgM 10mg% of the colostrum standard. The mean IgA and IgG concentration in uterine washings obtained from resistant, susceptible and control mares are illustrated (Figures 3.5, 3.6). Both the challenged and control mares showed an initial decline in the IgA levels, followed by a gradual, but steady increase. The highest peak in IgA concentrations occurred at 2 to 4 days post challenge (Figure 3.5, Appendices 1, 5, 7). This peak roughly coincided with the re-emergence of β haemolytic

streptococci and the neutrophil peak. The IgA levels in uterine washings obtained from susceptible mares showed a gradual decline, but remained slightly above the levels present in the uterus before challenge. There was, however, no significant difference (N.S.) between the mean IgA concentrations of uterine washings obtained from resistant and susceptible mares over the whole sampling period. There were significant differences, ($P<0.01$), between the IgA concentrations of the challenged (both resistant and susceptible) and control mares. Appendices 1 and 5 contain individual mare profiles of uterine IgA concentrations, before and after challenge, considered to by typical of the profiles seen with resistant, susceptible and control mares.

IgG levels followed the same general trend as the IgA but there were no significant differences (N.S.) between the IgG content in washings obtained from the three groups of mares (Figures 3.6, Appendix 8). IgM remained undetectable in all but 4 uterine washing samples from 4 different mares.

3.4.7 Bactericidal activity of uterine washings

The results of bactericidal assays showed that uterine washings obtained after challenge from resistant and susceptible mares used in Experiment 1, and the uterine fluid obtained from the two mares with severe chronic endometritis contained no bactericidal factor(s). There was no inhibition of growth of the streaked β haemolytic streptococci by any of the uterine washings placed on the plates.

Table 3.3

Leucocidal activity of uterine washings obtained during the "protein flush" 6 hours after bacterial challenge from control, resistant and susceptible mares.

	Mare group	% viable neutrophils after incubation for 0 hr	1 hrs	2 hrs	3 hrs	4 hrs
1	Resistant[a] mares n=4	*100	**95 ± 1.25	96.5 ± 1.88	97.25 ± 1.66	97.25 ± .85
2	Susceptible[a] mares n=4	100	94.5 ± 1.85	98.0 ± .40	93.76 ± 4.6	90.75 ± 7.0
3	) Control[b] mares n=2	100	96.5 ± .50	47.5 ±27.5	81.0 ± 6.0	75.0 ± 5.0
4	) Chronic[a] infection n=2	100	96.5 ± 1.5	81.5 ±11.5	97.0 ± 1.0	96.0 ± 1.5
5	) Hbss[a] buffer n=2	100	99.5 ± .5	98.5 ± .5	98.5 ± .5	97.5 ± .5

Mare group in the same column with different superscript(s) had neutrophil viability values that differed significantly ($P<0.05$).

Group 1) = Uterine washings obtained from resistant mares at 6 hrs after challenge; Group 2) = Uterine washings obtained from susceptible mares at 6 hrs after challenge; Group 3) = Uterine washings obtained from the unchallenged control mares at 6 hrs after the begining of observation period; Group 4) = Uterine fluid obtained at post mortem from mares 188(1) and 190(1), which had severe chronic streptococcal endometritis and Group 5) = Hanks balanced salt solution.

3, 4, and 5 served as controls.

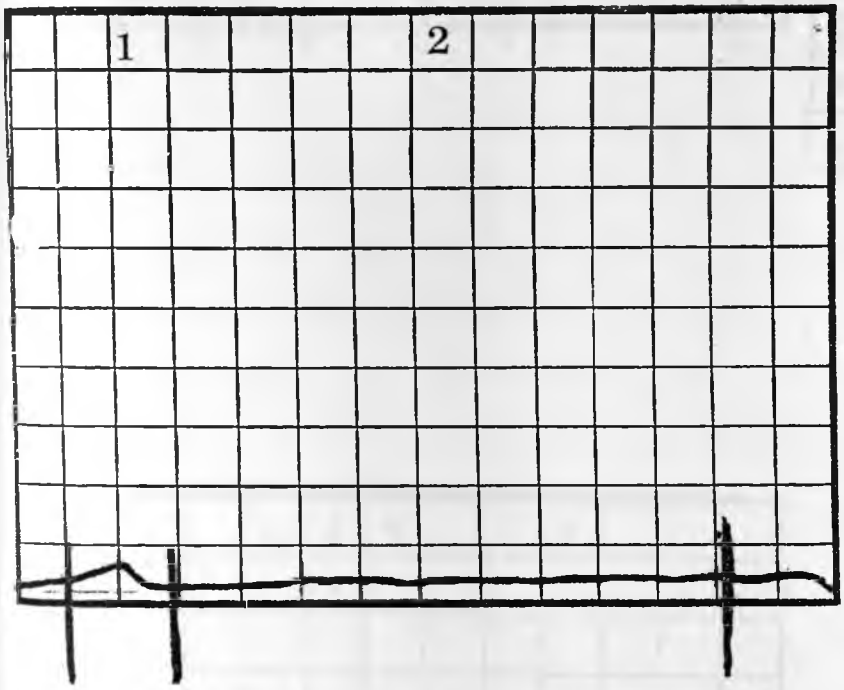
* = Mean (%) viable neutrophils before incubation

** = the mean (%) viable neutrophil after incubation

Figure 3.7A, B and C

Representative densitometer tracings of cellulose acetate electrophoresis pre-challenge uterine fluid (A), uterine fluid obtained at 12 hours after challenge (B), and pre-challenge serum (C). Densitometer tracings of electrophoresis of uterine fluid and washings obtained after challenge from resistant and susceptible mare were similar. Homologous and pooled serum were used as internal controls. Note the relative concentration of 2, B_1 and B_2 globulins in uterine fluid are at least twice as high in as those in serum.

A

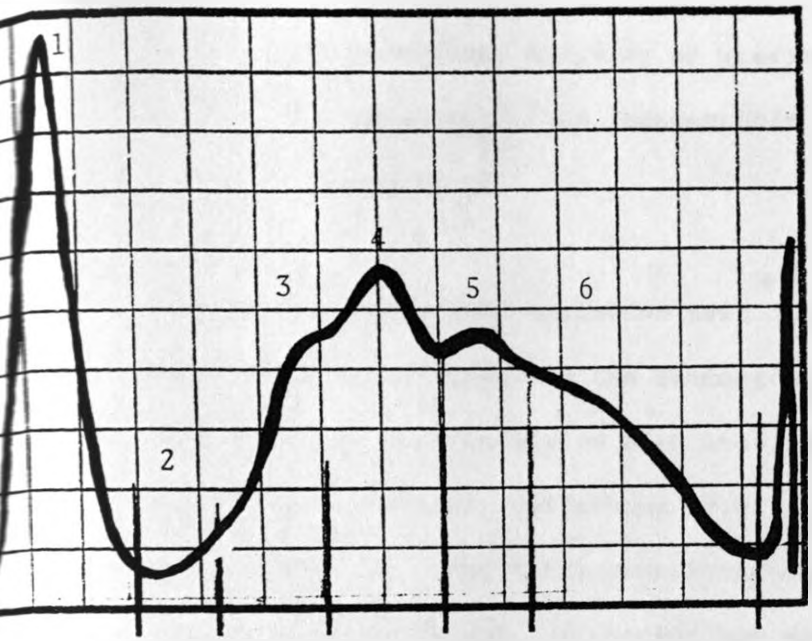


TEST NO.	15
TOTAL CONC.	4.00

Prechallenge

Uterine fluid

		RELATIVE PERCENT	CONC.
1	Albumin	51.03	2.20
2	Globulin	42.91	1.71
3			
4			
5			
6	A/G RATIO	1.33	

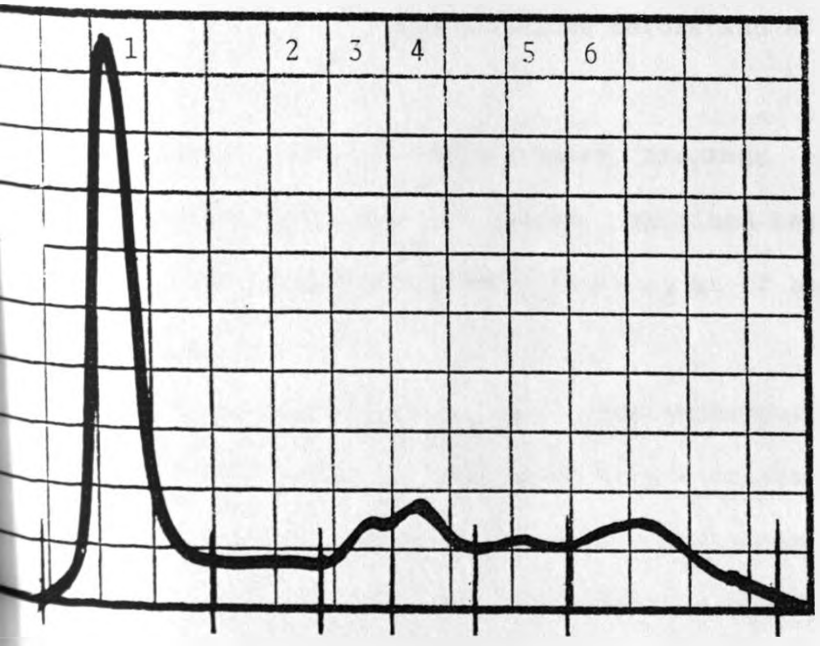


TEST NO.	18
TOTAL CONC.	68.00

Uterine Fluid

		RELATIVE PERCENT	CONC.
1	Albumin	26.39	11.94
2	α1	2.22	1.50
3	α2	12.81	8.71
4	β1	23.53	16.00
5	β2	14.12	9.60
6	γ	20.99	14.23
A/G RATIO		0.36	

C



TEST NO.	1. Serum
TOTAL CONC.	56.00

Serum

		RELATIVE PERCENT	CONC.
1	Albumin	51.08	28.60
2	α1	6.88	3.85
3	α2	7.87	4.40
4	β1	9.91	5.54
5	β2	8.59	4.81
6	γ	15.68	8.18
A/G RATIO		1.04	

3.4.8 Leucocidal activity of uterine washings obtained from resistant and susceptible mares at 6 hours after challenge.

Using the trypan blue exclusion test it was observed that 99-100% of the neutrophils used in the leucocidal activity test were viable before they were incubated with uterine fluid and washings. There was no significant difference (N.S.) between the percent viable neutrophils in test tubes containing uterine washings obtained from either the resistant or susceptible mares after challenge or the uterine fluid obtained from the two chronically infected mares (Table, 3.3, Appendix 9). Uterine washings obtained from the resistant and susceptible mares at 6 hours and 10 days after challenge were observed to have no leucocidal activity (Table 3.3, Appendix 9).

3.4.9 Cellulose acetate electrophoresis of uterine washings and fluids obtained before and after challenge. (Experiment 2)

Representative densitometer tracings of cellulose acetate strip electrophoresis of serum, obtained before challenge, and uterine fluid, obtained without flushing at 12 hours after challenge are shown (figure 3.7).

Cellulose acetate strip electrophoresis of undiluted uterine fluid showed that, in comparison to pre-challenge uterine fluid, there was a definite change in content, composition and relative percentages of the various protein fractions in uterine fluid obtained at 12 hours after challenge (Figure 3.7 A,B).

The relative percentage of alfa and beta globulins and albumin in uterine fluid obtained 12 hours after challenge were twice as high as those observed in pre-challenge serum (Figure 3.7.C).

3.5 DISCUSSION

The clinical signs of endometritis observed in this study were similar to those previously reported (Hughes and Loy, 1969; 1975; Peterson et al., 1969).

Based on clinical and laboratory (bacteriology and cytology) findings, "resistant" mares were defined as those that resolved infection by around the 4th day of challenge. In contrast, infection in mares considered to have lowered resistance to uterine infection ("susceptible") persisted for more than 10 days. Using these criteria 11 of the 32 challenged mares in Experiments 1 and 2 were designated as "susceptible" and 21 as "resistant".

In comparison to the challenge results, reproductive history was observed to be highly sensitive (Se = 0.92) and specific when classifying the mares into either resistant or susceptible status. Biopsy findings were observed to be less sensitive (Se = 0.58) and moderately specific (Sp = 0.7) at classifying the mares into either resistant or susceptible categories. The probability of occurrence of a false resistant or false susceptible was higher when biopsy findings were used to classify mares into either resistant or susceptible status, than when reproductive history was used (0.46 Vs 0.08 false resistant, 0.29 Vs 0.0 false susceptible respectively). In addition the proportion of susceptible mares (test negatives) that were truly

susceptible (negative) was only 70% for the biopsy findings, in comparison to 100% for the reproductive history. Thus, not all mares with moderate to severe chronic degenerative changes were susceptible to uterine infection on challenge, according to the challenge results. This observation suggests that while chronic endometrial degenerative changes may predispose a mare to recurrent uterine infection (Hughes and Loy, 1969; 1975; Peterson et al., 1969), other factor(s), possibly inherent to the individual mare, were equally as important. The use of reproductive history in predicting susceptibility of a mare to uterine infection has its limitations. The main limitation is the fact that reproductive history is often subjective and diffcult to verify, as it is dependent upon accurate observations and record keeping. Hence it is probably best to use a combination of reproductive history and biopsy findings to assess the ability of the mares' uterus to resolve bacterial endometritis.

In the present study there was a dramatic reduction in the number of B haemolytic streptococci isolated from uterine swabs and washings obtained from both resistant and susceptible mares between 2 and 6 hours post challenge. While it is possible that most of the inoculated β haemolytic streptococci had been killed by phagocytes and/or soluble bactericidal factors, this could have also been indicative of sequestration or firm adherence of the bacteria to specific binding sites on uterine luminal epithelial cells. Peterson et al., (1969) and Blanchard et al., (1981) suggested that the failure to recover pathogenic bacteria from mares with histologic endometritis could be related to the chronicity of the infection, with the invading organisms being deeply embeded in uterine tissues. It is, however, apparent from the results of the present study that this phenomena is

of constant occurrence in the acute phase of endometritis. This observation also sheds further doubts on the diagnostic value of a single uterine swab in the mare (Williamson et al., 1983).

The re-emergence of β haemolytic streptococci in both "susceptible" and "resistant" mares at 24 to 48 hours after challence coincided with a secondary cellular, protein and IgA response. The re-emergence may indicate the release of bacteria by dying phagocytes and/or their spontaneous detatchment from sites on uterine luminal epithelial cells. The persistence of the re-emerged β haemolytic streptococci in "susceptible" mares could have been due to either a subtle failure in intracellular killing or the inability of these mares to sustain a cellular response. If the susceptible mares were unable to sustain a cellular response and this was related in part, to the presence of leucocidal factor(s), the low leucocyte counts in these mares after 10 days, despite persistent infection, would be expected.

In contrast to the findings of Hughes and Loy, (1975), and Peterson et al., (1969) who observed that lymphocytes predominated by the seventh day of infection, in the present study neutrophils were observed to be the predominant cell type in all samples from mares with persistent infection, except for one mare at 14 days. These observations are in agreement with Bennett et al., (1980) who reported that uterine manipulation and/or intrauterine infusion of sterile isotonic solutions resulted in a predominantly neutrophilic response that persisted in 39% of the mares for more than 7 days. Therefore it may be that the discrepancy in the observations reported in this thesis and those reported by Hughes and Loy (1969; 1975) and Peterson et al., (1969), could be due to the differences in the frequency of sampling.

The serial sampling system adopted in the present study may have resulted in repeated uterine stimulation which resulted in a sustained neutrophilic response.

The decline of IgA levels in the uterine washings observed in both the challenged and control mares, at 2 to 4 hours after challenge, is difficult to explain other than to suggest that it could have been due to initial dilution effects of flushing. This initial decline was followed by a significantly greater increase in IgA concentration in uterine washings of resistant and susceptible mares, in comparison to control mares. This observation and the fact that there was a rapid fall in IgA concentration in the uterine washings of resistant mares immediately they overcame the infection, indicates that IgA was released in response to both uterine manipulation and the presence of bacteria but its release was more marked and persistent in the presence of bacteria.

These findings are in agreement with those of Mitchell et al., (1982), who observed a substantial increase in IgA in uterine secretions compared to that in serum and a corresponding fall in IgG with no substantial increase in IgM and indicated that an initial passive and preferential secretion was followed by local production of IgA. The data available from the present study suggests that both resistant and susceptible mares are capable of mounting cellular and humoral responses and that their reaction to bacterial challenge is similar.

In the present study most of the resistant mares eliminated the infused β haemolytic streptococci within 4 days. The four days that it took the resistant mares to eliminate the infection were not long

enough for specific antibodies directed against the infused β haemolytic streptococci to be produced and exert their opsonic and/or bactericidal effects. This observation would indicate that in the absence of specific antibodies, functional neutrophils are critical in the elimination of the infused β haemolytic streptococci. It is, however, unlikely that the mares designated as susceptible in the present experiments had gross neutrophil function disorders since these mares did not suffer from recurrent generalized infections as is the case of humans with gross neutrophil functional defects (Klebanoff, 1970; Clark and Kimbal, 1974).

In the present study a rapid increase in total protein, which reached a peak 6-12 hours following challenge, was observed in all experimental mares. During this protein "flush" there was a definite increase in albumin, and alfa and beta globulins in uterine fluids obtained from challenged mares. This initial protein increase observed in the challenged mares probably represented a non-specific humoral response to uterine stimulation. It is, however, likely that the larger protein peak observed in these mares 12 hours after challenge represented proteins released by dead and lysed bacteria, leucocytes and epithelial cells.

The movement of proteins, including immunoglobulins, across the membrance barrier may be either by passive diffusion, leakage or energy dependent active transfer (Kenney and Khaleel, 1975; Asbury et al., 1980; Blue et al., 1980).

Schumacher (1977), observed that cervical irritation in women was associated with increased concentrations of soluble proteins in

cervical secretions. This observation and the findings of the present study enhance the suggestion (Hughes and Loy, 1969, 1975; Peterson et al., 1969) that the influx of protein, in conjunction with neutrophils, into the mammalian uterus may be the second in the line of defence after the physical barriers (including the mucous and intact uterine luminal epithelium). In addition, these findings lend support to the suggestion by Williamson et al, (1983) that high protein concentration may be a useful adjunct to bacteriology, cytology and biopsy in diagnosing edometritis in the mare.

Qualitatively, the electrophoretic profiles obtained from uterine luminal fluid were similar to those derived from serum. Thus, the data suggests that uterine luminal fluid and its components are principally derived from serum. It is, however, probably that specific uterine protein(s) including some with micro-biocidal properties do occur in the equine uterine fluid, as suggested by Peterson et al. (1969) and Mitchell et al., (1982). The data obtained in the present study also indicates that it is highly unlikely that the uteri of susceptible mares were deficient in opsonins including immunoglobulins and components of the complement system as suggested by Asbury et al., (1982), since there was no significant difference in the soluble factors, including immunoglobulins A and G and the Beta globulins, in uterine washings obtained from both resistant and susceptible mare (see Experiment I).

Data obtained from the present study showed that there were no leucocidal factors in uterine washings or fluid obtained from both susceptible and resistant mares after challenge. This observation would indicate that in the absence of leucocidal factors, the lowered

leucocyte count in uterine washings obtained from infected susceptible mares 10 days after challenge, was most likely due to their lowered ability to sustain a cellular response.

Endometritis, the inflammation of the endometrium, represents a dynamic state consisting of several interdependent reactions involving leucocytes, platelets, blood and lymph vessels and tissues. These reactions are incited in the host by chemical, physical and/or infections irritants (Volanaskis et al., 1975). The establishment of the infectious irritants in the mare is often a sequel to trauma of the reproductive tract, fetal maceration and emphysema, retained after birth, abortions and poor management at the stud (Roberts, 1971; Hughes and Loy, 1969; 1975; Rossdale and Ricketts, 1980).

In the present study the immediate cellular response to experimental infusion with β haemolytic streptococci was an early (within 1 hour) intense predominantly neutrophilic infiltration into the endometrium and uterine lumen. This increase in neutrophils, was associated with a rapid decrease in the number of viable bacteria isolated from uterine washings, fluid and/or swabs.

Rebello et al., (1975), suggested that women who lacked the ability to produce IgA locally compensated for this lack of local IgA by extracting polymeric IgA from the plasma. In the present study it was observed that while all pre-challenge uterine washings had detectable concentrations of IgA, those obtained from susceptible mares had at least twice the levels of the resistant mares. This may have been due to the ability of the susceptible mares to produce IgA locally (Mitchell et al., 1986) or the persistence IgA from a previous

infection. The possibility of a compensatory mechanism, as suggested for women by Rebello et al., (1975) cannot be ruled out.

The findings of the present study, in agreement with those of Hughes and Loy (1969, 1975), Peterson et al., (1969) and Blue et al., (1982), suggest that neutrophil infiltration into the uterine lumen and the subsequent phagocytosis of the invading bacteria, the presence of soluble factors and cervical drainage play a central role in the elimination of the infecting organisms in the mare.

Both the resistant and susceptible mares in the present studies were capable of mounting similar cellular and humoral responses. These findings and the observations by Lie et al., (1981) and Mitchell et al., (1982) that susceptible mares were capable of locally producing specific immunoglobulins A and G, indicates that the presence of non specific and specific immunoglobulins and neutrophils in uterine lumen do not prevent the establishment, persistence and/or recurrence of uterine infections in mares with lowered resistance to uterine infection.

Studies are necessary to determine whether there is a decrease, with time, in the rate of intracellular killing by uterine phagocytes from chronically affected mares or an inherent inability of these mares to sustain a cellular response in the presence of persistent infection. It is also essential that more practical techniques of assessing lowered uterine resistance to infection (susceptibility) in the mare be established. Such techniques would not only allow direct comparison of data obtained from the various institutes interested in the study and management of equine endometritis and infertility but also enable a more objective classification to be introduced.

CHAPTER 4

PAGES

LIGHT AND ELECTRON MICROSCOPIC STUDIES OF THE TISSUE AND CELLULAR REACTION TO INTRAUTERINE INOCULATION WITH BETA HAEMOLYTIC STREPTOCOCCI IN MARES WITH VARYING RESISTANCE TO ENDOMETRITIS.

PAGE

CHAPTER 4.

LIGHT AND ELECTRON MICROSCOPIC STUDIES OF THE TISSUE AND CELLULAR REACTION TO INTRAUTERINE INOCULATION WITH B HEMOLYTIC STREPTOCOCCI IN MARES WITH VARYING RESISTANCE TO ENDOMETRITIS.

4.1 INTRODUCTION

Light miscroscopic examination of uterine biopsies has been used as a diagnostic and research tool to assess the response of the uterus to natural and experimental infection (Kenney, 1978; Samuel et al., 1979; Rossdale and Ricketts, 1980). Rossdale and Ricketts (1980), using a light microscope, reported that acute endometritis, in the mare, was characterized by PMN infiltration into the strata spongiosum and compactum and in between the luminal epithelia cells. These researchers further stated that the degree of vascular congestion and margination with PMN and the density of cellular infiltrate and the depth to which it extended was very variable. Rossdale and Ricketts (1980) in agreement with Ricketts et al., (1978) reported that they occasionally observed bacteria associated with the luminal epithelial cells in sections stained with Gram twort. Samuel et al., (1979) showed that scanning and transmission electron microscopy are useful adjuncts to conventional light miscroscopy in such studies.

4.2 AIMS.

The aim of the present investigations was to study the tissue and cellular changes that occur in the endometrium following infection and to establish the extent of vascular damage. It was also proposed to study the adherence of the infused β haemolytic streptococci to the luminal epithelial cells.

4.3 MATERIALS AND METHODS

4.3.1 Selection of mares.

Uterine biopsies obtained during the adjustment period (see section 2.1) from mares used in Experiments 1, 2 and 3, were used to establish the condition of the uterus before challenge. Then;

1) To study the tissue and cellular changes at specific intervals after experimental uterine infection uterine biopsies were obtained from mares at various intervals after the intrauterine infusion of β haemolytic streptococci.

2) To study the vascular changes during the infux of serum proteins observed in uterine washings 3-6 hours after challenge (see section 3.4.5) uterine biopsies were obtained from 6 challenged mares for transmission and scanning electron microscopic (TEM and SEM respectively) examination.

Uterine biopsies obtained in (1) and (2) above were examined for adhering bacteria by light microscopic examination of gram stained biospy sections. Transmission electron microscopic (TEM) examination of uterine biopsies was also undertaken to determine if bacterial adherence to the uterine luminal epithelial cells occurred during the early stages of infection.

4.3.2 Preparation and evaluation of uterine biopsies by routine light microscopic examination.

The technique of collection, processing and assessment of uterine biopsies was as described previously (see section 2.5). Uterine biopsies were obtained from 38 challenged and 3 control mares once before, and at least once between 24 hrs and 32 days intrauterine infusion of B haemolytic streptococci (Table 4.1). Uterine biopsies obtained before the bacterial challenge served as controls (see sections 2.1). Immediately following collection, the biopsies were carefully divided into 2 portions. The first portion of each biopsy obtained was placed in Bouins fixative, while the other was fixed in 10% formal saline. Formal saline fixed portions were stained with carbochromotrope - methylene green - pyronin, for the demonstration of plasma cells and eosinophils and duplicate sections were gram stained to check for the presence of bacteria. Sections from the Bouins fixed portions were routinely stained with hematoxylin eosin for histopathological examination.

Table 4.1

The number of mares from which biopsies for light microscopy were collected before and at the various sampling intervals after challenge.

Time of biopsy collection	Number of mares in each sampling interval
Before challenge	51
Biopsies obtained after challenge	
3 hrs	2
4 hrs	2
5 hrs	2
6 hrs	7
24 hrs	10
6 days	4
10 days	10
14 days	7
28 days	6
32 days	3

Uterine biopsies were obtained from 51 different mares for light microscopy.

4.3.3 Preparation and evaluation fo uterine biopsies by scanning electron microscopy (SEM).

Uterine biopsies for electron microscopy were obtained from 4 challenged mares and 2 unchallenged mares at 3, 4, 5 and 24 hrs after the infusion of β haemolytic streptococci or isotonic mannitol.

A portion of each biopsy was fixed for scanning electron microscrope examination (SEM). It was immersed in 2% acetic acid for 5 minutes immediately after collection, to coagulate the thick mucous layer that often covered the endometrium. The acid treated biospies were then washed throughly in physiological saline before being fixed as for the TEM and cut into pieces with a surface area of 2-3 mm^2. These pieces of tissue were immediately dehydrated through a graded series of alcohols. The sections were taken from 100% alcohol and placed into liquid carbon dioxide in an FL-9496 Balzers critical point drying apparatus for super drying (Balzers Union Aust. Ltd., NSW) (Ferenczy, 1976).

4.3.4 Preparation and evaluation of uterine biopsies by transmission electron microscopy (TEM).

The second portion of each biopsy for transmission electron microscopy was processed and examined as described by Samuel, Ricketts and Rossdale (1978).

A portion of the uterine biopsy was placed in fresh 2.5% gluteraldehyde in phosphate buffer at pH 7.2 for 10 minutes immediately after collection. These portions were subsequently cut into 1 mm cubes and washed thoroughly in physiological saline before being fixed for at least for 2 hrs at 4C in freshly prepared 0.1 M sodium cacodylate buffer (pH 7.4, at 4 C) containing 1% gluteraldehyde and 4% paraformaldehyde. After fixation, the sections were transferred into 0.1 M sodium cacodylate buffer with 5% glucose and stored at 4C prior to processing for examination by transmission electron microscope (TEM) (Samuel et al., 1978).

4.4. RESULTS

4.4.1 Histopathological findings.

The stage of the oestrous cycle at the time of sampling was assessed as described by Kenney (1978) (see section 6.3.1). All stages of the oestrous cycle were represented in biopsies as judged by the luminal epithelium, glandular and stromal structures present in biopsies (Appendix 10). Four mares showed evidence of mitotic activity in glandular epithelial cells during the follicular stage of the cycle. Histological findings observed in pre-challenge

uterine biopsies fell into three categories, (I, II, and III), which are based on Kenney's criteria (1978).

Category I

The endometrium which showed little or no degenerative and/or inflammatory changes was classified as being in category I. Twenty pre-challenge uterine biopsies were classified as being in category I.

Category II

The endometrial biopsies which showed a mild diffuse and/or moderate localized degenerative changes including slight to moderate periglandular fibrosis with accompanying glandular nesting and scattered lymphatic lacunae, were classified as being in category II. Twenty one of the pre-challenge uterine biopsies examined had these characteristics.

Category III

The type of biopsies placed in this category were those with severe diffuse fibrosis and widespread periglandular fibrosis with glandular nesting, widespread lymphatic lacunae and atrophy of the endometrium. In addition there were occassional eosinophils and mononuclear cells especially lymphocytes infiltrating into the endometrial stroma. Based on these characteristics ten of the biopsies examined were classified as category III.

Examples of biopsies placed in the different categories are shown in figures 4.1, A & B, and 4.2 A, B & C.

Figures 4.1 (A & B)

A photomicrograph of a normal uterine biopsy section of the equine uterus obtained before challenge. Note the well defined low cuboidal epithelium (EP) and glands, without periglandular fibrosis (arrows). This uterine biopsy was classified as being in catergory I. (H & E, A X 100, B X 200).

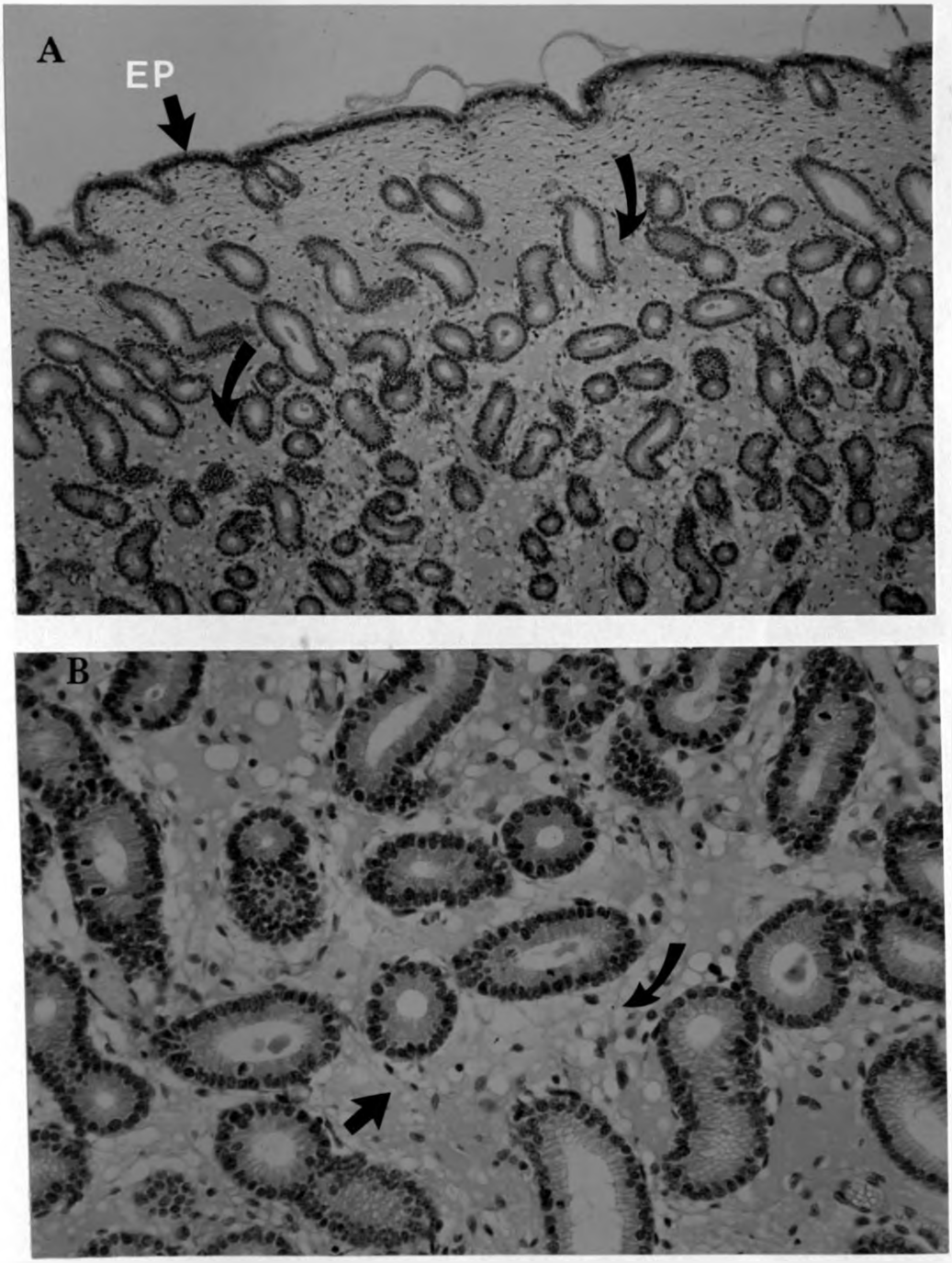


Figure 4.2 (A & B)

A photomicrograph of a uterine biopsy showing endometrial degenerative changes. This biopsy which was obtained before intrauterine infusion of Beta haemolytic streptococcus was classified as category III. Note the nesting of the endometrial glands and the glandular cysts (CY) resulting from peri and inter glandular fibrosis (F) (H & E, A, X 100, B X 200).

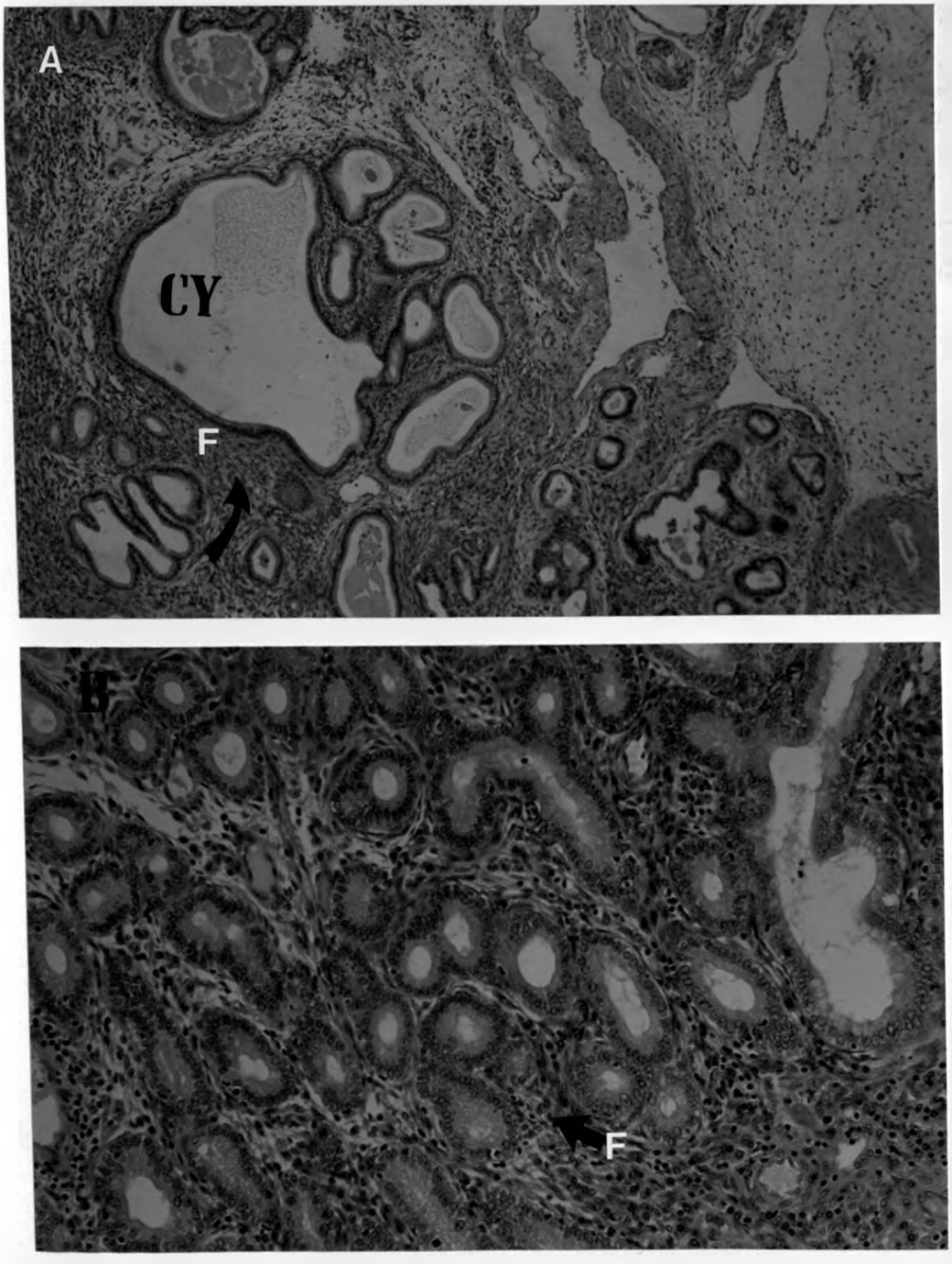
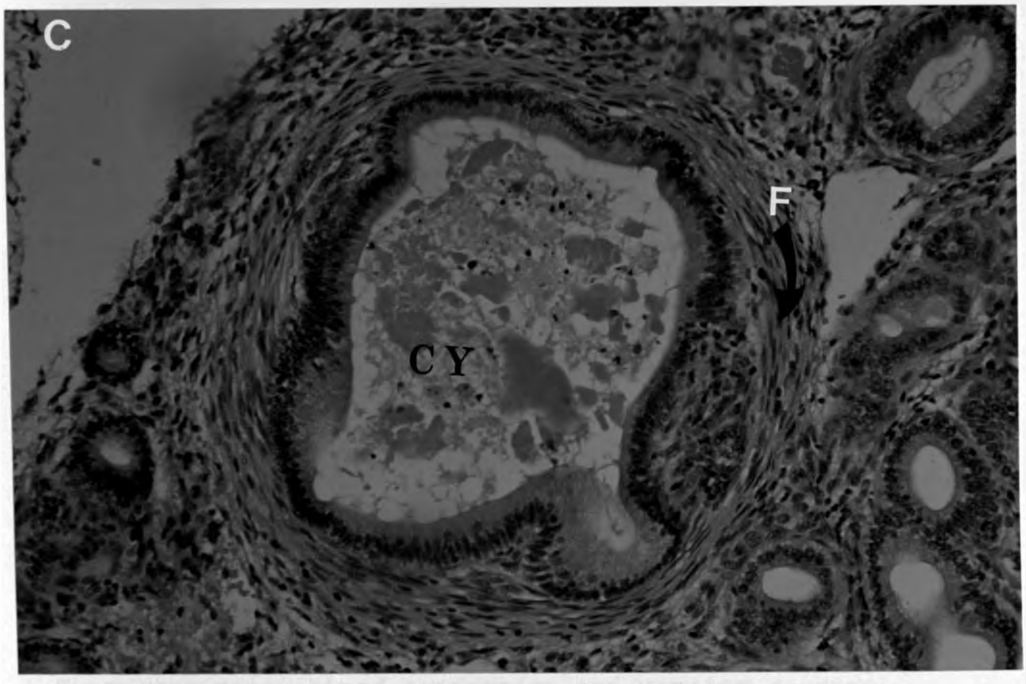


Figure 4.2 (C)

A photomicrograph of a uterine biopsy classified as being in category III. Cystic gland (CY), formed as a result of the moderate to severe periglandular fibrosis (F), contained both degenerating cellular debri and non cellular inspissated secretions (H & E, C X 200).



The following histopathological features were observed in uterine samples obtained from both resistant and susceptible mares (unless otherwise indicated) at 3-24 hrs, 2-6 days and 10-32 days after challenge:

4.4.1.1. Light microscope observations in uterine biopsies obtained at 3 to 24 hrs after challenge.

Category I and II mares

Biopsies obtained at 3, 4, 6 and 24 hrs following challenge showed widespread, severe acute inflammation, characterized infiltration of neutrophils into the strata compactum and spongiosum, uterine luminal epithelium and into the uterine lumen. The density of the infiltrating neutrophils and the stromal depths to which the netrophil infiltration extended (Figure 4.3 A) varied between mares.

There was generalized congestion and varying degrees of localized haemorrhage. Widespread vascular margination and perivascular cuffing with neutrophils was observed in most uterine biospies obtained at 3-24 hrs after challenge (Figure 4.3 B). In some sections neutrophils formed an amorphous layer over the surface of the endometrium and/or just below the basement membrane.

Eosinophils were observed in all caterogry I and II endometria obtained 6 hrs after challenge. The density

of the infiltrating eosinophils, which were mostly aggregated in the strata compactum and spongiosum, varied between mares. In the 2 types of endometria, the infiltrating eosinophils reached a peak 24 hrs post challenge.

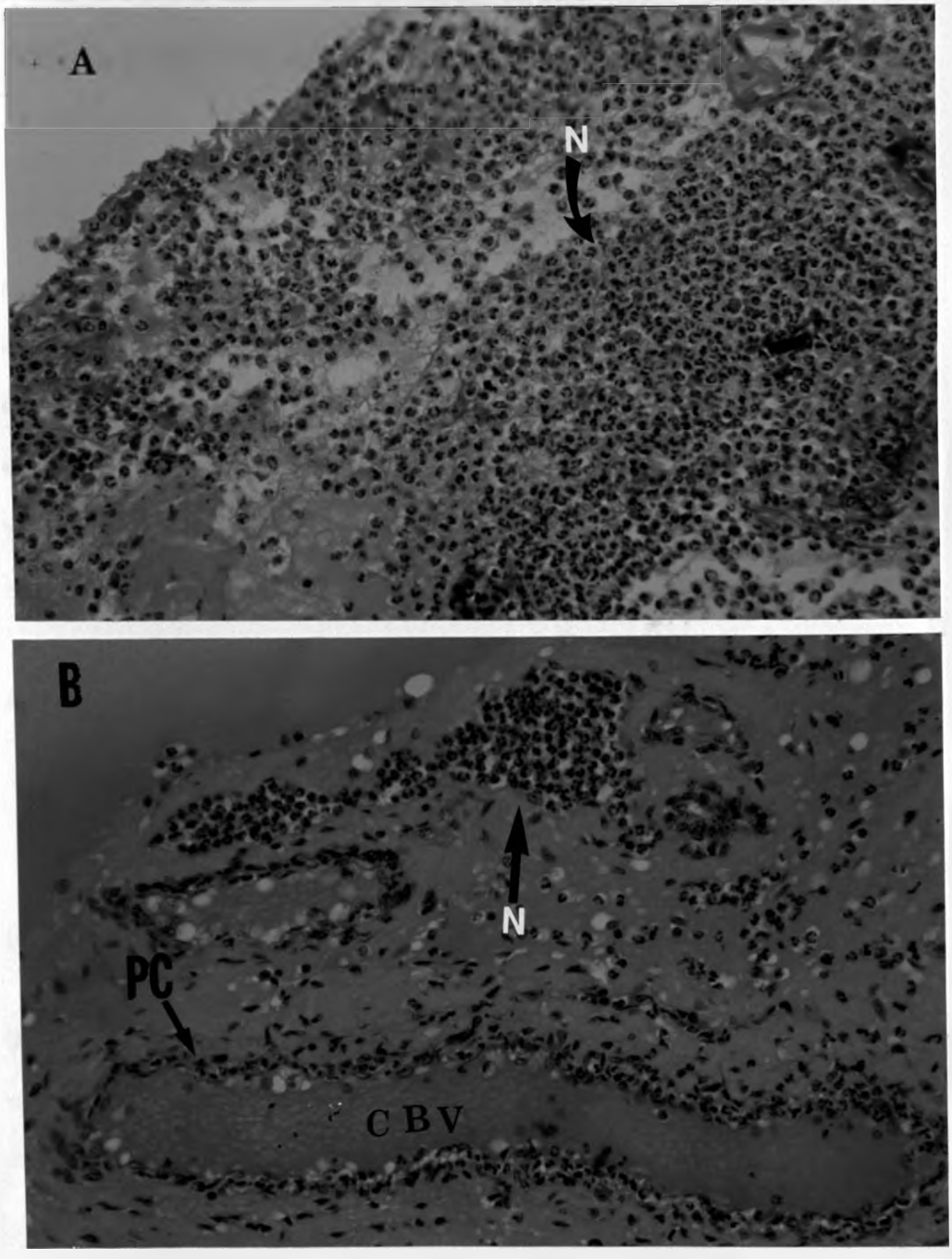
Category III mares

Polymorphonuclear cell and tissue reaction in biopsies obtained from category III mares between 3-24 hrs post challenge resembled that described for category I and II mares. Eosinophils, which were observed in pre-challenge uterine biopsies reached peak concentrations in biopsies obtained at 24 hrs post-challenge. Most eosinophils were aggregrated in strata compactum and spongiosum. Occasional lymphocytes and plasma cells were also observed in some of the sections.

No bacteria were seen adhering to uterine luminal epithelium in any of the Gram stained sections obtained from mares in either categories 3-24 hrs post challenge.

Figures 4.3 (A & B)

A photomicrograph of a uterine biopsy section representative of the uterine biopsies obtained during the acute phase (3 to 24 hrs) after challenge. Note the diffuse predominantly neutrophilic infiltration into the endometrial tissues (N), the diffuse stromal oedema and perivascular cuffing (PC) fo the congested blood vessel (CBV).
(H & E, A X 400, B X 400).



4.4.1.2 Light microscopic observations in uterine biopsies obtained at 2 to 6 (day(s) after challenge.

Category I and II mares

Biopsies obtained at 2, 4 and 6 days after challenge had similar characteristics to those obtained at 3 to 6 hrs except for slight diffuse monocytic infiltration and slight periglandular aggregation of lymphocytes which was seen in 7 of the 9 biopsy section (Figure 4.4.a).

Category III mares

Biopsies obtained from mares with category III endometrial at 2-6 days post challenge were characterized by a mild diffuse monocytic infiltration with slight periglandular aggregation of lymphocytes. Eosinophilic infiltration was a constant finding in all category III endometrial biopsies.

4.4.1.3. Light microscopic observations in uterine biopsies obtained 10 to 32 days after challenge (Category I, II, III).

Category I and II

Uterine biopsies obtained, 10 days after challenge from mares that had eliminated the infused bacteria had findings similar to their pre-challenge biopsies.

Category III

Cellular and tissue changes were similar to those observed in biopsies obtained between day 1 and 6 post challenge. Some degeneration of luminal epithelial cells, which varied from mild to severe degeneration of the apical cytoplasm with occasional ulceration was observed in a few of the biopsies. Eosinophils were observed in endometrium obtained 10 days after challenge (Figure 4.4.B) Plasma cells were observed less frequently than any other mononuclear cell even in mares which were still heavily infected 32 days after challenge (Figure 4.4.C).

Figure 4.4. (A)

A photomicrograph of a uterine biopsy section obtained 6 days after challenge showing cellular infiltration into uterine luminal epithelium (C) and stratum compactum (St) slight basal vacuolation (BS) of the luminal epithelium. Neutrophils were still the predominant inflammatory cell type (H & E. A X 400).

