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STUDY OF NEUTROPHIL DIAPEDESIS ACROSS A BOVINE MAMMARY EPITHELIUM IN VITRO

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

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SHORT TITLE:

Study of Neutrophil Transepithelial Migration *in vitro*

ABSTRACT

M.Sc. Animal Science

Yongqing Lin

Study of Neutrophil Diapedesis Across a Bovine Mammary Epithelium in vitro

Bovine mastitis due to bacterial infection is one of the most costly diseases affecting the dairy industry. The polymorphonuclear neutrophils (PMNs) present in milk have a central protective role against invading pathogens. However, the manner by which PMNs traverse the secretory epithelia and the relationship between PMN diapedesis and the epithelial damage are unclear. This in vitro study investigated the process and rate of bovine PMN transepithelial migration. The bovine mammary epithelial cell line, MAC-T, formed a confluent monolayer with characteristic tight junctions, polarity and functional barrier to the dye trypan blue. In the first series of experiments, neutrophils were added into the upper compartment of the culture insert and stimulated to migrate across the epithelium in an apical-to-basal direction by the addition of Staphylococcus aureus to the lower compartment. Light and transmission electron microscopy revealed the following series of events for PMN transmigration: (1) adherence of PMNs to the surface of the epithelium; (2) projection of pseudopods toward the intercellular junction; (3) migration between adjacent epithelial cells; and (4) re-approximation of epithelial cell membranes and re-formation

of tight junctions after PMNs crossed the epithelium. Morphologically, epithelial damage caused by PMN transmigration was not evident. In the second series of experiments, PMN diapedesis across the epithelium in a basal-to-apical direction was investigated. The rate of PMN migration was quantified by assaying the activity of neutrophil azurophilic granule marker, myeloperoxidase (MPO). The minimal number of PMNs detectable by the MPO assay was approximately 5×10^3 cells. Results showed that PMNs were able to migrate across the mammary epithelium in the physiologically relevant direction, and that their migration was greatly stimulated by the presence of zymosan activated serum (ZAS). The effects of ZAS were dose- and time-dependent. In the presence of 5% ZAS and 2×10^6 neutrophils per insert, approximately 2.4×10^5 neutrophils migrated across the epithelium in 120 min. In summary, this study for the first time described morphologically the process of PMN migration across bovine mammary epithelial cells, and has established a model system by which the process and rate of PMN diapedesis across a bovine mammary epithelium in a physiologically relevant direction can be studied in a controlled environment.

RÉSUMÉ

Étude sur la diapédèse des neutrophiles bovins à travers un épithélium mammaire in vitro

La mammite bovine causée par des infections bactériennes est l'une des maladies les plus coûteuses qui affectent l'industrie laitière. Les neutrophiles polymorphonucléaires (PMNs) présents dans le lait ont un rôle central dans la protection contre l'envahissement des pathogènes. Cependant, la façon par laquelle les PMNs traversent les épithéliums sécréteurs ainsi que la relation entre la diapédèse des PMNs et le dommage épithélial demeurent des sujets obscurs. Cette étude in vitro a examiné le processus et le taux de migration transépithéliale des PMNs bovins. La lignée de cellules épithéliales mammaires MAC-T formait une monocouche confluente démontrant les jonctions étanches, la polarité et l' exclusion de la teinture de trypan bleue, typiques de ces type de cellules. Dans la première série d'expériences, des neutrophiles ont été ajoutés au compartiment supérieur de la culture et stimulés afin de traverser l'épithélium dans la direction apicale-basale par l'addition de Staphylococcus aureus au compartiment inférieur. Des observations au microscope ordinaire et à transmission d'électrons ont permis d'identifier la séquence des événements de la transmigration des PMNs: (1) adhérence des PMNs à la surface de l'épithélium; (2) projection de pseudopodes vers la jonction intracellulaire; (3) migration entre les cellules épithéliales

adjacentes; et (4) rapprochement des membranes cellulaires épithéliales et re-formation des jonctions étanches suivant la migration des PMNs en travers l'épithélium. Au point de vue morphologique, il n'y avait pas de dommage épithélial évident causé par la transmigration des PMNs. La seconde série d'expériences avait pour but d'examiner la diapédése de PMNs à travers l'épithélium dans la direction inverse i.e. de basale à apicale. La vitesse de migration a été quantifiée en mesurant l'activité du myéloperoxidase (MPO), un marqueur de granules neutrophiles azurophiliques. Le nombre minimale de PMNs détectable par la détermination de la MPO était approximativement 5×10^3 cellules. Les résultats démontrent que les PMNs étaient capable de traverser l'épithélium mammaire dans la direction physiologiquement pertinente, et que leur migration était grandement stimulée par la présence de serum activé au zymosan (SAZ). Les effets du SAZ étaient dépendent de la dose ainsi que du temps. Lorsque le milieu de culture contenait du SAZ à 5% et 2×10^6 neutrophiles, environ 2.4×10^5 neutrophiles ont traversé l'épithélium en 120 minutes. En résumé, cette étude a décrit morphologiquement pour la première fois le processus de migration des PMNS à travers des cellules épithéliales mammaires bovines, et a établie un system model par lequel le processus ainsi que la vitesse de diapédèse des PMNs à travers l'épthélium mammaire dans une direction physiologiquement pertinente pourraient être étudiés dans un environement controllé.