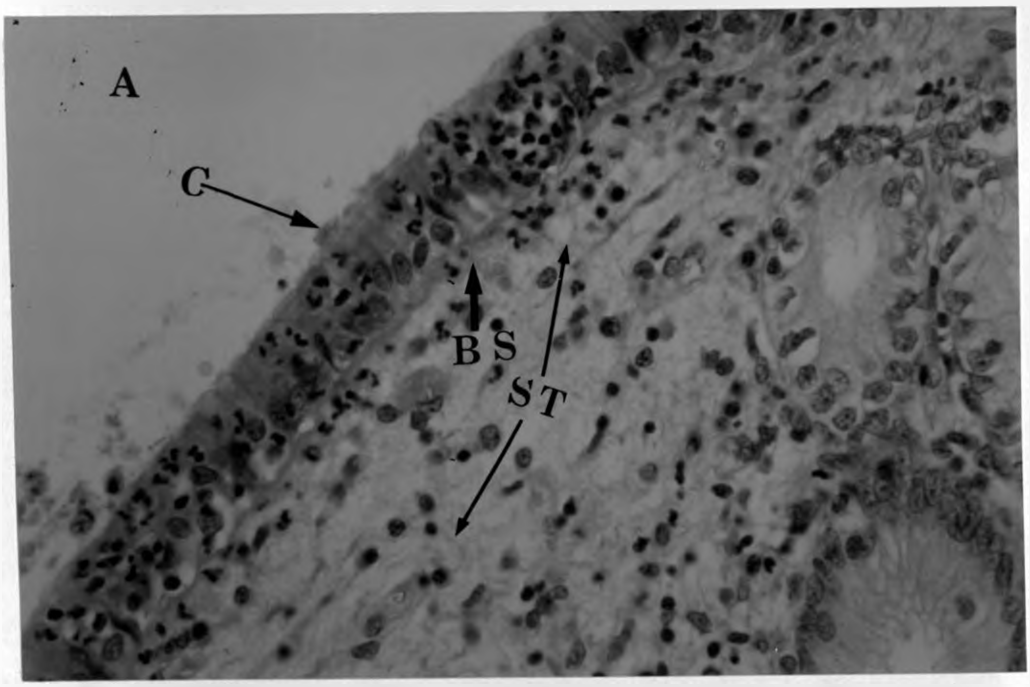


Figure 4.4. (B)

A photomicrograph of a uterine biopsy section representative of the uterine biopsies obtained from mares that were still infected 14 days after challenge. Note the diffuse massive stromal infiltration by eosinophils (E). The eosinophils were occasional companions of mononuclear cells, especially lymphocytes (Carbochromotrope Methylene green pyronin, B X 400).

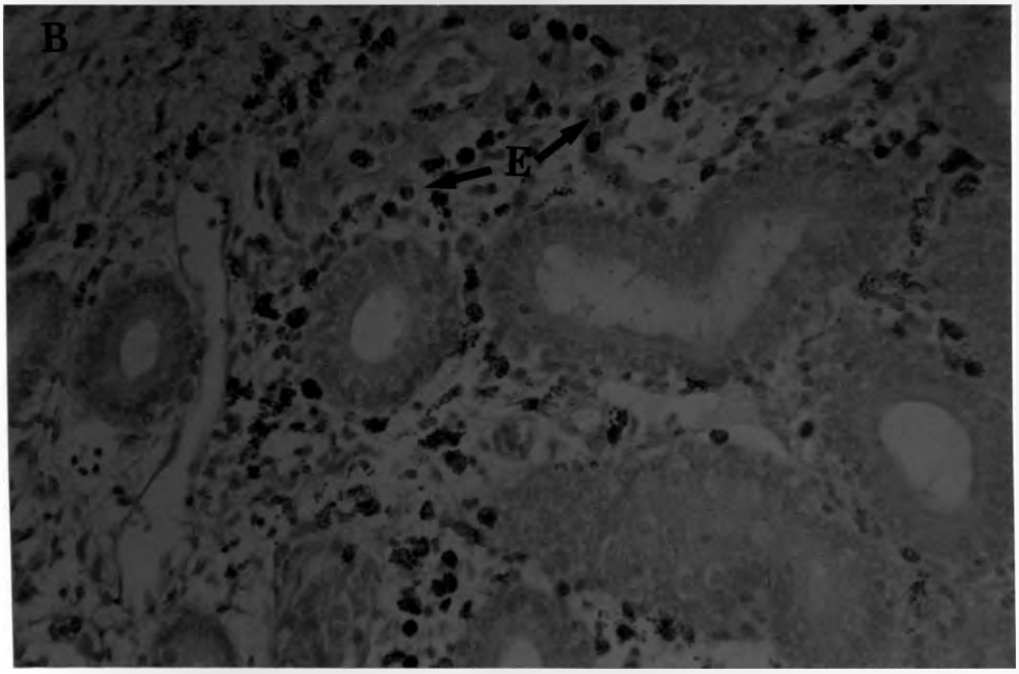
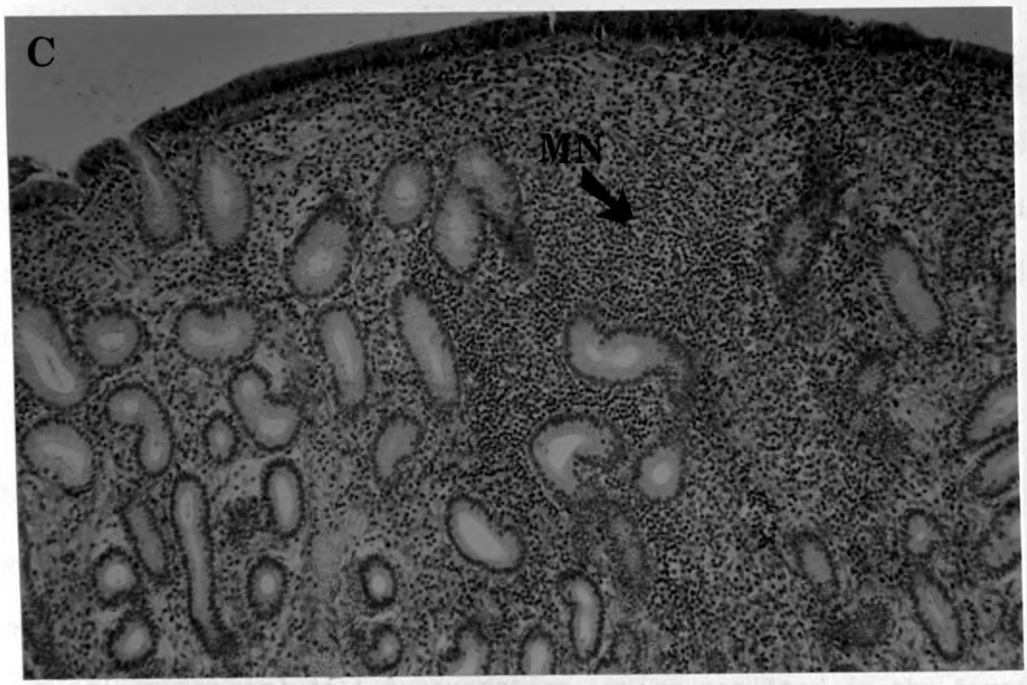


Figure 4.4. (C)

A photomicrograph of a uterine biopsy section representative of the uterine biopsies obtained from mares that were still infected 28 days after challenge. These biopsy sections showed diffuse, intense, predominantly lymphocytic infiltration into the endometrial tissue (H & E, C X 100).



4.4.2 Electron microscopic observations.

The nature of the predominantly neutrophilic infiltration into the strata compactum and spongiosum and into the uterine lumen was more clearly defined using scanning (SEM) and transmission (TEM) electron microscopy.

4.4.2.1. Scanning electron microscopy.

Uterine biopsies before challenge showed that the surface of the equine uterus was folded into long ridges and troughs. Under high magnification gland openings on the uterine surface were observed to be surrounded by ciliated cells (Figures 4.5 A, B & C). Where acid treatment did not completely remove the mucous covering the uterine luminal epithelial mucous flakes were seen on the surface of the uterus (Figure 4.5 A).

The effects of experimental inoculation with the β haemolytic streptococci varied between mares. The most common findings were matting of the cilia, occasional loss of cilia and varying degree of luminal epithelial cell desquamation, which resulted in the formation of "craters" (Figures 4.6 A, B). The loss of luminal epithelial cells, observed in uterine biopsies obtained 6-24 hrs after challenge, gave the uterine surface a "moth eaten appearance" (Figures 4.6 C & D).

Figure 4.5 (A)

A low power electronmicrograph of a uterine biopsy before intrauterine inoculation of streptococci showing the equine endometrium that was folded into long ridges (R) and troughs (T), which were covered by a layer of mucous (M) (SEM magnification, A X 40).

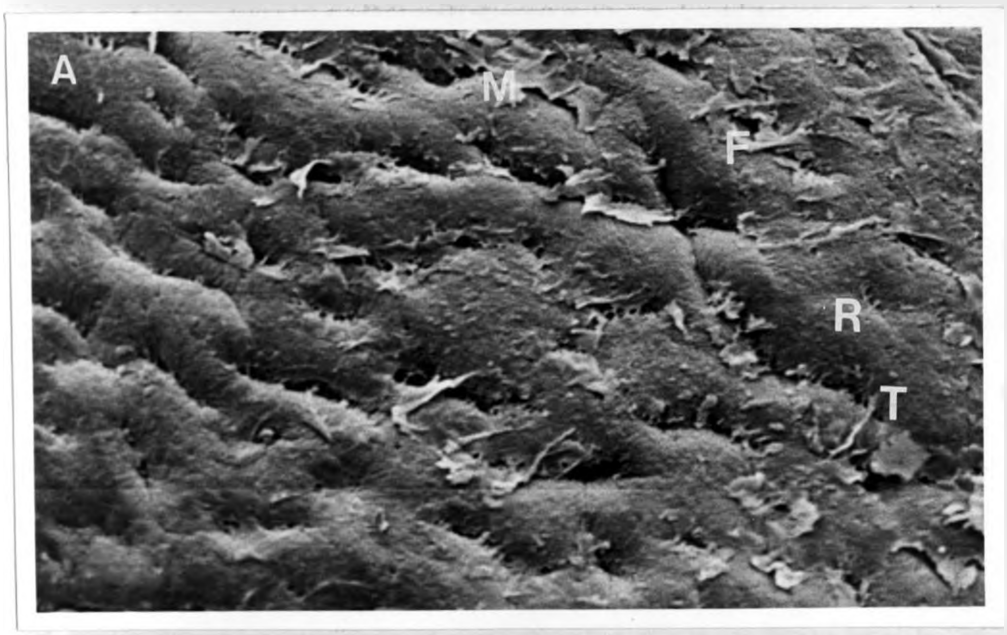
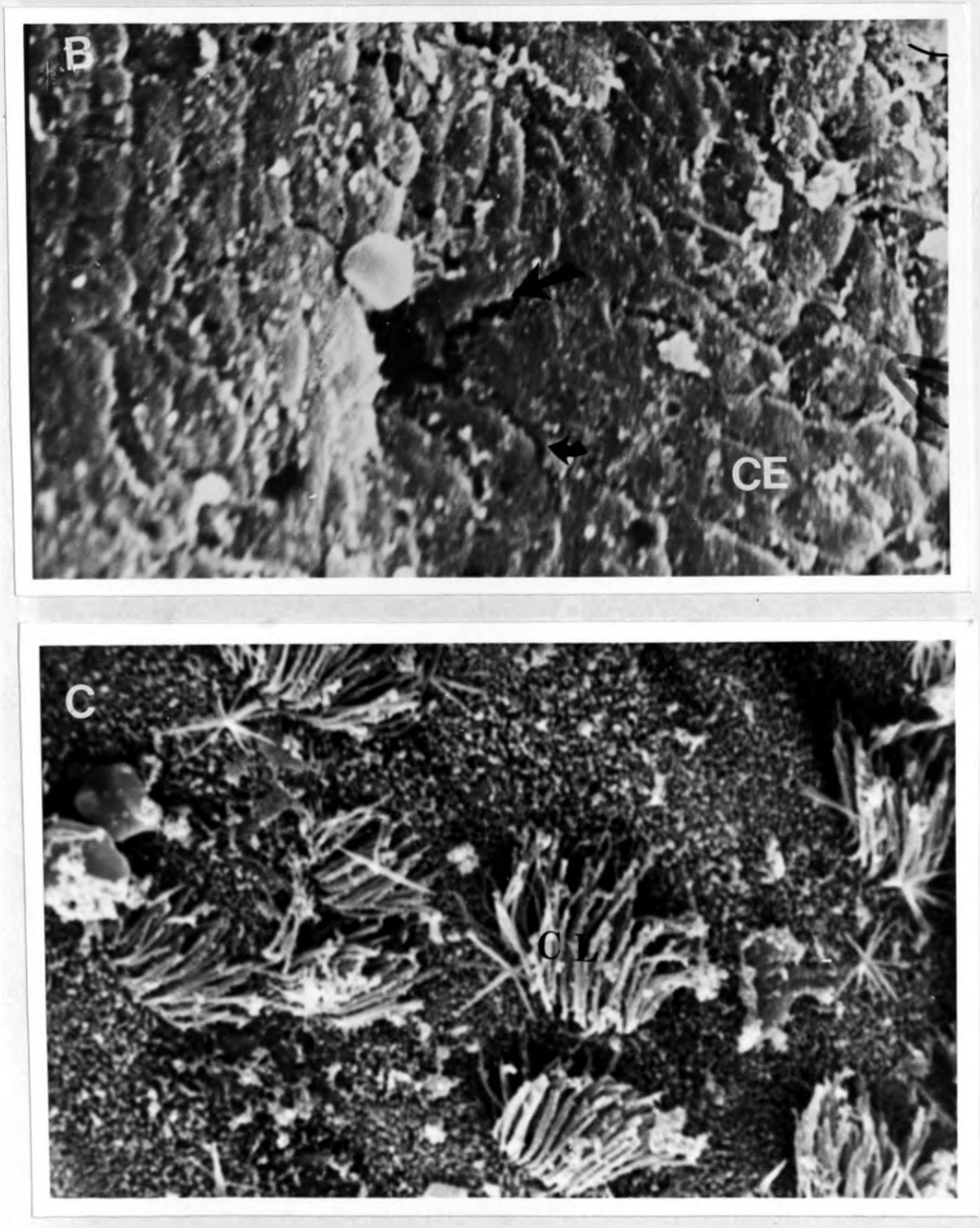


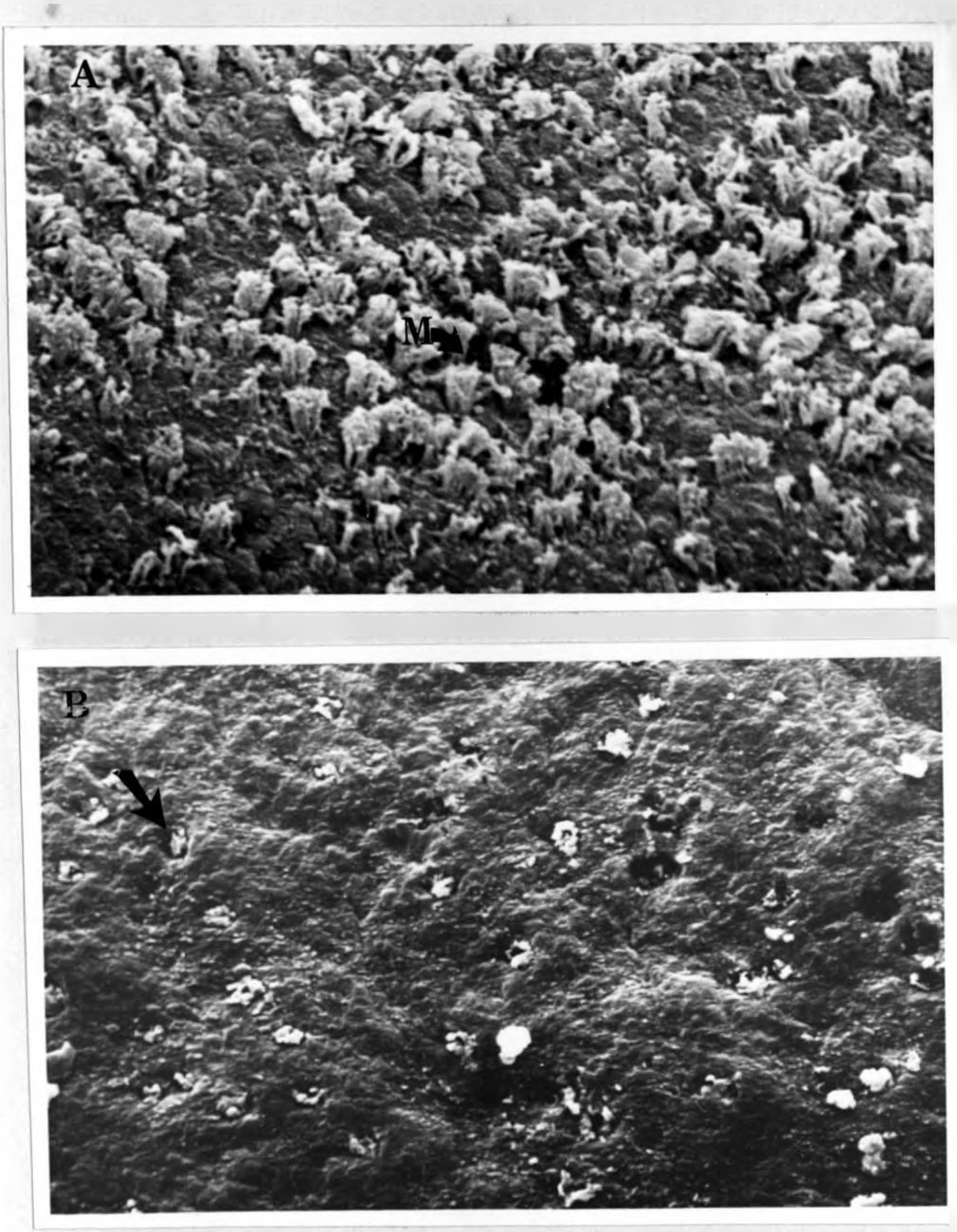
Figure 4.5 (B & C)

Electron micrographs of normal uterine biopsies representative of those obtained from both resistant and susceptible mares before challenge. Outlines of individual luminal epithelial cells (CE) and ciliated cells (CL) surrounding the mouths of glands were seen at higher magnifications. The fissures in tissue were caused by overdrying in the critical point drier (Arrows) (SEM magnification, B X 640, C X 5,000).



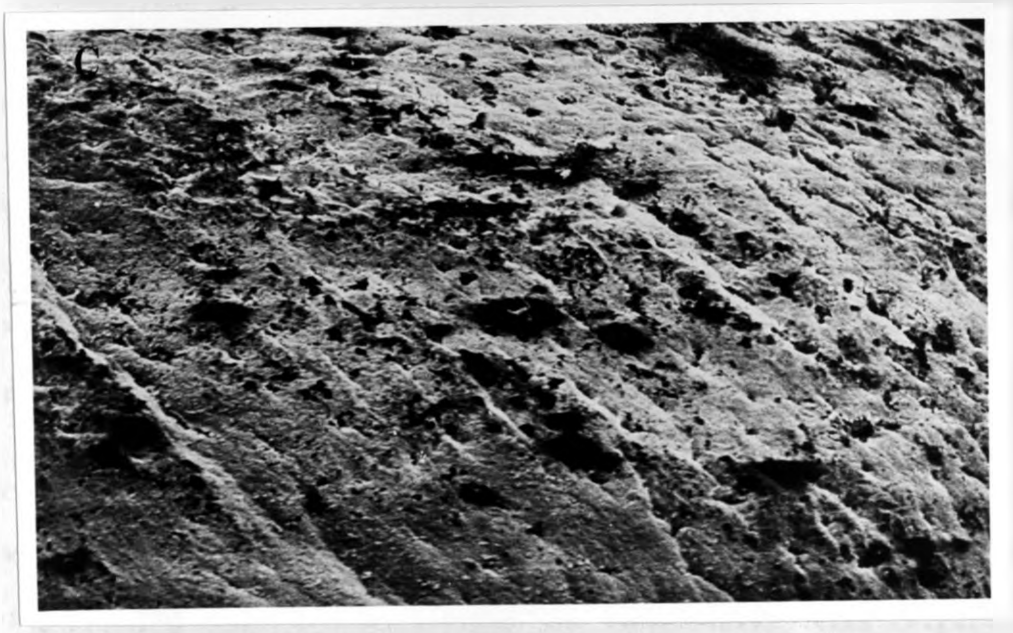
Figures 4.6 (A & B)

Photomicrographs of biopsies obtained within 4 hours of challenge showed that the immediate tissue response to experimental infection was matting of the cilia (M) and the loss of cilia around the mouths of glands (Arrows) (SEM magnification, A X 2,500, B X 640).



Figures 4.6 (C)

Electron micrographs of biopsies obtained 24 hours after intrauterine inoculation of Beta haemolytic streptococci. The uterine biopsies had "moth eaten" appearance which was due to craters left by the loss of uterine luminal epithelial cells (SEM magnification, C X 80).



4.4.2.2. Transmission electron microscopy.

Electronmicrographs typical of pre-challenge uterine biopsies obtained from resistant and susceptible mares are shown in Figures 4.7 A.

Transmission electron microscopic examination of uterine biopsies obtained at 3 and 6 hrs after inoculation with B haemolytic streptococci showed diffuse severe neutrophilic infiltration (Figure 4.8), and damaged cilia, without any apparent disruption of luminal epithelial cell (Figures 4.9 A & B).

Neutrophils seen migrating towards the uterine lumen moved through the intercellular spaces between the uterine luminal epithelial cells (Figures 4.9 A & B). Occasionally neutrophils were observed in uterine luminal epithelial cells (Figure 4.9 C).

Most of the blood vessels seen in TEM sections obtained after challenge, were congested. Some of the congested blood vessels showed evidence of gapping (fenestrations) between the endothelial cells (Figure 4.10 A). Occasionally erythrocytes were observed in the space between the endothelial cells and the basement membrane (Figures 4.10 B & C). Uterine biopsies obtained from unchallenged mares, which served as controls, did not show similar features. As with the light microscopic sections, no bacteria were seen adhering to the luminal epithelial cells using the TEM. Intracellular bacteria were only seen in neutrophils in cytological smears made from samples collected at 3-24 hrs after challenge (see section 2.2.2) (Figure 4.11 A & B).

Figure 4.7 (A)

Transmission electron micrographs of uterine biopsies obtained before challenge. Uterine biopsies obtained from both resistant and susceptible mares before challenge had intact uterine luminal epithelium and cilia. Note the absence of basal vacuolation and cellular infiltration (TEM magnification, A X 11,000).

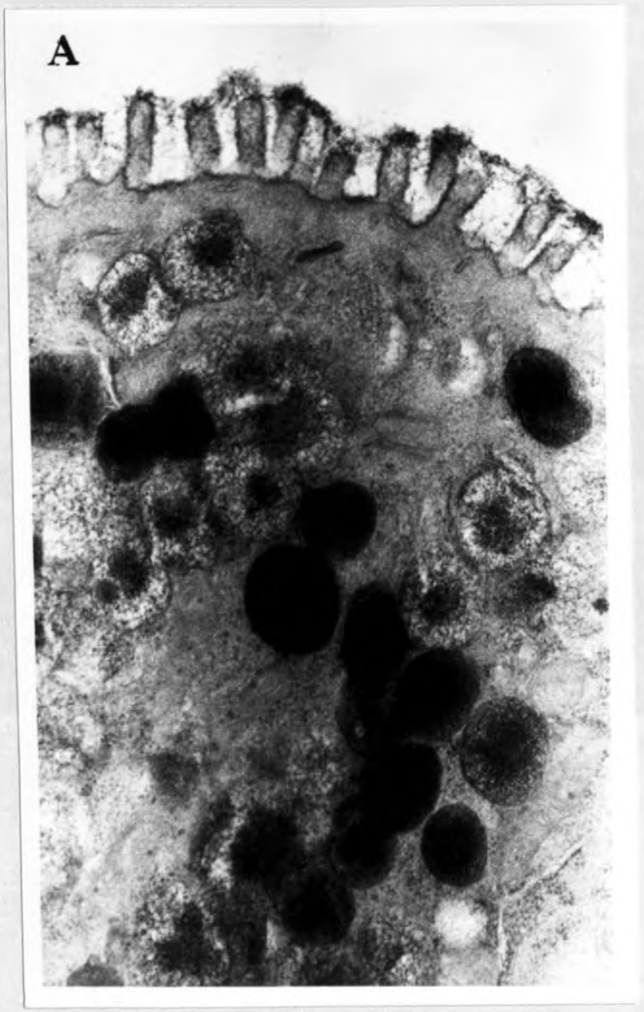
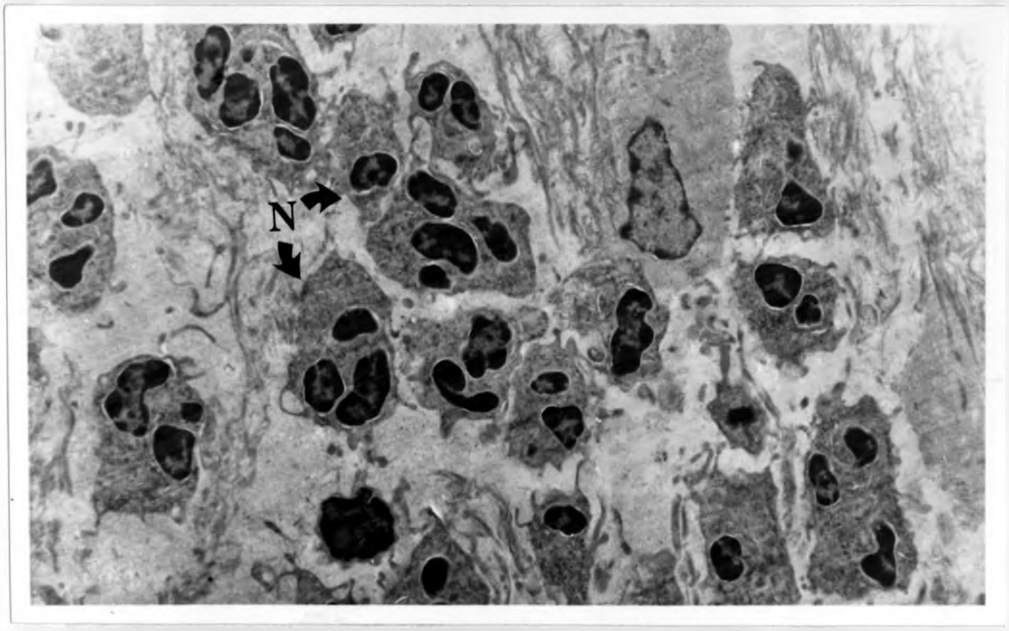


Figure 4.8

Electron micrograph of a uterine biopsy obtained four hours after challenge. Uterine biopsies obtained from both resistant and susceptible mares four hours after challenge showed the same features. A diffuse, severe, predominantly neutrophilic infiltration (N) into the endometrial stroma was observed in all sections obtained after challenge (TEM magnification, X 5,700).



Figures 4.9 (A & B)

Transmission electron micrographs of a uterine biopsy obtained 3 hours after challenge. Note the loss of cilia in some areas (Arrows). Most neutrophils migrating towards the uterine lumen were observed to migrate in intercellular spaces (IN) rather than through the luminal epithelial cells. The micrographs also show basal vacuoles (BV) above the basal lamina (TEM magnification, A X 3,400, B X 7,100).

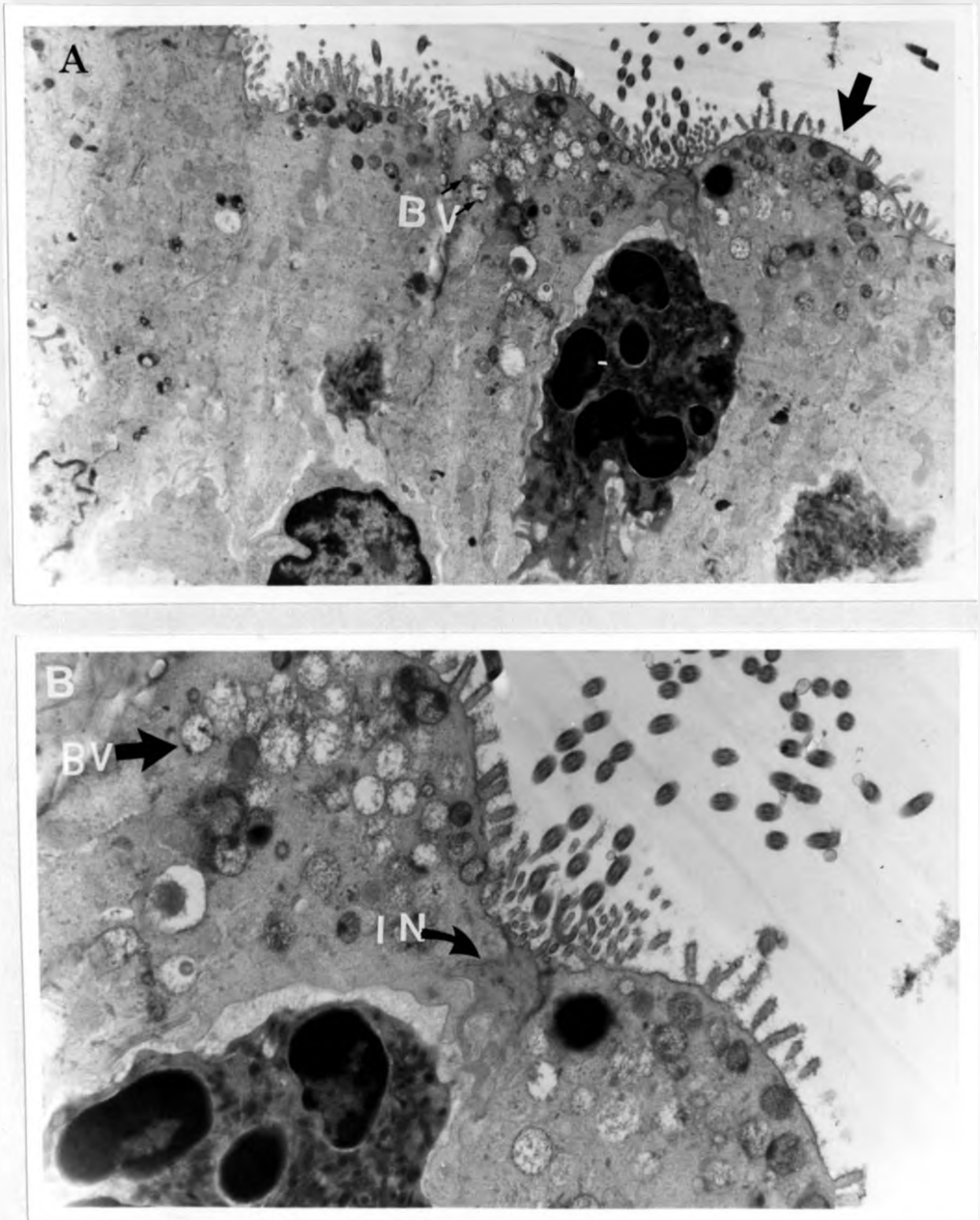


Figure 4.9 (C)

Transmission electron micrograph of a uterine biopsy obtained 3 hours after challenge. The micrograph shows a nuetrophil migrating through a uterine luminal epithelial cells (LEP) (TEM magnification C X 3,400).

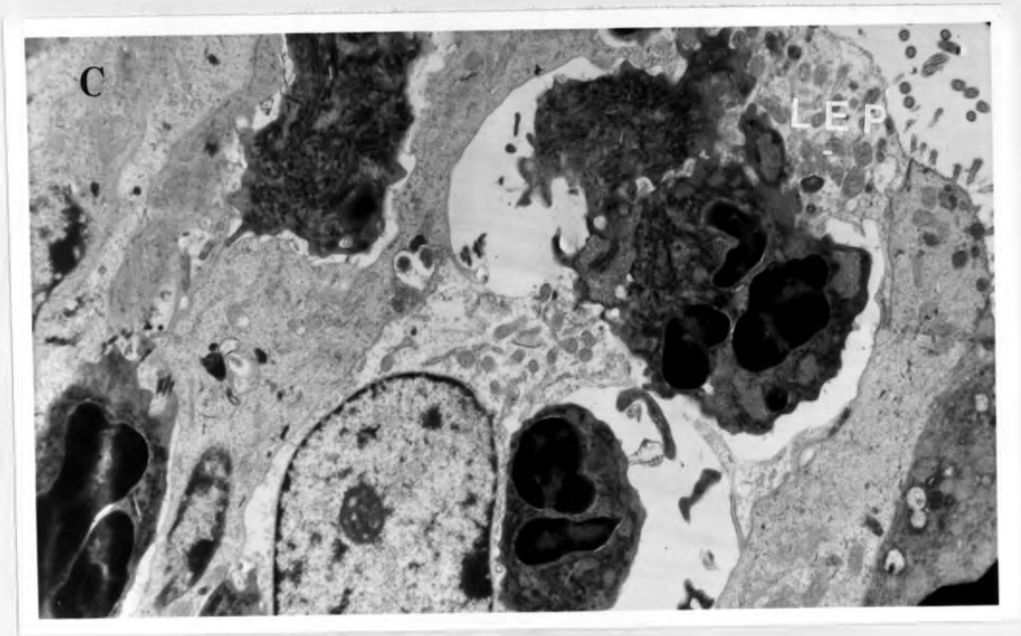


Figure 4.10 (A)

Transmission electron micrographs of a uterine biopsy, obtained at 4 hours after challenge, showing congested blood vessels with vascular fenestrations. Part of an erythrocyte (Er) can be seen in between the basement membrane (BM) and the vacular endothelial cell (En) (TEM magnification, A X 11,000).

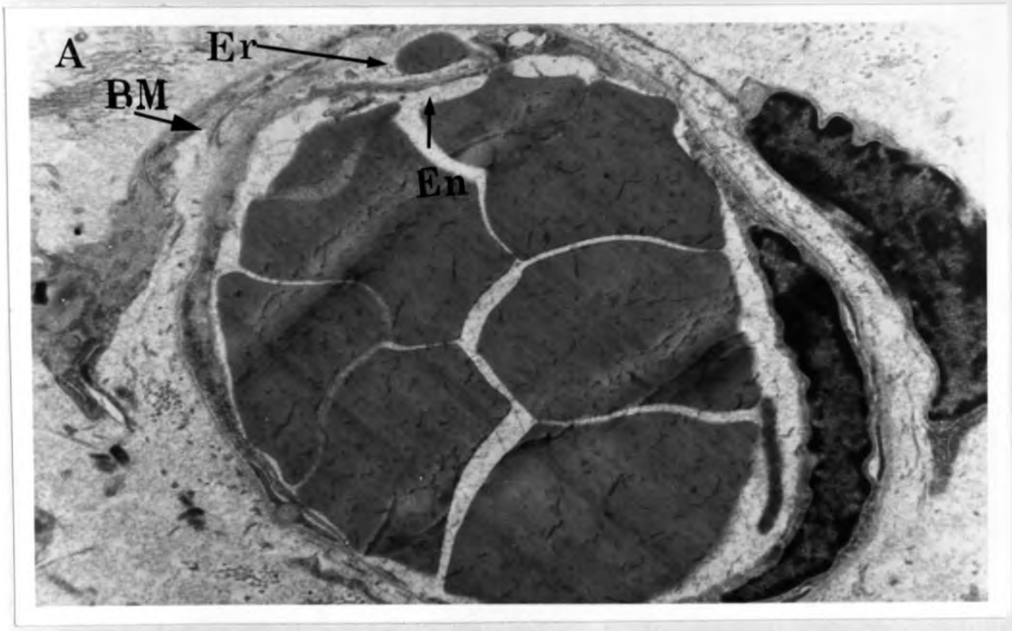
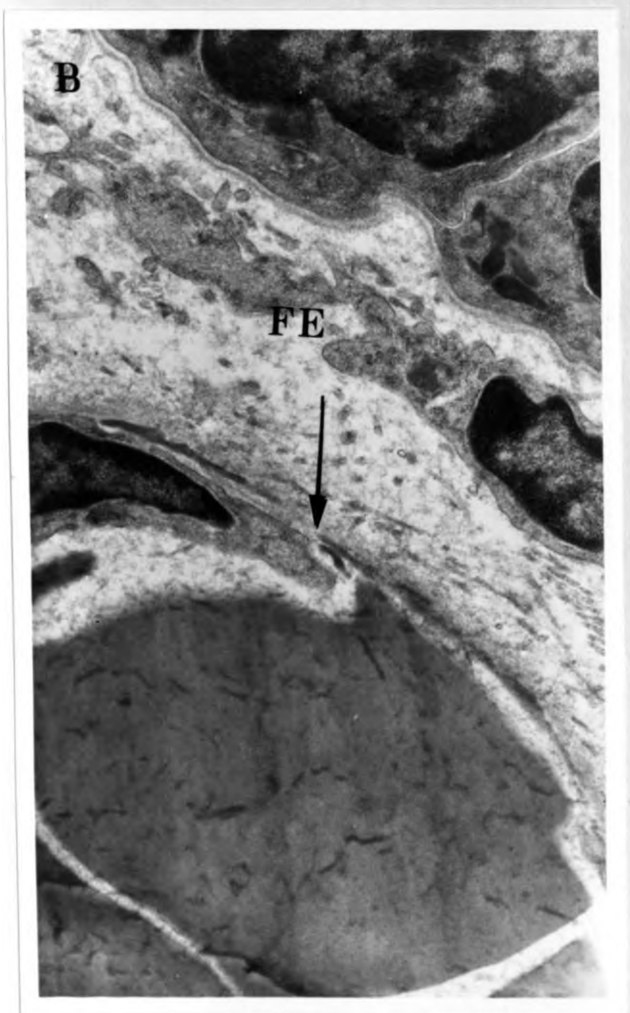


Figure 4.10 (B & C)

Electron micrograph of uterine biopsies obtained 4 hours after challenge, showing congested blood vessels. Note the gap between the endothelial cells. These gaps are referred to as vascular fenestrations in the text (FE) (TEM magnification, B X 34,000, C X 5,700).



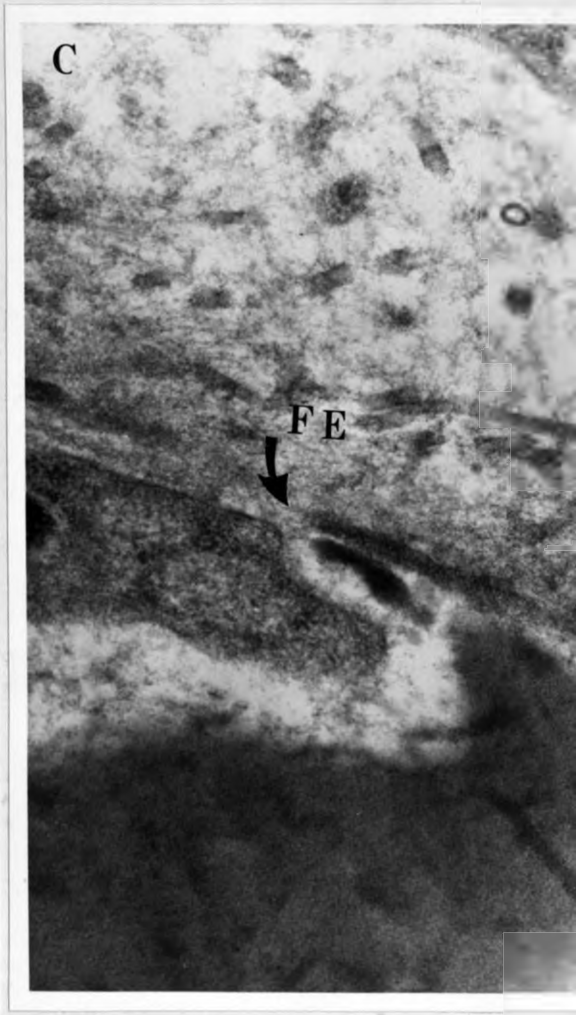


Figure 4.11 (A)

Uterine smears made during the acute phase of the infection, especially within the first 24 hours after challenge, showed a predominantly neutrophilic (N) response with few red blood cells (Rb) (Wrights' stain, A X 400).

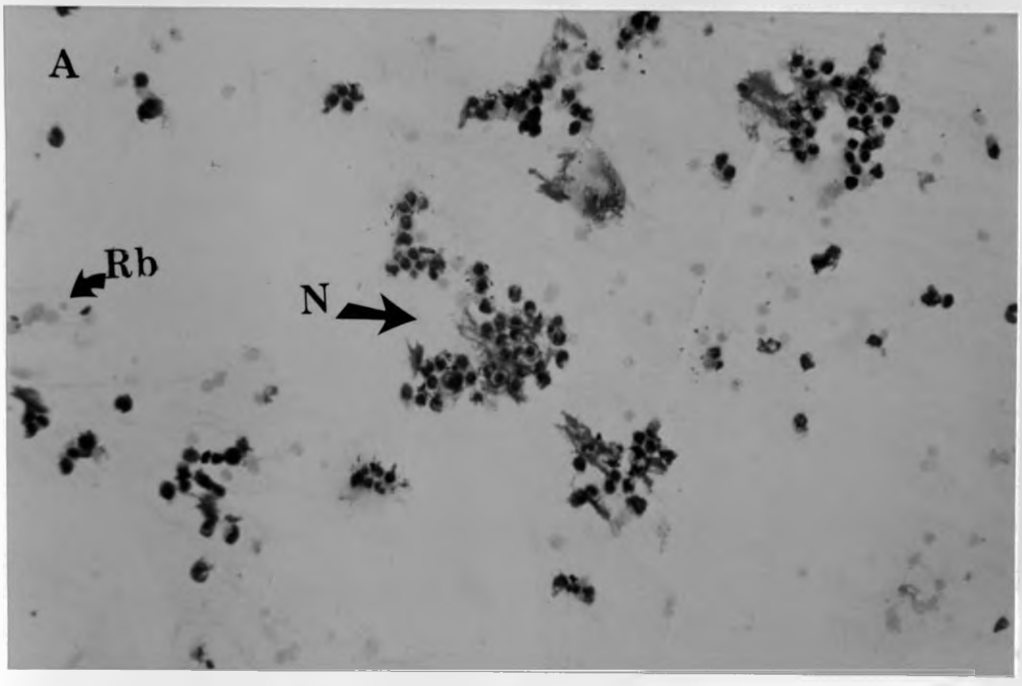
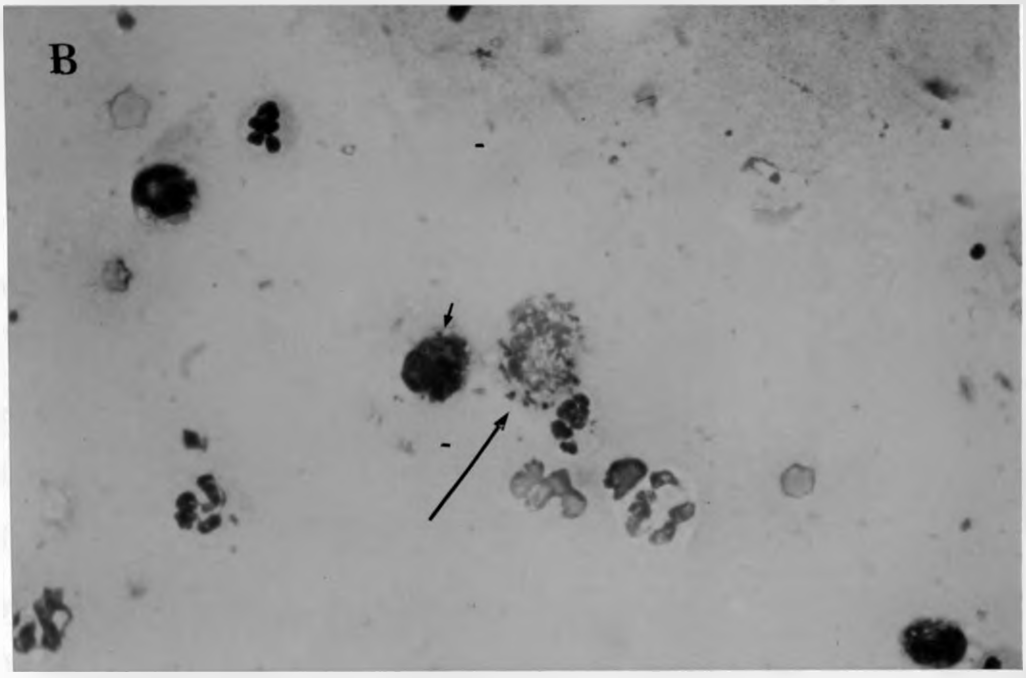


Figure 4.11 (B)

Uterine cytology smears made in the first 24 hours of challenge showed some neutrophils with 2 or more intracellular bacteria (Arrows) (Wright's stain, B X 1,000).



4.5 DISCUSSION

In all tissues and organs the portion of vascular system involved in an inflammmatory reaction is the microcirculation or the terminal vascular bed, which includes terminal arterioles, capillaries, venules and arteriovenous shunts. Accordingly, the principal anatomic barrier between the circulation and the interstitial tissues are the endothelium and basement membrane (Lamm and Stetson, 1972). In the present study vascular congestion, margination with neutrophils and perivascular cuffing was observed in all biopsies obtained following challenge. TEM examination of biopsies obtained 3 hrs after challenge revealed endothelial fenestrations in terminal venules and arterioles, rather than gross damage to the terminal vascular bed. These fenestrations were not observed in uterine biopsies obtained from unchallenged mares. Thus it is most likely that the 6 hrs influx of serum protein ("protein flush" see 3.4.5) and the cellular infiltrates into the endometrial tissue and uterine lumen left the blood vessels through these fenestrations. However, since no change in the appearance of the basement membrane was observed in biopsies obtained 24 hrs after challenge, it is suggested that the passage of proteins and inflammatory cells 24 hrs after challenge was most probably accomplished through the intact membrane and/or some naturally occuring discontinuities in the endothelium (Lamm and Stetson, 1972).

The descriptions of the endometrial histopathology and cytology previously reported in the mare proved to be consistent with the findings of the present study (Solomon, Schultz and Fahning, 1972; Kenney, 1978; Munyua, 1982). In the present studies light microscopic examinations of biopsies obtained following challenge indicated that

the effects of the intrauterine inoculation of β haemolytic streptococci on the luminal epithelium were varied, including a breakdown of the surface membranes and the loss of groups or individual uterine luminal epithelial cells. SEM examination of biopsies obtained at the same interval following challenge revealed damaged cilia and eroded luminal epithelial cells. This tissue damage was most likely related to the persisting inflammatory reaction and infection.

Eosinophils have been reported in equine uterine biopsies by a number of investigators including Solomon et al., (1972), Kenney, (1978), Gordon and Sartin, (1978) and Munyua, (1982). These investigators, however, did not indicate how soon after infection the eosinophils appeared, nor did they attach any significance to their presence. In the present study the eosinophils, which were observed in prechallenge category III uterine biopsies and in all biopsies obtained 6 hours following challenge, seemed to migrate along with neutrophils. In biopsies obtained from infected mares at 10 or more days after challenge eosinophils were seen in large numbers. The presence of eosinophils in pre-challenge category III uterine biopsies most probably indicated a recent infection(s), the persistence of antigen-antibody complexes from a previous infection, or the persistence of cellular and/or soluble antigenic stimuli. Likewise the aggregations of eosinophils in the strata compactum and spongiosum, in biopsies obtained after challenge may have been in response to similar stimuli.

The absence of plasma cells in biospies obtained from mares that were still infected 10-32 days post challenge is difficult

to explain other than assume that repeated sampling may have influenced the expected change in the predominant infiltrating cell type(s).

Evidence available from the present study indicates that mares classified as either resistant or susceptible to uterine infection, on the basis of persistence of bacteria after challenge, initially showed similar tissue and cellular changes following uterine challenge with β haemotylic streptococci. It was therefore concluded that the subsequent tissue damage was related to the inability, of the susceptible mares, to resolve the uterine infection (endometritis),

CHAPTER 5

PAGE

CHEMOTACTIC PHAGOCYTIC AND INTRACELLULAR KILLING ABILITY OF CIRCULATING AND UTERINE NEUTROPHILS OBTAINED FROM A GROUP OF MARES WITH DIFFERING RESISTANCE TO ENDOMETRITIS.

PAGE

CHAPTER 5

CHEMOTACTIC, PHAGOCYTIC AND INTRACELLULAR KILLING ABILITY OF CIRCULATING AND UTERINE NEUTROPHILS OBTAINED FROM A GROUP OF MARES WITH DIFFERING RESISTANCE TO ENDOMETRITIS.

5.1. INTRODUCTION

Hughes and Loy (1969; 1975) and Peterson, McFeely and David (1969), regarded the initial neutrophil response to be critical in resolution of endometritis. These workers suggested that mares may be predisposed to endometritis by a breakdown in humoral and/or cellular response. Asbury et al., (1982), Williamson et al., (1984) and Munyua et al., (1984), however, have shown that there is no significant difference between susceptible and resistant mares in the numbers of neutrophils released into the uterus in experimentally induced endometritis. Asbury et al., (1982), also reported that there was no significant difference in the phagocytic ability of circulating neutrophils obtained from resistant and susceptible mares, and suggested that uteri of mares susceptible to infection were deficient in opsonins, to a degree which allows bacteria to become established and produce endometritis. Munyua et al., (1984) suggested that the lowered resistant to endometritis observed in these mares may be related to their inability to sustain an inflammatory response.

5.2. AIMS

The present study was designed to assess the chemotactic, phagocytic and intracellular killing ability of neutrophils harvested from

uterine washings and blood obtained from resistant and susceptible mares used in Experiments 1 and 3 (chapter 3).

5.3 MATERIALS AND METHODS

5.3.1. Selection of mares.

Twenty one of the mares used in Experiments 1 and 3 were randomly selected for inclusion in the present study. Uterine samples were collected at the same times as those in Experiments 1 and 3 (see section 3.3.5). The mares were retrospectively classified as being either resistant (n=11) or susceptible (n=10) depending on their ability to resolve an experimentally induced endometritis (see section 3.4).

5.3.2 Preparation of opsonins for chemotactic and phagocytic assays.

In the present study homologous uterine washings, and homologous whole and heat inactivated serum were used as sources of opsonins. Serum was inactivated by heating for 30 minutes at 56C. By contrast, activated serum was used as the source of chemoattractant in the chemotaxis study.

Uterine washings, a source of opsonins, were obtained as described previously (see section 3.3.12).

Homologous serum, prepared from freshly collected coagulated venous blood, was divided into several 2 ml aliquots. To obtain serum lacking in complement (heat inactivated serum) a 2 ml aliquot was

heated to 56C for 30 minutes.

When activated serum was required 1 ml of this fresh serum was incubated with 25 mg of Zymosan A (Sigma Chemicals, Perth, WA) at 37C for 30 minutes. The Zymosan was then removed by centrifugation at 500 g for 10 minutes.

5.3.3. Isolation of neutrophils.

5.3.3.1. Isolation of peripheral (circulating) neutrophils for chemotactic and phagocytic assays.

Neutrophil suspensions were prepared following the method described by Washburn et al., (1982).

After the collection of blood (30 ml) in heparinized tubes, erythrocytes were sedimented in 10-12 ml of 6% dextran saline solution in an inverted syringe at an angle of 45-50 degrees. After this sedimentation of erythrocytes the plasma-dextran-saline supernatant was decanted through an 18 gauge needle into 20 ml centrifuge tubes, and spun at 500 g for 10 minutes at 4C. The resultant pellet was washed twice in Hanks' buffered salt solution (Hbss.) and any contaminating erythrocytes were lysed by adding 4 ml of buffered ammonium chloride with EDTA (8.32 g of NH_4CL, 0.84 g of $NaHCO_3$, 43.2 mg of EDTA, and 1,000 ml of distilled water). Isotonicity was restored with 4 ml of sodium citrate saline solution (1 part of 0.075 M sodium citrate to 4 parts of phosphate buffered saline solution) and the neutrophils were centrifuged at 500 g for 5 minutes. The resulting pellet was suspended in 5 ml of Hbss and layered on to 3-4 ml of sepalymph (Veta PTY. Israel) and centrifuged at 400 g for 40 minutes.

The bottom layer containing neutrophils was resuspended in 2 ml Hbss and the concentration of neutrophils adjusted to X 10^6/ml after a total count on a electronic particle counter (Coulter electronics, WA) and a differential count. Viability of the isolated granulocytes was determined by trypan blue exclusion (Rabinowitz, 1964; Majeski and Alexander, 1976).

5.3.3.2. Isolation of uterine neutrophils for chemotactic, phagocytic and intracellular killing ability assays.

Neutrophils for chemotactic, phagocytic and intracellular killing ability assays were harvested from uterine washings obtained at 2, 3, 4, 6 and 9 hrs after challenge. To isolate uterine neutrophils, uterine washings (4 ml) were centrifuged at 500 g for 10 minutes and the resulting pellet washed with sterile saline solution. The pelleting and washing procedure was repeated twice before the predominantly neutrophil pellet was re-suspended in 2 ml Hbss with carbonate containing 2.0% bovine serum albumin to obtain a concentration of at least 1 x 10^5 PMN/ml.

5.3.4. Preparation of agarose plates for use in chemotactic assays.

Agarose gels containing 0.5% agarose type 111 (Sigma chemicals, WA.), 2% bovine serum albumin (Commonwealth Serum Laboratories, WA) and Hbss with carbonate were poured into 35 x 10 mm tissue culture dishes (Flow Laboratories, WA.) and allowed to solidify at room temperature for at least 1 hour. The agarose plate were subsequently

refrigerated for at least 1 hour to increase gel firmness. When sufficiently firm a template was used to punch 1 set of three 2.4 mm wells which were 3.0 mm apart, in the agarose gel. The agarose gel in the wells was then removed by applying a gentle vacuum through a pasteur pippete.

5.3.5 Evaluation of chemotactic ability of uterine and peripheral neutrophils.

Chemotaxis, defined as directional migration, was studied in 8 of the 21 mares selected for inclusion in the present study. Three of these mares were retrospectively classified as resistant and 5 as susceptible. The method used to measure chemotaxis was as described in detail by Tannous et al., (1982).

To measure directional migration zymosan activated serum (ZAS), used as the chemoattractant (10 µl), was added to the middle wells, while a similar volume (10 µl) Hbss with bovine serum albumin was added to the middle wells of control plates to measure the random migration. The plates were then incubated at 37C in an atmosphere containing 5% carbon dioxide and 100% humidity. After the incubation the outer wells were filled with a 10 µl uterine or peripheral neutrophil suspension (x 10^5 PMN/ml) and the plates incubated as described above for a further 3 hours.

The distance migrated by the neutrophils from the outer wells towards the middle wells containing the chemoattractant (ZAS) or the Hbss was determined with the 10x objective of a phase contrast microscope fitted with a grid previously calibrated using a stage micrometer.

Chemotactic differential (CD) was assessed by determining the difference between random migration and directional migration (MA-MR).

Chemotaxis was evaluated by calculating a migration (chemotactic) index:- chemotactic index (CI) being the ratio of distance migrated towards the attractant (MA) compound to random migration (MR) (distance towards the Hbss-BSA wells) i.e. MA/MR.

5.3.6. Evaluation of phagocytic ability of uterine and peripheral neutrophils

The invitro phagocytic activity of peripheral neutrophils obtained from 5 of the 21 mares (3 susceptible and 2 resistant) was evaluated using a modification of the method described by Root, Resenthal and Balestra (1972). Staphylococcus aureus, (0.5 ml suspension containing x 10^6 colony forming units per ml), were opsonized for 30 minutes using either 0.5 ml of whole serum, heat inactivated serum, uterine washings or Hbss (control). Neutrophil suspensions (1 ml) were added to the opsonized bacteria suspension and the mixture incubated at 37C for 1 hour in a shaking water bath set at 60 cycles per minute. The tubes were then centrifuged at 400 g for 10 minutes and the resulting pellet used to prepare cytological smears. These smears were stained with Wrights' stain (Sigma chemicals Co. Perth, WA) for routine light microscopic examination. During the microscopic examination only solitary, whole neutrophils with intact cell membrane and containing at least 2 intracellular bacteria were counted. The number of neutrophils with and without intracellular bacteria and the number of bacteria in each neutrophil were recorded.

The phagocytic index was taken as the (mean number of intracellular bacteria per neutrophil incubated in the presence of either serum or uterine washings) minus (the background effect), which was the mean number bacteria in neutrophils incubated in the presence of only Hbss (i.e. in the absence of an opsonic source). At least 50 neutrophils with more than 2 intracellular bacteria were counted per smear.

Percent phagocytosis was calculated from the number of neutrophils with at least 2 intracellular bacteria among the first 200 neutrophils counted.

To assess phagocytic ability of uterine neutrophils obtained at various interval after challenge, cytological smears made from pelleted neutrophils (see section 5.3.3.2) were prepared for light microscopic examination and assessed as described for the peripheral neutrophils.

5.3.7 Viability of neutrophils and β haemolytic streptococci in liquid nitrogen and after shaking with beads.

In preliminary trials the β haemolytic streptococci used throughout the present studies were found to survive a single freeze-thaw cycle in liquid nitrogen followed by shaking for 60 seconds on a vortex shaker with 0.22 mm glass beads (Appendix 11 a). A single freeze-thaw cycle followed by a 30 seconds shaking with beads resulted in the disruption of more than 96% of the neutrophils in suspension (Appendix 11b).

5.3.8 Evaluation of intracellular killing ability of uterine neutrophils.

The intracellular killing ability of uterine neutrophils obtained from

16 of the 21 mares (7 susceptible and 9 resistant) selected for inclusion in the present study was measured using neutrophils harvested at 2 and 4 hrs (n=8) and 3,6 and 9 hrs (n=8) after challenge. Washed uterine neutrophils suspended in 2 ml Hbss with carbonate were divided into two 1 ml aliquots (see section 5.3.3.2).

Uterine neutrophils in the first aliquot were disrupted immediately by rapid single freeze and thaw cycle in liquid nitrogen followed by 30 seconds vigorous shaking on a vortex with 0.2 mm glass beads. Five µl of the serial dilutions of the disrupted neutrophil suspension (ranging from 1:100 to 1:10,000) were plated onto blood agar plates and incubated overnight at 37°C . The second aliquot was washed as with the first and then incubated at 37°C for 1 hour before the neutrophils in suspension were lysed and plated as for aliquot 1. The plates were read after 24 hrs incubation and the number of colonies of bacteria recorded. The percentage of killed bacteria was calculated by comparing the number of colony forming units of bacteria which grew from following incubation of the neutrophils for 1 hr.

Results of the intracellular killing assay in preliminary trial using neutrophils harvested from uterine washings obtained 2-4 hrs after challenge with β haemolytic streptococci are shown (Table 5.1). The neutrophils harvested from uterine washings obtained from susceptible mares killed 80-100% of the haemolytic streptococci while those obtained from resistant

Table 5.1 Intracellular killing ability of neutrophils harvested from uterine washings collected at 2 and 4 hours after inoculation with Streptococci

Washings Collected At:	Resistant Mares						Susceptible Mares	
	160	48	155	67(2)	153	160(2)	183	186
2 hours	89.35%*	50%	70%	56%	66%	86%	80%	93%
4 hours	70.59%	81%	55%	ND	53%	ND	97.88%	100%

1. *% bacteria killed after 1 hours' incubation of uterine neutrophils

2. Neutrophils harvested at 24 hours from mare 160 killed 83.3% of Streptococci

3. There was significant difference ($p<0.01$) between the intracellular killing ability of uterine neutrophils from resistant (68.75) and susceptible (92.50) mares.

4. ND - Not Done due to insufficient neutrophils being harvested

mares killed 53-89% of the bacteria. The difference between the intracellular killing ability of uterine washings obtained from susceptible mares and resistant mares was significant ($P < 0.01$, Table 5.1).

5.4 RESULTS

A total of 21 mares were used in present study. Using the challenge results (see Section 3.4) 10 of these mares were classified as susceptible and 11 as resistant to uterine infection.

5.4.1. Chemotactic ability of peripheral and uterine neutrophils obtained from resistant and susceptible mares.

5.4.1.1. Chemotactic differential of peripheral and uterine neutrophils.

The mean chemotactic differential of circulating neutrophils obtained from resistant mares (1.60 ± 0.06), did not differ significantly (N.S) from that of the susceptible mares (1.66 ± 0.03). The chemotactic differential (1.64 ± 0.03) of the neutrophils harvested from circulation was significantly higher ($P<0.05$) than the chemotactic differential measured using pooled neutrophils harvested from uterine washings (0.09 ± 0.09) (Table 5.2, Appendix 12).

The mean chemotactic differential of neutrophils harvested from uterine washings obtained from resistant mares at 3 and 6 hours after challenge (1.31 ± 0.13 and 1.10 ± 0.08 respectively) did not differ significantly (N.S.) from that observed in neutrophils harvested from uterine washings of susceptible mares at similar time intervals (1.06 ± 0.09 and 0.78 ± 0.083) (Table 5.2,. The mean chemotactic differential of neutrophils harvested from uterine washings obtained from resistant mares 9 hrs after challenge (0.87 ± 0.35) was 49.43% more than that observed in neutrophils harvested from uterine washings obtained from susceptible mares (0.43 ± 0.26) 9 hrs after challenge (Table 5.2, Appendix 13).

5.4.1.2. Chemotactic index of peripheral and uterine neutrophils

The mean chemotactic index of the neutrophils harvested from heparinized blood obtained from resistant mares (2.25 ± 0.33) did not differ significantly (N.S.) from that observed in neutrophils harvested from blood obtained from susceptible mares (2.78 ± 0.19).

Table 5.2.

The mean (± SE) chemotactic differential of uterine neutrophils obtained from resistant and susceptible mares after intrauterine inoculation with β haemolytic streptococci.

Mare classification	Time (hrs) after challenge		
	3	6	9
Resistant mares n=10	1.31±0.13	1.10±0.08	0.87±0.35
Susceptible mares n=11	1.06±0.09	0.78±0.08	0.43±0.26

Table 5.3

The mean (± SE) chemotactic index of uterine neutrophils obtained from resistant and susceptible mares after uterine inoculation with β haemolytic streptococci.

Mare classification	Time (hrs) after challenge		
	3	6	9
Resistant mares n=11	1.47±0.01	1.62±0.13	2.04±0.43
Susceptible mares n=10	1.99±0.16	1.39±0.07	1.12±0.25

The mean chemotactic index of neutrophils harvested from uterine washings obtained from resistant and susceptible mares at 3 & 6 hrs after challenge (1.47 ± 0.10 and 1.62 ± 0.13 respectively) did not differ significantly (N.S) from that observed in uterine neutrophils obtained from susceptible mares at similar intervals (1.99 ± 0.16, 1.39 ± 0.07 respectively) (Table 5.3, Appendix 13).

The mean chemotactic index of neutrophils harvested from uterine washings obtained from resistant mares 9 hours after challenge (2.04 ± 0.43) was 54.90% more than that observed in neutrophils harvested from uterine washings obtained from susceptible mares (0.43 ± 0.26) 9 hrs after challenge (Table 5.3, Appendix 13).

5.5.1 Phagocytic ability of peripheral neutrophils incubated with S. aureus

Phagocytic index and phagocytosis % of peripheral neutrophils harvested from 5 mares (3 susceptible and 2 resistant) are shown (Table 5.4, 5.5). The percentage of neutrophils containing at least 2 bacteria in the first 200 neutrophils counted (% phagocytosis), ranged from 80-100 % in whole serum, 80-98% in heat inactivated serum, and 10-60% in 0, 24 and 48 hrs uterine washings. The percentage of neutrophils with bacteria in control tubes (Hbss), which were used to assess the background effect, were observed to be between 34-72% (Table 5.4 Appendix 14).

Both fresh and heat inactivated homologous serum enhanced the phagocytic activity of the neutrophils. There was no significant difference (N.S.) between the whole and heat inactivated serum enhanced

Table 5.4

The mean (mean ± SE) phagocytosis (%) of peripheral neutrophils incubated with Staphylococcus aureus in the presence of whole pooled serum, heat inactivated serum, uterine washings and Hanks buffer as sources of opsonins.

Mare classification	Whole serum	HIS	UW 0 hrs	UW 24 hrs	UW 48 hrs	Hanks
Resistant n=10	87±7.02	83±7.04	47± 5.01	39±17.30	46±26.02	55±11.05
Susceptible n=11	98±0.60	90±5.39	43±16.67	39± 9.26	21± 2.40	50±13.11

HIS = Heat inactivated serum; UW = Uterine washings.

Table 5.5

The phagocytic index (mean ± SE) of peripheral neutrophils incubated with Staphylococcus aureus in the presence of whole pooled serum heat inactivated serum, uterine washings and Hanks buffer as sources of opsonins.

Mare classification	Whole serum	HIS	UW 0 hrs	UW 24 hrs	UW 48 hrs	Hanks
Resistant mares n=11	19.05±4.15	15.6±6.3	5.7±1.7	6.6±1.75	6.05±1.75	5.7±1.3
Susceptible mares n=10	16.89±2.95	16.27± 2.27	7.47± 0.69	5.57± 0.74	5.97±0.23	6.87± 0.42

Table 5.6

The phagocytic index (mean ± SE) of uterine neutrophils obtained from resistant and susceptible mares after intrauterine inoculation with β haemolytic streptococci.

Mare classification	Phagocytic index of uterine neutrophils harvested at		
	3 hrs	6 hrs	9 hrs
Resistant n=11	12.51±3.34	8.08±0.64	7.28±1.25
Susceptible n=10	7.30±0.77	8.49±0.98	7.54±1.40

phagocytic index of both susceptible and resistant mares (Table 5.4). Uterine washings collected from both resistant and susceptible mares before (0 hrs) and 24 hrs after challenge did not, substantially enhance phagocytosis (Tables 5.5, Appendix 15).

5.5.2 Phagocytic ability of uterine neutrophils

Phagocytosed bacteria were observed in most neutrophils harvested from uterine washings obtained after challenge There was no significant difference (N.S.) between the phagocytic index of uterine neutrophils isolated from resistant and susceptible mares (Table 5.6).

5.6.1 Intracellular killing ability of uterine neutrophils

Intracellular killing ability of uterine neutrophils obtained between 3 and 9 hrs after intra-uterine infusion of β haemolytic streptococci, are presented (Table 5.7). Neutrophils harvested from uterine washings obtained from resistant mares 3 hrs after challenge had a significantly higher ($P < 0.05$) intercellular killing ability than those obtained from susceptible mares. There were wide individual variations in the intracellular killing ability of nuetrophils harvested from uterine washings of the susceptible resistant mares.

Table 5.7 Intracellular killing ability of uterine neutrophils (expressed as a %) obtained from resistant and susceptible mares, after intrauterine inoculation with Streptococcus Zooepidemicus

Washings Collected at	Resistant Mares			Susceptible Mares				
	93	201	203	108	202	204	206	207
3 hours	98*	99.5	97	98	70	99.2	0	0
6 hours	33	0	0	0	0	ND	17	40
9 hours	ND	0	0	40	21.43	ND	25	42.86

1. *% bacteria killed after 1 hours' incubation of uterine neutrophils

2. ND Not done due to insufficient neutrophils being harvested

The ability to kill the ingested bacteria decreased sharply in neutrophils obtained from both resistant and susceptible mares at 6 to 9 hrs (Table 5.7).

5.7 DISCUSSION

Uterine neutrophils used in the present study were harvested at specific intervals after intrauterine infusion of viable β haemolytic streptococci. Therefore, compared to the peripheral neutrophils, uterine neutrophils could be considered to be biologically active, since chemotaxis into the uterine lumen is a requirement for their isolation. Hence it is possible that the differences observed in chemotaxis between uterine (isolated after uterine challenge) and peripheral neutrophils (isolated from blood) are due to the effects of different isolation procedures rather than gross chemotactic disorders in the uterine neutrophils.

In the present studies the chemotactic differential/index of uterine neutrophils isolated from resistant and susceptible mares did not differ significantly. This observation and the finding that there was no significant difference between the number of neutrophils in uterine washings obtained from resistant and susceptible mares (Chapter 3), suggest that chemotactic dysfunction is unlikely to be the cause of the lowered resistance to bacterial endometritis observed in the mares designated as susceptible in the present investigations.

Using pooled peripheral neutrophils, whole and heat inactivated serum was observed to have a greater ability of enhancing phagocytosis than Hanks buffer and uterine washings. Hanks buffer, which contained no opsonins, and uterine washings, which were obtained from both resistant and susceptible mares before and after uterine inoculation with β haemolytic streptococci were, however, observed to have a comparable ability of enhancing phagocytosis. These observations were taken to indicate that while pooled whole and heat inactivated serum significantly enhanced phagocytosis, uterine washings obtained from both resistant and susceptible mares did not. In contrast to this, Asbury et al., (1982) using the chemiluminescence technique, observed that before inoculation, resistant mares had substances in their uterine secretions that opsonized bacteria resulting in effective phagocytosis while uterine contents of susceptible mares were not effective in opsonization. These investigators further stated that following inoculation, the ability of uterine contents to opsonize bacteria was similar between groups. Asbury et al., (1982) also observed that addition of serum to uterine washings significantly enhanced opsonization of bacteria in both groups. The discrepancy between conclusion drawn from these two studies could have been partly due to the different techniques used to assess phagocytic function of peripheral neutrophils In the present study, unlike that undertaken by Asbury et al., (1982), an attempt was made to differentiate between the ability of harvested peripheral neutrophils to phagocytize bacteria in the presence and absence of an opsonic source. The difference between the phagocytic index obtained in the presence of an opsonic source (whole and heat in activated serum and uterine washings) and that obtained in the absence of opsonins (Hanks

buffer) was taken to be the true indicator of the ability of that opsonic source to enhance phagocytosis. The discrepancy observed could also be partly due to the differences in the procedure used to obtain uterine washings and variations in the dilutions of uterine fluid collected from different mares. Despite these discrepancies; however, the observations reported by Asbury et al., (1982), and the data available from the present study suggests that peripheral neutrophils obtained from mares with lowered resistance to bacterial endometritis have phagocytic ability comparable to that of peripheral neutrophils obtained from resistant mares. Therefore it is unlikely that lowered phagocytic ability of peripheral neutrophils was the cause of the variation in sussceptibility to endometritis observed in these 2 groups of mares.

Intracellular killing ability of uterine neutrophils obtained from the susceptible mares 2-4 post challenge (preliminary trial page **163**) was significantly different from that recorded for the resistant mares. In the actual trial, however, uterine neutrophils harvested from uterine washings obtained from resistant mares had a significantly higher killing ability. These inconsistent results are difficult to explain other than assume that they could have been due to the relatively short functional integrity of neutrophils (Miller, 1976; Fuenfer et.al., 1976) hence artifactual.

Thus, data available from the present study indicates that a difference in chemotactic differential and index, phagocytic index and/or the subsequent intracellular killing ability of uterine neutrophils was unlikely to be responsible for the lowered resistance to bacterial endometritis in the mares designated as susceptible.

CHAPTER 6

THE EFFECT OF THE STAGE OF REPRODUCTIVE CYCLE AND NATURAL MODULATORS OF INFLAMMATION, CERULOPLASMIN AND TRYPSIN INHIBITOR, ON THE RESOLUTION OF EQUINE ENDOMETRITIS.

PAGE

CHAPTER 6.

THE EFFECT OF THE STAGE OF THE OESTROUS CYCLE AND NATURAL MODULATORS OF INFLAMMATION, CERULOPLASMIN AND TRYPSIN INHIBITOR, ON THE RESOLUTION OF EQUINE ENDOMETRITIS.

6.1 INTRODUCTION

The degree of influence on ovarian steroids on the establishment and the subsequent persistence of invading bacteria in the equine uterus, is at present unclear. Hughes and Loy (1969, 1975), Peterson et al., (1969), Munyua et al., (1984) and Williamson et al., (1884), showed that the clinical response to experimental uterine infection in mares was similar, in extent and duration regardless of the stage of the reproductive cycle. In contrast Ganjam et al., (1982) and Washburn et al., (1882), using ovariectomized mares treated with progresterone or oestrogen to simulate levels found in cycling mares, observed that under the influence of oestrogens the equine uterus was more resistant to infection. Both groups of investigators, however, concluded that the initial neutrophilic response was central to the subsequent elimination of the invading bacteria.

Secretory responses by neutrophils are critical for the initiation, development and regulation of acute inflammation (Becker and Showell, 1974: Wright and Gallin, 1979). The chemotactic factors which promote direct migration of neutrophils and stimulate granule exocytosis have been shown to trigger oxidative metabolism at the plasma membrane (Lehmeyer et al., 1979). During the oxidative metabolism superoxide anions, hydroxy radicals and singlet oxygen are formed as a consequence of the spontaneous dismutation of superoxide anions, or by interactions between superoxide anions and hydrogen peroxide. These highly reactive

radicals have been implicated as mediators of inflammation, causing damage to biomembranes (Goldstein et al.,1979; Conforti et al., 1983). Denko, (1979) and Goldstein et al., (1979) showed that cells and tissues were protected from injury by these highly reactive radicals by superoxide dismutase, plasma protease inhibitors such as alfa-1 antitrypsin and alfa-2 macroglobulin, and antioxidants or free radicals such as ceruloplasmin.

6.2. AIMS

The aims of the present investigation were to assess the influence of the stage of the oestrous cycle on the elimination of the inoculated β haemolytic streptococci, and to study the pattern of change of natural modulators, ceruloplasmin and Trypsin inhibitor, of infection following bacterial challenge.

6.3 MATERIALS AND METHODS

To achieve these aims blood and uterine washing samples collected during Experiments 1 and 3 (see chapter 3) were used. For comparison, uterine fluids obtained at post mortem from 2 mares with chronic endometritis (see section 3.2.2.) were also analysed for the presence of natural modulators (ceruloplasmin and trypsin inhibitor) of inflammation.

6.3.1. Selection of experimental mares and uterine challenge

Twenty one of the mares used in Experiments 1 and 3 were used in the present trial. The mares were challenged by infusion of β haemolytic streptococci in 2 ml of isotonic mannitol (see section 3.2).

To determine the stage of reproductive cycle at challenge. Plasma progesterone levels, measured in paired plasma samples obtained 10 days apart, clinical examination findings, and, for defining anoestrus, histological findings in uterine biopsies obtained 10 days after challenge were used. This information was used to retrospectively assign the mares into either oestrus (n=8), dioestrus (n=7) and anoestrus (n=6) groups.

6.3.1.1. Characteristics of mares in oestrus.

The mares in oestrus at challenge were defined as those that had low progesterone levels (< 1.0 ng/ml) on day 0 and high progesterone levels (> 4.0 ng/ml) on day 10 of challenge. In addition these mares were observed to have a relaxed cervix and palpable follicle on one or both ovaries on day 0.

6.3.1.2. Characteristics of mares in dioestrus.

The mares considered to be in dioestrus had high progesterone levels (> 4.0 ng/ml) on day 0. On clinical examination mares in dioestrus were observed to have pale cervical and vaginal mucous membranes and a thickened closed cervix.

6.3.1.3. Characteristics of mares in anoestrus.

Mares were defined as being anoestrus if plasma progesterone levels were low (< 0.8 ng/ml) on day 0 and on the 10th day (< 0.95 ng/ml) after challenge. Clinically mares in anoestrus had inactive ovaries,

dry and pale vaginal and cervical mucous membranes. Uterine biopsies obtained from these mares at 10 days showed characteristic features of anoestrus including basopilic low cuboidal luminal uterine epithelium, non edematous lamina propria and high gland density with varying degrees of glandular atrophy.

6.3.2. Collection of uterine swabs and washings.

Serial uterine swabs for bacteriology and cytology, and uterine washings for quantitative and qualitative protein assays and neutrophil enumeration were obtained and processed as described in Experiments 1 and 3 (see sections 2.2.1, 2.2.2., 3.3.5., 3.3.6 and 3.3.7 respectively).

The numbers of β haemolytic streptococci recovered and the concentrations of neutrophils, protein, and immunoglobulins in uterine washings (reported in chapter 3), were grouped according to the stages of the oestrous cycle of individual mares at challenge. The mean values for the serial samples from each group were then compared.

6.3.3. Determination of plasma progesterone levels by enzyme immunoassay.

Plasma samples for progesterone assay were obtained from mares selected for inclusion in the present trial. Heparinized venous blood was obtained by venupuncture from the 21 selected mares before (day 0) and 1, 2, 4, 10, 14 and 28 days after challenge. Plasma was separated from the cellular components of blood by centrifuging the heparinized blood samples at 3,000 g for 15 minutes before the supernatant fluid was decanted and aliquoted into several

1 ml fractions. All aliquots were stored frozen until required for assay.

Progesterone levels were determined using a solid phase enzyme immunoassay, identical to that established and described by Munro and Stabenfeldt (1984). Dr. Stabenfeldt provided most of the major assay constituents including the progesterone antibody and conjugate. The progesterone antisera for the Enzyme immunoassay (EIA) was raised in rabbits immunised against progesterone-11 a-hemisuccinate-BSA. The antisera was purified by ammonium sulphate precipitation and BSA absorption and then diluted in PBS with 0.1% BSA to 1:2000 titre. Horseradish peroxidase, which was used to prepare the labelled ligand, was coupled to the 3-carboxymethyloxime derivative of progesterone to yield 3-O-carboxymethyloxime. The substrate used was 2, 2-azino-di-3-etheylbenzthiazoline sulfonic acid, a diammonium salt (ABIS) (Sigma Chemicals Co. WA). The substrate was prepared 15 minutes before it was needed by adding 25 µl of 40nM ABTS and 80 µl 0.5M H202 to 24.67 ml 0.05M citrate (pH 4.0) to give a final molarity of 0.4mM ABTS, 1.5mM H_2O_2 and 0.05M citrate.

To extract progesterone, plasma (100 µl) was mixed with 2.0 ml petroleum ether and shaken on a disc shaker for 30 minutes. After shaking, the plasma was frozen in a dry ice-ethanol mixture and the supernatant was decanted into dry labelled tubes and air dried under pressure. Prior to the assay the extracted progesterone was taken up in assay buffer (125 µl) and stored overnight. To establish the percentage of progesterone extracted, 3_H labelled progesterone (10 µl) was added to low progesterone plasma (100 µl) and the mixture treated as for progesterone extraction After drying the supernantant the recovered 3_H progesterone was taken up in 100µl assay buffer and mixed with scintillation cocktail for counting. The activity in these extracted tubes divided by

the activity in similar volume of $^{3}_{H}$ progesterone (10 µl) which was unextracted to calculate the percent progesterone recovery.

$$\text{Percent recovery} = \frac{\text{cpm } ^{3}_{H} \text{ progesterone in extracted tubes}}{\text{cpm in } ^{3}_{H} \text{ in progesterone tubes (unextracted).}}$$

Where

cpm = counts per minute, an expression of radioactivity measured using a scintilation counter.

6.3.4. Enzyme immunoassay procedure for measuring progesterone.

Polystyrene microtitre plates (Dynatec, Switzerland) were coated with 50 µl antibody in coating buffer at a 1:2000 dilution (containing approximately 1.0 ug of protein) incubated for at least 24 hours at 4C. The wells were emptied by inversion and washed 5 times with the washing solution to remove the antisera not bound to the polystyrene, and inverted to dry on paper towels. Progesterone standard was serially diluted in buffer to :- 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 pg/50 µl and added to the 10 center rows of the first microtitre plate. The remaining two rows in the standard curve plate received 50 µl of buffer and were used as controls. Extracted plasma samples were added in duplicate in 50 µl aliquots wells of separate plates. Immediately following this, 50 µl of the conjugate was added to all wells and the plates incubated for 2 hours at 25C. Separation of the bound steroid from the free steroid was accomplished by washing the free steroid from the bound antibody complex absorbed to the surface of the wells. The substrate, ABTS, was added and incubated for 1 hr at 25C. The reaction was stopped by the addition of the stop solution. The resulting

colour change, which was inversely proportional to the quantity of progesterone in the sample, was measured using a Multiscan spectrophotometer (Flow laboratories, WA) set at 405 nm Wavelength. The progesterone levels in samples were then read from the standard curve (Figure 6.1). Buffers and working solutions used through out the enzyme immunoassay procedures were prepared as described in detail elsewhere (Appendix 16).

6.3.4.1. Quality control

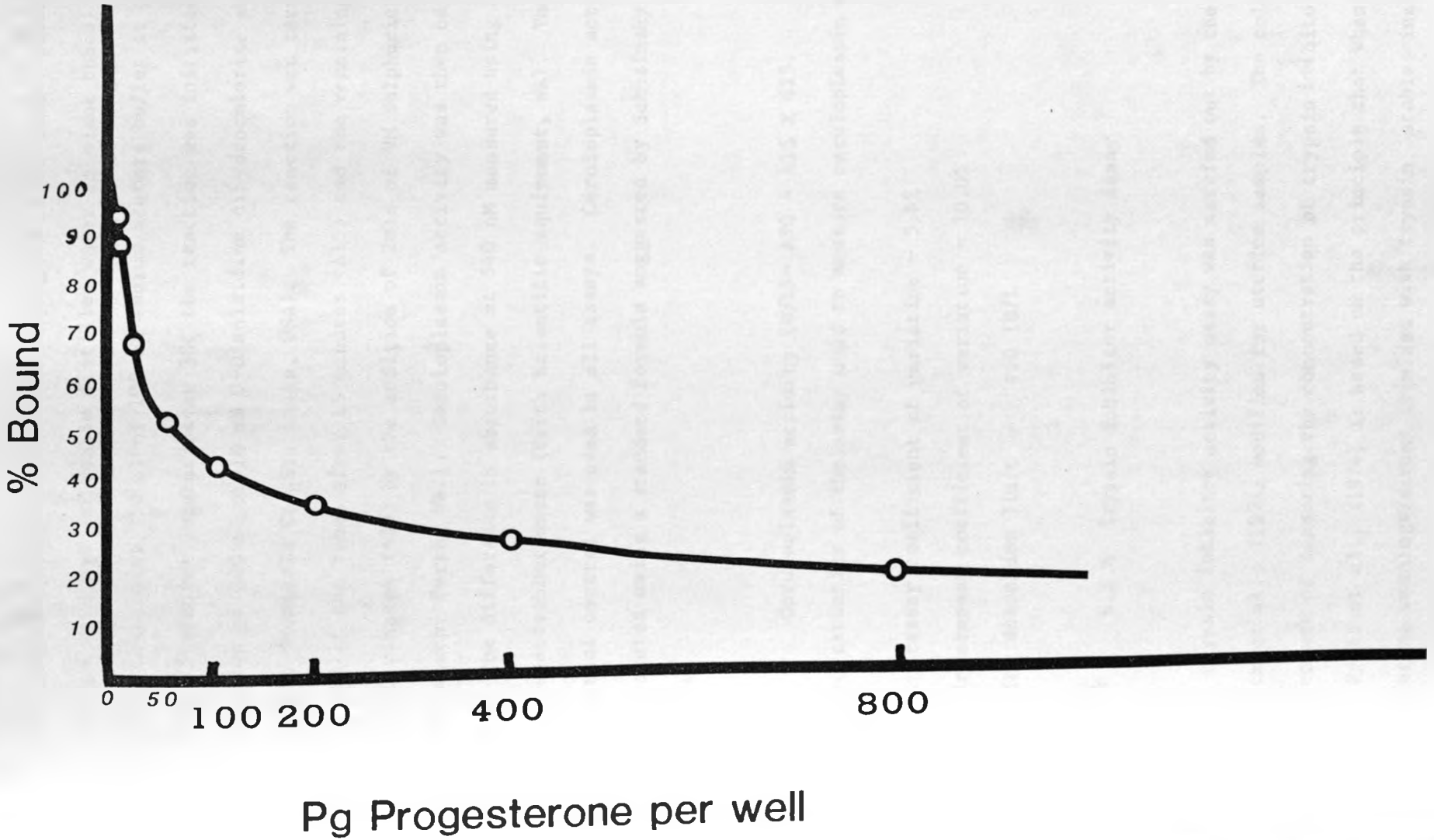
. Non-specific binding was measured using the two wells on the top left hand corner of each plate, which were not coated with antibody. Two pools of equine plasma (high and low progesterone) were included in each assay for the determination of intrassay coefficient of variation and to establish the sensitivity of our assay. The interassay and intrassay coefficients of variation were calculated by the methods of Rodbard (1974). Characteristics of the Enzyme immunoassay were:-

- Percent recovery - 88%
- Coefficient of variation
  Interassay - 6.6%
  Intrassay - 4.4%
- Sensitivity - 3.3 pg/well
- NSB - < 1 ng/well

6.3.5. Ceruloplasmin activity assay.

The ceruloplasmin assay was performed in quadruplicate as described in detail by Schosinsky, Lehmann and Beeler, (1974).

Figure 6.1 Enzyme Immunoassay standard curve for Plasma progesterone (representative curve)



The standard assay consisted of adding 50 µl of either plasma or uterine washings (or double distilled water to blank tubes) into 4 tubes containing 750 µl of 100 mM sodium acetate buffer at pH 5.6. After 5 minutes incubation at 30C the reaction was initiated by addition of 200 µl of 10 mM O-Dianisidine dihydrochloride substrate (Sigma Chemicals Co. St. Louis, USA). The reaction was terminated in two of the tubes after 15 minutes (A15) and the remaining 2 tubes at 30 minutes (A30) by the addition of 2mls of 9M sulphuric acid (Chemtech, Perth, WA.). Ceruloplasmin activity was then calculated from the difference in absorbance at 540 nM measured using a Stasar III spectrophotometer (Perth Scientific equipment, WA). The same internal control was used in all assays. Ceruloplasmin activity was calculated using a standard formula suggested by Schosinsky et al., (1974).

Ceruloplasmin activity (U/L)= A30 - A15 X 625.

Characteristics of the assay used to measure ceruloplasmin were:-

- intrassay coefficient of variation - 5.6%
- interassay coefficient of variation - 10.0%
- low detection limit - 1.0 IU/L

6.3.6 Trypsin inhibitor activity assay

The trypsin inhibitor activity assay was carried out by the method of Fritz et al., (1974) modified for uterine samples. The colorimetric procedure of measuring the concentration of trypsin inhibitor described by Fritz et al., (1974) is based on the principle that when the sample (uterine washings/serum) is mixed with trypsin, stable, inactive

complexes are formed if trypsin inhibitors are present. Any excess, unbound, trypsin can then be measured using a synthetic substrate, N-benzoylarginine-p-nitranilide (BAPNA - Sigma Chemical Co. Perth, WA). The hydrolysis of the substrate is followed by a direct increase in absorption at 405 nM (Fritz et al., 1974).

The samples, uterine washings or fluid or serum, were cleared by incubating 0.5 ml of the sample with an equal volume of 6% perchloric acid (G. Fredrick Smith Chemical Co. WA) at room temperature for 30 minutes followed by centrifugation at 3,000 g for 30 minutes. The pH in the supernatant fluid was adjusted to between pH 4.0 and pH 7.0 by dropwise addition of 5M K_2CO_3 solution and the resulting mixture was allowed to stand for one hour at 4C before centrifugation at 4,000g for 20 minutes. The trypsin inhibitor content in the supernatant potassium perchlorate was then measured as follows.

Uterine washing, uterine fluid or serum (0.1 ml), trypsin (0.1 ml, working solution 2.5 ug/ml) and substrate (0.1 ml, BAPNA) were added to flat bottomed microtitration plates (Flow labs,, WA). In addition to the 80 samples, each 96 cup micro titration plate included a blank row and a standard dilution series prepared from antitrypsin (25 ug/ml working solution) and pooled serum (serial dilutions 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 & 1:128). A trypsin control with no antitrypsin (Sigma Chemicals, Co. Oerth WA) was included in all assays. The plates were incubated at 37C for 3.5 hours, following which the absorbance was measured using a multiscan automatic plate reader (Flow laboratories, Perth WA) set at 405 nm. The concentration of the trypsin inhibitor activity was then read from the antitrypsin standard curve and expressed as µg antitrypsin (Figure 6.2).

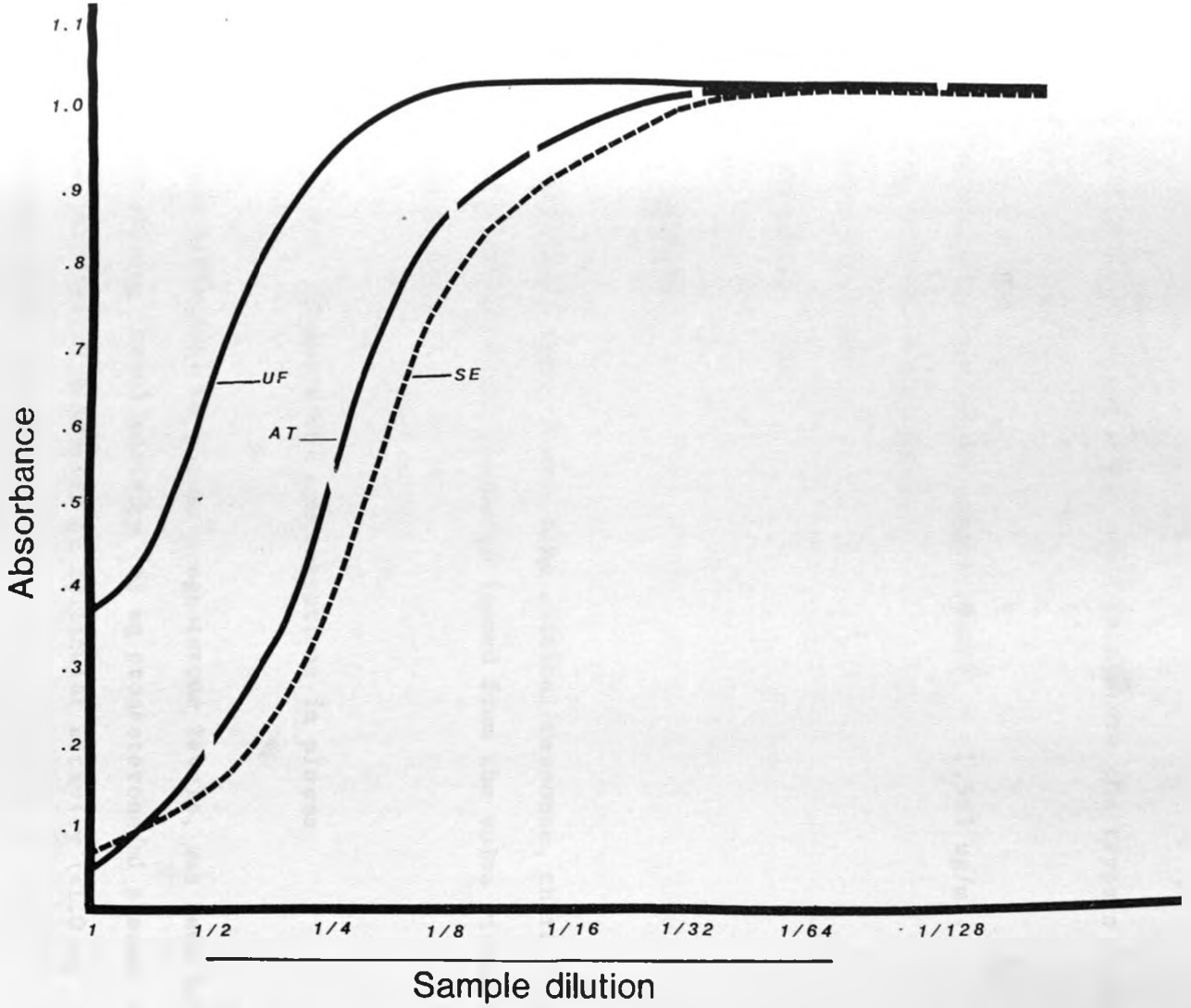


Figure 6.2 Dose response curves showing trypsin inhibitor effects of dilutions of uterine fluid collected from mares with chronic streptococcal endometritis (UF) pooled equine serum (SE) and Antitrypsin [(AN) 25µg/ml)] as measured by release of P-nitroaniline from N-benzoarginine-p-nitroanilide by excess trypsin

Throughout the trypsin inhibitor assays the inhibition was observed not to be linearly proportional to the inhibitor concentration up to 100% value, therefore the samples were diluted where necessary for the measurements to be made in the linear part of the inhibition curve (Figure 6.2).

Characteristics of the assay used to measure the trypsin inhibitors were:-

- lowest detection level (sensitivity) - 1.563 ug/ml.
- coefficient of variation.
  intrassay - 14%
  interassay - 12%

6.4. RESULTS

All challenged mares showed some clinical response, characterized by a copius mucopurulent discharge issued from the vulva within 24 hours of inoculation.

6.4.1 Progesterone concentrations in plasma

A clear difference in plasma progesterone levels, was seen between mares showing luteal activity (>2 ng progesterone/ml plasma) and those with inactive ovaries or follicular activity (<1.0 ng progesterone/ml plasma).

Based on the levels of plasma progesterone measured using the enzyme immunoassay, biopsy findings in samples obtained 10 days after the begining of the observation, and findings of clinical and physical

Table 6.1

The mean progesterone levels (ng/ml) in samples obtained from experimental mares before and after intrauterine inoculation of β haemolytic streptococci.

Stage of oestrous cycle at challenge	Plasma progesterone levels (ng/ml) in samples collected	
	Before (0hrs) & after (10 days) challenge	
Oestrus (n=8)	0.72 ± 0.18	5.37 ± 0.83
Dioestrus (n=7)	4.80 ± 7.54	1.35 ± 1.13
Anoestrus (n=6)	0.65 ± 0.23	0.95 ± 0.48

examination including palpation of the ovaries, 8 oestrus, 7 dioestrus and 6 anoestrus mares were identified for inclusion in this study. The mean plasma progesterone levels for the three groups of mares at 0 and 10 days after challenge are presented in Table 6.1.

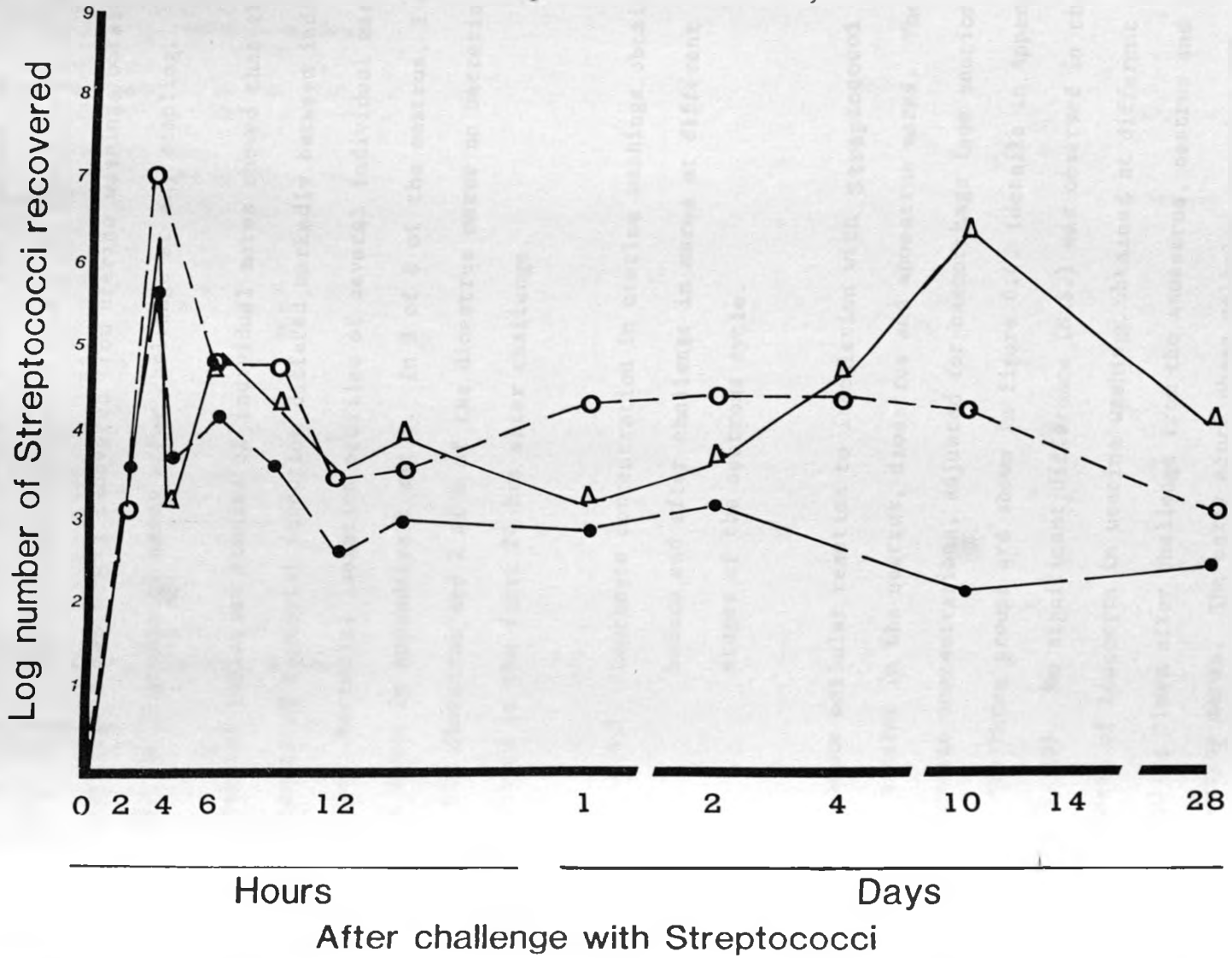
Progesterone concentrations in plasma collected from mares with luteal activity (in dioestrus) at challenge showed a marked decline in progesterone levels by the 4th day of challenge. In contrast plasma progesterone levels were observed to remain low in mares that were anoestrus at challenge (Appendices, 17, 18).