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CHAPTER I. GENERAL INTRODUCTION

Mastitis is one of the leading diseases and management problems in the dairy industry. Despite considerable efforts to control it for the past 20 years, mastitis continues to cause significant economic loss in dairy production worldwide. It is estimated that losses from mastitis exceed 2 billion dollars per year in the United States (Eberhart et al., 1987). On a per cow basis, this figure is consistent with the estimated losses incurred in the Canadian (Gill, 1990), European and Australian dairy industries (Blosser, 1978).

With the continuing challenge of economic losses resulting from mastitis, dairy scientists have become increasingly aware that large gaps between our knowledge of this disease and its control, still exist (Eberhart et al., 1987). Therefore, further research on the pathophysiological aspects of the disease, especially the roles of the factors involved in mastitis defense, is needed. A better understanding of this disease will lead to the development of more effective strategies to control mastitis.

Milk leukocytes, especially polymorphonuclear neutrophils (PMNs), are considered one of the major cellular components to protect against invading pathogens in the udder (Paape et al., 1979). In response to bacterial infection, PMNs migrate

rapidly, in large numbers, into the alveolar lumen of the infected mammary gland, where they phagocytose and kill microorganisms. The important role of neutrophils in mastitis defense was first demonstrated 26 years ago by Jain et al. (1968). Since then, considerable work (Paape et al., 1978; Daley et al., 1991) has been done on studying the phagocytosis and killing ability of bovine neutrophils. However, only a few studies have investigated the process of PMN diapedesis into milk. The manner by which PMNs migrate across the tissue barriers remains poorly elucidated.

The alveolar epithelia of the mammary gland is the major and final barrier for PMNs when they migrate from blood into milk. Thus, neutrophil transepithelial migration must be a crucial step of PMN diapedesis. Therefore, clarifying the process and cellular events of PMN transepithelial migration is fundamental to a better understanding of the roles that neutrophils play in mastitis resistance, and may also be a key to gain insight into the "mastitis mystery" (Daley and Hayes, 1992).

Progress in our understanding of the neutrophil diapedesis mechanism has been impeded by substantial methodological problems. Traditionally, an in vitro system, the under agarose migration method (Carroll, 1982), and an in vivo system, the mammary gland infection model (Harmon and

Heald, 1982), have been the major systems to study diapedesis of bovine neutrophils. These models, however, do not allow us to monitor the rate of PMN migration *in vitro* or to identify the factors affecting PMN diapedesis *in vivo*.

With the establishment of a bovine epithelial cell line, denoted MAC-T (Mammary Alveolar Cell-large T antigen) (Huynh *et al.*, 1991), it is now possible to study bovine neutrophil migration across a bovine mammary epithelium *in vitro*. A previous study (Macdonald *et al.*, 1994) has demonstrated that MAC-T cells formed a confluent monolayer with characteristic tight junctional complex, apical-basal polarity and significant transepithelial electrical resistance. Moreover, they found no significant change in the transepithelial electrical resistance, when the MAC-T monolayer was challenged with either *Staphylococcus aureus* or neutrophils.

Since the process and rate of transmigration are two major aspects of PMN diapedesis, this project was conducted to address these issues, using a MAC-T cell epithelial monolayer *in vitro* system. This study firstly investigated the process of bovine PMN transepithelial migration. The PMNs were allowed to transmigrate across the epithelium under the stimulation of a chemoattractant. The migration process was observed at an electron microscopic level. Secondly, a microassay system has been established to quantify the rate of bovine PMN

transepithelial migration in a basal-to-apical direction, using an enzyme assay.

CHAPTER II. LITERATURE REVIEW

1. BOVINE MASTITIS

1.1. Overview

Mastitis is classically defined as an inflammation of the mammary gland. Although any type of injury to the mammary tissues may be expected to induce an inflammatory response, bovine mastitis is most frequently associated with bacterial infection (Eberhart et al. 1987). The disease has a clinical and more commonly, a subclinical manifestation.

Normally, mastitis develops as a result of passage of pathogenic bacteria through the streak canal, followed by their multiplication within the milk. The interaction between the invading pathogens and the cow's immune systems induces the inflammatory response in the infected mammary gland (Bramley and Dodd, 1984). Bacteria isolated from mastitic cows are commonly widespread in the cow's environment and are predominantly Staphylococci, Streptococci and coliforms (Nickerson, 1993). Together these bacteria comprise about 84% of the isolates from clinical cases and about 90% from subclinical cases (Sandgren, 1991).

The severity of mastitis is determined by the nature of the invading pathogens, the condition of the cow's immune systems, and to some extent by the stress placed on the

mammary gland by milking practices. The clinical signs of mastitis are, in reality, an expression of the host defense intended to destroy the invader and to make way for repairing the damaged tissues.