6.4.2. Bacteriological culture of uterine swabs and washings, in mares grouped according to their stage of the oestrous cycle.

The bacterial results, grouped according to susceptiblity or resistance to uterine infection have been presented in chapter 3 (see section 3.4.2.). No bacteria were cultured from any of the prechallenge uterine swabs or washings obtained from the three groups of mares.

The mean numbers of β haemolytic streptococci recovered after challenge, grouped according to the mares stage of reproductive cycle at challenge, are shown in Figure 6.3 and appendix 19. In most of the mares low numbers of β haemolytic streptococci (cfu) were recovered at 4 hrs after challenge (Figure 6.3, Appendices 1, 5). This was followed by a sharp increase in the numbers of bacteria recovered at 6 hrs after challenge. The numbers of β haemolytic streptococci recovered from uterine washings obtained from all three groups of mares had decreased markedly by 12 hrs and rose gradually over the next 28 days after challenge. One mare (No/ 142) in oestrus, 2 mares in

Figure 6.3 The mean number of Streptococci /ml recovered from uteri of oestrus (-Δ-Δ-), dioestrus (-o-o-) and anoestrus (-●-●-) mares following uterine challenge with Streptococci.
(See Appendix 19 for standard error)



dioestrus (mares 35 and 145) and one anoestrus mare (mare 55) had positive bacteriology (swabs and uterine washings) 28 days after challenge but majority (resistant mares) had cleared the infection. There was no significant difference (N.S.) in the numbers of B haemolytic streptococci recovered from uterine washings obtained from the 3 groups of mares after challenge at any sampling.

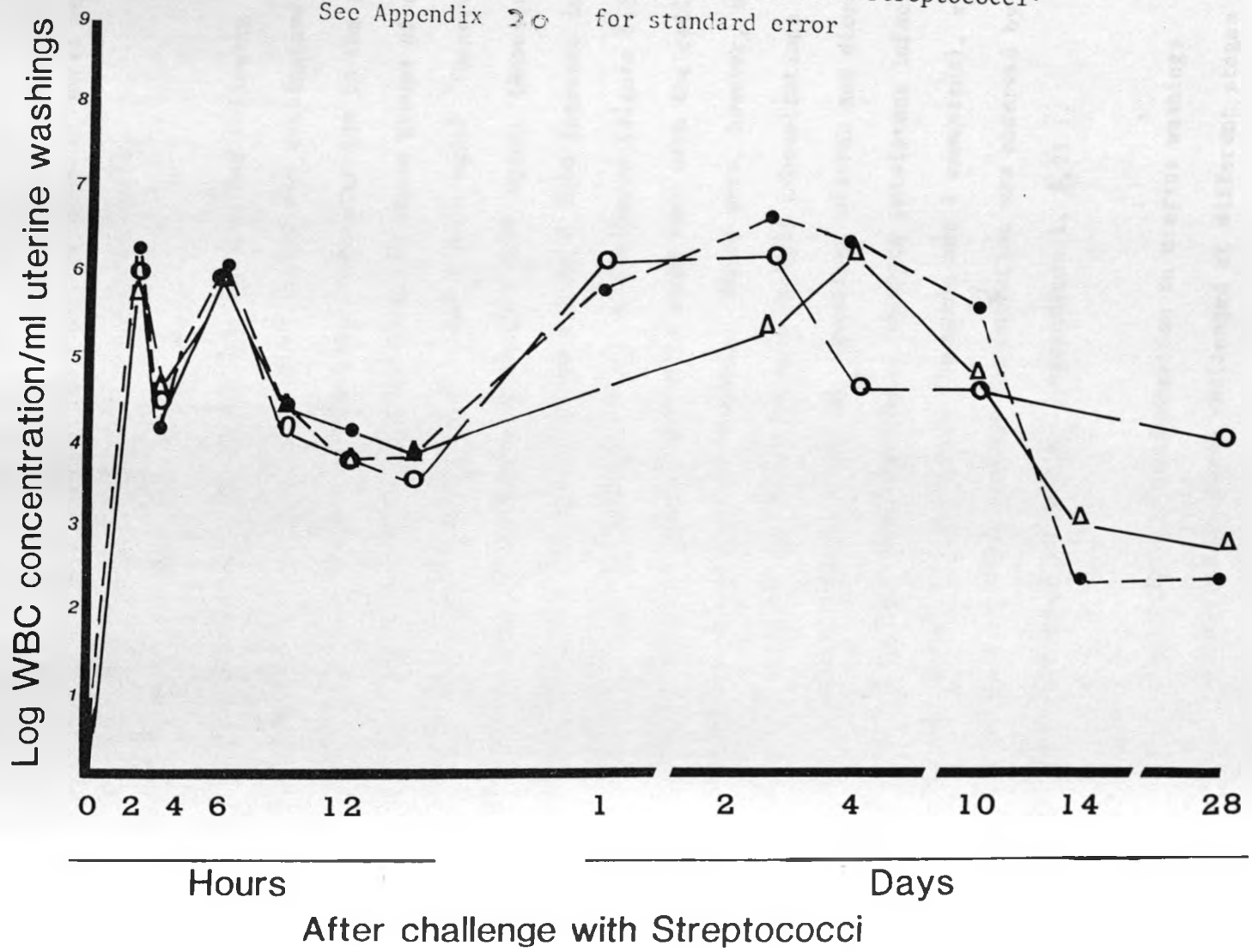
Bacterial isolation studies of individual mares showed that the dynamics of bacterial infection differed markedly between individual mares. Bacterial isolation profiles of several individual mares are shown in Appendices 1 and 5. In 3 of 8 of the oestrus, 2 of 7 of the dioestrus and 2 of 6 of the dioestrus mares no bacteria were isolated in the first 24 hrs after challenge.

6.4.3. Leucocyte concentration in uterine washings obtained before and after challenge in mares at different stages of the oestrous cycle.

The acute cellular reaction to inoculation with **Streptococci** was similar in the oestrus, dioestrus and anoestrus mares. The mean Leucocyte concentrations, adjusted for haemorrhage (see section 3.3.6), for the three groups are shown in figure 6.4. (details in Appendices 1, 5, 20). No significant difference (N.S.) was observed in the numbers of leucocyte in uterine washings obtained at different sampling times after challenge from the anoestrus, oestrus and dioestrus mares. The early acute phase cellular reaction was accompanied by a rapid decline in the number of β haemolytic streptococci recovered from uterine washings and swabs (Figures 6.4., Appendices 1, 5, 20).

Four mares, 1 in oestrus, 2 in dioestrus and 1 in anoestrus, showed

Figure 6.4 The mean leucocyte counts/ml uterine washing obtained from oestrus (-Δ-Δ-), dioestrus (-●-●-) and anoestrus (-o-o-) mares following intrauterine inoculation with Streptococci.
See Appendix 20 for standard error



a marked decline in neutrophils concentration 28 days after challenge inspite of persisting infection (Figure 6.4, Appendices 1, 5, 20).

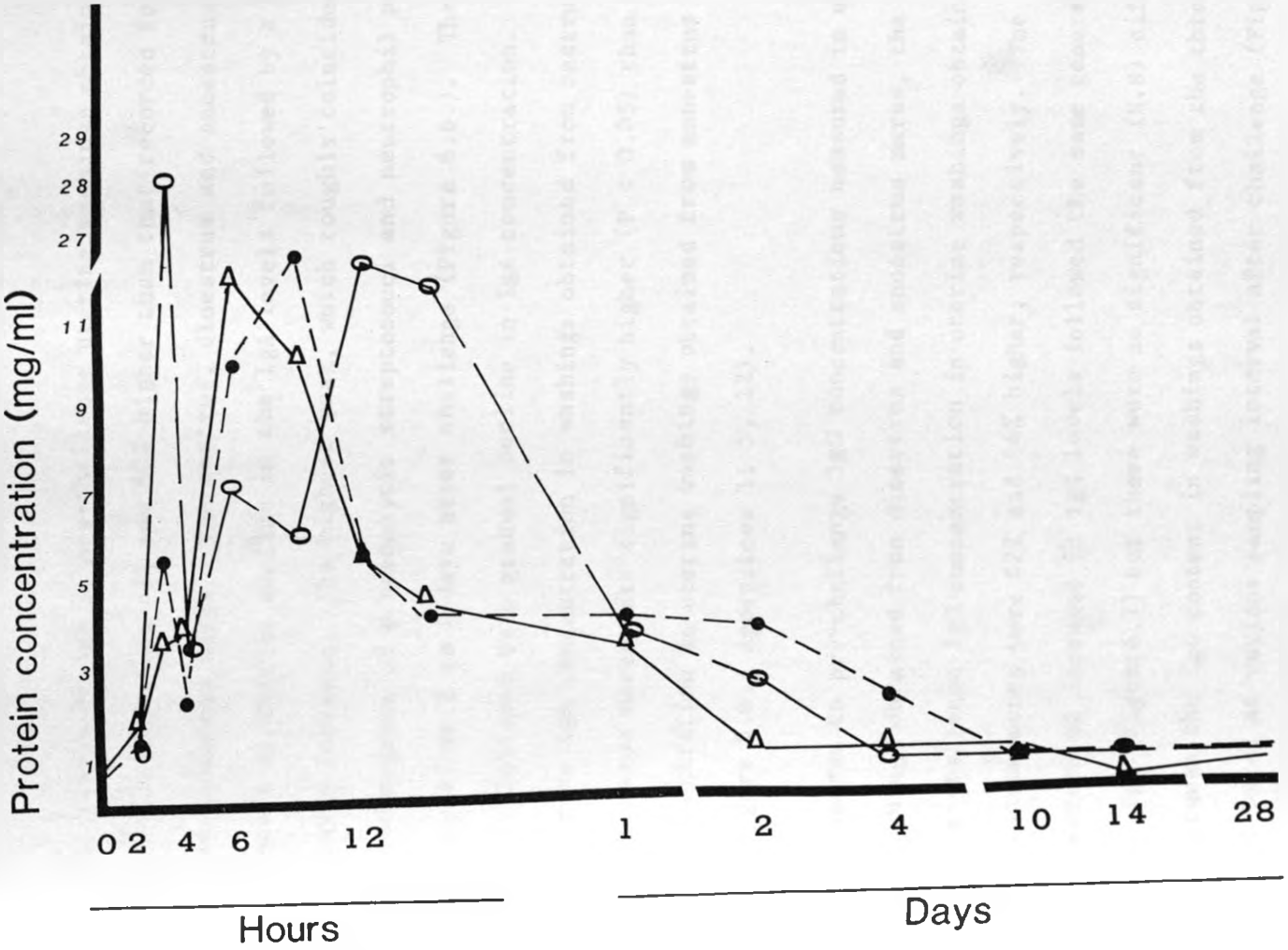
6.4.4 Protein in uterine washings and serum of mares at different stages of the oestrous cycle.

Serial uterine washings and serum samples obtained following challenge were subjected to both quantitative and qualitative measurements of proteins. The protein concentration in the pre-challenge uterine washings obtained from the three groups of mares did not differ significantly (N.S) 0.63 ± 0.14 mg/ml (Oestrus), 0.86 ± 0.39 mg/ml (Dioestrus), and 0.53 ± 0.06 mg/ml, (Anoestrus). Mares in all the three groups showed a 2 to 3 fold increase in protein concentrations within 3-6 hrs of challenge (Figure 6.5). This initial protein "flush" generally coincided with the cellular (predominantly neutrophilic) response. There were, however, no significant differences (N.S.) between protein concentrations in uterine washings obtained from the anoestrus, oestrus and dioestrus mares at the various sampling times. Despite persistent infection in the four mares, (1 oestrus, 2 dioestrus and 1 anoestrus), a marked decrease in total protein concentration was observed by 28 days after challenge (Figure 6.5, Appendices 1, 5,21).

6.4.5 Immunoglobulin concentration in uterine washings obtained from mares challenged at different stages of the oestrous cycle.

Immunoglobulins (Ig) A, G and occassionally M were detected in most of the uterine washings tested. The sensitivity of the respective

Figure 6.5 The mean protein concentration (mg/ml) in uterine washings obtained from oestrus (-Δ-Δ-), dioestrus (-●-●-) and anoestrus (-o-o-) mares following challenge with Streptococci.
(See Appendix 2.6 for Standard error)



immunoglobulin assays was, IgA 22 mg%, IgG 15 mg% and IgM 10 mg% of the colostrum standard. The mean IgA and IgG concentrations in uterine washings obtained from oestrus, dioestrus and anoestrus mares are illustrated in Figure 6.6. and Appendices 1, 5, & 22.

The levels of IgA in pre-challenge uterine washings obtained from oestrus mares was 42% and 80% higher than that recorded for dioestrus and anoestrus mares. The oestrus, dioestrus and anoestrus, mares all showed an initial decline in the IgA levels followed by a gradual but steady increase. The highest peak, which roughly coincided with the re-emergence of β haemolytic streptococci and neutrophil peak, occured at 2 to 4 days after challenge (Figure 6.6.). These peaks were followed by a gradual decline in IgA concentration. The mean uterine IgA concentration in washings obtained from oestrus and dioestrus mares were significantly higher ($P < 0.05$) than mean IgA concentration in uterine washings obtained from anoestrus mares. (Figure 6.6., Appendices 1, 5, 22).

Compared to pre-challenge IgG concentrations measured in uterine washings obtained from dioestrus and anoestrus mares, the levels of pre-challenge IgG concentration in uterine washings obtained from oestrus mares were 42% and 54% higher, respectively. The pattern of increase in IgG levels followed the same general trend as IgA (Figure 6.7) but there were no significant (N.S) differences between the IgG content in washings obtained from the three groups of mares at various sampling interval after challenge (Figure 6.7, Appendices 1, 5, 23).

Figure 6.6 The mean IgA concentration (mg%) in uterine washings of oestrus (-Δ-Δ-), dioestrus (-●-●-) and anoestrus (-o-o-) mares following challenge with streptococci.
(See Appendix 22 for standard error)

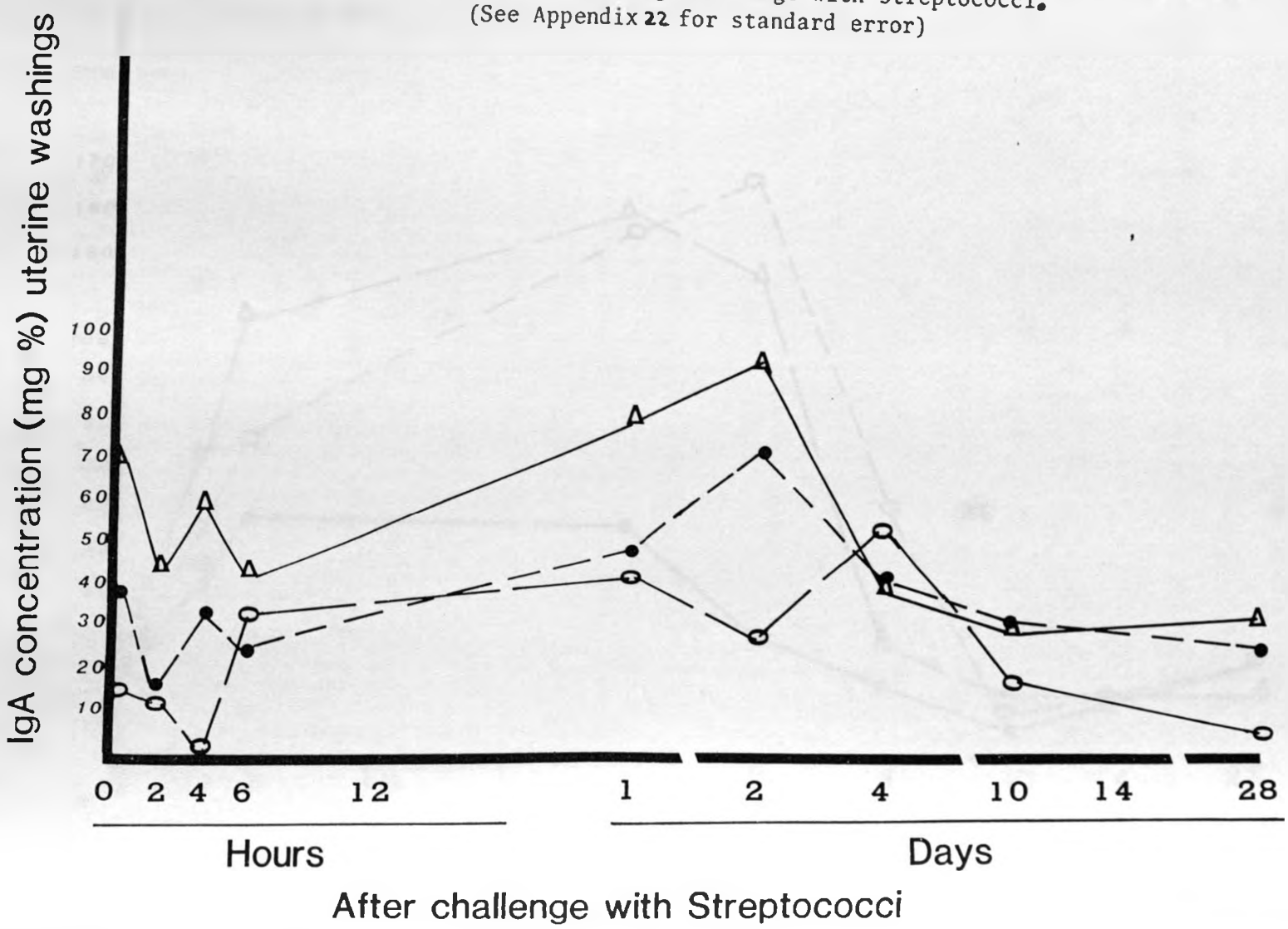
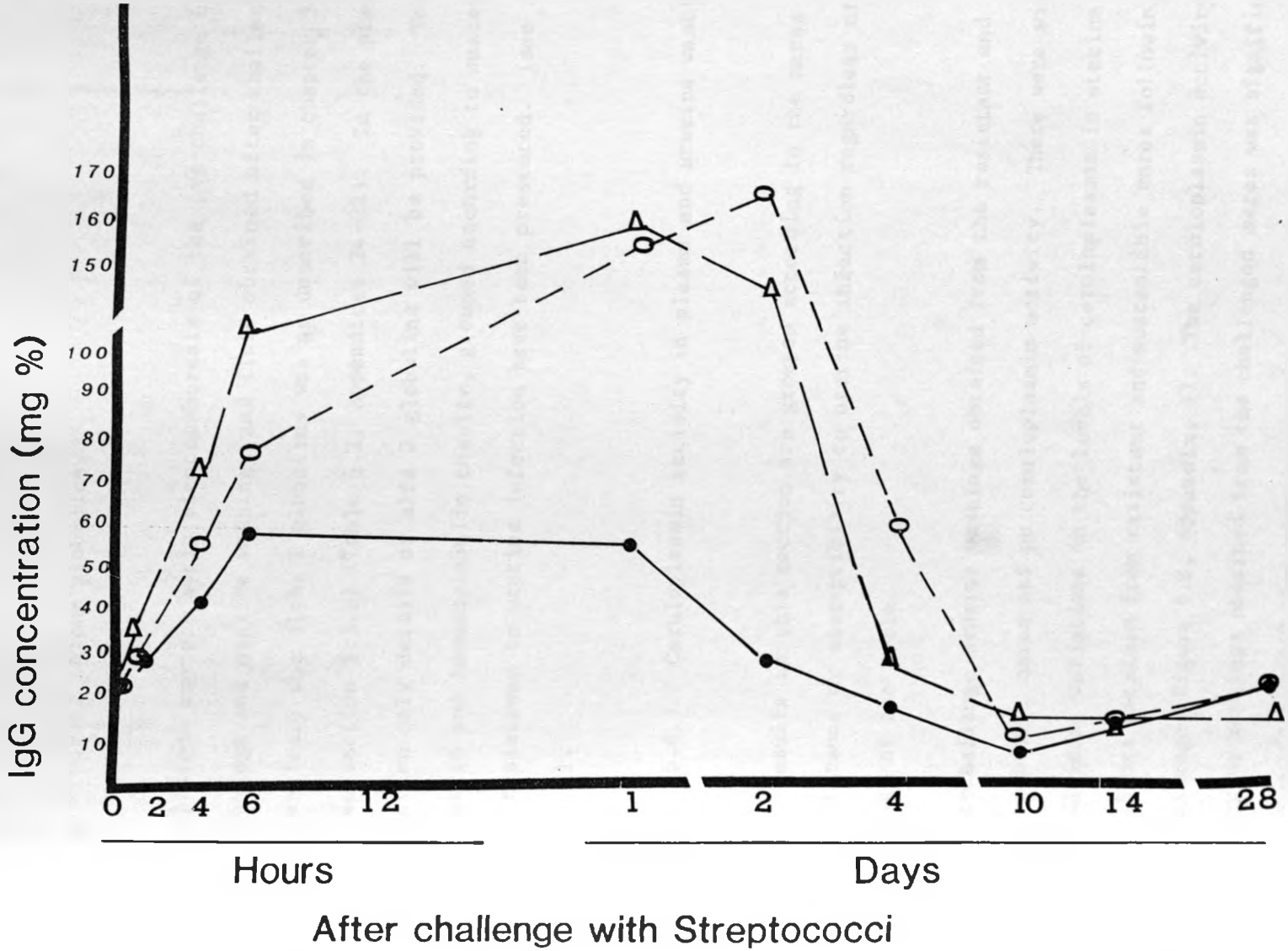


Figure 6.7 The mean IgG concentration (mg%) in uterine washings obtained from oestrus (-▲▲-), dioestrus (-o-o-) and anoestrus (●-●-●) mares, following challenge with Streptococci.
(See Appendix 23 for standard error)



THE OCCURENCE OF NATURAL MODULATORS OF INFLAMMATION IN UTERINE WASHINGS.

6.4.6. Alpha globulins.

Cellulose acetate strip electrophoresis of the pre-challenge uterine washings and uterine washings and fluid obtained after challenge to quantitate the alpha 2 globulins was as described in chapter 3 (see section 3.3.8) (Table 6.2. Appendices 24-31). In the present section only details of alfa 2 globulins will be provided. Total protein and immunoglobulin results, grouped according to susceptibility or resistance to uterine infection have been presented. (see section 3.4.1).

6.4.7. Ceruloplasmin activity in plasma and uterine washings.

The results in this section are grouped according to the mares resistance or susceptibility to uterine infection regardless of the stage of the cycle.

Pre-challenge uterine washings obtained from the resistant and susceptible mares had no ceruloplasmin activity. There were wide individual variations in the levels of ceruloplasmin in uterine washings obtained from resistant and susceptible mares following challenge (Figure 6.8, Appendix, 1). The ceruloplasmin activity in uterine washings obtained from the challenged mares was significantly higher ($P < 0.05$) than that measured in uterine washings obtained from the unchallenged control mares (Figure 6.8, Appendix, 1). No ceruloplasmin activity was detected in uterine washings obtained throughout the observation period, in 2 of the 3 unchallenged control

Figure 6.8 The mean ceruloplasmin activity (iu/ℓ) in uterine washings obtained from resistant (-o-o-o-), susceptible (●—●) and control mares (-Δ-Δ-) following intrauterine inoculation with β haemolytic Streptococci. See Appendix I for standard error)

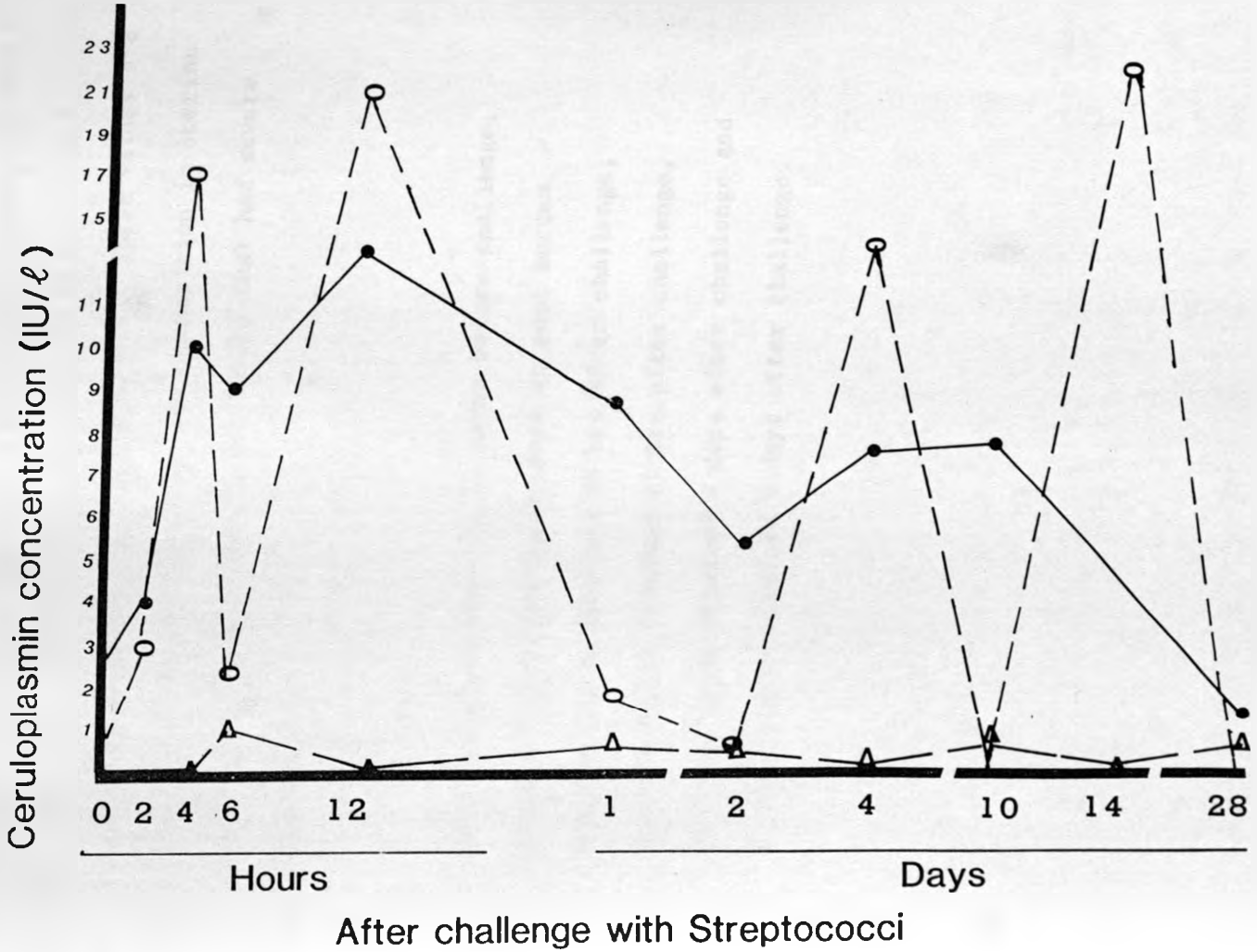


Table 6.2.

The mean protein concentration and composition in serum obtained before challenge and uterine fluid obtained 12 hrs to 9 days after intrauterine inoculation of Streptococci. These values are compared to the protein concentration and composition in uterine fluid obtained from 2 mares, 188(2) and 190(2) that had severe (natural) endometritis.

I) Serum obtained from the 21 mares before challenge,

II) Uterine fluid from the 2 mares at post mortem

III) Uterine fluids obtained 12 hrs after challenge,

IV) Uterine fluids obtained 24 hrs after challenge,

V) Uterine fluids obtained 4 days after challenge and

VI) Uterine fluids obtained 9 days after challenge.

Table 6.2 continued.

Protein mg/ml	Albumin mg/ml	GLOBULINS (mg/ml) Alfa 1	Alfa 2	Beta 1	Beta 2	Gamma
I) Before challenge						
62.91 ±1.01	28.91 ±0.43	2.74 ±0.37	7.93 ±0.50	7.22 ±0.48	6.21 ±0.29	8.75 ±0.50
II) Infected mares- PM						
13.0 ±6.0	3.58 ±1.92	0.29 ±1.19	1.75 ±0.71	2.37 ±1.04	2.87 ±1.70	2.13 ±0.44
III) 12h after challenge						
70.0 ±2.0	15.78 ±3.84	1.60 ±1.0	9.09 ±0.32	15.61 ±0.41	9.55 ±0.05	15.40 ±1.11
IV) 24h after challenge						
38.33 ±3.18	14.58 ±0.14	0.99 ±0.08	4.81 ±0.67	6.26 ±1.78	5.18 ±0.39	6.48 ±0.97
V) 4d after challenge						
47.0	17.32	6.92	13.25	10.33	6.85	3.0
VI) 9d after challenge						
10.0	1.69 ±0.17	0.69 ±0.17	1.93 ±0,98	2.53 ±0.35	2.53 ±0.35	3.13 ±0.68

Table 6.3

The mean concentration of uterine trypsin inhibitors in uterine washings obtained from challenged mares and unchallenged control mares.

Mare group	Time of collection of uterine washings after challenge.						
	0 hrs	4 hrs	6 hrs	24 hrs	2 days	4 days	10 days
Res.	19.66 ±0.12	20.25	19.30 ±0.50	18.87	19.85 ±0.13	19.64 ±0.39	20.11
Sus.	19.41 ±0.18	-	20.18 ±0.07	19.50 ±0.27	18.74 ±0.14	19.50 ±0.21	-
Cont.	19.74 ±0.45	19.43	19.63 ±0.48	-	19.98 ±0.28	19.98	-

Res. = resistant mares, Suc. = Susceptible mares and

Cont.= Control mares.

mares (187 & 188) Figure 6.8, Appendix, 1).

6.4.8. The levels of trypsin inhibitors in uterine washings and plasma.

The mean trypsin inhibitor concentrations measured in uterine washings from challenged and unchallenged mares are shown (Table 6.3, Appendix 32). The mean trypsin inhibitor content in plasma obtained from the mares before and after challenge was 19.76 ± 0.11 ug/ml. The trypsin inhibitor concentrations in uterine washing and plasma samples obtained from the challenged resistant and susceptible, and unchallenged control mares, before or after challenge remained almost constant and did not differ significantly (N.S.).

6.5. DISCUSSION

The clinical signs of endometritis observed in this study following intrauterine infusion of β haemolytic streptococci, were similar to those previously reported (Hughes and Loy, 1969; 1975; Peterson *et al.*, 1969). All mares challenged in the present study, irrespective of the stage of the reproductive cycle at challenge, showed a similar clinical response in extent and duration. Clinically these signs were characterized by a mucopurulent discharge from the vulva within 24 hrs of challenge.

Within the three groups of mares (oestrus, dioestrus and anoestrus),

there were great individual variations in the pattern of recovery of the inoculated β haemolytic streptococci on swabbing. Despite these individual variations, however, the numbers of β haemolytic streptococci recovered from uterine washings obtained after challenge decreased sharply in all mares within 3 to 24 hours after challenge. This sharp decline in the numbers of isolated β haemolytic streptococci was followed by an increase in the numbers of β haemolytic streptococci isolated from the uterine lumen of most mares. The β haemolytic streptococci persisted beyond 10 days in those mares previously classified as susceptible to endometritis, regardless of the stage of the reproductive cycle at which they were infected. Likewise mares previously classified as resistant to uterine infection, cleared the infused bacteria within 4 days.

The re-emergence of the β haemolytic streptococci coincided with a secondary increase in soluble and cellular factors in all three groups of mares at 24 to 48 hours after challenge. The persistence of the re-emerged β haemolytic streptococci in the four mares challenged at different stages of the reproductive cycle may have been due to the inability of these particular mares to sustain a cellular response, rather than to the stage of the reproductive cycle at challenge. These observation indicated that the cellular and humoral responses, including the secretion of IgA and IgG, following uterine inoculation of pathogenic streptococci are not greatly influenced by ovarian steroid hormones in the mare. The well characterized response to bacterial challenge, of cellular infiltration (predominantly neutrophils) into the uterine lumen and a significant increase in the concentrations of uterine proteins was similar in the three groups of mares and most probably represented a non-specific humoral response to uterine stimulation.

In the present study it was observed that all pre-challenge uterine washings had detectable concentrations of IgA and IgG. The inital decline of IgA in the uterine washings observed in all three groups of mares at around 2 to 4 hrs after challenge may have been due to the dilution effects of flushing, hence artifactual. This initial decline was followed by a marked increase in IgA concentration in uterine washings of both oestrus and dioestrus mares, and to a lesser extent the anoestrus mares. There was, however, no significant differences in IgG concentrations after challenge between the three groups of mares. The rapid fall in IgA concentration in the uterine washings of the mares that overcame the infection indicated that IgA was released in response to uterine manipulation and the presence of bacteria but their release was more marked and persistent than the presence of bacteria. This difference between mares was related to their susceptibility to uterine infection, not to the stage of the reproductive cycle at challenge.

Ceruloplasmin and alfa 2 globulins, both documented modulators of inflammation, were observed in uterine washings obtained from both resistant and susceptible mares only after challenge, and one in unchallenged mare 2 to 3 hrs after the infusion of isotonic mannitol. This presence of these naturally occuring modulators of inflammation in both resistant and susceptible mares indicates that they are unlikely to be the factors which are responsible for the differences in susceptiblity. In addition the low levels of ceruloplasmin and alfa 2 globulins observed in uterine washings obtained from most unchallenged mares indicated that the degree and extent of damage caused by uterine manipulation only was not severe enough to provoke their leakage or release. In contrast, trypsin inhibitors, which are also modulators of inflammation, were detected in pre-challenge

uterine washings obtained from all mares. The concentration of trypsin inhibitors in uterine washings obtained from challenged and unchallenged mares remained almost constant throughout the observation period, and were comparable to those measured in plasma. The detection of trypsin inhibitors in the uterine secretions and washings before and after challenge indicated the trypsin inhibitors may be involved in the protection of uterine luminal epithelium and tissues from the effects of the products of neutrophil extracellular degranulation. This opinion is in agreement with that by Klinkhamer, (1963), Teir et al., (1963), Weissmann et al., (1972) and Wright, (1982). These investigators showed that protection against degranulation products of the neutrophils was especially crucial in pathologic conditions in which there is large scale invasion of tissues by bacteria. They showed that the presence of large number of bacteria provides sufficient stimulus for the extracellular release of these antimicrobial components from phagocytic vacuoles, which are also cytotoxic.

Data available from the present study indicates that cellular and humoral responses in the mare may not be greatly influenced by ovarian steroids and that the stage of the reproductive cycle at challenge was not critical for the successful resolution of endometritis. Further studies are, however, necessary to determine whether there are variations in the quality and absolute values of modulators of inflammation and uterine opsonins, and the ability of the susceptible mares to sustain a cellular response.

CHAPTER 7

CHAPTER 7

CONCLUSION

The experiments described in this thesis were designed to establish a technique of identifying mares with lowered resistance to streptococcal endometritis and to study the pathogenesis of equine streptococcal endometritis, including the factors influencing the elimination of the experimentally infused β haemolytic streptococci.

Acute uterine infection was induced in 32 mares, which were tentatively classified as either resistant or susceptible to endometritis depending on their reproductive history and the degree and extent of endometrial degeneration. Infection was achieved by the intrauterine inoculation of β haemolytic streptococci suspended in sterile isotonic mannitol solution. Three control mares were infused with sterile isotonic mannitol solution. Serial uterine swabs, washings and biopsies and blood samples were obtained before (0 hrs) and at fixed time intervals after intrauterine infusion of the B haemolytic streptococci inoculum (2, 3, 4, 6, 9, 12 and 15 hrs and 1, 2, 4, 6, 10, 14 and 28 days).

The findings of physical examination and the results obtained from laboratory analysis of the serial uterine samples were used to assess the degree, extent and duration of the immune responses, endometrial damage and the persistence of the infused β haemolytic streptococci in the uterine lumen.

Based on the results of the experimental intrauterine inoculation with β haemolytic streptococci (challenge results) resistant mares were defined as those that eliminated the infused bacterial by the 4th day

of challenge. Using the same criterion, susceptible mares were defined as those that needed more than 10 days to eliminate the infused β haemolytic streptococci. Some of the mares tentatively classified as susceptible on the basis of their poor endometrial biopsies were reclassified as resistant based on the challenge results. Similarly some of the mares tentatively classified as susceptible on the basis of their reproductive histories were reclassified as resistant after the challenge experiments. These observations indicated that while chronic degenerative endometrial changes may predispose a mare to uterine infection, other factors, most probably inherent to the individual mare, were more important. A reliable reproductive history of the mares, especially with reference to previous recurrent uterine infections, was found to be a fairly accurate basis for declaring mares either susceptible or resistant to endometritis. For practical and economic reasons, however, it is suggested that a combination of reproductive history and biopsy findings, despite their low specificity, be used to screen and classify mares.

The immediate cellular response to intrauterine inoculation of the β haemolytic streptococci was characterized by a predominantly neutrophilic response which was similar in both challenged and unchallenged control mares. There was no significant difference in the numbers of neutrophils in uterine washings obtained from resistant and susceptible mares in serial uterine samples obtained after challenge. Neutrophil function tests indicated that there was no significant difference in the chemotactic, phagocytic and intracellular killing ability of both circulating and uterine neutrophils obtained from resistant and susceptible mares. It is suggested that the failure by the susceptible mares to eliminate the infused β haemolytic

streptococci was not due to a deficiency in neutrophils or gross neutrophil functional disorders.

Coinciding with acute phase cellular reaction was an influx of serum proteins including immunoglobulins A, G, G(T) and traces of M. Immunoglobulin A and G reached a peak in uterine washings obtained from both challenged and control mares after 6 to 24 hours. IgA and IgG concentrations in uterine washings obtained from the challenged mares were significantly higher than those measured in uterine washings obtained from control mares. There was, however, no significant difference between the total protein, IgA and IgG concentrations in uterine washings and fluid obtained from resistant and susceptible mares. These findings and the observation that persisting infection resulted in continuing high levels of IgA indicated that the IgA initially entered the uterine lumen by passive diffusion and/or active secretion, and was subsequently produced locally in response to the establishment of the infection in susceptible mares. Therefore it is unlikely that the lowered resistance to uterine infection observed in the mares designated as susceptible was due to a deficiency in IgA or IgG.

The concentrations of ceruloplasmin and alfa 2 globulins, both documented modulators of inflammation, reached their highest levels between 6 and 24 hours after challenge. In contrast the concentrations of trypsin inhibitors (also documented modulators of inflammatory), remained unchanged. The concentrations of natural modulators of inflammation in uterine washings obtained from resistant and susceptible mares did not differ significantly.

In the present study the stage of the reproductive cycle at challenge was determined by using circulating progesterone levels measured by the enzyme immunoassay (EIA), clinical and physical examination of the mares and histological findings in biopsies obtained at 10 days after the begining of the experiments. It was observed that the stage of the cycle did not influence the duration and extent of the response to streptococcal challenge or the persistence of the infused bacteria.

Light and electron microscopic studies showed that the extent of cellular infiltration into the endometrium and the extent of uterine luminal epithelial damage after challenge was similar in both resistant and susceptible mares. This observation and the findings that the concentrations of natural modulators of inflammation (ceruloplasmin, alfa 2 globulins and trypsin inhibitors) in uterine washings obtained from resistant and susceptible mares did not differ significantly, suggested that excessive endometrial damage was unlikely to be the cause of the recurrent uterine infections observed in susceptible mares.

From the data obtained in the present investigations it was concluded that repeated uterine infections, in mares with lowered resistance to endometritis (susceptible), occurred in the presence of IgA and that although these mares were capable of subsequently mounting a local immune response that was comparable to that mounted by the resistant mares, they were still unable to eliminate the infused β haemolytic streptococci. This inability to resolve the experimentally induced endometritis was, however, not due to gross disorders in neutrophil function, lack of immunoglobulins or natural modulators of inflammation or to the stage of the reproductive cycle at challenge.

FUTURE RESEARCH

In my opinion future research should be aimed at -

1. establishing a more objective method of classifying mares into either resistant or susceptible categories.

2. establishing whether susceptible mares are unable to eliminate the invading bacteria due to

    a) time dependent inherent neutrophil functional disorder(s)
    or
    b) an inability to sustain a cellular response
    of both (a) and (b).

3. defining the exact role of

    a) Immunoglobulins IgA, IgG and IgG(T)
    b) Ceruloplasmin
    c) Trypsin inhibitors

in the prevention of establishment and/or subsequent resolution of uterine infections in the mare.

APPENDIX 1

Total protein, ceruloplasmin, Immunoglobulin A and log leucocyte concentrations and concurrent C.F.U. of bacteria in Uterine washings obtained from resistant, susceptible and control mares.

KEY

TFD	=	Too few for a differential count
CFU	=	Colony forming units
O or -	=	Under (Immunoglobulins (A, G ,M)-no reaction (Cytology-negative or absent (Bacteriology - no growth (Ceruloplasmin - no activity (Trypsin inhibitors - no activity
+	=	Positive or present
NAD	=	Nothing abnormal diagnosed
A loopful	=	The standard loops used in this study held 10 µl of fluid

MARE IDENTIFICATION: 35 FINAL (After Challenge) CLASSIFICATION: SUSCEPTIBLE

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.15	1.14		1.60		4.14	1.04	0.37	0.09		0.27
IgA levels (mg% colostrum)	0	33		33		50	33	44	33		25
Leucocyte concentration per ml of uterine washing	0	$2x10^7$		$2x10^7$		$1x10^8$	$8x10^7$	$2x10^5$	$1x10^6$		$<10^2$
Neutrophil concentration per ml uterine washing	T.F.D.	$2x10^7$		$2x10^7$		$1.9x10^7$	$5.8x10^7$		$9x10^5$		
Eosinophil concentration per ml uterine washing	T.F.D.	0		0		0	$3.2x10^6$		$1x10^4$		
Lymphocyte concentration per ml of uterine washing	T.F.D.	$2x10^5$		$4x10^5$		$5x10^6$	$1.9x10^7$		$9x10^4$		
Bacterial C.F.U. per uterine swab	0	0		0		150	80	0	80		0
Bacterial C.F.U. per ml uterine washing	0	90		0		1000	80	0	1000		20
Plasma ceruloplasmin activity in lu/?	0	0		3.125		4.375	0.625	1.25	0		0
Uterine ceruloplasmin activity in lu/?											

MARE IDENTIFICATION: 186 FINAL (After Challenge) CLASSIFICATION: SUSCEPTIBLE

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.61	4.35	4.06	7.26	2.80	0.91	0.53	0.37	0.32	0.20	
IgA levels (mg% colostrum)			N O T		D O N E						
Leucocyte concentration per ml of uterine washing	0	$1.3x10^4$	$3.4x10^4$	$8x10^4$	$1x10^5$	$2.0x10^2$	$3x10^3$	$2x10^3$	$1x10^3$	$2x10^3$	
Neutrophil concentration per ml uterine washing	T.F.D.	$1.3x10^4$	$3.4x10^4$	$7.7x10^4$	$1x10^5$	$1.9x10^3$	$2.9x10^3$		$8x10^2$	$4x10^2$	
Eosinophil concentration per ml uterine washing		0	0	0	0	0	0		$5x10^1$	$1x10^2$	
Lymphocyte concentration per ml of uterine washing		0	0	$3.2x10^3$	0	$6x10^1$	$6x10^1$		$1.5x10^2$	$1.5x10^3$	
Bacterial C.F.U. per uterine swab	0	50	50	0	0	0	0	1000	-	0	
Bacterial C.F.U. per ml uterine washing	0	50	50	0	0	0	0	1000+	1000+	0	
Plasma ceruloplasmin activity in Iu/l	86.563					113.125	111.563	104.375	99.375	101.875	
Uterine ceruloplasmin activity in Iu/l	0	25.0	34.375	8.125	4.375	0	11.25	12.5	28.125	16.25	

MARE IDENTIFICATION: 55 FINAL (After Challenge) CLASSIFICATION: SUSCEPTIBLE

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.41	0.96		1.35		1.68	0.27	0.42	0.53		0.24
IgA levels (mg% colostrum)	33	33		33		54.5	33	38	33		25
Leucocyte concentration per ml of uterine washing	0	$1x10^6$		$1x10^7$		$2x10^5$	$2x10^6$	$4x10^5$	$8x10^5$		$1x10^5$
Neutrophil concentration per ml uterine washing	T.F.D.	$8.9x10^5$		$9.3x10^6$		$1.7x10^5$	$2x10^6$		$7.4x10^5$		
Eosinophil concentration per ml uterine washing		0		0		0	0		$4.8x10^4$		
Lymphocyte concentration per ml of uterine washing		$1.1x10^5$		$7x10^5$		$3.2x10^4$	0		$1.6x10^4$		
Bacterial C.F.U. per uterine swab	0	0		23		27	0	0	70		20
Bacterial C.F.U. per ml uterine washing	0	100		120		0	0	0	0		0
Plasma ceruloplasmin activity in Iu/ml											
Uterine ceruloplasmin activity in Iu/ml	1.25	1.25		0		0	1.875	0	0		0

MARE IDENTIFICATION: .....188..... FINAL (After Challenge) CLASSIFICATION: ....CONTROL.....

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.43	0.80	1.29	3.27		0.85	0.69	0.62	0.34	0.41	
IgA levels (mg% colostrum)			N O T		D O N E						
Leucocyte concentration per ml of uterine washing	0	$1.3x10^3$	$3.7x10^3$	$5.8x10^3$		$2.5x10^3$	$1.5x10^3$	$1x10^3$	$<10^2$	$<10^2$	
Neutrophil concentration per ml uterine washing	0	0	$3.7x10^3$	$5.8x10^3$		$2.5x10^3$	$1.4x10^3$				
Eosinophil concentration per ml uterine washing	T.F.D.		0	0		0					
Lymphocyte concentration per ml of uterine washing			$3.7x10^2$	0		5.10^2	$4.5x10^2$				
Bacterial C.F.U. per uterine swab	0	0	0	0		0	0	0	0	0	
Bacterial C.F.U. per ml uterine washing	0	0	0	0		0	0	0	0	0	
Plasma ceruloplasmin activity in lu/ml	86.88	78.75	79.69	73.13		77.81	6.75	78.75	63.75	96.25	
Uterine ceruloplasmin activity in lu/ml	0.00			No activity detected						0.00	

MARE IDENTIFICATION: 187 FINAL (After Challenge) CLASSIFICATION: CONTROL

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.40	0.95	3.90	3.50		2.50	2.77	0.74	0.35	(bloody) 1.41	
IgA levels (mg% colostrum)			N O	T	D O	N E					
Leucocyte concentration per ml of uterine washing	0	$1.4x10^3$	$3.8x10^3$	$1.4x10^5$		$5x10^3$	$7x10^3$	$2x10^3$	$<10^2$	$<10^2$	
Neutrophil concentration per ml uterine washing	T.F.D.		$3.8x10^3$	$1.4x10^5$		$4.9x10^3$	$7x10^3$	$2.0x10^3$			
Eosinophil concentration per ml uterine washing			0	0		0	70	0			
Lymphocyte concentration per ml of uterine washing			0	$1.4x10^3$		$1x10^2$	70	20			
Bacterial C.F.U. per uterine swab	0	0	0	0		0	0	0	0	0	
Bacterial C.F.U. per ml uterine washing	0	0	0	0		0	0	0	0	0	
Plasma ceruloplasmin activity in Iu/ℓ	77.5	84.38	80.63	85.31		84.38	73.13	78.75	75.0	77.5	
Uterine ceruloplasmin activity in Iu/ℓ	0.00			No activity detected						0.00	

MARE IDENTIFICATION: .....72..... FINAL (After Challenge) CLASSIFICATION: CONTROL .....

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.24	0.64		1.80		1.04	1.0	1.19	0.21		0.15
IgA levels (mg% colostrum)	33	33		38		35	54	33	0		0
Leucocyte concentration per ml of uterine washing	0	$3x10^5$		$1x10^5$		$2x10^6$	$2x10^6$	$2x10^6$	$7x10^5$		$5x10^4$
Neutrophil concentration per ml uterine washing	T.F.D.	$2.6x10^5$		$9x10^4$		$1.3x10^6$	$2x10^6$	$2x10^6$			
Eosinophil concentration per ml uterine washing	T.F.D.	0		0		0	0	0			
Lymphocyte concentration per ml of uterine washing	T.F.D.	$4.5x10^4$		$1x10^4$		$7x10^5$	0	0			
Bacterial C.F.U. per uterine swab	0	0		0		0	0	0	0		0
Bacterial C.F.U. per ml uterine washing	0	0		0		0	0	0	0		0
Plasma ceruloplasmin activity in lu/ℓ			NOT	DONE							
Uterine ceruloplasmin activity in lu/ℓ	0	0		3.125		1.875	1.25	0.625	1.875		1.25

MARE IDENTIFICATION: 144 FINAL (After Challenge) CLASSIFICATION: POOR CONTROL

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	No sample	1.33		3.38		1.74	10.66	6.71	0.05		No sample
IgA levels (mg% colostrum)	54.5	33		33		0	104.5	50	0		
Leucocyte concentration per ml of uterine washing	0	$2x10^6$		$4x10^6$		$7x10^7$	$7x10^5$	$2x10^8$	$5x10^5$		
Neutrophil concentration per ml uterine washing	T.F.D.	$1.9x10^6$		$4x10^6$		$7x10^7$	$7x10^5$	$2x10^8$	$4.9x10^5$		
Eosinophil concentration per ml uterine washing		$8x10^4$		0		0	0	0	$1x10^4$		
Lymphocyte concentration per ml of uterine washing		$5x10^5$		0		0	0	0	$5x10^3$		No sample
Bacterial C.F.U. per uterine swab	0	0		0		1000	20	0	0		0
Bacterial C.F.U. per ml uterine washing	0	0		35		1000	0	0	0		0
Plasma ceruloplasmin activity in lu/%			NOT DONE								
Uterine ceruloplasmin activity in lu/%	0	0		0.625		1.25	0	1.25	1.875		1.875

MARE IDENTIFICATION: 130 FINAL (After Challenge) CLASSIFICATION: POOR CONTROL

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.28	1.0	1.52	2.96		7.68	1.62	1.9	0.72		0.57
IgA levels (mg% colostrum)	0	33	33	33		63	35	33	0		33
Leucocyte concentration per ml of uterine washing	0	$5x10^4$	$8.3x10^4$	$1.1x10^4$		$6.3x10^4$	$5.8x10^5$	$5.5x10^4$	$<10^2$		$5x10^3$
Neutrophil concentration per ml uterine washing	T.F.D.		$8.3x10^4$	$1.1x10^4$		$6.2x10^4$	$5.2x10^5$				
Eosinophil concentration per ml uterine washing			0	0		$1.2x10^3$	$5.8x10^3$				
Lymphocyte concentration per ml of uterine washing			0	0		0	$5.2x10^4$				
Bacterial C.F.U. per uterine swab		0	0	0		25	60	0	0		0
Bacterial C.F.U. per ml uterine washing				NOT	DONE						
Plasma ceruloplasmin activity in lu/ml	78.75	NOT	DONE			93.75	99.375	72.5	82.5		76.875
Uterine ceruloplasmin activity in lu/ml	4.375	5.0	6.875	6.875		5.0	3.75	3.75	4.375		4.375

MARE IDENTIFICATION: 154 FINAL (After Challenge) CLASSIFICATION: POOR CONTROL

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	1.2	2.18	3.86	3.42		1.46	2.0	10.88	0.65		0.96
IgA levels (mg colostrum)	33	37	37	48		33	48	140	33		33
Leucocyte concentration per ml of uterine washing	0	$2.1x10^5$	$2x10^5$	$6.5x10^5$		$2x10^5$	$4.6x10^6$	$1.5x10^7$	$1.0x10^4$		$1x10^4$
Neutrophil concentration per ml uterine washing	T.F.D.	$2.0x10^5$	$2x10^5$	$6.5x10^5$		$1.9x10^5$	$4.1x10^6$	$1.5x10^7$			
Eosinophil concentration per ml uterine washing		0	0	0		$2x10^3$	$3.7x10^5$	0			
Lymphocyte concentration per ml of uterine washing		$8x10^3$	$2x10^3$	0		$6x10^3$	$9.2x10^4$	0			
Bacterial C.F.U. per uterine swab	0	0	0	0		40	0	0	0		0
Bacterial C.F.U. per ml uterine washing				N O T	D O N E						
Plasma ceruloplasmin activity in Iu	71.25		NOT DONE			89.375	88.75	90.0	101.875		104.375
Uterine ceruloplasmin activity in Iu/ml	4.375	8.75	8.75	18.75		8.75	8.125	18.75	0		6.25

MARE IDENTIFICATION: 145 FINAL (After Challenge) CLASSIFICATION: SUSCEPTIBLE

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.44	1.06		1.41		2.13	6.80	2.68	0.24		0.47
IgA levels (mg% colostrum)	126.5	33		No reaction		33	67.5	33	46		40
Leucocyte concentration per ml of uterine washing	0	$7x10^6$		$1x10^7$		$3x10^9$	$2x10^8$	$3x10^5$	6x10		$<10^2$
Neutrophil concentration per ml uterine washing	T.F.D.	$6.5x10^6$		$1x10^7$		$2.9x10^9$	$1.9x10^8$	$2.79x10^8$	$5.6x10^5$		
Eosinophil concentration per ml uterine washing		0		0		0	0	0	3x10		
Lymphocyte concentration per ml of uterine washing		$4.9x10^5$		0		$6x10^8$	$1.4x10^7$	$2.1x10^7$	$6x10^3$		
Bacterial C.F.U. per uterine swab	0	0		0		1000+	500	100	100		0
Bacterial C.F.U. per ml uterine washing	0	0		0		1000+	1000+	1000+	500		60
Plasma ceruloplasmin activity in lu/ml											
Uterine ceruloplasmin activity in lu/ml	0	0		2.5		1.25	5.0	3.75	0		0

MARE IDENTIFICATION: 142 FINAL (After Challenge) CLASSIFICATION: SUSCEPTIBLE

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.66	0.67		5.24		3.70	2.32	1.64	0.85		0.83
IgA levels (mg% colostrum)	96	78.5		54.5		109	161	56.5	50		62
Leucocyte concentration per ml of uterine washing	0	$2x10^6$		$2x10^8$		$4x10^5$	$9x10^5$	$2x10^8$	$8x10^5$		$4x10^6$
Neutrophil concentration per ml uterine washing	T.F.D.	$1.9x10^6$		$1.9x10^8$		$4x10^5$	$3.7x10^5$	$1.8x10^8$	$6.5x10^5$		$3.4x10^6$
Eosinophil concentration per ml uterine washing		$2x10^4$		$2x10^6$		0	0	$1.6x10^7$	$1.1x10^5$		$8x10^4$
Lymphocyte concentration per ml of uterine washing		$4x10^4$		$6x10^6$		0	$3.2x10^4$	$4x10^6$	$4x10^4$		$4.8x10^5$
Bacterial C.F.U. per uterine swab	0	0		0		0	0	1000	300		500
Bacterial C.F.U. per ml uterine washing	0	0		0		0	0	1000	1000		500
Plasma ceruloplasmin activity in lu/ℓ											
Uterine ceruloplasmin activity in lu/ℓ	0	0.625		1.875		0	1.875	0	0		0

MARE IDENTIFICATION: 183 FINAL (After Challenge) CLASSIFICATION: SUSCEPTIBLE

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.42	1.01	3.25	5.20	6.46	1.76	1.07	1.05			
IgA levels (mg% colostrum)				NOT	DONE						
Leucocyte concentration per ml of uterine washing	0	2.2×10^3	5.2×10^4	6.2×10^4	6.6×10^4	8.1×10^4	6.6×10^3	1.8×10^3	No sample		
Neutrophil concentration per ml uterine washing	T.F.D.	2.2×10^3	5.2×10^4	6×10^4	6.2×10^4	7.8×10^4					
Eosinophil concentration per ml uterine washing	T.F.D.	0	0	0	2×10^3	1.6×10^3					
Lymphocyte concentration per ml of uterine washing	T.F.D.	0	0	2×10^3	2×10^3	1.8×10^2					
Bacterial C.F.U. per uterine swab	0	500	500	100	0	0	300	300	50		
Bacterial C.F.U. per ml uterine washing	0	1000	1000	500	20	0	20	250	No sample		
Plasma ceruloplasmin activity in lu/ℓ	98.75					118.438	102.5	106.875	101.75	104.375	
Uterine ceruloplasmin activity in lu/ℓ	0	13.125	16.875	9.375	21.25	1.875	26.25	53.125	ND	43.75	

MARE IDENTIFICATION: 67 FINAL (After Challenge) CLASSIFICATION: RESISTANT

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.36	0.83		1.90		0.40	0.28	0.38	0.38		0.74
IgA levels (mg% colostrum)	0	0		33		0	0	41.5	0		0
Leucocyte concentration per ml of uterine washing	0	$5x10^6$		$1x10^7$		$7x10^5$	$5x10^5$	$1x10^6$	$3x10^5$		$2x10^5$
Neutrophil concentration per ml uterine washing	T.F.D.	$5x10^6$		$1x10^7$		$7x10^5$	$4.4x10^5$	$1x10^6$			
Eosinophil concentration per ml uterine washing		0		0		0	0	0			
Lymphocyte concentration per ml of uterine washing		0		0		0	$6x10^4$	0			
Bacterial C.F.U. per uterine swab	0	0		0		0	0	0	0		0
Bacterial C.F.U. per ml uterine washing	0	0		0		0	0	0	0		0
Plasma ceruloplasmin activity in lu/l											
Uterine ceruloplasmin activity in lu/l	0	0		0		1.25	0	1.25	1.25		0.625

MARE IDENTIFICATION: 48 FINAL (After Challenge) CLASSIFICATION: RESISTANT

DATA SUMMARY SHEET

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.33	0.67	20.69	15.72	0.65	0.43	0.84	0.27	0.23	0.16	
IgA levels (mg% colostrum)				NOT	DONE						
Leucocyte concentration per ml of uterine washing	0	$9x10^3$	$1.4x10^5$	$1.3x10^5$	$1x10^4$	$2x10^5$	$2x10^3$	$5.3x10^3$	$4x10^2$	$2x10^2$	
Neutrophil concentration per ml uterine washing	T.F.D.	$9x10^3$	$1.4x10^5$	$1.3x10^4$	$1x10^4$						
Eosinophil concentration per ml uterine washing	T.F.D.	0	0	0	0						
Lymphocyte concentration per ml of uterine washing	T.F.D.	0	0	$2.6x10^3$	0						
Bacterial C.F.U. per uterine swab	0	1000	1000	1000	0	0	0	0	0	0	
Bacterial C.F.U. per ml uterine washing	0	1000	1000	42	0	0	0	0	0	0	
Plasma ceruloplasmin activity in Iu/-	73.75		NOT	DONE		78.435	78.438	78.438	106.875	108.75	
Uterine ceruloplasmin activity in Iu/-	0	0	0	20	20	18.75	18.125	20.0	25.625	24.375	

MARE IDENTIFICATION ..... 153 FINAL (After Challenge) CLASSIFICATION ..... RESISTANT

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	1.15	1.52	2.20	2.68		1.52	1.26	1.62	1.42		0.72
IgA levels (mg% colostrum)	81	54.5	59	70		89	89	33	33		33
Leucocyte concentration per ml of uterine washing	0	$2.3x10^6$	$5x10^5$	$3.8x10^5$		$2.5x10^4$	$2.1x10^5$	$1.5x10^5$	$7.5x10^3$		$<10^2$
Neutrophil concentration per ml uterine washing	T.F.D.	$2.3x10^6$	$4.8x10^5$	$3.8x10^5$		$2.4x10^4$	$2.1x10^5$	$1.5x10^5$			
Eosinophil concentration per ml uterine washing		0	0			$5x10^2$	0	0			
Lymphocyte concentration per ml of uterine washing		0	$2.5x10^4$			$5x10^2$	0	$3x10^4$			
Bacterial C.F.U. per uterine swab	0	0	0	0		0	200	150	0		0
Bacterial C.F.U. per ml uterine washing			N O T	D O N E							
Plasma ceruloplasmin activity in Iu/ℓ	90.625					116.875	94.375	97.50	99.375		95.0
Uterine ceruloplasmin activity in Iu/ℓ	6.25	6.25	7.5	9.375		8.125	5.0	5.0	3.125		6.25

MARE IDENTIFICATION ....39..... FINAL (After Challenge) CLASSIFICATION ....RESISTANT.....

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.14	0.57		1.40		0.74	0.27	0.59	0.10		0.31
IgA levels (mg% colostrum)	33	0		0		38	33	33	0		0
Leucocyte concentration per ml of uterine washing	0	$7x10^6$		$1x10^7$		$8x10^5$	$2x10^5$	$1x10^6$	$3x10^5$		$<10^2$
Neutrophil concentration per ml uterine washing	T.F.D.	$6.4x10^6$		$1x10^7$		$7.9x10^5$		$1x10^6$			
Eosinophil concentration per ml uterine washing	T.F.D.	0		0		0	$2x10^5$	0			
Lymphocyte concentration per ml of uterine washing	T.F.D.	$5.6x10^5$		0		$8x10^3$		0			
Bacterial C.F.U. per uterine swab	0	0		0		0	0	0	0		0
Bacterial C.F.U. per ml uterine washing	0	0		0		0	100	20	0		0
Plasma ceruloplasmin activity in Iu/ℓ			N O T		D O N E						
Uterine ceruloplasmin activity in Iu/ℓ	0	0		0		0	0	0	0		0

MARE IDENTIFICATION ...68..... FINAL (After Challenge) CLASSIFICATION ..... RESISTANT

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.66	3.14	4.92	6.40		3.80	1.35	1.25	0.64		0.40
IgA levels (mg% colostrum)	33	0	33	33		46	33	33	33		35
Leucocyte concentration per ml of uterine washing	0	$1.5x10^5$	$5x10^4$	$6x10^5$		$3x10^6$	$3.2x10^5$	$5x10^3$	$5x10^3$		$3x10^3$
Neutrophil concentration per ml uterine washing	T.F.D.		$4.8x10^4$	$6x10^5$		$2.9x10^6$	$2.9x10^5$				
Eosinophil concentration per ml uterine washing			0	0		$3x10^4$	$2.5x10^4$				
Lymphocyte concentration per ml of uterine washing			$2x10^3$	0		$9x10^4$	$6x10^3$				
Bacterial C.F.U. per uterine swab	0	80	90	500		25	0	0	0		0
Bacterial C.F.U. per ml uterine washing			N O T	D O N E							
Plasma ceruloplasmin activity in Iu/ℓ	98.125					146.25	129.375	141.25	88.125		90.625
Uterine ceruloplasmin activity in Iu/ℓ	19.688	22.188	24.375			25.625	0	0	0		0

MARE IDENTIFICATION 71 FINAL (After Challenge) CLASSIFICATION RESISTANT

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.29	0.96		4.14		1.30	1.35	4.45	0.40		0.83
IgA levels (mg% colostrum)	33	0		33		33	33	41.5	0		0
Leucocyte concentration per ml of uterine washing	0	$1x10^6$		$9x10^7$		$3x10^6$	$2x10^5$	$1x10^7$	$5x10^5$		$2x10^4$
Neutrophil concentration per ml uterine washing	T.F.D.	$9.5x10^6$		$8.1x10^7$		$3x10^6$	$1.9x10^5$	$5.2x10^6$			
Eosinophil concentration per ml uterine washing		0		0		0	$6x10^3$	$4x10^5$			
Lymphocyte concentration per ml of uterine washing		$5x10^4$		$4.5x10^6$		0	$4x10^3$	$4.4x10^6$			
Bacterial C.F.U. per uterine swab	0	0		0		0	50	200	0		0
Bacterial C.F.U. per ml uterine washing	0	0		0		0	0	120	0		0
Plasma ceruloplasmin activity in Iu/ℓ				N O T		D O N E					
Uterine ceruloplasmin activity in Iu/ℓ	0.625	0.625		3.75		1.25	2.5	0	5.0		0

DATA SUMMARY SHEET

MARE IDENTIFICATION ....160..... FINAL (After Challenge) CLASSIFICATION ..RESISTANT.....

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.74	1.23	2.58	3.18		4.92	5.24	1.12	0.82		1.38
IgA levels (mg% colostrum)	0	0	0	33		52	37	70	0		0
Leucocyte concentration per ml of uterine washing	0	$8.5x10^4$	$1.3x10^5$	$1.3x10^5$		$2.4x10^5$	$3.6x10^5$	$3x10^4$	$2.5x10^3$		$<10^2$
Neutrophil concentration per ml uterine washing	T.F.D.	$8.3x10^4$	$1.3x10^5$	$1.3x10^5$		$2.4x10^5$	$3.6x10^5$	$3x10^4$			
Eosinophil concentration per ml uterine washing	T.F.D.	0	0	0		0	0	0			
Lymphocyte concentration per ml of uterine washing	T.F.D.	$1.7x10^3$	0	0		$4.8x10^3$	0	0			
Bacterial C.F.U. per uterine swab	0	0	0	30		0	40	0	0		0
Bacterial C.F.U. per ml uterine washing				NOT		DONE					
Plasma ceruloplasmin activity in Iu/ℓ	82.188	N	O T	D O N E		102.50	101.75	111.563	116.875		78.438
Uterine ceruloplasmin activity in Iu/ℓ	0	1.875	20.625	20.0		22.5	21.875	31.875	31.875		16.875

MARE IDENTIFICATION ....161.....

FINAL (After Challenge) CLASSIFICATION ..... RESISTANT

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.31	0.88	8.68	6.08	0.75	0.63	0.43	0.97	No sample		
IgA levels (mg% colostrum)											
Leucocyte concentration per ml of uterine washing	0	$2.4x10^4$	$1.3x10^5$	$4.5x10^4$	$1.0x10^4$	$1.6x10^3$	$6x10^2$	$2.2x10^3$	No sample		
Neutrophil concentration per ml uterine washing	T.F.D.	$2.4x10^4$	$1.3x10^5$	$4.5x10^4$	$9.4x10^3$	$1.6x10^3$	$6x10^2$				
Eosinophil concentration per ml uterine washing		0	0	0	$2x10^2$	0	0				
Lymphocyte concentration per ml of uterine washing		0	0	$4.5x10^2$	$4x10^2$	$1.6x10^1$	0				
Bacterial C.F.U. per uterine swab	0	500	50	0	0	0	500	0	0		
Bacterial C.F.U. per ml uterine washing	0	100	100	0	0	0	50	0	0		
Plasma ceruloplasmin activity in Iu/ℓ	90.625	N O T	D O N E			96.875	76.25	104.35	123.124	-	141.25
Uterine ceruloplasmin activity in Iu/ℓ	0	5.0	8.125	6.875	5.0	5.0	5.0	9.375	9.375	-	9.375

MARE IDENTIFICATION 141

FINAL (After Challenge) CLASSIFICATION ..... RESISTANT

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.37	1.52		3.52		4.64	6.0	0.95	0.42		0.24
IgA levels (mg% colostrum)	41.5	33		33		78.5	67.8	78.5	50		0
Leucocyte concentration per ml of uterine washing	0	$5x10^6$		$2x10^7$		$5x10^8$	$9x10^7$	$4x10^5$	$4x10^5$		$1x10^4$
Neutrophil concentration per ml uterine washing	T.F.D.	$4.5x10^6$		$2x10^7$		$4.9x10^8$	$7.4x10^7$	$4x10^5$			
Eosinophil concentration per ml uterine washing		0		0		0	$9x10^5$	$1.6x10^4$			
Lymphocyte concentration per ml of uterine washing		$5x10^5$		$4x10^5$		$1.5x10^7$	$1.2x10^7$	$8x10^4$			
Bacterial C.F.U. per uterine swab	0	0		0		0	40	0	0		0
Bacterial C.F.U. per ml uterine washing	0	0		0		0	0	0	0		0
Plasma ceruloplasmin activity in Iu/ℓ				N O T		D O N E					
Uterine ceruloplasmin activity in Iu/ℓ	0	0		0		4.375	0	0.625	0		0

MARE IDENTIFICATION ....155..... FINAL (After Challenge) CLASSIFICATION ..... RESISTANT

	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	4 days	10 days	14 days	28 days
Protein in mg/ml of uterine washing	0.66	1.30	2.42	1.92		4.92	6.60	1.04	1.71		1.38
IgA levels (mg% colostrum)	0	0	33	33		81	109	41.5	44		33
Leucocyte concentration per ml of uterine washing	0	$7x10^4$	$1.3x10^5$	$2x10^5$		$28x10^5$	$1.2x10^5$	$8x10^4$	$6.5x10^4$		$4x10^4$
Neutrophil concentration per ml uterine washing	T.F.D.	$6.4x10^4$	$5.2x10^4$	$6x10^4$		$6.2x10^4$	$7.8x10^4$				
Eosinophil concentration per ml uterine washing	T.F.D.	0	0	0		$2x10^3$	$1.6x10^3$				
Lymphocyte concentration per ml of uterine washing	T.F.D.	$4x10^3$	$1.3x10^5$	$2x10^3$		$2x10^3$	$8x10^2$				
Bacterial C.F.U. per uterine swab	0	80	0	0		20	50	0	0		0
Bacterial C.F.U. per ml uterine washing			N O T			D O N E					
Plasma ceruloplasmin activity in Iu/ℓ	103.75					109.375	106.875	108.75	130.0		113.125
Uterine ceruloplasmin activity in Iu/ℓ	0	5.0	8.125	1.25		1.25	0.625	10.0	3.75		0

APPENDIX 2

Total protein, ceruloplasmin, Immunoglobulin A and log leucocyte concentrations and concurrent C.F.U. of bacteria in Uterine washings obtained from resistant, susceptible and control mares.

KEY

TFD = Too few for a differential count

CFU = Colony forming units

0 or - = Under (Immunoglobulins (A, G, M) - no reaction
(Cytology-negative or absent
(Bacteriology - no growth
(Ceruloplasmin - no activity
Trypsin inhibitors - no activity

\+ = Positive or present

NAD = Nothing abnormal diagnosed

A loopful = The standard loops used in this study held 10 µl of fluid

MARE NUMBER ..... 202 ..... AFTER CHALLENGE CLASSIFICATION ..... SUSCEPTIBLE .....

SUMMARY SHEET

	00 hours	3 hours	6 hours	9 hours	12 hours	15 hours	24 hours	10 days
Protein in mg/ml of uterine washing	1.14		27.13		4.97	6.86	2.48	ND
Leucocyte concentration per ml of uterine washing	0	4.35	5.82	5.15	4.71	4.24	3.48	
Percent Neutrophils	T.F.D.	99	100	98	99	97	100	90
Percent Eosinophils		0	0	2	1	2	0	5
Percent Lymphocytes		1	0	0	0	1	0	5
Bacterial C.F.U. per uterine swab	0	2000	200	0	0	0	0	200
Bacterial C.F.U. per loopful of uterine washing	0	$5x10^5$	$1.2x10^5$	$4.2x10^3$	$1x10^2$	$1.8x10^3$	0	$1x10^5$

MARE NUMBER ..... 207 ..... AFTER CHALLENGE CLASSIFICATION .. SUSCEPTIBLE .....

SUMMARY SHEET

	00 hours	3 hours	6 hours	9 hours	12 hours	15 hours	24 hours	10 days
Protein in mg/ml of uterine washing	0.36	4.94	18.75	19.25	6.41	5.00	7.94	1.06
Leucocyte concentration per ml of uterine washing	0	4.14	4.49	4.72	4.35	3.36	3.34	ND
Percent Neutrophils	0	100	100	100	99	98	98	90
Percent Eosinophils	T.F.D.	0	0	0	1	2	1	5
Percent Lymphocytes		0	0	0	0	0	1	5
Bacterial C.F.U. per uterine swab	0	500	1500	1000	200	59	0	0
Bacterial C.F.U. per loopful of uterine washing	0	$1x10^7$	$1.2x10^5$	$9x10^4$	$6.5x10^3$	$1x10^4$	$4x10^3$	$8x10^3$

MARE NUMBER ..... 204 ..... AFTER CHALLENGE CLASSIFICATION ..... SUSCEPTIBLE .....

SUMMARY SHEET

	00 hours	3 hours	6 hours	9 hours	12 hours	15 hours	24 hours	10 days
Protein in mg/ml of uterine washing	0	0.15	2.13	2.86	1.40	1.63	3.82	3.08
Leucocyte concentration per ml of uterine washing	0	4.15	4.48	3.63	3.58	3.32	3.87	
Percent Neutrophils	T.F.D.	100	100	99	100	99	98	100
Percent Eosinophils		0	0	1	0	0	2	0
Percent Lymphocytes		0	0	0	0	1	0	0
Bacterial C.F.U. per uterine swab	0	150	28	0	0	0	0	200
Bacterial C.F.U. per loopful of uterine washing	0	9.5×10^3	5×10^2	0	0	0	2.5×10^2	1×10^7

MARE NUMBER 108 ..... AFTER CHALLENGE CLASSIFICATION ..SUSCEPTIBLE.....

SUMMARY SHEET

	00 hours	3 hours	6 hours	9 hours	12 hours	15 hours	24 hours	10 days
Protein in mg/ml of uterine washing	3.19	9.41	9.25	14.81	4.57	3.84	1.85	0.5
Leucocyte concentration per ml of uterine washing	0	3.94	4.46	4.78	3.52	3.65	3.97	
Percent Neutrophils	T.F.D.	100	100	99	97	98	100	85
Percent Eosinophils	T.F.D.	0	0	1	2	2	0	5
Percent Lymphocytes	T.F.D.	0	0	0	1	0	0	10
Bacterial C.F.U. per uterine swab	0	1500	1500	22	17	18	1000	85
Bacterial C.F.U. per loopful of uterine washing	0	$1x10^7$	$3.2x10^4$	$1.0x10^4$	$5.5x10^2$	$2x10^2$	$1.2x10^4$	$8.5x10^3$

MARE NUMBER ..... 206 ..... AFTER CHALLENGE CLASSIFICATION ... SUSCEPTIBLE .....

SUMMARY SHEET

	00 hours T.F.D.	3 hours	6 hours	9 hours	12 hours	15 hours	24 hours	10 days
Protein in mg/ml of uterine washing	0.49	28.00	26.13	6.14	ND	11.75	6.75	
Leucocyte concentration per ml of uterine washing	0	4.53	4.34	4.16	3.94	3.54	4.03	
Percent Neutrophils		100	100	100	100	97	99	95
Percent Eosinophils		0	0	0	0	1	1	3
Percent Lymphocytes		0	0	0	0	2	0	2
Bacterial C.F.U. per uterine swab	0	1000	500	500	28	0	70	0
Bacterial C.F.U. per loopful of uterine washing	0	4.7×10^5	3.1×10^4	4.5×10^3	4.2×10^2	1×10^3	0	1×10^3

MARE NUMBER ..... 201 ..... AFTER CHALLENGE CLASSIFICATION ... RESISTANT .....

SUMMARY SHEET

	00 hours	3 hours	6 hours	9 hours	12 hours	15 hours	24 hours	10 days
Protein in mg/ml of uterine washing	0.83	5.75	35.25	26.38	ND	7.37	12.00	0.38
Leucocyte concentration per ml of uterine washing	0	4.23	5.03	4.92	4.48	4.50	3.30	
Percent Neutrophils	T.F.D.	100	100	100	98	96	99	0
Percent Eosinophils		0	0	0	2	4	1	0
Percent Lymphocytes		0	0	0	0	0	0	0
Bacterial C.F.U. per uterine swab	0	500	500	250	0	0	110	0
Bacterial C.F.U. per loopful of uterine washing	0	$1x10^7$	$5.5x10^5$	$2.4x10^5$	$2.1x10^4$	$3.8x10^4$	$1.5x10^3$	0

MARE NUMBER ..... 203 AFTER CHALLENGE CLASSIFICATION ..... RESISTANT .....

SUMMARY SHEET

	00 hours	3 hours	6 hours	9 hours	12 hours	15 hours	24 hours	10 days
Protein in mg/ml of uterine washing	0.70	2.60	32.25	19.20	6.14	3.46	ND	0.49
Leucocyte concentration per ml of uterine washing	0	4.30	6.67	4.68	4.35	4.47	4.02	
Percent Neutrophils	T.F.D.	100	100	99	98	98	99	0
Percent Eosinophils		0	0	1	0	2	1	0
Percent Lymphocytes		0	0	0	2	0	0	0
Bacterial C.F.U. per uterine swab	0	1500	500	185	23	0	0	0
Bacterial C.F.U. per loopful of uterine washing	0	$1x10^7$	$2.5x10^5$	$1x10^5$	$3.2x10^3$	$9x10^2$	$5x10^2$	0

MARE NUMBER ....93..... AFTER CHALLENGE CLASSIFICATION ....RESISTANT.....

SUMMARY SHEET

	00 hours	3 hours	6 hours	9 hours	12 hours	15 hours	24 hours	10 days
Protein in mg/ml of uterine washing	0.34	2.54	5.91	1.49	0.79	1.24	2.08	1.34
Leucocyte concentration per ml of uterine washing	0	4.70	4.46	3.85	3.74	3.62	3.96	
Percent Neutrophils	T.F.D.	100	99	98	98	100	100	
Percent Eosinophils		0	0	1	1	0	0	
Percent Lymphocytes		0	1	1	1	0	0	
Bacterial C.F.U. per uterine swab	0	300	22	0	0	0	0	0
Bacterial C.F.U. per loopful of uterine washing	0	$3x10^5$	$7x10^3$	0	0	0	0	0

Appendix 3 Log leucocyte concentration/ml of uterine washings collected from susceptible, resistant and control mares before and after infection

	0 hrs	2 hrs	4 hrs	6 hrs	24 hrs	48 hrs	4 days	10 days	14 days	28 days
Resistant[a] (n=11)	0	*7.3±0.18	3.95	7.7±0.56	7.4±0.36	6.6±0.26	6.9±0.25	5.81±0.12	2.3	4.0±0.81
Susceptible[a] (n=5)	0	6.3±0.36	4.6± 0.1	7.4±0.98	8.4±0.94	8.2±0.72	8.0±0.98	6.7±0.27	3.3	3.44±1.88
Controls[a] (n=3)	0	4.1±0.69	3.6	5.0±0.69	4.8±1.21	4.2±1.21	4.4±1.21	3.3±1.33	2.0	4.7

* mean ± standard error (SE)

Values in row with same superscripts did not differ significantly ($p>0.05$)

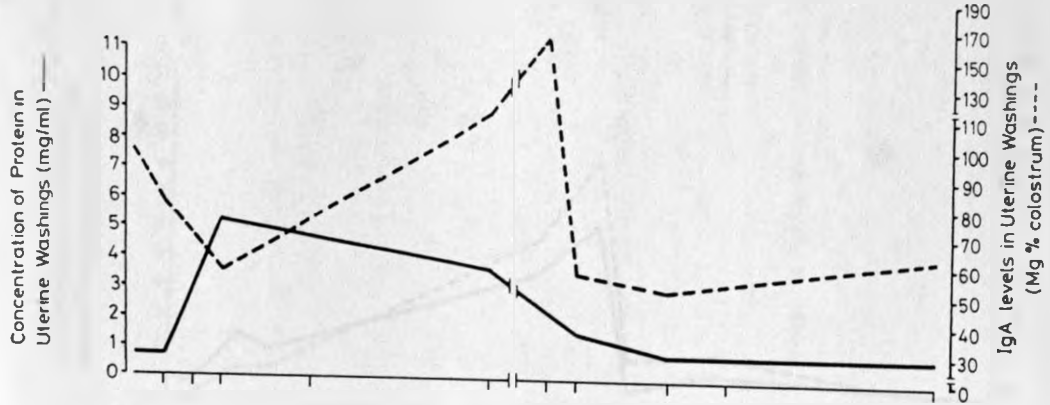
Appendix 4 Log concentrations of β haemolytic Streptococci (per ml) in uterine washings obtained from resistant and susceptible mares after the induction of acute endometritis with β haemolytic Streptococci

Mare Identification Number	Uterine Washing Obtained At:							
	0 hrs	3 hrs	6 hrs	9 hrs	12 hrs	15 hrs	24 hrs	10 days
39(2)	0	5.74	3.85	0	0	0	0	0
201	0	7.0	5.74	5.38	4.32	4.58	3.18	0
203	0	7.0	5.40	5.0	3.51	2.95	2.70	0
Mean ± SE	0	6.58±0.42	5.0±.58	3.46±1.73	2.61±1.33	2.51±1.34	1.96±0.99	0
180(2)	0	7.0	4.51	4.0	2.74	2.3	4.08	3.93
202	0	5.70	5.08	3.62	2.0	3.26	0	5.0
204	0	3.98	2.70	0	0	0	2.4	7.0
206	0	5.67	4.49	3.65	2.62	3.0	0	3.0
207	0	7.0	5.08	4.95	3.81	4.0	3.60	3.90
Mean ± SE	0	5.87±0.56	4.36±.44	3.24±.85	2.23±.63	2.54±0.68	2.02±0.87	4.60±0.69

Concentration of Neutrophils (——)
Eosinophils (·····) and Lymphocytes (- -
per ml of Uterine Washings
too few for a differential count (---)
Total ß- Hemolytic Streptococcus
Colony forming units (cfu) per
Loopful of Uterine Washings (——)
and Uterine Swab (----)
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10⁹ cfu)

APPENDIX 5

Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 142 - susceptible.



Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (- - -) per ml of Uterine Washings.
too few for a differential count (---)

10^{2} 10^{3} 10^{4} 10^{5} 10^{6} 10^{7} 10^{8}

Total β- Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (----)

0 100 200 300 400 500 600 700 800 900 1000

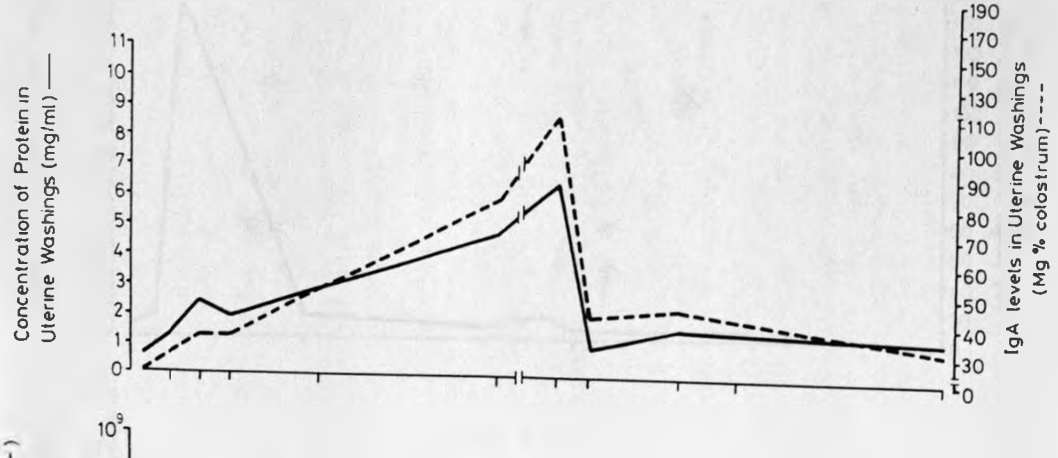
0 2 4 6 8 10 12 14 16 18 20 22 24 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28

Hours —— Days

Time post inoculation with β Hemolytic Streptococcus (at least $X10^{8}$ cfu)

APPENDIX 5

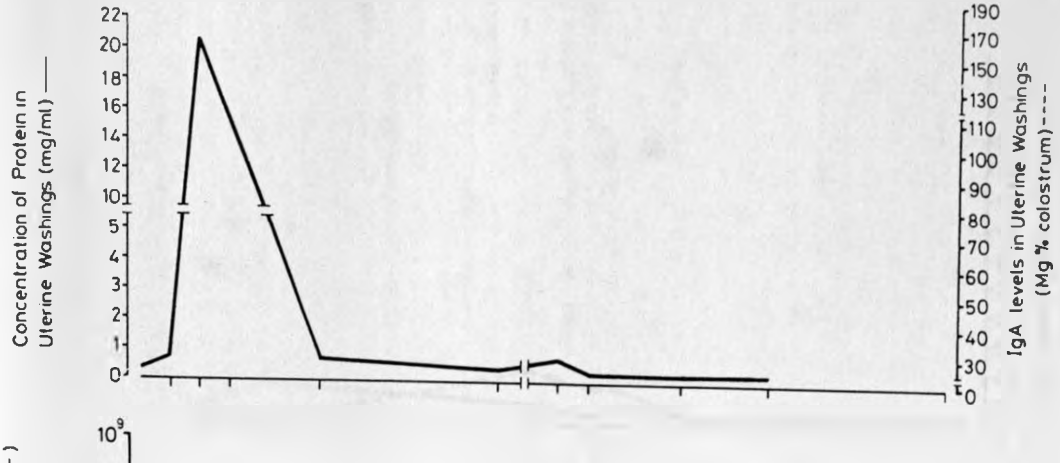
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 155 - resistant.

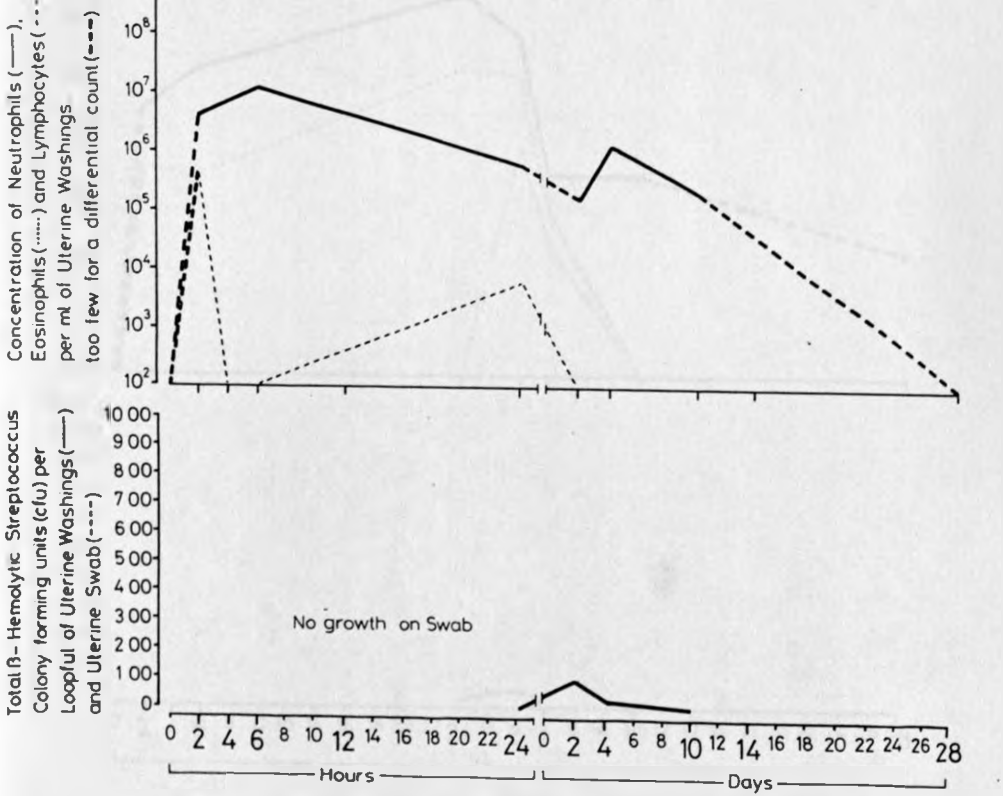


Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (- -) per ml of Uterine Washings
too few for a differential count (•••)
10^2
10^3
10^4
10^5
10^6
10^7
10^8
Total ß- Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (----)
0
100
200
300
400
500
600
700
800
900
1000
0 2 4 6 8 10 12 14 16 18 20 22 24 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10^9 cfu)

APPENDIX 3

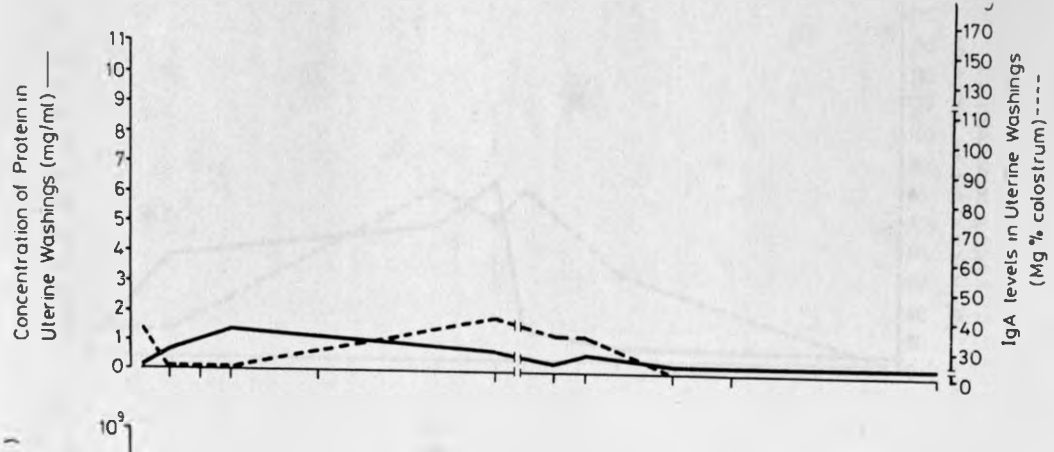
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 48 - resistant.

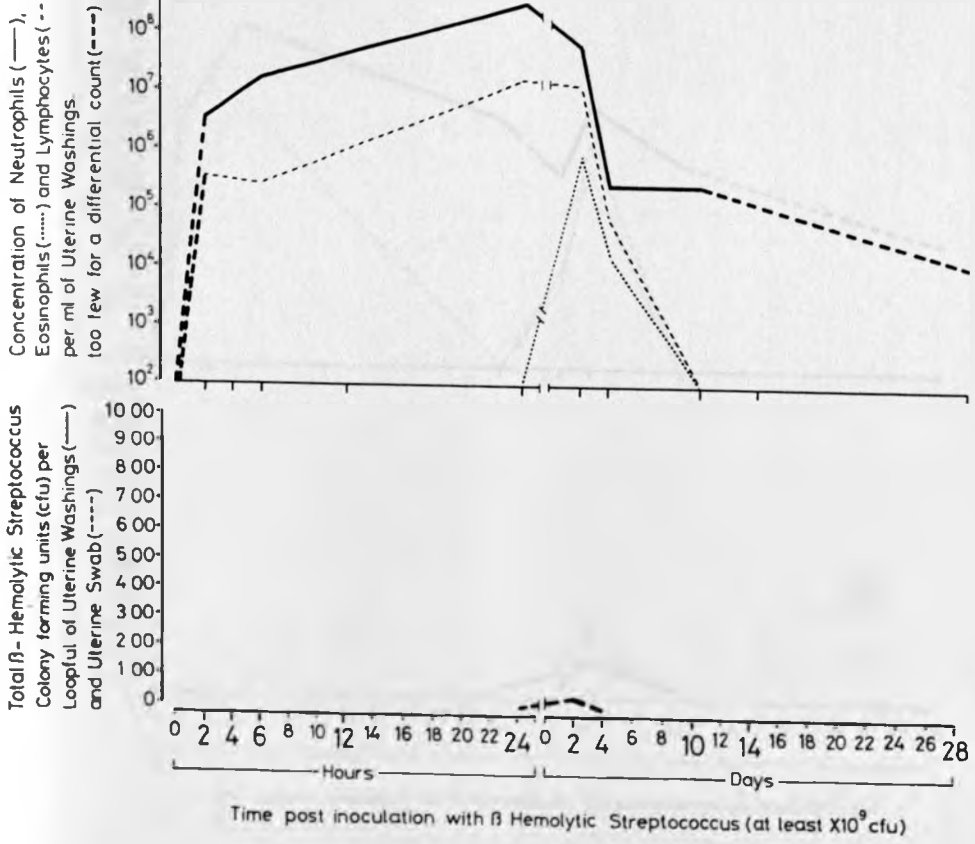



Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (- - -) per ml of Uterine Washings too few for a differential count (- - -)
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
Total ß- Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (- - -)
10 00
9 00
8 00
7 00
6 00
5 00
4 00
3 00
2 00
1 00
0
No growth on Swab
0 2 4 6 8 10 12 14 16 18 20 22 24 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10⁹ cfu)

APPENDIX 5

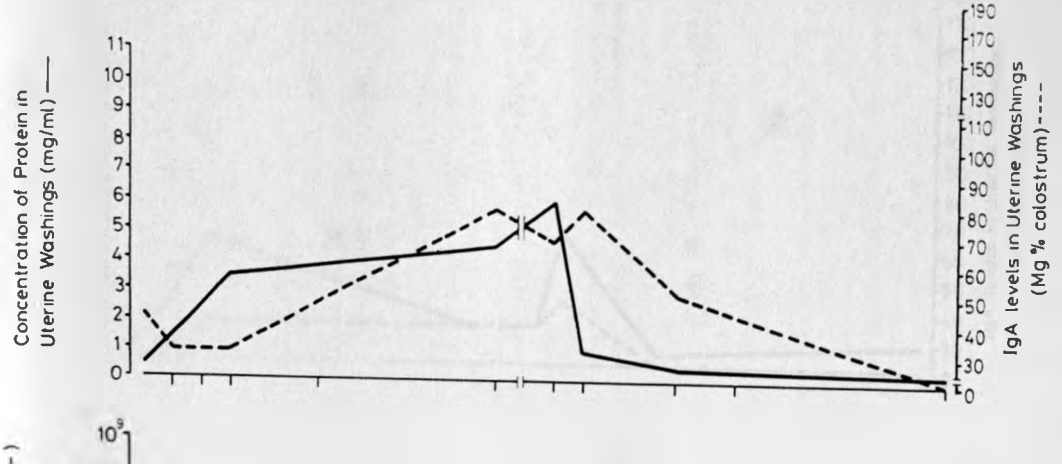
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 39 - resistant.

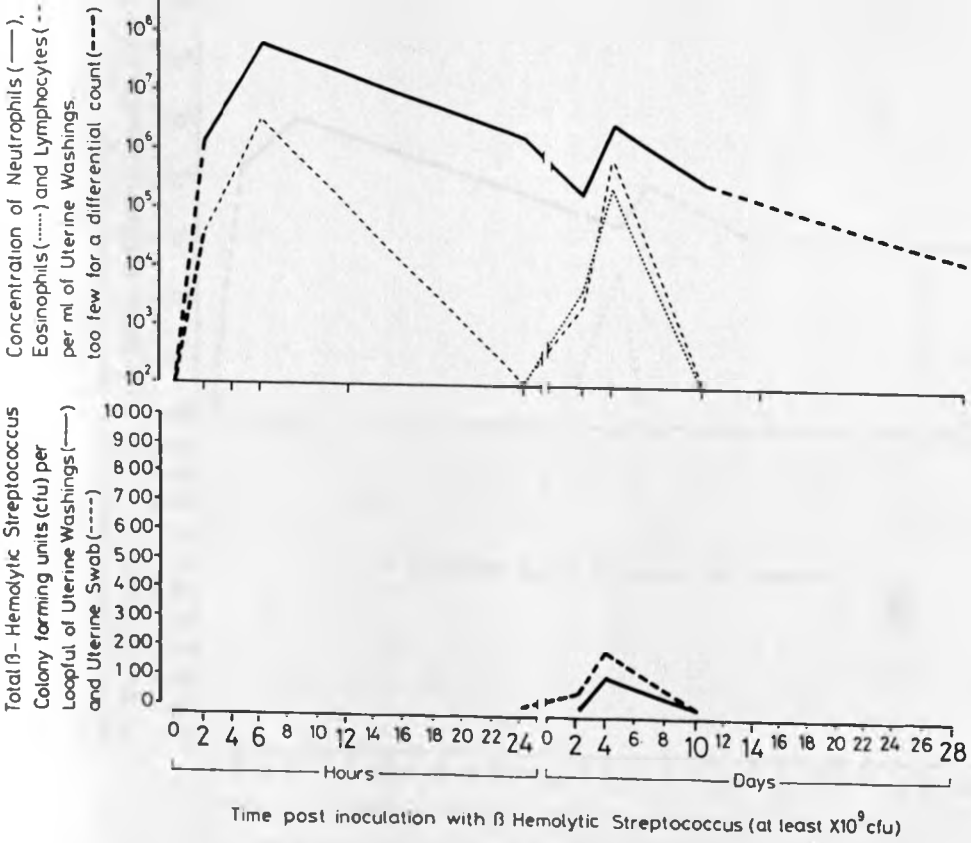



Concentration of Neutrophils (——),
Eosinophils (·····) and Lymphocytes (- - -)
per ml of Uterine Washings
too few for a differential count (- - -)
Total ß- Hemolytic Streptococcus
Colony forming units (cfu) per
Loopful of Uterine Washings (——)
and Uterine Swab (- - - -)
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10⁹ cfu)

APPENDIX 5

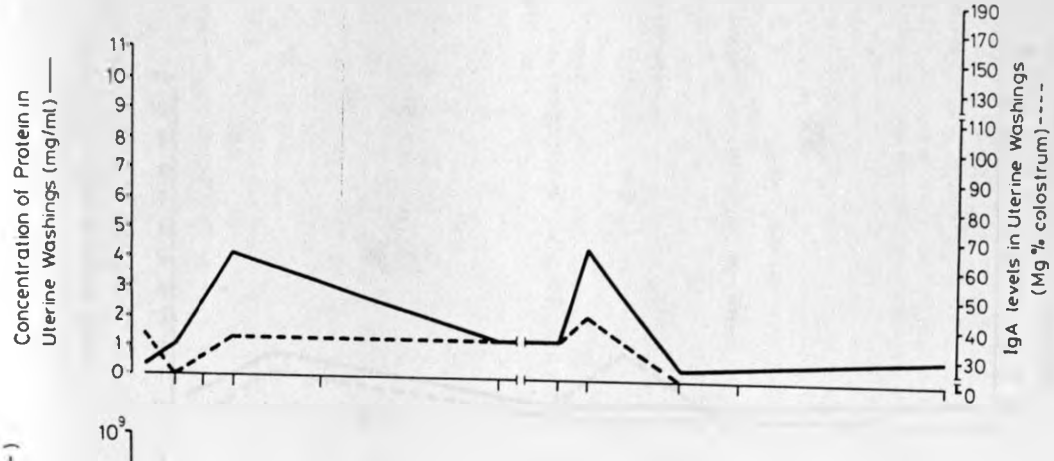
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 141 - resistant.

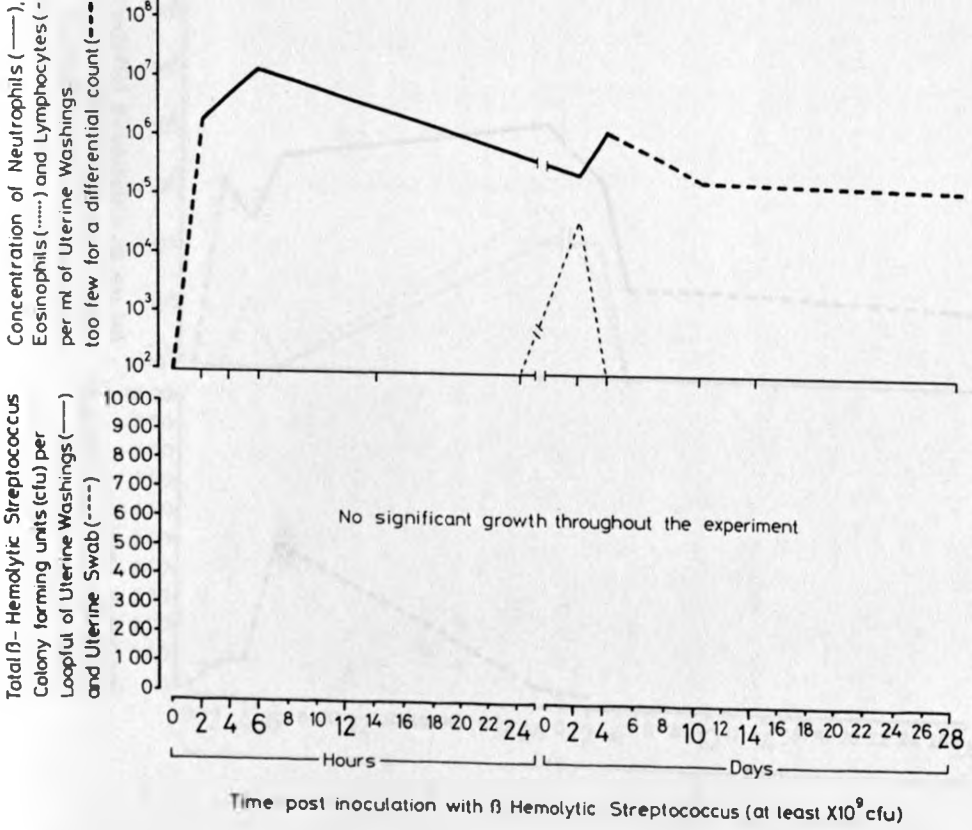



Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (- - -) per ml of Uterine Washings too few for a differential count (- - -)
Total ß-Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (- - - -)
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10⁹ cfu)

APPENDIX 5

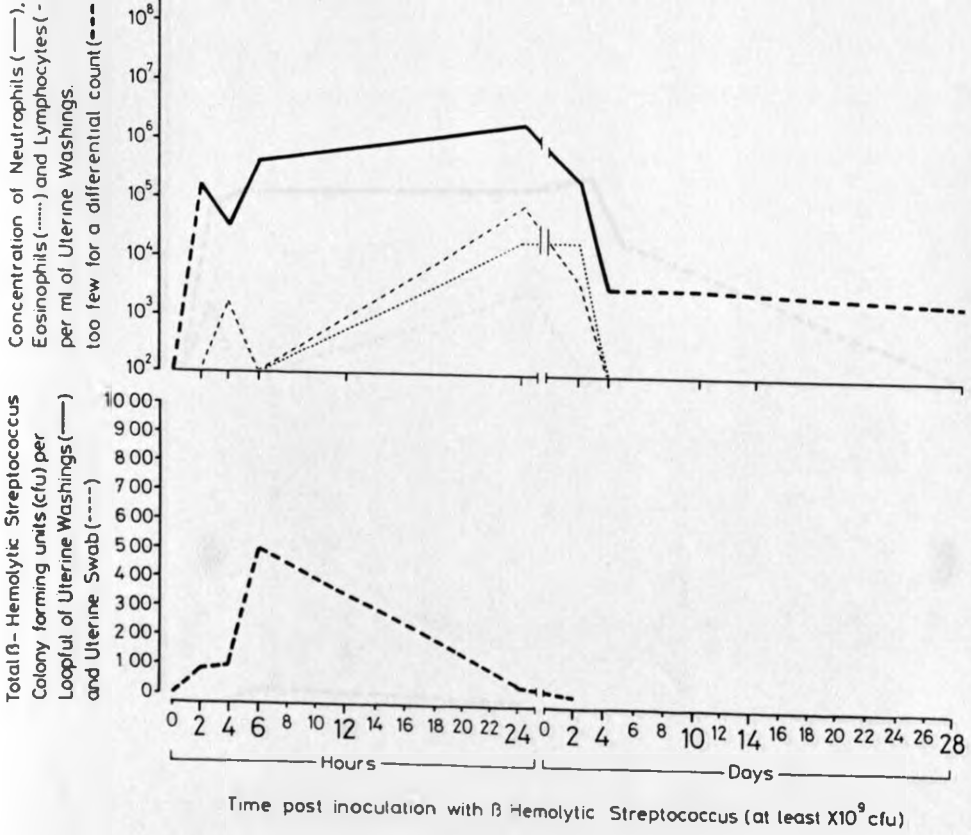
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 71 - resistant.




Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (- - -) per ml of Uterine Washings too few for a differential count
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Total ß- Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (----)
10 00
9 00
8 00
7 00
6 00
5 00
4 00
3 00
2 00
1 00
0
No significant growth throughout the experiment
0 2 4 6 8 10 12 14 16 18 20 22 24
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10^9 cfu)

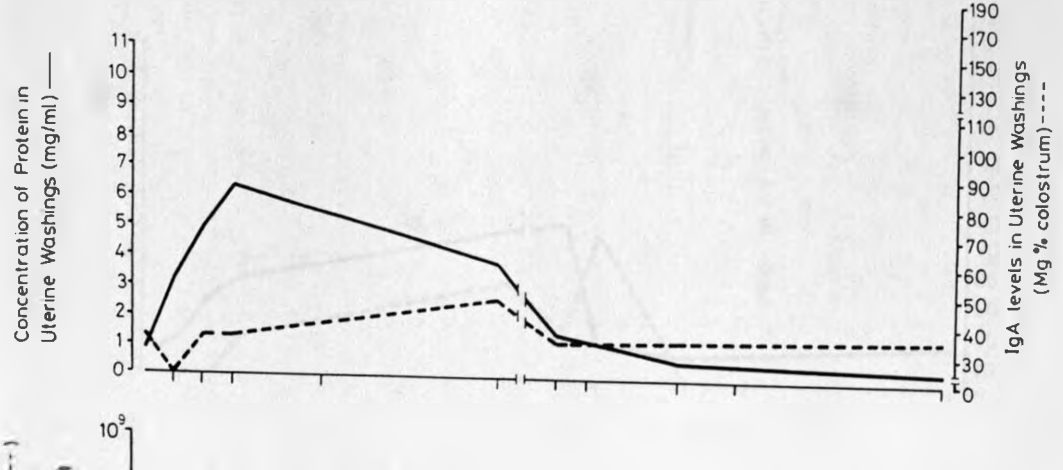
APPENDIX E

Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 68 - resistant.


Concentration of Neutrophils (——), Eosinophils (-----) and Lymphocytes (- - - -) per ml of Uterine Washings. too few for a differential count (• • • •)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Total ß- Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (- - - -)
10 00
9 00
8 00
7 00
6 00
5 00
4 00
3 00
2 00
1 00
0
0 2 4 6 8 10 12 14 16 18 20 22 24 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10^9 cfu)

APPENDIX 5

Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 67 - resistant.



Concentration of Neutrophils (——),
Eosinophils (·····) and Lymphocytes (-
per ml of Uterine Washings
too few for a differential count (---

10^2 10^3 10^4 10^5 10^6 10^7 10^8

Total ß- Hemolytic Streptococcus
Colony forming units (cfu) per
Loopful of Uterine Washings (——)
and Uterine Swab (----)

0 100 200 300 400 500 600 700 800 900 1000

0 2 4 6 8 10 12 14 16 18 20 22 24 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28

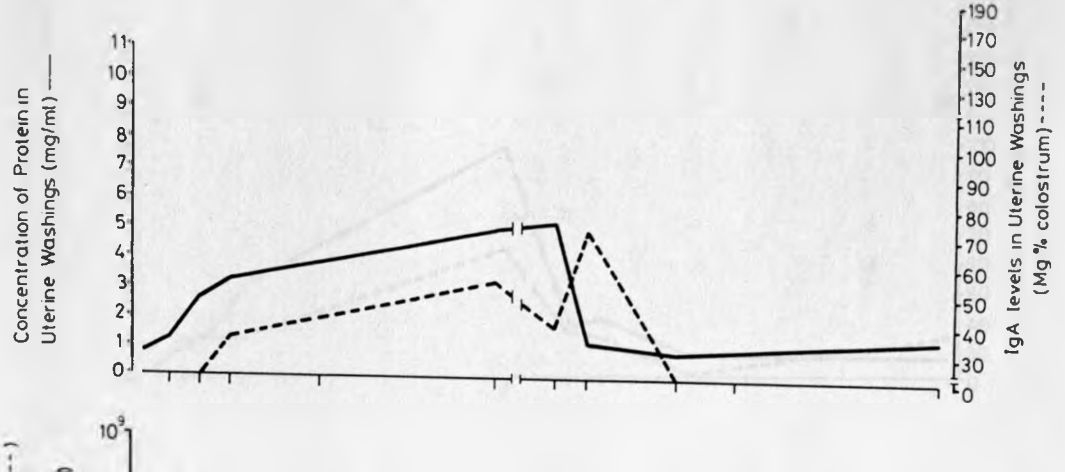
Hours

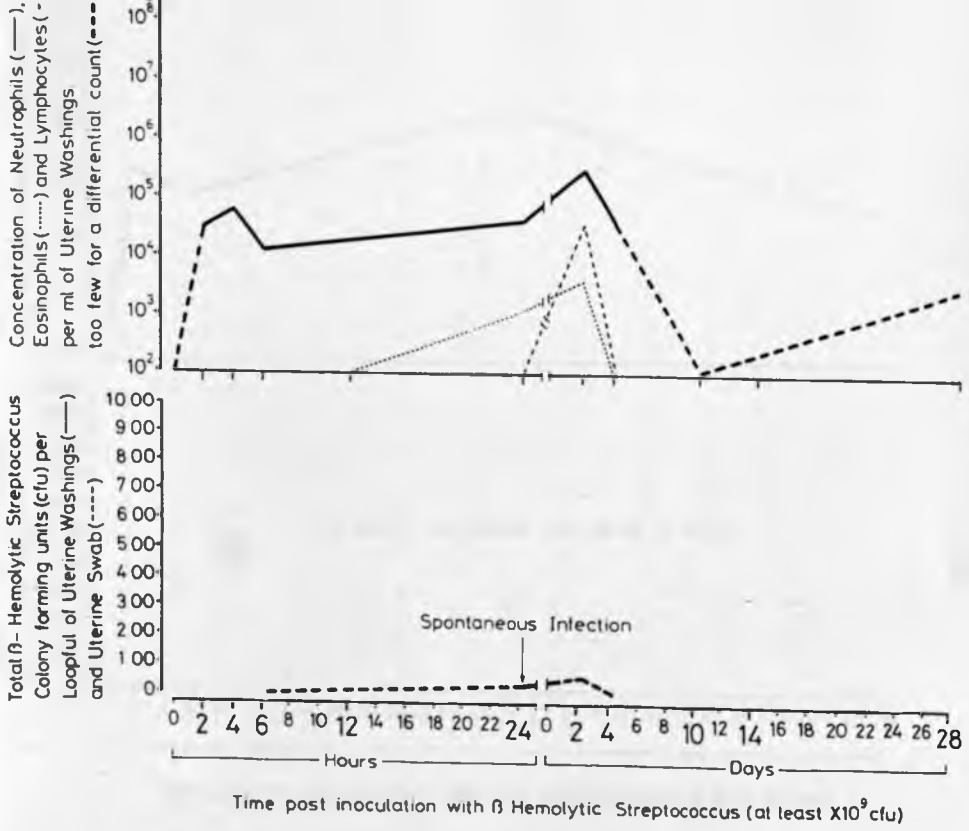
Days

Time post inoculation with ß Hemolytic Streptococcus (at least X10^9 cfu)

APPENDIX 5

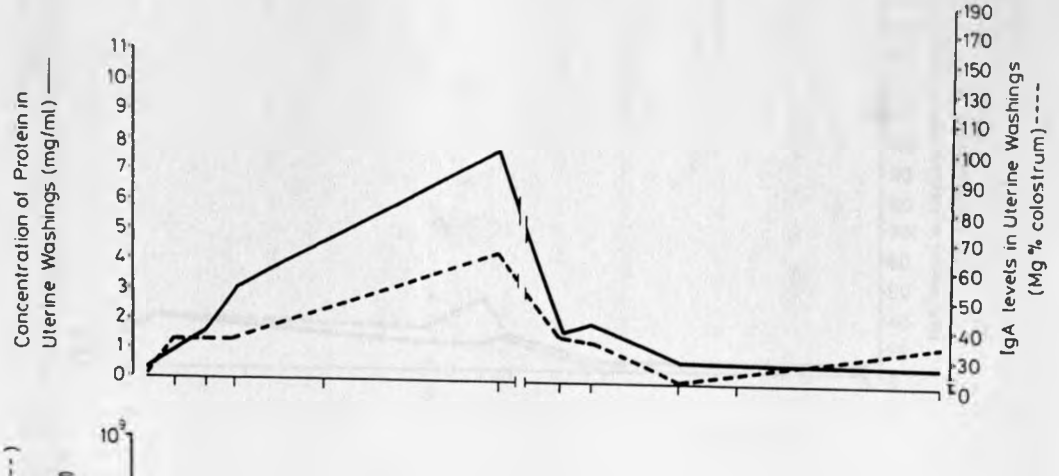
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 160 - resistant.

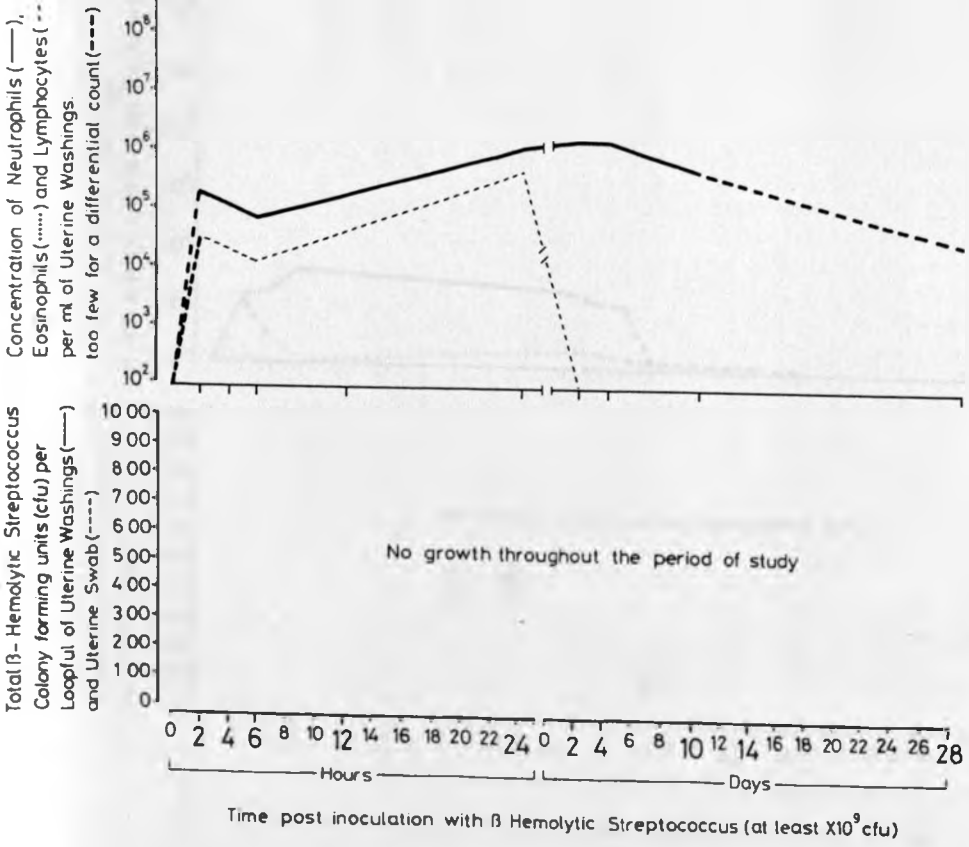



Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (- - -) per ml of Uterine Washings too few for a differential count
Total β- Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (- - - -)
Spontaneous Infection
Hours
Days
Time post inoculation with β Hemolytic Streptococcus (at least X10⁹ cfu)

APPENDIX B

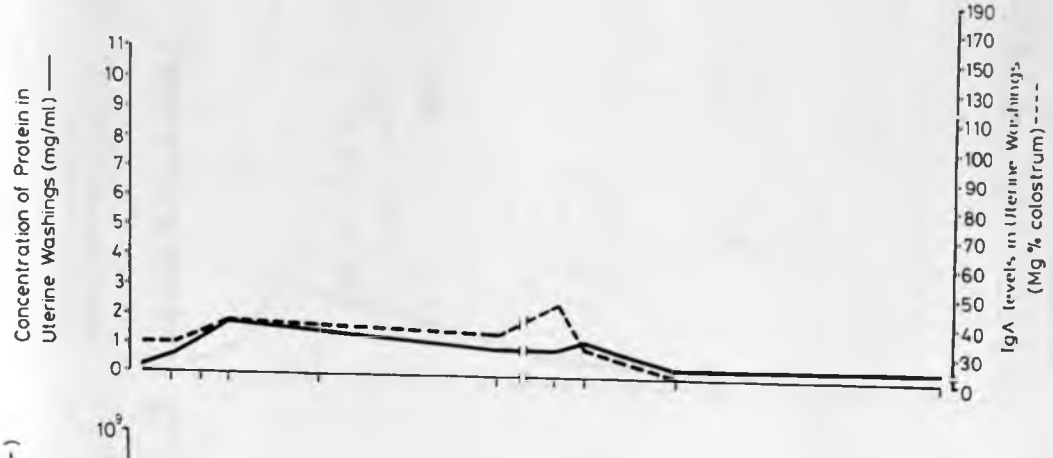
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 130 - susceptible.

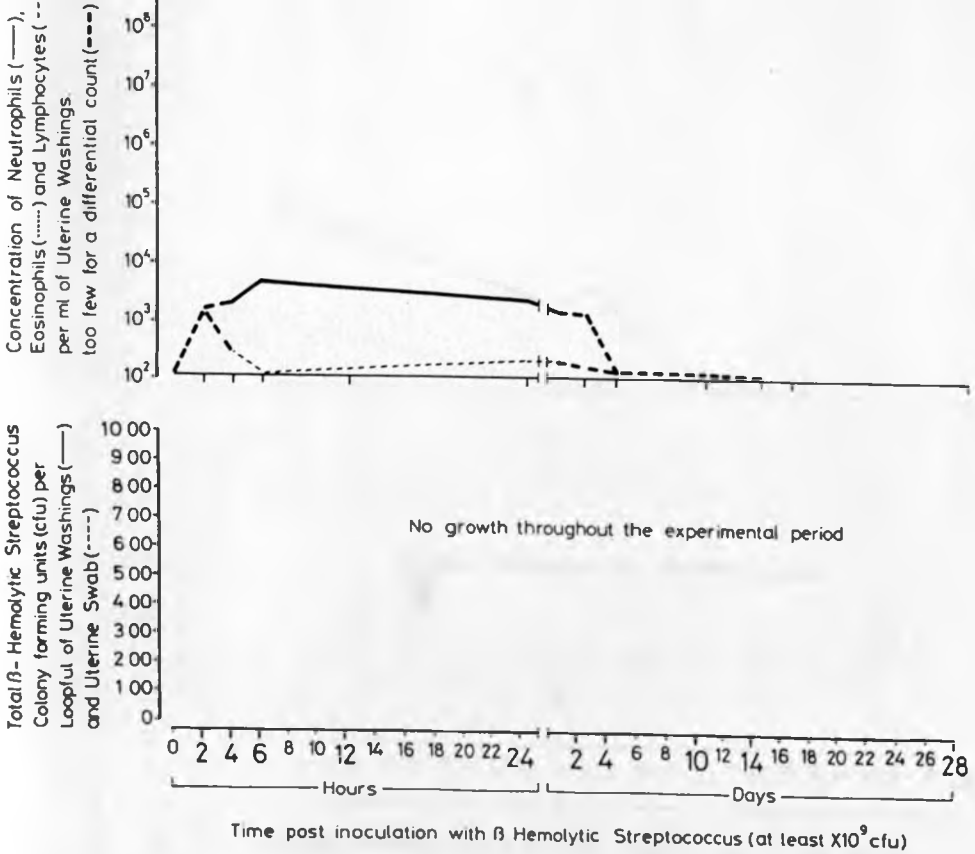



Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (- - -) per ml of Uterine Washings too few for a differential count (- - -)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Total ß- Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (- - - -)
10 00
9 00
8 00
7 00
6 00
5 00
4 00
3 00
2 00
1 00
0
No growth throughout the period of study
0 2 4 6 8 10 12 14 16 18 20 22 24
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10^9 cfu)

APPENDIX 5

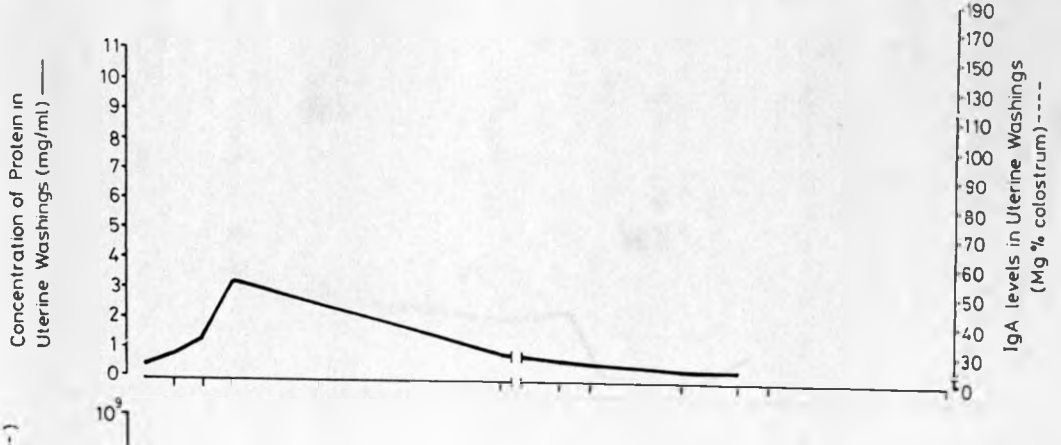
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 72 - control.

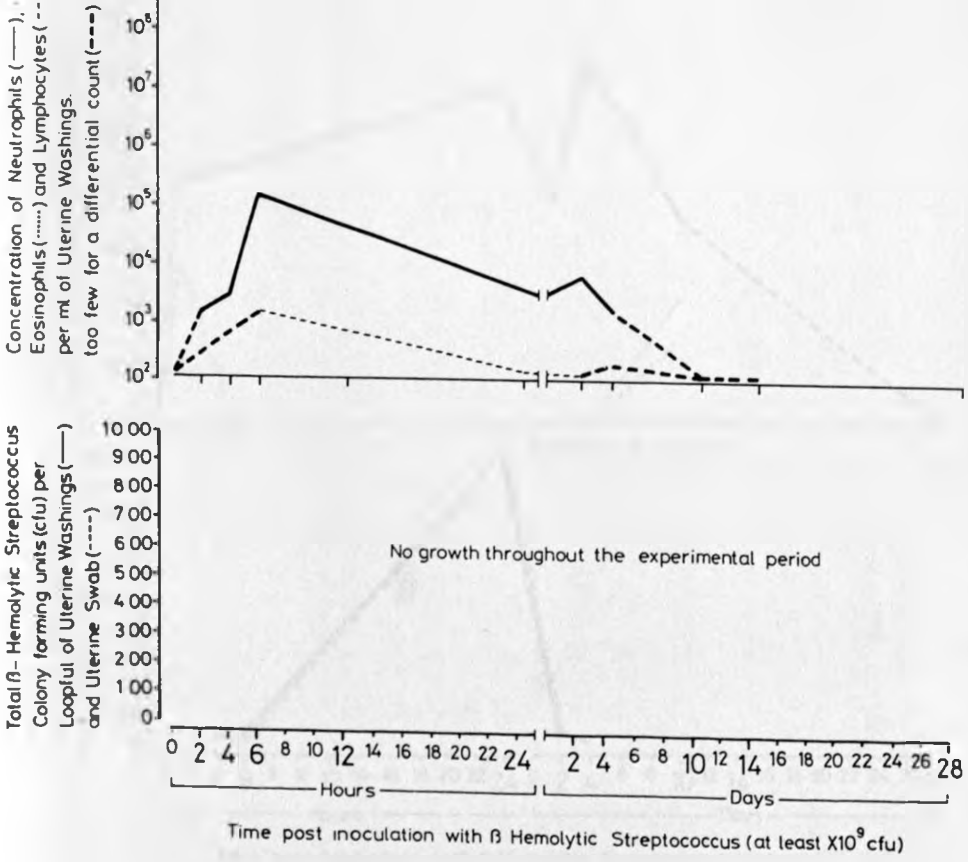



Concentration of Neutrophils (——),
Eosinophils (·····) and Lymphocytes (- - -)
per ml of Uterine Washings.
too few for a differential count (▪▪▪)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Total ß- Hemolytic Streptococcus
Colony forming units (cfu) per
Loopful of Uterine Washings (——)
and Uterine Swab (- - - -)
10 00
9 00
8 00
7 00
6 00
5 00
4 00
3 00
2 00
1 00
0
No growth throughout the experimental period
0 2 4 6 8 10 12 14 16 18 20 22 24 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10^9 cfu)

APPENDIX 5

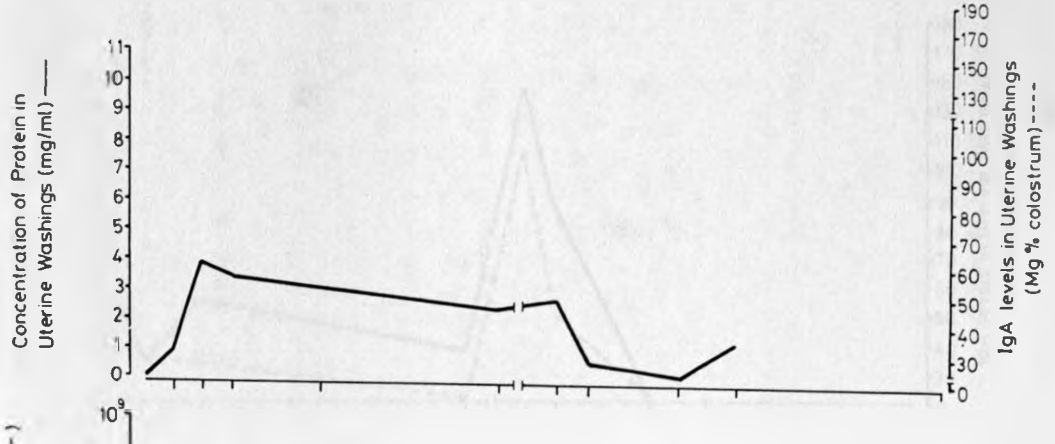
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 188 - control.




Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (- - -) per ml of Uterine Washings.
too few for a differential count (---)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Total ß- Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (----)
10 00
9 00
8 00
7 00
6 00
5 00
4 00
3 00
2 00
1 00
0
No growth throughout the experimental period
0 2 4 6 8 10 12 14 16 18 20 22 24
2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10^9 cfu)

APPENDIX 5

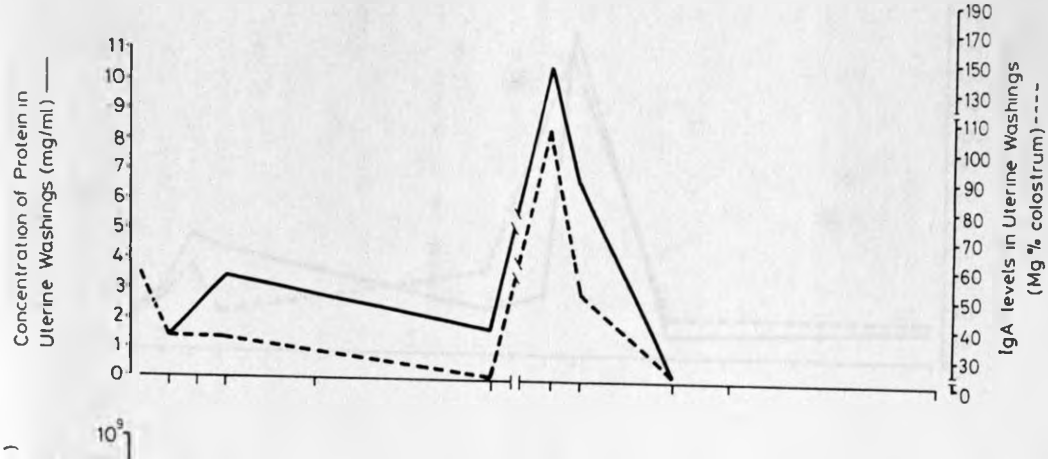
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 187 - control.

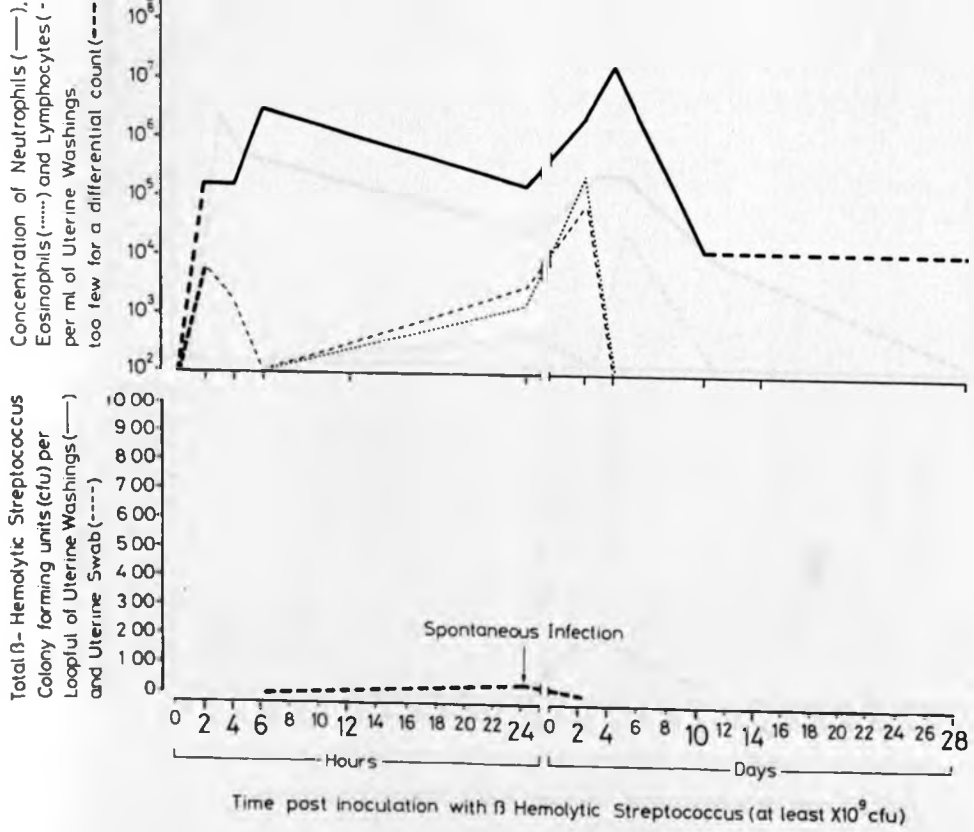


Concentration of Neutrophils (——),
Eosinophils (·····) and Lymphocytes (- - -)
per ml of Uterine Washings.
too few for a differential count (•••)
10^{2} 10^{3} 10^{4} 10^{5} 10^{6} 10^{7} 10^{8}
Spontaneous Infection
Total ß- Hemolytic Streptococcus
Colony forming units (cfu) per
Loopful of Uterine Washings (——)
and Uterine Swab (----)
0 100 200 300 400 500 600 700 800 900 1000
0 2 4 6 8 10 12 14 16 18 20 22 24
Hours
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10^{9} cfu)

APPENDIX 5

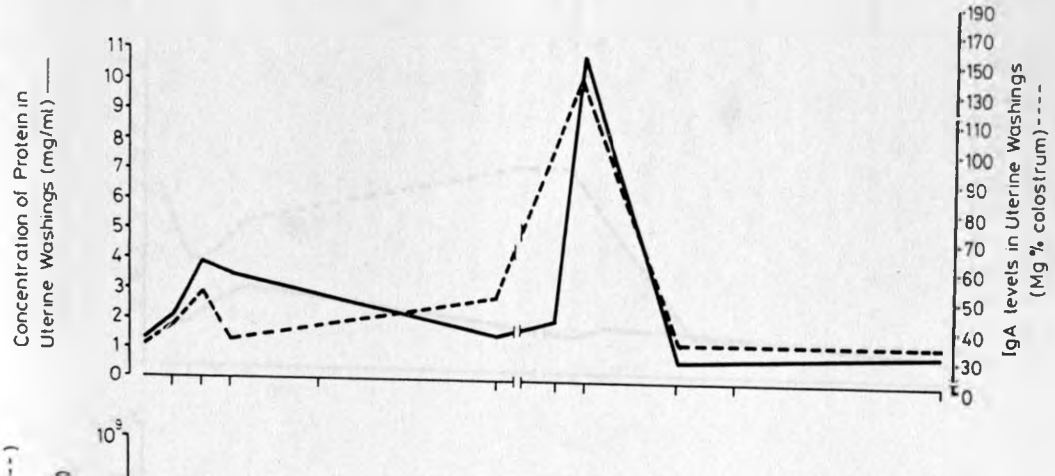
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 144 - susceptible.

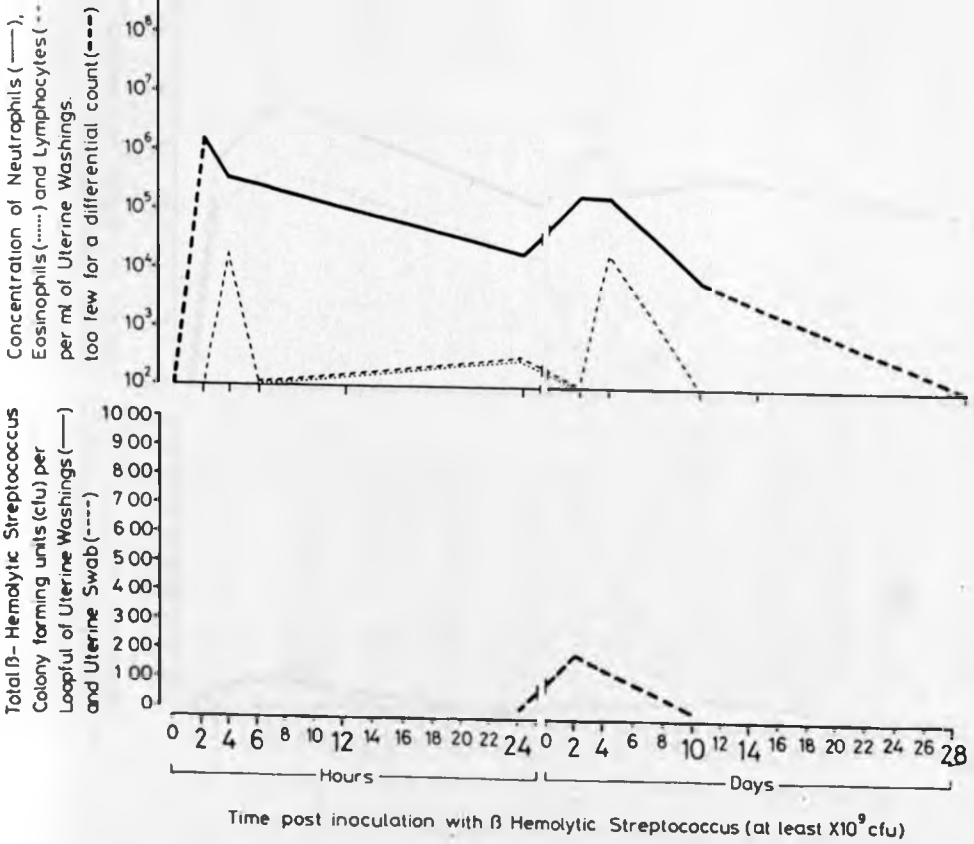



Concentration of Neutrophils (——),
Eosinophils (·····) and Lymphocytes (- -
per ml of Uterine Washings
too few for a differential count (- - -
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Total β- Hemolytic Streptococcus
Colony forming units (cfu) per
Loopful of Uterine Washings (——)
and Uterine Swab (- - - -)
1000
900
800
700
600
500
400
300
200
100
0
Spontaneous Infection
0 2 4 6 8 10 12 14 16 18 20 22 24
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with β Hemolytic Streptococcus (at least X10^9 cfu)

APPENDIX 5

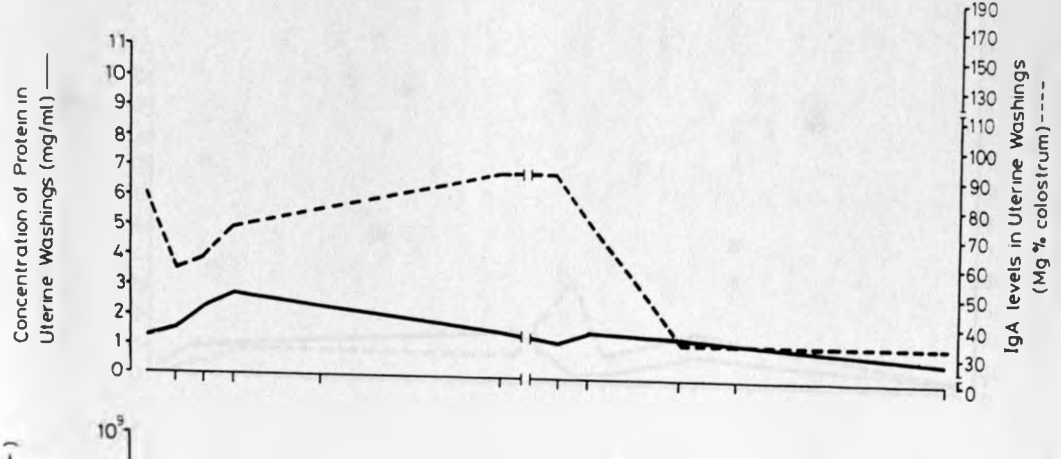
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 154 - susceptible.




Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (- - -) per ml of Uterine Washings. too few for a differential count
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Total ß- Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (- - - -)
10 00
9 00
8 00
7 00
6 00
5 00
4 00
3 00
2 00
1 00
0
0 2 4 6 8 10 12 14 16 18 20 22 24 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10^9 cfu)

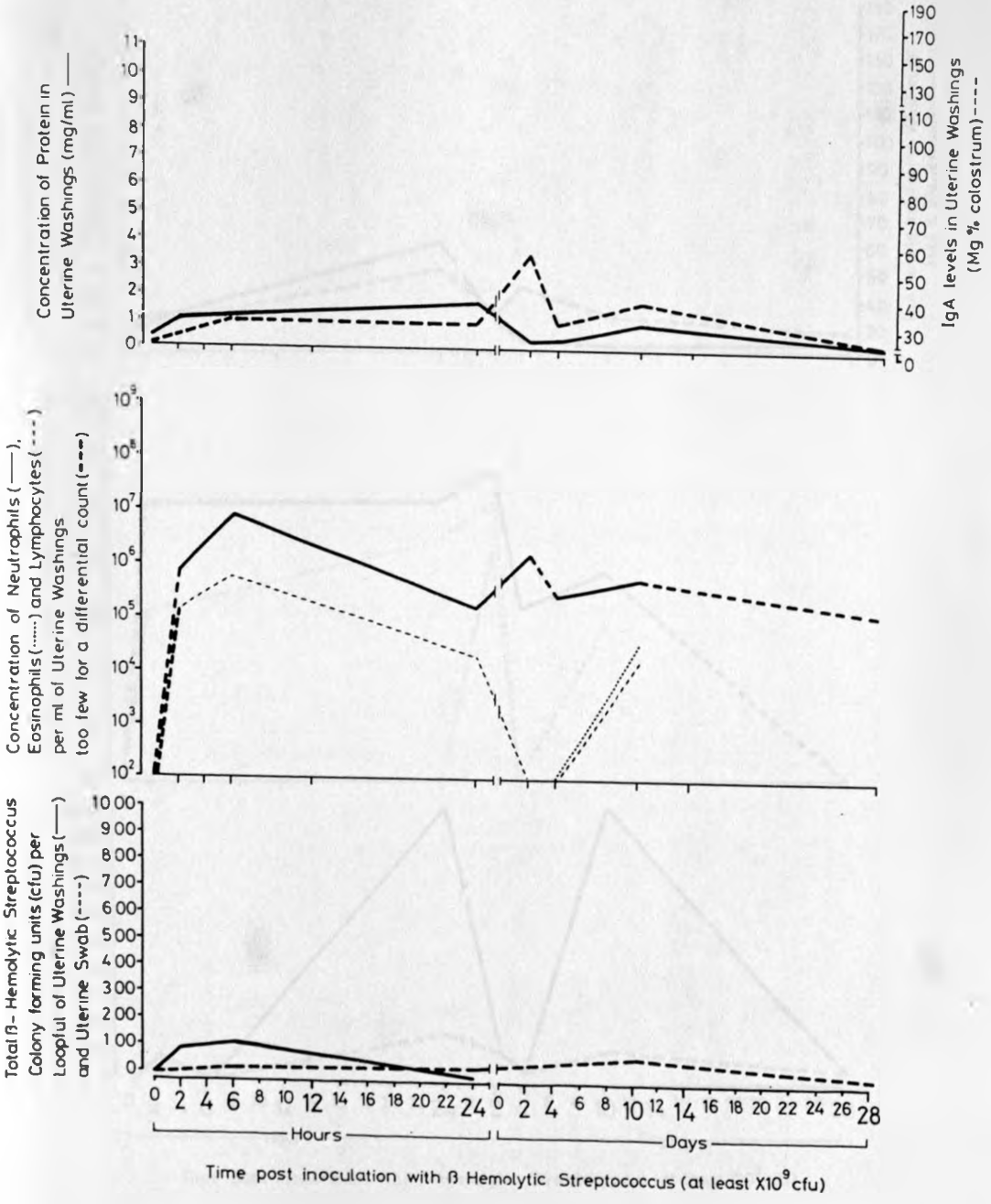
APPENDIX 5

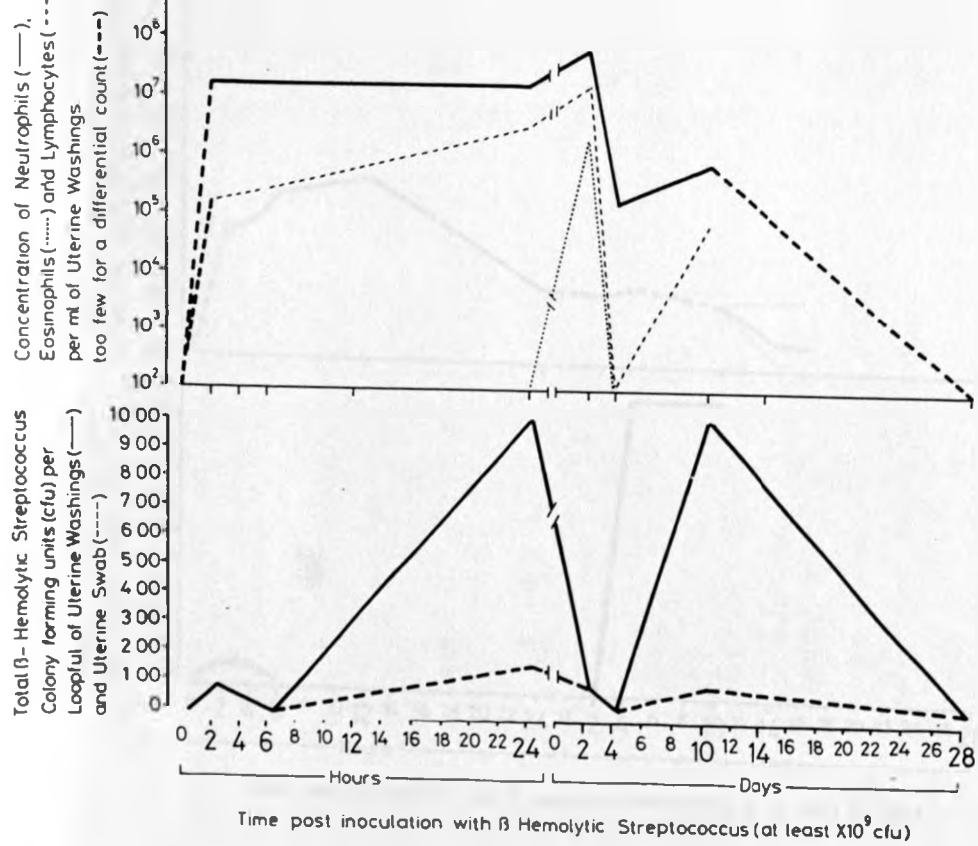
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 153 - resistant.



APPENDIX 5

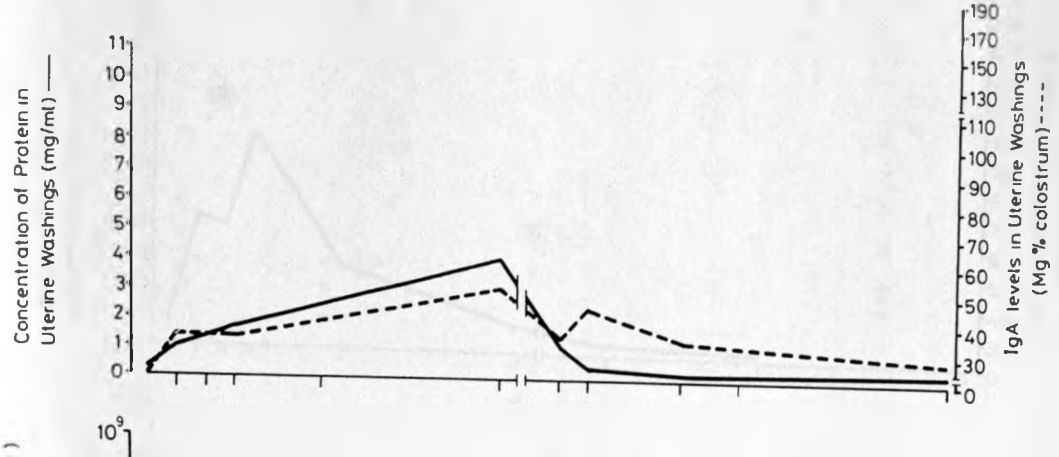
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 55 - susceptible.

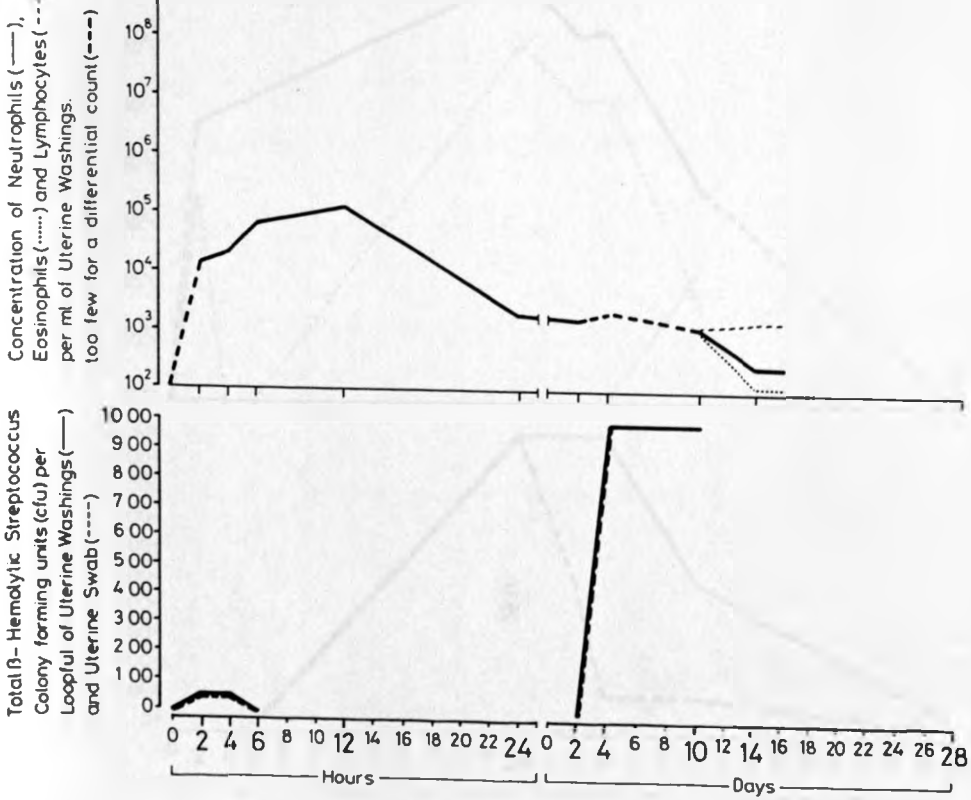



Concentration of Neutrophils (——),
Eosinophils (·····) and Lymphocytes (- - -)
per ml of Uterine Washings
too few for a differential count (- - -)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Total ß- Hemolytic Streptococcus
Colony forming units (cfu) per
Loopful of Uterine Washings (——)
and Uterine Swab (- - -)
10 00
9 00
8 00
7 00
6 00
5 00
4 00
3 00
2 00
1 00
0
0 2 4 6 8 10 12 14 16 18 20 22 24 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with ß Hemolytic Streptococcus (at least X10^9 cfu)

APPENDIX 5

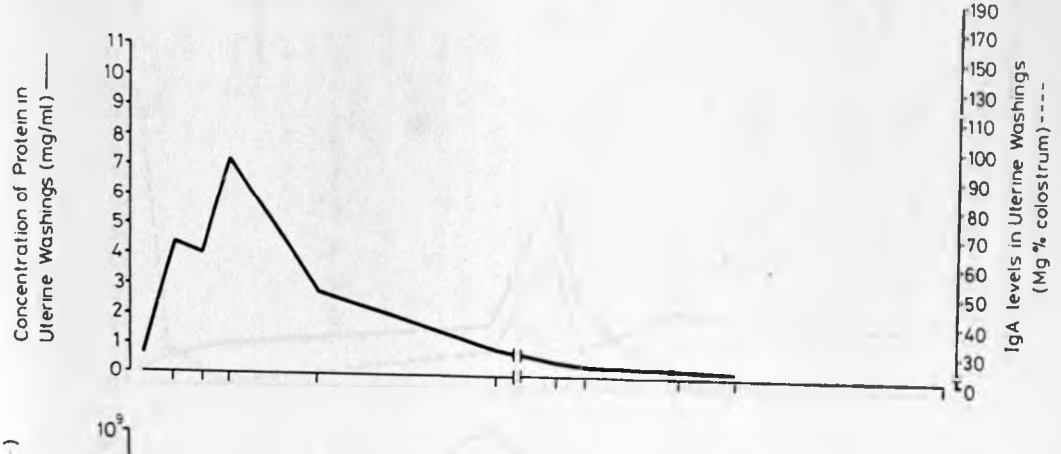
Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 35 - susceptible.

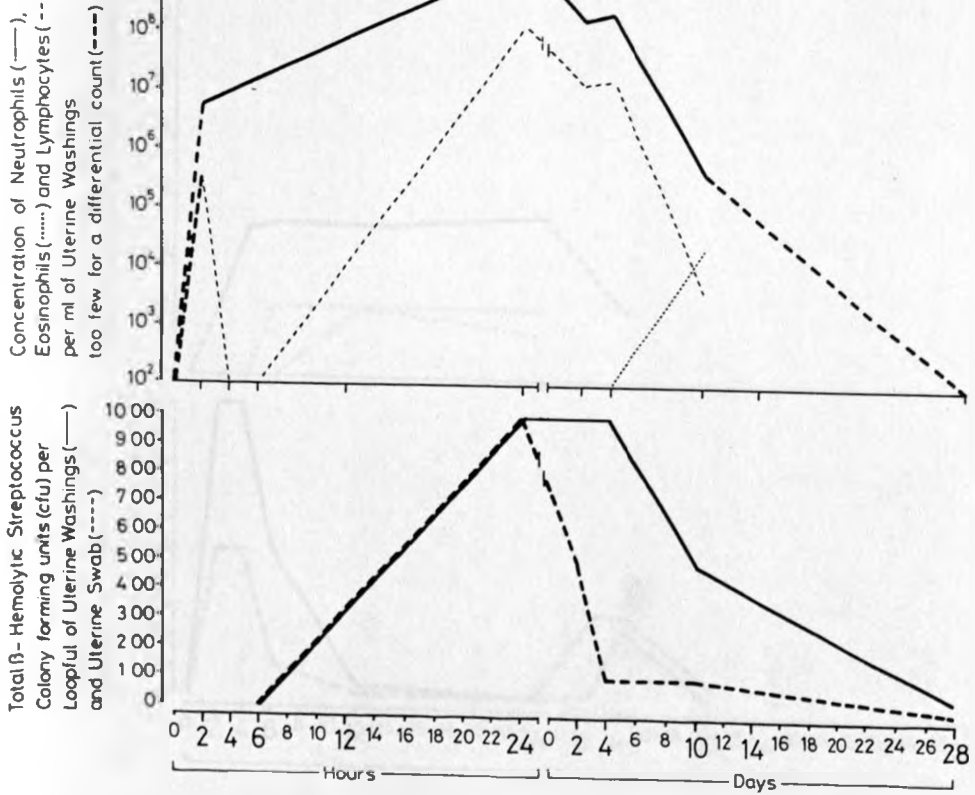



Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (– – –) per ml of Uterine Washings. too few for a differential count (– – –)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Total β-Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (– – –)
10 00
9 00
8 00
7 00
6 00
5 00
4 00
3 00
2 00
1 00
0
0 2 4 6 8 10 12 14 16 18 20 22 24 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours
Days
Time post inoculation with β Hemolytic Streptococcus (at least X10^9 cfu)

APPENDIX 5

Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 186 - susceptible.

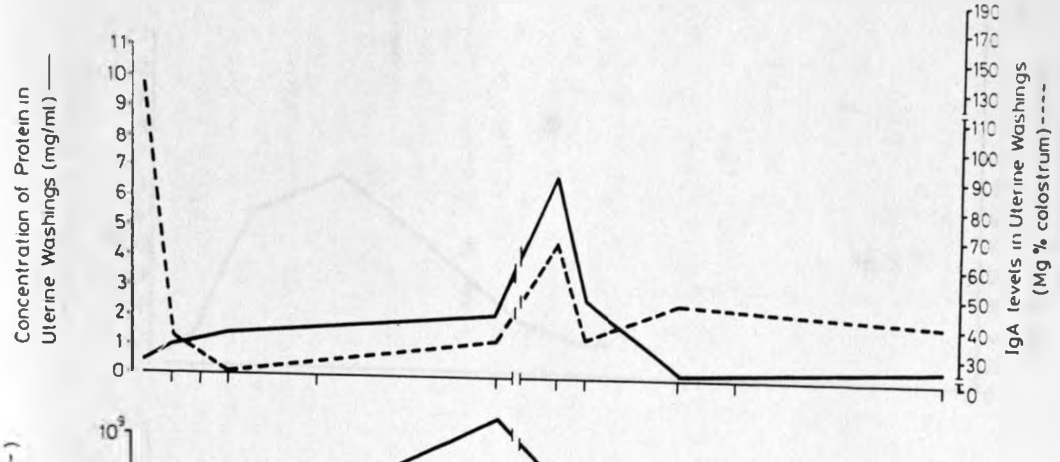




Time post inoculation with ß Hemolytic Streptococcus (at least $X10^9$ cfu)

APPENDIX 5

Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 145 - susceptible.



Concentration of Neutrophils (——), Eosinophils (·····) and Lymphocytes (- - -) per ml of Uterine Washings.
too few for a differential count (---)

10^2 10^3 10^4 10^5 10^6 10^7 10^8

Total ß- Hemolytic Streptococcus Colony forming units (cfu) per Loopful of Uterine Washings (——) and Uterine Swab (----)

0 100 200 300 400 500 600 700 800 900 1000

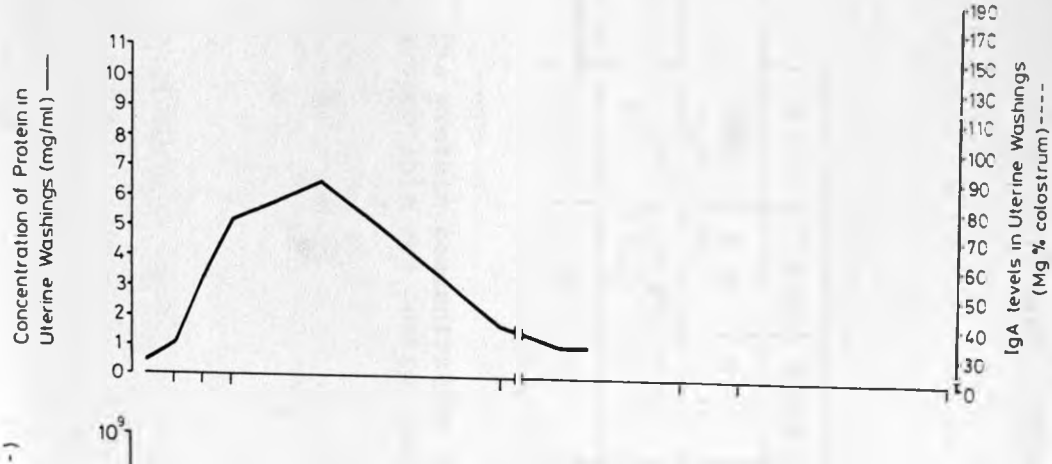
0 2 4 6 8 10 12 14 16 18 20 22 24 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28

Hours — Days

Time post inoculation with ß Hemolytic Streptococcus (at least X10^9 cfu)

APPENDIX 5

Graphical presentation of cellular and protein responses to intrauterine inoculation with Beta haemolytic streptococci (except control mares) in mare 56 - susceptible.



Appendix 6(a) The mean protein concentrations (mg/ml) in uterine washings obtained from challenged, resistant and susceptible and control mares.

	0 hrs	2 hrs	4 hrs	6 hrs	12 hrs	24 hrs	48 hrs	4 days	10 days	14 days	28 days
Resistant n=10	0.50 ± 0.30	1.26 ±0.74	6.92 ±7.18	4.69 ±4.22	0.7 ±0.07	2.33 ±1.98	2.36 ±2.53	1.26 ±1.19	0.68 ±0.55	0.16 -	0.75 ±0.44
Susceptible n=6	0.45 ± [illegible]	1.53 ±1.57	3.70 ±3.57	3.68 ±2.55	3.0 ±2.60	2.39 ±1.26	2.01 ±2.45	1.09 ±0.75	0.41 ±0.30	0.20 -	0.45 ±0.27
Controls n=5	0.35 ± [illegible]	0.80 ±0.16	2.6 ±1.55	2.86 ±[illegible]	-	1.46 ±0.1	1.50 ±1.12	0.85 ±0.30	0.3 ±0.06	0.91 ±0.71	0.15 -

The protein concentration (mg/ml) in uterine washings obtained from resistant, susceptible and control mares did not differ significantly ($p > 0.05$)

Appendix 6(b) The mean protein concentrations (mg/ml) in uterine washings obtained from challenged, resistant and susceptible mares

	0 hrs	3 hrs	6 hrs	9 hrs	12 hrs	15 hrs	24 hrs	10 days
Resistant n=3	0.62 ± 0.14	3.63 ± 1.06	24.47 ± 9.32	15.59 ± 7.49	8.03 ± 5.23	4.02 ± 1.79	7.14 ± 2.86	0.62 ± 0.38
Susceptible n=5	1.07 ± 0.56	11.73 ± 4.55	16.82 ± 4.74	15.17 ± 5.46	6.10 ± 1.94	5.82 ± 1.71	4.57 ± 1.19	3.53 ± 2.31

The protein concentration (mg/ml) in uterine washings obtained from resistant and susceptible mares did not differ significantly ($p>0.05$)

Appendix 7 The mean IgA concentration (mg%) in uterine washings obtained from resistant and susceptible mares

	0 hrs	2 hrs	4 hrs	6 hrs	24 hrs	48 hrs	4 days	10 days	14 days	28 days
Resistant[a] n= 8	27.69 ± 9.84	10.88 ± 7.39	31.25 ± 8.55	33.5 ± 6.63	52.19 ± 10.53	50.23 ± 12.58	46.5 ± 6.27	20.00 ± 7.81	not done	9.71 ± 5.87
Susceptible[a] n=4	63.88 ±33.31	44.38 ± 13.14	not done	30.13 ± 12.99	61.63 ± 19.00	73.63 ± 34.92	42.88 ± 5.86	40.5 ± 5.09	not done	38 ±10.10
Controls[b] n=	11.0 ± 9.51	11.0 ± 9.51	5.0 ± 3.54	12.7 ± 10.97	11.66 ± 10.10	18.0 ± 15.59	11.0 ± 9.51	-	10	-

Group in the same column with different superscripts differ significantly ($p < 0.01$)

Appendix 8(a) The mean IgG concentrations (mg%) in uterine washing obtained from challenged, resistant, susceptible and unchallenged control

	0 hrs	2 hrs	4 hrs	6 hrs	24 hrs	48 hrs	4 days	10 days	28 days
Resistant[a] n = 8	21.71 ± 5.79	32.63 ± 4.14	65.50 ±12.43	102 ± 22.95	160 ± 56.58	149 ± 64.50	37 ± 15.22	13.13 ± 2.19	16.39 ± 1.31
Susceptible[a] n = 4	25.0 ± 4.69	26.25 ± 2.25	not done	49.0 ± 10.25	77.75 ± 15.45	70.25 ±24.85	35.75 ± 10.06	20.00 ± 1.92	24.25 ± 5.44
Controls[a] n = 3	5.70 ± 5.66	7.67 ± 7.67	46.0 ± 21.23	51.0 ± 10.20	51.67 ±17.29	24.0 ± 12.22	5.7 ± 5.66	5.7 ±5.66	5.7 ± 5.66

There was no significant difference ($p > 0.05$) between the IgG concentration in uterine washings obtained from resistant, susceptible and control mares

APPENDIX:8(b) IgG concentrations in uterine washings following intrauterine infusion of Beta haemolytic streptococci

Sampling Time After Challenge

Mare Identification No.	00 hours		2 hours	4 hours	6 hours	12 hours	24 hours	2 days	4 days	10 days	14 days	28 days
Resistant mares:												
153	58		32	28	59	-	63	29	10	10	-	10
155	12		32	54	112	-	400	430	20	17	-	21
67(1)	17		54	112	200	-	140	31	14	10	-	14
160(1)	14		40	68	190	-	420	430	40	17	-	14
141	17		39	-	82	-	158	190	38	17	-	17
39	17		18	-	25	-	35	18	17	NR	-	17
71	17		25	-	108	-	47	47	140	17	-	21
67(2)	17		21	-	40	-	17	17	17	17	-	17
$\bar{X}$ ± SE	21.71 ± 16.12		32.63 ± 11.7	65.5 ± 35.15	102 ± 64.9	-	160 ± 160.06	149 ± 182.4	37 ± 43.04	13.13 ± 6.18	-	16.38 ± 3.70

APPENDIX: 8(b) IgG concentrations in uterine washings following intrauterine infusion of Beta haemolytic streptococci

Sampling Time After Challenge

Mare Identification No.	00 hours	2 hours	4 hours	6 hours	12 hours	24 hours	2 days	4 days	10 days	14 days	28 days
Susceptible mares:											
142	31	26	-	70	-	80	68	33	25	-	40
145	35	21	-	26	-	49	140	65	21	-	23
35	17	32	-	62	-	120	48	22	17	-	17
55	17	26	-	38	-	62	25	23	17	-	17
$\bar{X}$ ± SE	25 ± 9.38	26.25 ± 4.5	-	49 ± 20.49	-	77.75 ± 30.90	70.25 ± 49.70	35.75 ± 20.12	20 ± 3.83	-	24.25 ± 10.87
Control mares:											
72	17	23	-	43		38	32	17	17	-	17
188	NR	NR	NR	40		NR	NR	NR	NR	NR	-
187	0	0	NR	72		38	40	0	0	NR	-
$\bar{X}$ ± SE	5.7 ± 9.8	7.67 ± 13.28	NR	51.62 ± 17.67		25.33 ± 29.94	24 ± 21.17	5.7 ± 9.8	5.7 ± 9.8	NR	5.7 ± 9.8

Appendix 9(a) Leucocidal activity of uterine washings obtained from resistant and susceptible mares following intrauterine inoculation with Streptococci zooepidemicus

Mare Identification	% Viable Neutrophils After Incubation For:				
	0 hr	1 hr	2 hrs	3 hrs	4 hrs
Resistant					
39(2)	100	99	96	97	97
67(2)	100	96	95	92	95
201	100	95	90	98	99
203	100	93	99	99	98
$\bar{x}$ ± SE	100	95.75±1.25	95 ±1.88	95.5±1.6	97.25±.85
Susceptible					
202	100	99	99	98	98
204	100	90	98	80	70
206	100	95	97	98	95
207	100	94	98	99	100
$\bar{x}$ ± SE	100	94.5±1.85	98 ± .4	93.76±4.6	90.75±7.0
Control I					
187	100	96	20	75	80
188	100	97	75	87	70
$\bar{x}$ ± SE	100	96.5±0.5	47.5±27.5	81 ± 6.0	75 ±5.0
Control II					
188 (1)	100	98	70	96	95
190 (2)	100	95	93	98	98
$\bar{x}$ ± SE	100	96.5±1.5	81.5±11.5	97 ± 1.0	96.5±1.5
Control III					
HBSS	100	100	99	99	98
Buffer	100	99	98	98	97
$\bar{x}$ ± SE	100	99.5±0.5	98.5±0.5	98.5±0.5	97.5±0.5

Appendix 9(b) Leucocidal activity of uterine washings obtained from resistant and susceptible mares following intrauterine inoculation with Streptococcus zooepidemicus

Incubation Time	Mare Identification Number													
	39(2)	67(2)	201	203	202	204	206	207	187	188	188(1)	190(1)	HBSS	Buffer
0 hrs	*100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
1 hr	**99%	96%	95%	93%	99%	90%	95%	94%	96%	97%	98%	95%	100%	99%
2 hrs	96%	95%	90%	99%	99%	98%	97%	98%	20%	75%	70%	93%	99%	98%
3 hrs	97%	92%	98%	99%	98%	80%	98%	99%	75%	87%	96%	98%	99%	98%
4 hrs	97%	95%	99%	98%	98%	70%	95%	100%	80%	70%	95%	98%	98%	97%

* Percent (%) viable neutrophils before incubation
** Percent (%) viable neutrophils after incubation with uterine washings

APPENDIX 10

A summary of light microscopic examination of uterine biopsies obtained before and after challenge. Unchallenged control mares remained uninfected throughout the experimental period.

Mare No.	Age in years	Histopathological findings	stage of cycle
Uterine biopsies obtained 32 days after challenge.			
141	-	Mild chronic endometritis and mild degenerative changes (Category II)	dioestrus
142	-	Mild acute endometritis and severe degenerative changes (Category III)	dioestrus
144	-	Mild acute endometritis and moderate degenerative changes (Category III)	dioestrus
Uterine biopsies obtained 28 days after challenge.			
153	13 yrs	Mild acute endometritis and mild degenerative changes (Category III)	dioestrus
154	13 yrs	Mild to moderate degenerative changes (Category II)	proestrus
56	5 yrs	Mild degerative changes (Category I)	dioestrus
67	4 yrs	Mild acute endometritis and mild degerative changes (Category I)	dioestrus
130	6 yrs	Mild degerative changes (Category II)	dioestrus
160	6 yrs	Mild chronic endometritis (Category I)	proestrus

APPENDIX 10 (continued)

Uterine biopsies obtained 14 days after challenge.

174	-	Mild acute endometritis and mild degenerative changes (Category II)	-
173	11 yrs	Moderate acute endometritis & mild degenerative changes (Category II)	dioestrus
172	6 yrs	Severe acute endometritis moderate degenerative changes (Category III)	oestrus
73	13 yrs	Acute moderate endometritis and severe degenerative changes (Category III)	-
180	6 yrs	Mild chronic endometritis and moderate degenerative changes (Category II)	-
48	-	Normal (Category I)	proestrus
186	-	Mild endometritis and mild degenerative changes (Category II)	-

Uterine biopsies obtained 10 days after challenge.

72	12 yrs	mild chronic endometritis and mild degenerative changes (Category I)	proestrus
35	-	Mild acute endometritis and degenerative changes (Category II)	dioestrus
55	10 yrs	Mild degenerative changes (Category I)	proestrus
141	-	Mild degenerative changes (Category I)	proestrus
39	6 yrs	Mild degenerative changes (Category I)	dioestrus
71	6 yrs	Moderate acute endometritis & moderate degenerative changes (Category II)	proestrus
67(1)	4 yrs	Normal (Category I)	proestrus
142	-	Severe chronic endometritis & severe degenerative changes (Category III)	dioestrus
145	-	Mild acute endometritis & moderate degenerative changes (Category III)	proestrus

APPENDIX 10 (continued)

144	-	Moderate chronic endometritis and mild degenerative changes (Category II)	dioestrus

Uterine biopsies obtained 6 days after challenge.

155	6 yrs	Mild acute endometritis mild degenerative changes (Category II)	dioestrus
154	13 yrs	Mild degenerative changes (Category II)	dioestrus
153	13 yrs	Mild degenerative changes (Category II)	dioestrus
170	6 yrs	Moderate acute endometritis and moderate degenerative changes (Category III)	oestrus

Uterine biopsies obtained before and at 5 and 6 hrs after challenge.

170	6 yrs	Moderate degenerative changes (Category II)	-
131	-	Severe acute endometritis	-
160[1]	-	Normal (Category I)	proestrus
160[2]	-	Severe acute endometritis	-
160[3]	-	Severe acute endometritis	-
48(2)[1]	-	Mild degenerative changes	dioestrus
49	-	Severe acute endometritis	-
49	-	Severe acute endometritis	-

Uterine biopsies obtained before[1] and at 4[2], 6[3] and 24[4] hrs after challenge.

191	10 yrs	Moderate to severe degenerative changes (Category III)	-
191	-	Severe acute endometritis	
191	-	Severe acute endometritis	-
191	-	Moderate to severe acute endometritis	-
184	10 yrs	Normal (Category I)	proestrus
184	-	Severe acute endometritis	-
184	-	Severe acute endometritis	-
184	-	Moderate acute endometritis	-

APPENDIX 10 (continued)

107	5 yrs	Normal (Category I)	oestrus
107	-	Severe acute endometritis	-
1 107	-	Severe acute endometritis	-
107	-	Moderate acute endometritis	-

Uterine biopsies obtained before[1] and 3[2], 6[3] and 24[4] hrs after challenge.

109	7 yrs	Mild degenerative changes (Category I)	dioestrus
109	-	Severe acute endometritis	-
109	-	Severe acute endometritis	-
109	-	Moderate to severe acute endometritis	-

Uterine biopsies obtained before[1] and[2] 24 hrs after challenge.

201[1]	2-4 yrs	Normal (Category I)	oestrus
201[2]	-	Severe acute endometritis	-
202[1]	2-4 yrs	Normal (Category)	oestrus
202[2]	-	Severe acute endometritis	-
203[1]	2-4 yrs	Normal (Category I)	-
203[2]	-	Severe acute endometritis	-
204[1]	11-20 yrs	Mild to moderate degenerative changes (Category II)	-
204[2]	-	Severe acute endometritis	-
205[1]	20 yrs	Mild degenerative changes	-
205[2]	-	Severe acute endometritis	-
206[1]	11-20 yrs	Moderate to severe degenerative changes (Category II)	-
206[2]	-	Severe acute endometritis	-
207[1]	11-20 yrs	Moderate degenerative changes (Category II)	-
207[2]	-	Severe acute endometritis	-

APPENDIX 11a

The viability of neutrophils following a single freeze thaw cycle in liquid nitrogen and shaking with beads.

Treatment	Percentage of neutrophils disrupted.
Single freeze-thaw cycle	86.88±2.70
Shaking with beads for	
15 seconds	64.35±23.35
30 seconds	84.55±7.25
60 seconds	84.55±7.25
Single freeze thaw cycle and shaking with beads for 15 seconds	95.9±4.1

APPENDIX 11b

The number (logarithm) of viable B haemolytic streptococci colony forming units after incubation at 37C in Hanks buffered salt solution for 30 to 60 minutes.

	colony forming units after incubation		
Log No. of Streptococci	0 minutes	30 minutes	60 minutes
	8.31±0.03	8.37±0.001	8.41±0.001

Appendix 12 Natural log chemotactic differential of uterine neutrophils obtained from resistant and susceptible mares after intrautrine inoculation with Streptococci

Time of Sampling	Mare Identification							
	201	203	39	202	204	206	207	180
3 hours	1.3	1.54	1.096	1.15	1.097	0.699	1.176	1.176
6 hours	1.253	1.0	1.06	1.114	0.699	0.699	0.699	0.699
9 hours	0.176	1.24	1.189	1.173	0	0.964	0	0
$\bar{x}$ ± SE	1.243± 0.037	1.26± 0.16	1.115± 0.039	1.146± 0.017	0.599± 0.320	0.787± 0.089	0.625± 0.342	0.625± 0.342

Appendix 13 Chemotactic index of uterine neutrophils obtained from resistant and susceptible mares after intrauterine inoculation with Streptococci

Time of Sampling	Mare Identification							
	201	203	39	202	204	206	207	180
3 hours	1.5	1.29	1.63	2.06	2.0	1.38	2.0	2.5
6 hours	1.59	1.4	1.86	1.65	1.4	1.3	1.4	1.22
9 hours	1.83	1.43	2.86	1.77	0.67	1.69	0.61	0.86
	1.64± 0.099	1.373± 0.043	2.12± 0.378	1.827± 0.122	1.592± 0.295	1.457± 0.119	1.34± 0.403	1.537± 0.498

Appendix 14 The Phagocytosis (%) of peripheral neutrophils incubated with Staphylococcus aureus in the presence of whole pooled serum, heat inactivated serum, uterine washings and Hanks buffer as sources of opsonins

Mare Number	Whole Serum	Heat Inactivated Serum	Uterine Washings			* Hanks Buffer
			0 hrs	24 hrs	48 hrs	
35 (S)	100%	98%	60%	56%	44%	*60%
55 (S)	98%	80%	60%	38%	22%	72%
144(S)	96%	92%	10%	24%	16%	24%
72 (R)	94%	90%	52%	56%	72%	66%
141(R)	80%	76%	42%	22%	20%	44%

*Phagocytosis percent of incubations containing Hanks buffer was used to assess the ability of neutrophils to take up the bacteria in the absence of opsonins

R = resistant. S = susceptible.

Appendix 15(a) The phagocytic index of peripheral neutrophils incubated with Staphylococcus aureus in the presence of whole pooled serum heat inactivated serum, uterine washings and Hanks buffer as sources of opsonins

Mare Number	Whole Serum	Heat Inactivated Serum	Uterine Washings			*Hanks Buffer
			0 hrs	24 hrs	48 hrs	
35 Susceptible	22.6±10.7	20.7±8.8	8.7±5.9	4.1±3.1	6.2±8.0	*6.5±4.2
55 Susceptible	12.8±6.73	14.9±9.1	7.4±3.5	6.2±4.9	6.2±5.3	7.7±4.6
144 Susceptible	15.2±9.2	13.2±7.3	6.3±4.5	6.4±3.5	5.5±3.0	6.4±3.4
141 Resistant	23.2±11.5	21.9±10.3	7.4±4.16	8.3±6.8	7.8±4.5	7.0±6.4
72 Resistant	14.9±7.8	9.3±4.0	4.0±2.2	4.9±3.5	4.3±2.2	4.4±3.2

*The phagocytic index of the incubations containing Hanks buffer was used to assess the ability of neutrophils to take up the bacteria in the absence of opsonins

Appendix 15(b) The phagocytic index of uterine neutrophils obtained from resistant and susceptible mares after intrauterine inoculation with Streptococcus

Time of Sampling	Phagocytic Index of Mares							
	39(2)	180(2)	201	202	203	204	206	207
3 hours	7.22±3.69	4.8±1.88	18.69±4.37	6.71±2.78	11.61±5.36	9.43±4.5	8.18±4.0	7.36±3.14
6 hours	6.82±2.79	11.52±5.18	8.56±2.90	7.63±3.79	8.86±4.06	6.5±2.9	6.78±4.58	10.0±2.09
9 hours	0	4.76±2.53	8.53±3.16	8.59±4.49	6.0±3.17	0	0	9.27±5.04

APPENDIX 16

Buffers and working solutions of the EIA.

The plate coating and antibody dilution buffer at 0.05 M and pH 9.6, was made by dissolving Na_2CO_3 1.59 g and $NaHCO_3$ 2.93 g (without the 0.02% NaN_3) in 1000 ml of distilled water and stored at 4C.

The PBS stock solution consisted of Solution A (27.8 g $NaH_2PO_4.H_2O$) and Solution B (28.4 g Na_2HPO_4). To prepare the PBS working solution, which was stored at 4C, the stock solutions were miexed as follows:- 195 ml Solution A, 305 ml Solution B, 8.7 g NaCl and 1.0 g BSA made up to 500 ml with distilled water. This PBS working solution was used for the dilution of the conjugate and progesterone standards in plasma samples. The washing stock solution contained 1.5 M NaCl (87.66 g in 1000 ml distilled H_2O), and 0.5% Tween 20 (5.0 ml in 1000 ml distilled H_2O), which was diluted 10 fold before use.

The substrate stock solutions contained,

a) 0.05 M Citrate ($H_3C_6H_5O_7$), pH 4.0, - 9.605 g which was added to 1000 ml distilled H_2O and pH adjusted with NaOH (5N) before being stored at 4C.

b) 40 mM ABTS.-0.5487 g was added to 25 ml distilled water and stored at 4C.

c) Fresh batches of 0.5 M 2% H_2O_2 were prepared montly by diluting 0.5 ml 8 M (30%) solution of H_2O_2 with 7.0 ml distilled H_2O (total volume 7.5 mls) before being stored at 4C.

Substrate working solution.

Desired Volume (ml)	Citric Acid	ABTS	H2O2
25	24.6	250ul	80ul
50	49.4	0.5	160ul
100	98.7	1.0	320ul

The stock stop solution was made up of, a). 0.15 M H and 0.006 M NaOH. - 5.2 ml 28.8 M HF or 6.24 g of 48% HF were added to 6.0 ml 1.0 M NaOH and made up to 1000 ml with distilled water before being stored at room temperature. b). 1.0 M Edetic acid - 38.0 g edetic acid, the tetrasodium salt, were added to 100 ml water and stored at room temperature.

Working stop solutions.

Desired Volume (ml)	HF solution	EDTA solution
25	25 ml	25ul
50	50 ml	50ul
100	100 ml	100ul

(10 mls of Substrate and Stop solutions are required for each 96 well plate).

APPENDIX 17

Plasma progesterone levels (ng/ml) in samples obtained from experimental mares before (0 hrs) and 10 days after intrauterine inoculation of Streptococci.

Mare Number	Progesterone (ng/ml) in plasma obtained at	
	0 hrs	10 days.
153(R)	0.39	3.9
39(R)	0.21	Biopsy*
142(R)	0.12	Biopsy
186(S	0.12	Biopsy
39(2)R	0.681	8.18
201(R)	1.23	4.32
202(S)	1.13	6.36
204(S)	1.30	4.09
71(R)	2.0	Biopsy
145(S)	2.9	Biopsy
144(S)	2.2	Biopsy
155(R)	3.0	7.0
35(S)	2.8	Biopsy
181	4.6	10.45
203(S)	10.45	5.0
207(S)	10.45	7.7
16	0.46	1.6
68	1.2	0.01
55(S)	0.15	Biopsy
141(S)	1.17	Biopsy
67(R)	0.12	Biopsy
206(S)	1.30	1.25

APPENDIX 17 (Continued)

(1) Mares classified as either R (Resistant) or S (Susceptible) following challenge with Streptococci.

(2) Progesterone values > 2.0 ng/ml were interpreted to indicate luteal phase. Progesterone values < 1.0 were interpreted as indicating follicular phase.

*Biopsy - Biopsies obtained for light miroscopic examination 10 days after challenge.

APPENDIX 18

Plasma progesterone levels (ng/ml) before and following uterine manipulation and challenge with Betahaemolytic Streptococci.

Mare Number	Plasma progesterone (ng/ml)					
	0 hrs	1 day	2 days	4 days	10 days	28 days
155(2)	3.0	4.4	2.7	0.33	0.70	0.37
130	1.9	1.3	0.2	0.21	1.1	1.0
154	1.2	2.4	2.6	1.3	1.0	0.44
67(2)	1.2	0.28	0.02	0.06	0.01	0.09
153	0.39	0.23	0.07	0.71	3.9	2.9
160	0.46	0.28	0.21	0.0	1.6	0.30
188	0.0	0.0	0.0	0.0	0.0	0.0
187	0.0.	0.0	0.0	0.0	0.0	0.0

Progesterone values > 2.0 ng/ml were interpreted to indicate luteal phase. Progesterone values < 1.8 ng/ml and < 1.0 ng/ml were interpreted as indicating follicular phase and seasonal anoestrus respectively.

Appendix 19 The mean log number of Streptococcus zooepidemicus cfu recovered from uterine washings obtained from mares challenged at oestrus, dioestrus and anoestrus

Stage of Cycle	Uterine Washings Obtained At:												
	0 hrs	2 hrs	3 hrs	4 hrs	6 hrs	9 hrs	12 hrs	15 hrs	1 day	2 days	4 days	10 days	28 days
Oestrus n = 8	0	3.01 ±0.70	6.43 ±2.44	3.22 ±2.0	4.93 ±1.9	4.78 ±2.54	3.62 ±1.76	4.0 ±2.14	3.16 ±1.25	3.7 ±1.99	4.73 ±2.36	6.11 ±2.32	4.22 ±2.58
Dioestrus n = 7	0	3.63 ±1.85	7.0 ±0.0	not done	4.76 ±1.88	4.80 ±2.71	3.53 ±2.0	3.57 ±2.16	4.44 ±1.64	4.47 ±2.34	4.48 ±2.34	4.38 ±1.73	3.18 ±1.74
Anoestrus n = 6	0	3.56 ±1.65	5.67 ± 0.0	3.65 ±2.69	4.20 ±1.70	3.65 ±0.0	2.62 ± 0.0	3.0 ±3.0	2.94 ±1.28	3.20 ±1.49	0 ± 0	2.22 ±1.07	2.52 ±1.19

Appendix 20 The mean leucocyte concentration/ml uterine washings in utering washings obtained from mares challenged at oestrus, dioestrus and anoestrus

Stage of Cycle	Uterine Washing Samples Obtained At:													
	0 hrs	2 hrs	3 hrs	4 hrs	6 hrs	9 hrs	12 hrs	15 hrs	24 hrs	2 days	4 days	10 days	14 days	28 days
Oestrus n = 8	0	5.65 ±0.60	4.36 ±0.12	4.92 ±0.39	5.73 ±0.47	4.39 ±0.76	4.13 ±0.28	3.92 ±0.27	4.60 ±0.38	5.26 ±0.66	6.20 ±1.67	4.75 ±0.57	3.0 ±0.0	2.67 ±0.67
Dioestrus n = 7	0	6.25 ±0.54	4.13 ±0.10	5.11 ±0.0	6.17 ±0.53	4.72 ±0.03	3.82 ±0.33	3.83 ±0.33	5.80 ±0.85	6.65 ±0.85	6.42 ±0.83	5.57 ±0.26	2.3 ±0.0	2.77 ±0.72
Anoestrus n = 6	0	5.60 ±0.33	4.53 ±4.53	4.84 ±0.14	5.89 ±0.46	4.16 ±0.0	3.94 ±0.0	3.54 ±0.0	6.06 ±0.65	6.17 ±0.47	4.6 ±0.43	4.46 ±0.53	Not Done	4.03 ±0.52

Appendix 21 The mean protein concentration (mg/ml) in uterine washings obtained from mares challenged at oestrus, dioestrus and anoestrus

Stage of Cycle	Uterine Washing Samples Collected At:													
	0 hrs	2 hrs	3 hrs	4 hrs	6 hrs	9 hrs	12 hrs	15 hrs	24 hrs	2 days	4 days	10 days	14 days	28 days
Oestrus n = 8	0.63 ±0.14	1.77 ±0.87	3.47 ±1.15	3.83 ±0.89	11.79 ±5.14	10.04 ±8.18	5.57 ±3.16	4.28 ±1.65	3.41 ±1.29	1.10 ±0.46	1.06 ±0.34	1.07 ±0.39	0.20 ±0.20	0.62 ±0.16
Dioestrus n = 7	0.86 ±0.39	1.08 ±0.08	5.66 ±3.48	2.42 ±0.00	9.87 ±4.42	17.75 ±1.47	5.71 ±0.57	4.1 ±0.46	3.88 ±1.07	3.76 ±1.72	2.15 ±0.90	.70 ±0.19	not done	0.73 ±0.25
Anoestrus n = 6	0.53 ±0.06	1.54 ±0.42	28.0 ±0.0	3.75 ±1.17	7.08 ±3.88	6.14 ±0.0	13.13 ±0.0	11.75 ±0.0	3.70 ±0.94	2.63 ±1.24	0.83 ±0.18	0.56 ±0.07	not done	0.69 ±0.23

Appendix 22 The mean IgA concentration (mg%) in uterine washings obtained from mares challenged at oestrus, dioestrus and anoestrus

Stage of Cycle	Uterine Washings Obtained At:								
	0 hrs	2 hrs	4 hrs	6 hrs	1 day	2 days	4 days	10 days	28 days
Oestrus n = 8	*4.15±0.33	2.79±1.40	4.08	2.75±1.38	4.27±0.32	4.36±0.46	3.68±0.18	2.47±1.24	2.54±2.22
Dioestrus n = 7	2.08±1.23	1.75±1.01	3.50	2.62±0.87	3.83±0.21	3.97±0.29	3.68±0.06	2.78±0.93	2.60±0.87
Anoestrus n = 6	1.44±0.88	1.40±0.86	0	3.50	2.46±1.01	1.78±0.92	3.94±0.15	1.48±0.85	0.64±0.60

* mean ± SE

Appendix 23 The mean IgG concentration (mg%) in uterine washings obtained from mares challenged at oestrus, dioestrus and anoestrus

Stage of Cycle	Uterine Washings Obtained At:								
	0 hrs	2 hrs	4 hrs	6 hrs	1 day	2 days	4 days	10 days	28 days
Oestrus n = 8	*3.44±0.36	3.20±0.17	3.60±0.33	4.01±0.24	3.97±0.21	3.27±0.20	2.79±0.22	1.15±0.94	2.94±0.40
Dioestrus n = 7	2.97±0.23	3.99	**ND	4.20±0.34	4.63±0.50	4.68±0.53	3.80±0.47	2.89±0.05	3.07±0.02
Anoestrus n = 6	2.80±0.04	3.53±0.17	4.21±0.23	4.29±0.47	4.86±0.36	4.16±0.63	3.19±0.21	2.73±0.11	2.76±0.05

* mean ± SE

** ND - Not Done

APPENDIX 24

Protein concentration and composition in uterine washings obtained before intrauterine inoculation of Streptococci.

Mare	Protein	Albumin	GLOBULINS				
			Alfa		Beta		Gamma
No	mg/ml	mg/ml	1	2	1	2	
55	5.0	2.24		2.75			
67	5.0	1.62		3.37			
141	5.0	No Fractions Distinguishable (NFD)					
141	4.0	1.44		2.55			
180	5.0	NFD					
48	5.0	NFD					
161	6.0	NFD					
186	6.0	NFD					
160	1.0	NFD					
153	5.0	NFD					
39	1.0	NFD					
155	1.0	NFD					
68	1.0	NFD					

APPENDIX 24 (continued)

142	4.0	<———	NFD	———>
71	1.0	<———	NFD	———>
183	2.0	<———	NFD	———>
35	0.1	<———	NFD	———>
*72	2.0	<———	NFD	———>
*187	9.0	<———	NFD	———>
*188(2)	1.0	<———	NFD	———>

*CONTROL MARES - Mares that were not challenged and remained uninfected throughout the observation period.

APPENDIX 25

Protein concentration and composition in uterine washings obtained 4 hrs after intrauterine inoculation of Streptococci.

Mare No	Protein mg/ml	Albumin mg/ml	GLOBULINS					
			Alfa		Beta		Gamma	
			1	2	1		2	
48	26.0	12.18	1.03	2.68	3.70		2.20	4.10
161	15.0	6.35	0.85	2.58	2.71		0.97	1.51
*187	1.0	<——— NFD ———>						

APPENDIX 26

Protein concentration and composition in uterine washings obtained 12 hrs after intrauterine inoculation of Streptococci.

Mare Nc	Protein mg/ml	Albumin mg/ml	GLOBULINS					
			Alfa	Alfa	Beta		Gamma	
			1	2	1		2	
48	6.0	<——— NFD ———>						
161	6.0	<——— NFD ———>						
180	8.0	<——— NFD ———>						
183	25	10.64	1.01	2.79	1.34		5.57	0.18

APPENDIX 27

Protein concentration and composition in uterine washings obtained 6 hrs after intrauterine inoculation of Streptococci.

Mare	Protein	Albumin	GLOBULINS				
			Alfa		Beta		Gamma
No	mg/ml	mg/ml	1	2	1	2	
48	19.0	8.77	0.78	1.85	2.69	1.85	3.04
153	39.0	16.27	1.19	5.05	5.89	3.45	7.12
71	14.0	6.85	0.71	1.66	1.83	1.96	0.96
142	21.0	10.03	1.24	2.43	2.73	2.03	2.50
39	6.0	<——— NFD ———>					
68	10.0	<——— NFD ———>					
186	8.0	<——— NFD ———>					
67(1)	7.0	<——— NFD ———>					
*72	6.0	<——— NFD ———>					
*188(2)	2.0	<——— NFD ———>					

APPENDIX 23

Protein concentration and composition in uterine washings obtained 24 hrs after intrauterine inoculation of Streptococci.

Mare	Protein	Albumin	GLOBULINS				
No	mg/ml	mg/ml	Alfa		Beta		Gamma
			1	2	1	2	
55	7.0	3.18	———	3.81		———	
67	5.0	1.70	———	3.29		———	
141	13.0	5.25	0.35	1.78	2.06	1.98	1.56
130	18.0	7.18	0.57	1.72	1.84	1.46	5.19
35	21.0	7.88	0.97	3.46	2.84	2.76	3.05
72	6.0	<———		NFD		———>	
48	6.0	<———		NFD		———>	
161	6.0	<———		NFD		———>	
186	6.0	<———		NFD		———>	

APPENDIX 29

Protein concentration and composition in uterine washings obtained 48 hrs after intrauterine inoculation of Streptococci.

Mare	Protein	Albumin	GLOBULINS				
			Alfa		Beta		Gamma
Nc	mg/ml	mg/ml	1	2	1	2	
55	5.0	2.29	←	2.70		→	
67	5.0	1.99	←	3.01		→	
141	12.0	5.44	0.51	1.39	19.4	1.13	0.97
145	13.0	5.50	0.61	2.63	1.77	1.39	1.01
*72	6.0	2.54	←	3.45		→	
160(1)	17.0	4.21	0.81	3.01	4.05	1.59	3.30
48	15.0	7.37	←	2.40	→2.17	→	→3.04
68	1.0	←	NFD				→
153	5.0	←	NFD				→
39	2.0	←	NFD				→
*187	10	←	NFD				→
*188(2)	2.0	←	NFD				→
186	2.0	←	NFD				→
155	8.0	←	NFD				→
183	3.0	←	NFD				→
48	2.0	←	NFD				→

APPENDIX 30

Protein concentration and composition in uterine washings obtained 4 days after intrauterine inoculation of Streptococci.

Mare No	Protein mg/ml	Albumin mg/ml	GLOBULINS				
			Alfa		Beta		Gamma
			1	2	1	2	
71	17.0	7.55	1.19	1.60	2.59	2.46	1.58
55	4.0	2.28	←	1	1.71.		→
67	5.0	1.94	←		3.05		→
72	5.0	1.14	←		3.25		→
160	1.0	←		NFD			→
145	19.0	8.57	←		10.42		→
130	1.0	←		NFD			→
141	5.0	←		NFD			→

1) unchallenged control mares.

2) Individual protein fractions were not distinguishable in uterine washings obtained from both challenged, resistant and susceptible, and unchallenged control mares at 6, 10, 14 and 28 days after challenge.

APPENDIX 31

Protein concentration and composition in serum obtained from resistant and susceptible mares before (0 hrs) and 24 hrs (*) after challenge.

Mare	Protein	Albumin	GLOBULINS				
			Alfa		Beta		Gamma
No	mg/ml	mg/ml	1	2	1	2	
145	55.0	33.20	2.53	3.83	2.03	7.00	6.38
144	64.0	31.66	4.13	6.09	6.73	7.52	7.85
142	56.0	26.43	2.93	6.60	8.01	4.71	7.29
141	59.0	28.77	4.81	6.18	8.12	3.72	8.00
72	65.0	30.90	3.83	6.57	7.89	6.53	9.26
71	67.0	31.49	4.10	6.52	8.85	6.09	9.92
67	65.0	28.35	3.06	9.09	7.73	6.68	10.07
55	62.0	29.22	3.91	5.54	8.54	4.86	9.88
39	61.0	28.98	1.75	7.96	7.77	5.56	8.96
35	61.0	26.09	1.49	9.70	8.41	6.74	8.54
160(2)	56.0	28.60	3.85	4.40	5.54	4.81	8.18
48	60.0	28.62	1.90	7.34	7.54	4.61	9.96
68	64.0	29.47	1.87	9.19	6.23	7.97	9.30
173	70.0	28.31	1.45	10.54	11.62	6.44	11.62
155	68.0	27.11	1.97	10.74	7.68	7.18	13.30
188(2)	67.0	31.68	3.43	6.22	5.53	6.57	13.56
187(2)	64.0	30.10	3.01	6.33	4.99	6.50	13.04
*154	65.0	30.88	1.15	8.59	5.56	8.69	10.10
*153	66.0	28.95	8.73	7.60	8.34	6.03	12.65
* 48	60.0	29.70	1.87	7.18	7.06	4.90	9.27

APPENDIX 31

*173	73.0	28.68	1.67	12.95	11.49	6.54	11.64
*172	60.0	26.08	2.12	9.70	8.64	5.50	7.93
*170	64.0	25.58	1.95	10.30	7.78	8.42	9.93

*Samples obtained 24 hours after challenge.

APPENDIX 31

Protein concentration and composition in uterine washings obtained from mares used in Experiment 3, before and after intrauterine inoculation of Streptococci.

Mare	Protein	Albumin					
			Alfa		Beta		Gamma
Nc	mg/ml	mg/ml	1	2	1	2	
No 201							
0 hr	0.83		← NFD →				
3 hrs	5.75	2.27	0.26	0.88	— 1.61 →		0.71
6 hrs	35.25	13.11	← 8.60 →		— 8.54 →		4.98
9 hrs	26.38	9.58	← 5.34 →		— 9.13 →		2.31
15 hrs	7.37	2.77	← 1.60 →		— 1.88 →		1.10
24 hrs	12.0	5.88	← 2.12 →		— 2.95 →		1.34
10 days	0.38		← NFD →				
No 202							
0 hr	1.14		← NFD →				
6 hrs	27.13	10.29	← 4.48 →		— 7.17 →		5.17
12 hrs	4.97	1.94	← 0.90 →		— 1.48 →		0.64
15 hrs	6.86	2.54	← 1.08 →		— 2.13 →		1.09
24 hrs	2.48	0.66	← 0.12 →		— 1.58 →		0.10

APPENDIX 31 (continued

No 203

0 hr	0.70		<—— NFD ——>	
3 hrs	2.60	1.07	<— 0.11 —> <— 0.78 —>	0.61
6 hrs	32.25	12.89	<— 5.86 —> <— 7.83 —>	5.95
9 hrs	19.20	3.81	0.24 7.02<— 3.22 —>	4.88
12 hrs	6.14	2.39	<— 1.76 —><— 0.89 —>	1.08
15 hrs	3.46	1.28	<— 1.02 —><— 0.72 —>	0.42
10 days	0.49		<—— NFD ——>	

No 204

0 hr	0.15		<—— NFD ——>	
3 hrs	2.13	0.66	<— 0.26 —><— 0.66 —>	0.53
6 hrs	2.86	0.99	<— 0.61 —><— 0.50 —>	0.75
12 hrs	1.40		<—— NFD ——>	
15 hrs	1.63		<—— NFD ——>	
24 hrs	3.82	1.67	<— 0.58 —><— 0.74 —>	0.81
10 days	3.08	1.04	<— 0.21 —><— 0.85 —>	0.96

No 206

0 hr	0.49		<—— NFD ——>	
3 hrs	28.00	13.34	<— 4.12 —><— 4.37 —>	6.16
6 hrs	26.13	4.27	<— 2.42 —><— 6.92 —>	12.50
9 hrs	6.14	2.63	<— 1.01 —><— 0.91 —>	1.56
15 hrs	11.75	5.32	<— 2.61 —><— 1.58 —>	2.21
24 hrs	6.75	3.17	<— 0.94 —><— 1.04 —>	1.57
10 days	0.44		<—— NFD ——>	

APPENDIX 31

No 207

0 hr	0.36		<——— NFD ———>	
3 hrs	4.94	1.90	<— 1.95 —> <— 0.70 —>	0.37
6 hrs	18.75	8.93	<— 2.77 —> <— 3.10 —>	3.93
9 hrs	19.25	9.37	<— 2.15 —> <— 2.95 —>	4.76
12 hrs	6.41	2.78	<— 0.82 —> <— 1.07 —>	1.71
15 hrs	5.0	2.36	<— 0.68 —> 0.60 0.40	0.67
24 hrs	7.94	3.79	<— 0.81 —> <— 1.39 —>	1.39
10 days	1.06		<——— NFD ———>	

93

0 hr	0.34		<——— NFD ———>	
3 hrs	2.54		<——— NFD ———>	
6 hrs	5.91	2.45	<— 0.92 —> <— 1.00 —>	1.52
9 hrs	1.49		<——— NFD ———>	
12 hrs	0.79		<——— ———>	
15 hrs	1.24		<——— ———>	
24 hrs	2.08		<——— ———>	
10 days	1.34		<——— ———>	

No 180

0 hr	3.19	0.96	<— 0.65 —><— 0.96 —>	0.60
3 hrs	9.44	2.99	<— 2.15 —><— 2.85 —>	1.43
6 hrs	9.25	2.91	<— 2.54 —><— 2.15 —>	1.64
9 hrs	14.81	4.04	<— 2.14 —><— 6.58 —>	2.03
12 hrs	4.57	1.61	<— 0.75 —><— 1.45 —>	0.74
15 hrs	3.84	1.27	<— 0.51 —><— 1.00 —>	1.04
24 hrs	1.85		<——— NFD ———>	
10 days	0.50		<——— NFD ———>	

REFERENCES.

Alexander J.W., Windhorst D.B. and Good R.A. (1968). Improved tests for the evaluation of neutrophil function in human disease. J. lab & Clin. Med. 72(1) 137.

Alexander J.W. and Wilson B.A., (1970). Neutrophil dysfunction and sepsis in injury. Surg. Gynaecol. Obstetr. 130:431.

Alper C.A., Abrahamson N., Johnston R.B., Jandl J. and Rosen F.S., (1970a). Increased susceptibility to infection associated with abnormalities of the complement mediated functions of the third component (C3). N. Engl. J. Med. 282:349.

Alper C.A., Abrahamson N., Johnston R.B., Jandl J. and Rosen F.S., (1970b). Studies, in vivo and invitro, on an abnormality in the metabolism of C3 in a patient with increased susceptibility to infection. J. Clin. Invest. 49:1975.

Alper C.A., Colten H.R., Rosen F.S., Rabson A.R., MacNab G.M. and Gear J.S.S., (1972). Homologous deficiency of C3 in a patient with repeated infections. Lancet 2:1179.

Aly R., Shinefield H.I., Straub W.B. and Maibach H.I., (1977). Bacterial adherence to nasal mucosal cells. Infect. Immun. 17:546.

Allen R.C., Sjernholm R.L. and Steele R.H., (1972). Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leucocytes and its particles in bactericidal activity. Biochem. Biophys. Res. Commun. 47:679.

Andersen B.R. and Brendzel A.M., (1978). Use of a unique chemiluminescence spectrometer in a study of factors influencing granulocyte light emission. J. Immunol. Neth. 19:279.

Andersen B.R. and Amirault H.J., (1979). Important variables in granulocyte chemiluminescence. Proc. Soc. Exp. Biol. Med. 162:139.

Aronson M., Medalia O., Schori L., Mirelman D., Sharon N. and Ofelc I., (1979). Prevention of colonization of the urinary tract of mice with methyl alfa-D-Mannopyranoside. J. Infect. Dis. 139:329.

Asbury C.A., Halliwell R.E.W., Foster G.W. and Longino S.J., (1980). Immunoglobutis in uterine secretions of mares with differing resistance to endometritis. Theriogenology, 14:(4). 299.

Asbury C.A., Schultz K.T., Klesius P.H., Foster G.W. and Washburn S.M., (1982). Factors affecting phagocytosis of bacteria by neutrophis in mares uterus. J. Reprod. fert. Suppl. 32:151.

Baehner R.L. and Nathan D.G., (1968). Quantitative nitroblue tetrazolium test in chromic granulomatous disease. N. Engl. J. Med. 278:971.

Bains A.M., (1966). The role of infection in infertility in thoroughbred mares. Vet. Red. 78:168.

Baggiolini M., De Duve and Masson P.L., (1970). Association of lactoferrin with specific granules in rabbit heterophil leucocytes. J. Exp. Med. 131:559.

Baker C.J., Goroff D.K., Albert S., Crocket V.A., Zinner S.H., Evrard K.R., Rosner B. and McCormick W., (1977). Vaginal colonization with group B streptococcus: A study of college women. J. Infect. Dis. 135:392.

Barrett-Connor E., (1971). Bacterial infection and sickle cell anaemia; an analysis of 250 infections in 166 patients and a review of literautre. Medicine, 50:97.

Bainton D.F., (1973). Sequential degranulation of the two types of polymorphonuclear leucocyte granules during phagocytosis of micro-organisms. J. Cell Biol. 58:249.

Baehner R.L., (1974). Molecular basis for functional disorders of phagocytes - medical progress. J. Pediatr., 84:(3). 317.

Bradford M.M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem., 72:248.

Belcher R.W. and Czarnetzki B. (1973). A simple screening test for chronic granulomatous disease. Am. J. Clin. Pathol. 60:450.

Bennett D.G., Poland H.J., Kaneps A.J. and Synder S.P., (1980). Reaction of the equine endometrium to intra-uterine infusion. 26th conven. Am. Ass. Equ. Pract. 135.

Beachey E.H., (1981). Bacterial adherence. Adhesin-receptor interactions mediating the attachment of bacteria to mucosal surface. J. Infect. Dis. 143:325.

Becker E.L. and Howell H.J., (1974). The ability of chemotactic factors to induce lysosomal enzyme release. J. Immunol. 112:2055.

Belding M.W., Klebanoff S.J. and Ray G.G., (1970). Peroxidase-mediated virucidal systems. Science 167:195.

Bjorkstein B., (1974). The influence of technical factors on the NBT test. Scand. J. Haematol. 12:46.

Bjorksten B. and Kaijser B., (1978). Interaction of human serum and neutrophils with Escherichia coli strains isolated from urine of patients with polynephritis or asymptomatic bacteriuria. Infect. Immunol. 22:308.

Bloom F., (1953). Canine medicine. Edt. Hask P.H. Chapter 9, 244-250 and Chapter 24:547.

Black W.G., Uberg L.C., Kidder H.E., McNuh S.H. and Comar L.E., (1953). Inflammatory response of the bovine endometrium. Am. J. Vet. Res. 14:179.

Bloom F., (1954). Pathology of cat and dog. Am. Vet. Public. Evanston.

Blume R.S. and Wolff S.M., (1972). The Chediak-Higashi syndrome. Studies in 4 patients and a review of literature. Medicine 51:247.

Blanchard T.T., (1981). Erroneous diagnosis of endometritis in the mare. Proc. Annual meet. Soc. Theriogenol. 247.

Blanchard T.T., Garcia M.C., Hurtgen J.P. and Kenney R.M., (1981). Comparison of two techniques for obtaining endometrial bacteriologic cultures in the mare. Theriogenology. 16:(1). 85.

Blue M.G., Brady A.A., Davidson J.W. and Kenney R.M., (1982). Studies on the composition and antbacterial activity of uterine fluid form mares. J. Reprod. Fert. Supp. 32:143.

Boxer G.H., Holmsen H., Robkin L., Bang N.U., Boxer L.A. and Baehner R.L., (1977). Abnormal platelet function in Chediak-Higashi syndrome. Br. J. Haematol. 35:521.

Bockenstedt L.K. and Goetzl E.J., (1980). Constituents of human neutrophils that mediate enhanced adherence to surfaces. Purification and identification as acidic proteins of the specific granules. J. Clin. Invest. 65:1372.

Botta G.A., (1979). Hormonal and type-dependent adhesion of Group B Streptococci to human vaginal cells. Infect. Immun. 25:1084.

Boxer L.A., Hedley-Whyte E.T. and Stossel T.P., (1974). Neutrophil actin dysfunction and abnormal neutrophils behaviour. N. Engl. J. Med. 291:1093.

Broome A.W.J., Lamming G.E. and Smith W., (1959). Studies on relationship between ovarian hormones and uterine infection in role of granulocyte system in uterine defence. J. Endocri. 19:274.

Bronza P.J., Senior R.M., Krentzer D.L. and Ward P.A., (1977). Chemotactic factor inactivators of human granulocytes J. Clin. Invest. 60:1280.

Burke T.J., (1976). Feline reproduction. The veterinary clinics of North America. Feline Medicine 317.

Buxton A and Fraser G., (1977). Sensitivity testing: In Ann. Microbiol. 1, 86-87 Publ. Blackwell Scientific Publications, London.

Callin C. and Quie P.G., (1978). Leucocyte chemotaxis. Raven Press 1978.

Chretien, J.H. and Garagusi V.F. (1971). Suppressed reduction of nitroblue tetrazolium by polymorphonuclear neutrophils from patients receiving steroids. Experimentia. 27:1343.

Clark R.A. and Kimbal H.R., (1971). Defective granulocyte.chemotaxis in the Chediak-Higashi syndrome. J. Clin. Invest. 50:2645.

Clark R.A. and Klebanoff S.J., (1977). Myeloperoxidase - H202 - halide system: cytotoxic effect on human blood leucocytes. Blood 50:65.

Clark R.A. and Klebanoff S.J., (1979). Chemotactic factor inactivation by the myeloperoxidase hydrogen peroxide-halide system. J. Clin. Invest. 64:914.

Cleat P.H. and Coid C., (1981). Phagocytic and bacteriacidal activity of human neutrophils against two isolates of Group B streptococci type 1c of differing pathogenicity. Brit. J. Exp. Path. 62:393.

Cleat P.H. and Coid C.R., (1982). An alternative role for specific in neutrophil bactericidal activity against highly pathogenic Group B streptococci. Br. J. Exp. Path. 63:452.

Clipperfield E.J. and Evans B.A., (1972). Influence of local infection and immunoglobulin formation in human endocervix. Clin. Path. Immunol. 11:219.

Coles E.H., (1974; 1980). Veterinary Clinical Pathology 2nd Ed. pp 392-393. W.B. Saunders Co., U.K. U.S.A.

Colby E.M., (1978). Feline reproduction. Scientific proceedings of 44th Annu. Meeting of A.A.H.A. 141-144.

Deibel R.H. and Seeley H.W., (1974). Family II Streptococcaceae: In Bergey's manual of Determinative Bacteriology (8th Edn). 490-528. Edts. Buchanan R.E. and Gibbons N.E. Publ. The Williams and Wilkins Co. Baltimore, USA.

De Coster R., Dieteven G. and Josse M., (1979). Aspects of clinique histologique bacteriologue et hormonal de metrite chronique chez la chiene (Bitch). Ann. Med. Vet. 123:233.

Dimmock W.W., (1928). Breeding problems in mares. Cornell Vet., 25 (Quoted by Peterson et al., 1969).

Dogamelli M.Z., Aydin N., Alancom E., (1978). A study of defence mechanisms of the uterine against infection during different phase of the oestrous cycle. Veteriner Fakultesis Dergisi, 25:(4). 674.

Easmon C.S.F., Cole P.J., Williams A.J. and Hastings M., (1980). The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescence. Immunol. 41:67.

Everitt J.T., Fetter A.W., Kenney R.M. and Della-Ferra M.A., (1981). Porcine necrotizing staphylococcal endometritis. Vet. Path. 18:(1). 125.

Farstad W., (1982). Bacterial findings in the genital tracts of bitches with reproductive disorders. Nordisk Veterinarmedi., An. 34:(12). 451.

Feigin R.D. and Pickering L.K., (1973). The role of nitroblue tetrazolium test in diagnosis of infections. South. Med. J. 68:237.

Ferenczy A, (1976). Scanning electron microscopy of the female reproductive tract. In: Biomedical research applications of scanning electron microscopy. Vol. 2. pp. 127-167. Ed. Hodges G.M. and Hallowes R.C., Academic Press, Lond. Toronto, N.Y., Sydney.

Finn C.A. and Porter D.G., (1975). The uterus: A hand book of reproductive biology. Vol. 1. p38. Unwin Brothers Ltd., England.

Fleming A. Allison V.D., (1922). Observations on bacteriolytic substance (lysozyme) found in secretions and tissues. Brit. J. Exp. Pathol. 3:252.

Forslin L., Danielsson D. and Falk V., (1979). Variations inn attachment of Nelsseria gonorrhoeae to vaginal epithelial cells during the menstrual cycle and early pregnancy. Med. Microbiol. Immun. 167:231.

Freeman R. and King B., (1972). Infective complications of in dwelling intravenous catheters and the monitoring of infections by the Nitro-blue tetrazolium test. Lancet, 1. 992.

Freter R., (1980). Prospects for preventing the association of harmful bacteria with host mucosal surfaces. In E.H. Beachey (ed). Bacterial adherence. Chapman and Hall, Lon. N.Y. p439.

Fuenfer M.M., Scott K. and Polk H.C., (1976). Evaluation of the phagocytic bactericidal capacity of neutrophils. A current review. J. Surg. Res. 21:207.

Fukuyama M., Kamimura T., Itoh I., Yoshida T. and Kudoh K.K., (1978). A study of anaerobic infection in domestic animals. II anaerobic bacterial flora in genital organs of healthy cows. Bulletin of Azabu Vet. College. 3 (2):279.

Gadek J.E., Fells G.A., Wright D.G. and Crystal R.G., (1980). Human neutrophil elastase functions as a type III collagenase. Biochem. Biophys. Res. Commun. 95:1815.

Ganjam V.K., McLeod C., Klesius P.H., Washburn S.M., Kwapien R., Brown B.G. and Atteberger M.H., (1980). Effect of ovarian hormones on the pathophysiological mechanisms involved in resistance vs susceptibility to uterine infections in the mare. Proc. 27th Ann. conv. Am. Ass. Equine Pract. p141.

Ganjam V.K., McLeod C., Klesius P.H., Washburn S.M., Kwapien R., Brown B. and Fazeli M.H., (1982). Effect of ovarian hormones on the phagocytic response of ovariectomized mares. J. Reprod. Fert. Suppl. 32:169.

Gifford R.H. and Malawista S.E., (1970). A simple rapid micromethod for detecting chronic granulomatous disease of childhood. J. Lab. Clin. Med. 75:511.

Giron D.J., (1971). Inhibition by estrone of antiviral protection and interferone elicited inducers in mice. Infec. Immun. 3:318.

Gibbs R.S., O'Dell T.N., McGregor R.R., Schartz R.H. and Marton H., (1975). Puerperal metritis: A prospective microbiological study. Am. J. Obst. G., 121:(7). 919.

Gibbons R.J., (1977). Adherence of bacteria to host tissue. In D. Schlessinger (ed). Microbiology. Am. Sco. Microbiol. Washington 395.

Gibbons R.J. and Van Houte J., (1980). Bacterial adherence and the formation of dental plaques. In E.H. Beachey (ed). Bacterial adherence. Chapman and Hall, London, N.Y. p60.

Goldstein I.M., (1976a). Polymorphonuclear leucocytes, lysosomes and immune tissue injury. Prog. Allergy, 20:301.

Goldstein I.M., (1976b). Pharmacologic modulation of lysosomal enzyme release from PMN leucocytes. J. Invest. Dermatol, 67:622.

Gordon L.R. and Sartin E.M., (1978). Endometrial bipsy as an aid to diagnosis and prognosis in equine infertility. J. Equ. Vet. Med. Surg. 1(10): 328.

Granelli-Piperno A., Vassali J.D. and Reich E., (1977). Secretion of plasminogen activator by human polymorphonuclear leucocytes. Modulation by corticosteroids glucocorticoids and other effectors. J. Exp. Med. 146:1693.

Gullberg R. (1974). Possible antimicrobiol function of the large molecular size. Vitamin B-binding protein. Scand. J. Gastroenterol. 9 (supp. 29):19.

Hawk H.W., Turner G.P. and Sykes J.F., (1960a). Effect of ovarian hormones on uterine defence mechanism during early stages of induced infection. Am. J. Vet. Res. 21:644.

Hawk H.W., Turner B.S., Sykes J.F., (1960b). Ovarian hormones versus uterine defence. Am. J. Vet. Res. 1:644.

Hawkins C., (1974). Neutrophilic leucocytes in immunologic reactions in vitro. III pharmacologic modulation of lysosomal constituent release. Clin. Immunol. Immunopathol. 2:141.

Hafez E.S.E., (1980). Reproduction in farm animals. 4th ed. Lea and Febiger, Philadelphia, U.S.A.

Harlan J.M., Killen P.D., Havker L.A. and Striker G.E., (1980). Endothelial denudation mediated by neutrophil derived neutral proteases. Clin. Res. 28:547a.

Hallgren R. and Venge P., (1976). Cationic proteins of human granulocytes. Effects on human platelet aggregation and serotonin release. Inflammation 1:359.

Hastings M.J.G., Petricevic I., Williams A.J., Cole P.J. and Easmon C.S.F., (1982). The effect of culture media on the production and measurement of luminol-dependent chemiluminescence. Brit. J. Exp. Path. 63:147.

Heap R.B. and Lamming G.E., (1960). Studies on uterine environment of different species. I. Influence of ovarian hormones on chemical composition of uterine secretions. J. Endocrin. 20(4):XXIII-XXIV of Proceedings.

Hemming V.G., Hall, R.T., Rhodes P.G., Shigeoka A.O. and Hill H.R., (1976). Assessment of Group B. Streptococcal opsonins in human and rabbit serum by neutrophil chemiluminescence. J. Clin. Invest. 58:1379.

Hersh E.M. and Bodey G.P., (1960). Leucocytes mechanisms in inflammation. Ann. Rev. Med., 21:105.

Henson P.M., (1971a). Interaction of cells with immune complexes: Adherence release of constituents and tissue injury. J. Exp. Med. 134:114.

Henson P.M., (1971b). The immunologic release of constituents from neutrophils leucocytes. II Mechanisms of release during phagocytosis and adherence to non-phagocytosable surfaces. J. Immunol., 107:1547.

Hicks R.G. and Bennett J.M. (1971). An improved cytochemical method for nitroblue tetrazolium reduction by neutrophils. Am. J. Med. Technol. 37:226.

Hirsch J.G. and Cohn Z.A., (1960). Degranulation of PMN leucocytes following phagocytosis of microorganisms. J. Exp. Med., 112:1005.

Hoffstein S., Zurier R.B. and Weissman G., (1974). Mechanisms of lysosomal enzyme release from human leucocytes III. Quantitative morphologic evidence for an effect of Immunol. Immunopathol. 3:301.

Holmes K.K., Counts G.W. and Beaty H.N., (1971). Disseminated gonococcal infection. Ann. Intern. Med. 74:979.

Holmes B., Page A.R. and Good R.A., (1967). Studies of the metabolic activity of leucocytes from patients with a genetic abnormality of phagocyte function. J. Clin. Invest. 46:1422.

Hughes J.P., Loy R.G., Asbury A.C. and Burd H.E., (1966). The occurence of pseudomonas in the reproductive tract of mares and its effect on fertility. Cornell Vet. 56:595.

Hughes J.P. and Loy R.G., (1969). Investigations on the effect of intra-uterine inoculation of Streptococcus zooepidemicus in the mare. Proc. 15th Ann. Mtg. Am. Assoc. Equine Pract. 289.

Hughes J.P. and Loy R.G., (1975). The relationship between infertility and stallion. Equine Vet. J. 7:155.

Hughes J.P., Staberfeldt G.H., Kindall H., Kennedy P.C., Edquist L.E., Neely, D.P. and Schalm O.W., (1979). Pyometra in the mare. J. Reprod. Fert. Supp. 27:191.

Jackobs J.D. and Miller M.E. (1982). Fatal Familial leineis disease. A deficiency of the opsonic activity of serum complement. Pediatrics., 49:225.

Janeway C.A. (1968). Progress in immunology. Syndromes of diminished resistance to infection. J. Pediatr. 72:885.

Janoff A., (1972). Human granulocyte elastase; further delineation of its role in connective tissue damage. Am. J. Pathol. 68:579.

Janoff A., (1975). At least three human neutrophil lysosomal proteases are capable of degrading joint connective tissue. Ann. N.Y. Acad. Sci. 256:402.

Jones G.W. and Rutter J.M. (1974). Contribution of the K88 antigen Escheridia coli to enteropathogenicity; protection agains disease by neutralising the adhesive properties of K88 antigen. Am. J. Clin. Nutr. 27:1441.

Kauder E. (1968). Leucocyte degranulation and vacuole formation in patients with chronic granulomatous disease of childhood. J. Clin. Invest. 47:1753.

Kallenius G. and Winberg J., (1978). Bacterial adherence to periurethral epithelial cells in girls prone to urinary tract infections. The Lancet, 540.

Kelly M.T., Martin R.R. and White A. (1971). Modulators of histamine release from platelets, lymphocytes and granulocytes. J. Clin. Invest. 50:1040.

Kenney R.M. and Khaleal S.A., (1975). Bacteriostatic activity of the mare uterus: A progress report on immunoglobulins. J. Reprod. Fert. Supp., 23:357.

Kenney R.M., (1978). Cyclic and pathological changes of the mare endometrium as detected by biopsy with a note on early embryonic death. J.A.V.M.A. 172(3):241.

Kelly J.K. and Fox H., (1979). The local defence system of the human endometrium. J. Reprod. Immunol., 1:39.

Kirkpatrick C.H., Green I., Rich R.R. and Schade A.L. (1971). Inhibition of growth of candida albicans by iron unsaturated lactoferrin ; relation to host-defense mechanisms in chronic mucocutaneous condidiasis. J. Infect. Dis. 124:539.

Klinkhamer J.M., (1963). Human oral leucocytes. Periodontics, 1:109.

Klebanoff S.J., (1967). Iodination of bacteria: A bactericidal mechanism. J. Exp. Med. 126:1063.

Klebanoff S.J., (1968). Myeloperoxidase-halide-hydrogen peroxidase antibacterial system. J. Bacteriol. 95:2131.

Klebanoff S.J. (1970). Localization and the role of myeloperoxidase of the mechanism of the halogenation reaction. In "biochemistry of the phagocytic process. Ed. Schultz, J. Amsterdam. pg89-110.

Klebanoff S.J. and Hamon C.B. (1972). Role of myeloperoxidase-mediated antimicrobial systems in intact leucocytes. J. Reticuloendothel. Soc. 12:170.

Klebanoff S.J. and Clark R.A., (1978). The neutrophil; function and clinical disorders. p 217-281, 489-783. North Holland, Amsterdam. North

Klebanoff S.J. (1982). Oxygen-dependent cytotoxic mechanisms of phagocytes. In: Advances in host defence mechanisms Vol. 1 edt. J.I. Gallin and A.S. Fauci, Raven Press, N.Y.

Knudsen O.D. (1964). Endometrial cytology as a diagnostic aid in mares. Cornell Vet. 54:415.

Lamm M.E. and Stetson C.A., (1972). Inflammation: In clinical immunobiology Vol. 1. pg 139-155 ed Bach F.H. and Good R.A. Pub. Academic Press. N.Y., Lon.

Lehmeyer J.E., Synderman R. and Johnston R.B., (1979). Stimulation of neutrophil oxidative metabolism chemotactic peptides: Influence of calcium ion concentration and cytochalasin B and comparison with stimulation by phorbol myristale acetate. Blood 54:35.

Liu I.K.M., Mitchell G., Perryman L.E. and Stewart E.W, (1981). Immunological defence mechanisms of the uterus in the mare. Proceedings of the annual meeting of society for theriogenology, 265.

Maaloe, O. (1946). On the relation between Alexin and opsonin. Minksgaard, Copenhage. Cited by Fuenfer et al 1976.

Malawista S.O. and Wiedlastine E., (1971). Colchicine-like effects on human blood leucocytes during phagocytosis. Blood 37:519.

Mancini G., Carbonara A.O. and Heremans J.P., (1965). Immunochemical quantitation of antigens by simple radial diffusion. Immunochem. 2:235.

Mandell G.L., (1970). Intraphagosomal pH of human polymorphonuclear neutrophils. Proc. Soc. Exp. Biol. Med. 134:447.

Mandell G.L., Rubin W. and Look E.W., (1970). The effect of NADH oxidase inhibitor (hydrocortisone). On polymorphonuclear leucocyte bactericidal activity. J. Clini. Invest. 49:1381.

Matula G. and Paterson P.Y., (1971). Spontaneous invitro reduction of nitroblue tetrazolium by neutrophils of adult patients with bacterial infection. N. Engl. J. Med. 285:311.

Majeski J.A. and Alexander J.W. (1976). The steroid effect on in vitro human neutrophils chemotactic response. J. Surgical Res. 21:265.

Mardh P.A. and Westrom L. (1976). Adherence of bacteria to vaginal epithelial cells. Infect. Immun. 13:616.

McCall C.E., Copper C.J. and De Chatelet L., (1971). Functional characteristics of human toxic neutrophils. J. Infect. Dis. 124:68.

McDonald L.E. (1969). Veterinary Endocrinology and Reproduction. Lea and Febinger, Philadelphia, USA.

McDonald L.E., (1980). Veterinary Endocrinology and Reproduction 3rd ed. Lea and Febiger (1980). Philadelphia, U.S.A. p 274.

Messner R.P. and Jelinek K., (1970). Receptors for human gammaglobulin on human neutrophils. J. Clin. Invest. 49:2165.

Miller M.E., Oski F.A. and Harris H.B., (1971). Lazy leucocyte syndrome, a new disorder of neutrophil function. Lancet I:665.

Miller D.S. and Beck S., (1975). Polymorphonuclear leucocyte phagocytosis quantitiation by a rapid radioactive method. J. Lab. Clin. Methods. 86:2 344.

Miller R.L., Webster M.E. and Melmon K.L., (1975). Interaction of leucocytes and endotoxin with the plasmin and kinin systems. Eur. J. Pharmacol. 33: 53.

Miller M.E., (1976). Neutrophil function: In clinical immunobiology vol. 3. pp 427-437 Ed. Bach F.H. and Good R.A. Academic Press, N.Y., Lon.

Movat H.Z., Habal F.M. and Macrnorine D.R.L., (1976). Generation of a vasoactive peptide by a neutral protease of human neutrophil leucocytes. Agents Actions 6:183.

Munyua S.J. (1982). A study of inflammatory responses in the endometrium of the mare. B.Sc. Honours thesis. Murdoch University. W.A.

Munyua S., Williamson P., Penhale J. and Murray J., (1984). Acute cellular and humoral responses to equine Streptococcal endometritis. Proc. of A.S.R.B., Melbourne August 27-31, page 60.

Myhre E.B. and Kronwall G., (1980). Demonstration of specific binding sites for human serum albumin in Group C and G Streptococci. Infect. Immun. 27:6.

Nathan D.G., Baehner R.L. and Weaver D.K. (1969). Failure of nitroblue tetrazolium reduction in phagocytic vacuoles of leucocytes in chronic granulomatons disease. J. Clin. Invest. 48:1895.

Nelson R.D., Quie P.G. and Simmons R.L., (1975). Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leucocytes and monocytes. J. Immunol. 115(6):1650.

Nieberk K. and Cohrs P., (1966). A textbook of special pathological anatomy in domestic animals, 734. Perganon (1966).

Novak E.R. and Woodruff J.D., (1967). Novake gynaecological and obstetric pathology with clinical and endocrine relations. W.B. Saunders Company (1967).

Ochs H.D. and Igo R.P., (1973). A simple screening method for detecting chronic granulomatous disease and female carriers. J. Pediatr. 83:77.

Ofek I. and Beachey E.H. (1980). Bacterial adherence. In G.H. Stollerman (ed). Advances in internal medicine Vol. 25 year book medical publishers, Chicago p503.

Osbaldson G.W., (1978). Bacteriological studies of reproductive disorders of the bitch. J.A.A.H.A. 14(3):363.

Park B.H., Fikrig S.M. and Smithwick E.M., (1968). Infection and nitroblue tetrazolium reduction by neutrophils. A diagnostic aid. Lancet 2:2.

Park B.H. and Good R.A., (1970). N.B.T. tested stimulated. Lancet 2:616.

Peterson F.B., McFeely R.A. and David J.S.E., (1969). Studies on the pathogenesis of endometritis in the mare. Proc. 15th Ann. Mtg. Am. Equine Pract. 279.

Pearman V., (1978). Biopsy and cytology as an aid to clinical diagnosis. Scientific presentation and seminar synopsis. 39 Ann. Meet. A.A.H.A., 504.

Perryman L.E., (1981). Immunological aspects of the fetus. Proceedings of the annual meeting of society for Theriogenology, 268.

Prescot T.W. (1973). Reproductive patterns in the domestic cat. Aust. Vet. J. 49:126.

Quie P.G., White J.G., Holmes B. and Good R.A., (1967). In vitro bactericidal capacity of human polymorphonuclear leucocytes: Diminished activity in chronic granulomatous disease of childhood. J. Clin. Invest. 46:668.

Rabinowitz T., (1964). Separation of lymphocytes, polymorphonuclear leucocytes and monocytes on glass columns, in chiding tissue culture observations. Blood 23 6:611.

Rausch A. and Moore T., (1975). Granule enzymes of polymorphonuclear neutrophils, and phylogenetic comparison. Blood 46:913.

Rebello R., Green F.H.Y. and Fox H., (1975). A study of the secretory immune system of the feminal genital tract. Brit. J. Obst. Gynec. 82:812.

Reiter B., Brock J.H. and Steel E.D., (1975). Inhibition of E. coli by bovine colostrum and post colostral milk. II. The bacteriostatic effect of lactoferrin on serum susceptible and serum resistant strain of E. coli. Immunology. 28:83.

Ricketts S.W., (1975). Endometrial biopsy as a guide to diagnosis of endometrial pathology in the mare. J. Reprod. Fert. Supp. 23:341.

Ricketts S.W., Rossdale P.D. and Samuel C.A., (1978). Endometrial biopsy studies in mares with contagious equine metritis 1977. Equine Vet. J. 10(3):160.

Roberts S.J., (1971). Veterinary obstetrics and genital disease. Ithaca N.Y. Edwards Bro. Inc. Ann. Abor. Michigan (530).

Root R.K., Rosethal A.S. and Balestra D.J., (1972). Abnormal bactericidal, metabolic and lysosomal functions of chediak Higashi syndrome leucocytes. J. Clin. Invest. 51:649.

Rodbard D., (1974). Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assay. Clin. Chem., 20:1255.

Rosen H. and Klebanoff S.J., (1976). Chemiluminescence and superoxide production by myeloperoxidase deficient leucocytes. J. Clin. Invest., 58:50.

Rossdale P.D. and Ricketts S.W., (1980). Equine Stud. Farm Medicine. A. Baillier Tindal 2nd Ed. page 54.

Rothwell D.J. and Doumas B.T., (1975). The effect of heparin and EDTA on the NBT test. J. Lab. Clin. Med. 85:950.

Salmon S.E., Cline M.J., Schultz J. and Lehrer R., (1970). Myeloperoxidase deficiency: Immunologic study of a genetic leucocyte defect. N. Engl. J. Med. 282:250.

Samuel C.A., Ricketts S.W., Rossdale P.D., Stevens D.H. and Thurle K.W., (1979). Scanning electron microscope studies of the endometrium of the cycling mare. J. Reprod. Fert. Supp. 27:287.

Santivatr D., Maheswavan S.K., Newman J.A. and Pomeroy B.S. (1982). Effect of infectious bursal diseases virus infection on the phagocytosis of Staphylococcus aureaus by mononuclear phagocytic cells of susceptible and resistant strains of chicken. Avian Diseases. 25:2 303.

Schwabe C.M., Riemann H.P. and Franti C.E., (1977). Mathematical approach: In epidemiology in Veterinary Practice. pp 73-76. Publ. Lea and Febiger, Philadelphia, USA.

Schaeffer A.J., Amundsen S.K. and Schmidt L.N., (1979). Adherence of Escherichia coli to human urinary tract epithelial cells. Infect. Immun. 24:753.

Schaeffer A.J., Jones J.M. and Dunn J.K. (1981). Association of in-vitro Escherichia coli adherence to vaginal and recurrent urinary tract infections. New Engl. J. Med. 304:1062.

Schopfer K. and Douglas S.D., (1978). Neutrophil function in children with kwashiokor. J. Lab. Clin. Med. 88:450.

Selvaraj R.J. and Bhat K.S., (1972). Metabolic and bactericidal activities of leucocytes in protein-calorie malnutrition. Am. J. Clin. Nutri. 25:186.

Selinger D. and Reed W.P., (1979). Pneumonococcal adherence to human epithelial cells. Infect. Immun. 23:545.

Schumacher G.F.B. and Yang S.L., (1971). Cyclic changes in immunoglobulins and specific antibodies in human and rhesus monkey cervical mucous. The Uterine Cervix in Reproduction Workshop Conference rottach-Egeru. Ed. Isler V. and G. Bettendorf. Georg Thieus Publishers, Stuttgart.

Sellwood R., Gibbons R., Gibbons R.A., Jones G.W. and Rutter J.M., (1975). Adhesion of enteropathogenic Escherichia coli to pig intestinal brush borders: the existence of two pig phenotypes. J. Med. Microbiol. 8:405.

Shin S.J., Lein D.H., Arousor A.L. and Nusbaun S.R. (1979). The bacteriological culture of organisms isolated and their interpretations. J. Reprod. Supp. 27:307.

Sigh H., Boyd E., Hutton M.M., Wilkinson P.C., Pebbles Brown D.A. and Ferguson-Smith M.A., (1972). Chromosomal mutation in bone marrow as a cause of acquired granulomatous disease and refractory microcytic anaemia. Lancet 4:873.

Sigma technical Bulletin, No. 840 1978 (Sigma Chemicals ST. Louis, U.S.A. Silverblatt

Silverblatt F.J. and Ofek I., (1978a). Influence of pili on the virulence of Proteus mirabilis in experimental hematogenous pyelonephritis. J. Infect. Dis. 138:664.

Silverblatt F.J. and Ofek I., (1978b). Effects of pili on susceptibility of Proteus mirabilis to phagocyosis and on adherence to bladder cells. In Ett. Kass and W. Brunfitt (ed). Infections of the urinary tract. University of Chicago Press. Chicago. p49.

Skarnes R.C. and Watson D.W., (1956). Characterization of leukin: An antibacterial factor from leucocytes active against gram positive pathogens. J. Exper. Med. 104:82.

Smith M.R. and Wood W.B., (1969). Heat labile opsonins to pneumococcus I. Participation of complement. J. Expe. Med. 130: 1209.

Smith H.A., Jones T.C. and Hunt R.D., (1972). Pathology. Lea and Febiger, U.S.A.

Sobel J.D., Schneider J., Kaye D. and Levinson M.E., (1981). Adherence of bacteria to vaginal epithelial cells at various times in the menstrual cycle. Infect. Immun. 32:194.

Spector T., (1978). Refinement of comassie blue method of protein quantitation. Anal. Biochem. 86:142.

Steerman R.L., Synderman R., Leikin S.L. and Colten H.R., (1971). Intrinsic defect of the polymorphonuclear leucocyte resulting in impaired chemotaxis and phagocytosis. Clin. Exp. Immunol. 9:939.

Stevens P. and Young L.S., (1977). Quantitative granulocyte chemiluminescence in the rapid detection of impaired opsonization of Escherichia coli. Infect. Immun. 16:796.

Stenson W.F. and Parker W.C., (1979). Metabolism of archidonic acid in ionophore stimulated neutrophils. Esterification of a hydroxylated metabolite into phospholipids. J. Clin. Invest. 64:1467.

Stenson W.F. and Parker C.W. (1980). Monohydroxyeicosatetranoic acids (HETES). induce degranulation of human neutrophils. J. Immunol. 124:2100.

Stjernholm R.L., Alten R.C., Steele R.H., Waring W.W. and Harris J.A. (1973). Impaired chemiluminescence during phagocytosis of opsonized bacteria. Infec. Immun. 7(2):313.

Stolterman G.H., (1975). Rheumatic fever and Streptococcol infections. Grune and Stratton. New York (47-62).

Sugarman B., (1980). Attachment of bacteria to mammalian surfaces. Infection 8:(4): 132.

Sugarman B. and Epps L.R., (1982). Effect of oestrogens on bacterial adherence to Hela-cells. Infect. Immun. 35(2):633.

Svanborg-Eden C. and Hansson H.A., (1978). Escherichia coli pili as possible mediators of attachment to human urinary tract epithelial cells. Infect. Immun. 21: 229.

Svanborg-Eden C. and Jodal U., (1979). Attachment of Escherichia coli to urinary sediment epithelial cells from urinary tract infection-prone and healthy children. Infect. Immun. 26:837.

Svanborg-Eden C. and Leffler H., (1980). Glycosphospholipids of human urinary epithelial cells as possible receptors for adhering Escherichia coli bacteria. Scand. J. Infect. Dis. 24:144.

Teir H., Rytomaa T., Cedarburg A and Kiviniemi K., (1963). Studies on the elimination of granulocytes in the intestinal tract of the rat. Acta. Pathol. Microbiol. Scand., 59:311.

Tomasi T.B., (1972). Secretory immunoglobulins. New Engl J. Med. 500.

Tylewska S.K., Hjerten S. and Wadstrom T., (1979). Contribution of M protein to the hydrophobic surface properties of Streptococcus pyogenes Fed. Euro. Microbiol. Soc. Microbiol.: Letters 6:249.

Valentine W.N. and Beck W.S., (1951). Biochemical studies on leucocytes: 1. Phosphate activity in health, leucocytosis and myelocytic leucemia. J. Lab. Clin. Med. 38:39-47.

Vaerman J.P. and Ferin J., (1974). Local immunological response in the vagina cervix and endometrium pp 281-301. In the Proc. 7th Symposium on immunological approaches to fertility control: Karolinska Symposia on Research methods in Reproductive endocrinology. University of Louvain, Brussels, Belgium.

Van Dyke K., Trush M., Wilson M. and Stealey P., (1977). Luminol dependent chemiluminescence analysis of cellularand humoral defects of phagocytosis using a chem-glo photometer. Microchem. J. 22:463.

Verhoef J., Peterson P.K., Quie P.G., (1977). Kinetics of staphylococcal opsonization attachment, ingestion and killing human PMN leucocytes: a quantitative assay using (3H). thymidine labelled bacteria. J. Immuno. Methods. 14:303.

Volanakis J.E., Freeman D.G. and Stroud R.M., (1975). Acute inflammation and its chemical mediators: The complement system. In Biologic basis of wound healing, ed. Menaker L., Harper and Row (Publ). N.Y., London, SF.

Waldman R.H. and Ganguly R. (1974). Special article: Immunity to infection on secretory surfaces. J. Infect. Dis., 130(4): 419.

Waldman R.H. and Ganguly R., (1976). Role of immune mechanisms on secretory surfaces in prevention of infections pg 29-48. In infections and the compromised host Ed. Allen J.C. (1976). Pub. The Williams and Wilkins Co., Baltimore.

Weissman G., Zurier R.B. and Hoffstein S., (1972). Leucocytic proteases and the immunologic release of lysosomal enzymes Am. J. Pathol. 68:538.

Weissman G., Zurier R.B. and Hoffstein S., (1973). Leucocytes as secretory organs of inflammation. Agents Actions 3:370.

Weissman G., Korchat H.M., Perez H.D., Smolen E., Goldstein I.M. and Hoffstein S., (1979). Leucocytes as secretory organs of inflammation. In: Advances in inflammation research Vol. I. Ed. by G. Weissman, B. Samuelsson, R. Paoletti. Raven Press, N.Y.

Weissman G., Smolen J.E. and Korchak H.M., (1980). Release of inflammatory mediators from stimulated neutrophils. N. Engl. J. Med., 202:27.

Williamson P., Dunning A., O'connor J. and Penhale J., (1983). Immunoglobulin levels, protein concentrations and alkaline phosphatase activity in uterine flushing from mares with endometritis. Theriogenology 19(3):441.

Williamson P., Penhale J., Munyua S. and Murray J., (1984). Acute reaction of the mare's uterus to bacterial infection. Proc. 10th Intern. Cong. Reprod. & AI., Illinois, June 10-14.

Wingfield N.J., Dibgy J., (1978). The techniques and clinical application of endometrial cytology in mares. Equine Vet. J. 10(3):167.

Winter A.J., Broome A.W., McNutt S.H. and Casida L.E., (1960). Variations in uterine responses to experimental infection due to hormonal state of ovaries. 1: The role of cervical drainage WBC numbers and non cellular factors in uterine bacericidal activity. Am. J. Vet. Res. 675.

Winter A.J., (1982). Microbial immunity in the reproductive tract. JAVMA 181(10):1069-1073.

Woods D.E., Straus D.C., Johanson W.G., Berry V.K. and Bass J.A., (1980). Role of pili in adherence of Pseudomonas aureginosa to mammalian buccal epithelial cells. Infect. Immun. 29:1146.

Woolcock J.B., (1980). Equine bacterial endometritis. Symposium on equine reproduction. The Vet. Clinics of North America (Large Animal practice). 2(2):241.

Wright D.G. and Malawista S.E., (1972). The mobilisation and extracellular release of granular enzymes from human leucocytes during phagocytosis. J. cell biol. 53:738.

Wright D.G. and Malawista S.E., (1973). Mobilization and extracellular release of granular enzymes from human leucocytes during phagocytosis. Inhibition; by colchicine and corticosteroids but not salicylates. Arthritis Rheum. 16:749.

Wright D.G. and Gallin J.J., (1975). Modulation of the inflammatory response by products release from the human polymophonuclear leucocytes during phagocytosis; generation and activation of the chemotactic factor CSA. Inflammation I:23.

Wright D.G. and Gallin J.J., (1977). A functional differentiation of human neutrophil granules; generation of C5a by specific (secondary). Granules product and inactivation of CSA by azurophil (primary). Granule products. J. Immunol. 119:1068.

Wright D.G. and Greenwald D., (1979). Increased motility and maturation of human blood monocytes stimulated by products released from neutrophil secondary granules. Blood 54 (suppl.). 95A.

Wright D.G. and Gallin J.I., (1979). Secretory responses of human neutrophils during adherence in vitro and during exudation in vivo. J. Immunol. 123:285-294.

Wright D.G. and Malawista S.E., (1972). The immobilisation and extracellular release of granular enzymes from human leucocytes during phagocytosis. J. Cell. Bio. 53:788.

Wright D.G., (1982). The neutrophil as a secretory organ of host defense. In advances in host defence mechanisms. Vol. 1. Ed. J.I. Gallin and A.S. Fauci. p 75-110. Raven Press. N.Y., U.S.A.

Yielding K.L. and Tomkins G.M., (1959). Inhibition of enzymatic oxidation of DPNH by steroid hormones. Proc. Nat. Acad. Sci. USA. 45:1730.

Zavy M.T., Bazer F.W., and Sharp D.C., (1978). A non surgical technique for the collection of uterine fluid from the mare. J. Anim. Sci. 47:672.

Zurier R.B., (1976). Prostaglandin release from human polymorphonuclear leucocytes. Adv. Prostaglandin thromboxane Res. 2:815.

Zeiyer H.I. and Spiznagel J.K., (1969). Cationic protein bearing granules of PMN: Separation of enzyme rich granule. Science 163:1063.