Mastitis is a complex disease in view of its pathogenesis, intensity of duration, immunity and therapy (Jain, 1979). Although a number of control practices based on milking hygiene, reducing exposure to environmental pathogens, dietary supplementation with essential micronutrients, antibiotic therapy, and vaccinations have been used to control the disease for the past decades, the incidence of mastitis remains high (Radostitis, 1994).

Major losses from mastitis have been classified into the following categories: a) realized (clinical) and unrealized (subclinical) reduced milk production; b) milk discarded because of antibiotic content or abnormal composition; c) veterinary service; d) medications and labour; and e) culling and mortality (Eberhart et al., 1987). Eberhart et al (1987) estimated that 70-80% of the losses from mastitis are associated with reduced milk production due to subclinical mastitis.

1.2. Defense mechanisms of the bovine udder

1.2.1. Leukocytes

Milk leukocytes and a small percentage of epithelial cells in milk are collectively termed somatic cells. The number and predominant types of somatic cells vary with the severity of mastitis and even with the period of lactation. In general, somatic cell count (SCC) in non-mastitis milk is less than 10^5 cells per millilitre, with the predominant cell types being macrophages (48%), lymphocytes (30%) and neutrophils (22%) (Nickerson, 1985). When the udder is infected, the SCC increases dramatically, due to a continuous migration of neutrophils into milk. Increased cell number is thus indicative of the existence of mastitis. Usually, somatic cell counts average 700,000 per millilitre in subclinically infected quarters, whereas a million per millilitre are common in clinical infections. Neutrophils constitute greater than 90% of SCC during mastitis (Kehrli and Shuster, 1994).

Milk leukocytes, especially neutrophils, are the major cellular immune component of the udder during mastitis (Nickerson, 1993). In the first stage of bacterial infection, the small numbers of somatic cells that are normally present attempt to resolve this infection immediately. If the bacteria are destroyed, then neither a noticeable increase in SCC nor clinical signs will occur (Kehrli and Shuster, 1994). If, however, the innate defense mechanisms of the udder lose the first battle with bacteria, then bacteria multiply rapidly. Bacterial products (e.g. toxins) and other factors (e.g.

interleukin 8 and leukotriene B4) released by various cell types in the gland are chemotactic (Sordillo, 1993). These factors elicit a rapid recruitment of additional blood leukocytes (mostly neutrophils) into the infected gland. After reaching the milk within the infected mammary gland, neutrophils function primarily by engulfing and killing invading bacteria (Nickerson, 1993). Therefore, the interaction of bacteria with milk leukocytes is critical to the establishment and outcome of mastitis.

Although leukocytes are known to play a protective role in defending the mammary gland, the relationship between SCC and the initiation and severity of mastitis has not been well established. Early studies found that a range of SCC from 400,000 to 600,000 cells/ml was critical to provide protection against an experimental challenge with pathogens (Schalm et al., 1964). This concept became the objective of several years of research into the use of polyethylene devices to increase the SCC to a level that provided protection to bacterial infection. However, later studies showed that 900,000 cells/ml were needed for protection (Schultze and Paape, 1984). Some cows with this high SCC may even remain subclinically infected in the same quarter for several lactations. Moreover, the environment of the mammary gland has been known to limit the phagocytic and killing ability of neutrophils (Paape et al., 1979) and macrophages (Politis et al., 1991).

1.2.2. Milk proteins

Some proteins in normal and mastitis milk also play an important role in the local defense against invading pathogens. Milk antibodies (IgG, IgA and IgM), for example, can opsonize bacteria, neutralize toxins, and prevent adhesion of bacteria to the mammary tissues (Nickerson, 1993). Lactoferrin derived from epithelial cells and leukocytes is effective against Staphylococci and coliforms because of its iron-chelating properties, which decrease iron availability for bacterial growth (Nuijens et al., 1993). Other milk proteins with antibacterial activity include lactoperoxidases, lysozymes and complements.

2. NEUTROPHILS

2.1. Overview

PMNs are bone marrow-derived, non-dividing end cells (Tizard, 1992). The development of a mature neutrophil from a myeloblast in bone marrow is completed in about 12 days (Bainton, 1988). When fully developed, PMNs leave the bone marrow and migrate into the bloodstream, where their half-life is about 10 hours. During this time, the PMNs circulate in the blood and survey the body by sensing signals from local inflammatory response (Tizard, 1992).

A characteristic of PMNs is the multilobed, irregular nucleus, which makes the cell extremely deformable (Bainton,

1988). It has been suggested that the multilobed shape of this organelle allows PMNs to move through small openings so that cells can enter tissue easily to perform their specialized role of phagocytosis (Paape et al., 1979).

The presence of enzyme-rich granules in the cytoplasm also distinguishes this cell from other cells. Ruminant PMNs contain three types of granules while other species contain two (Sandgren, 1991). Like PMNs of other species, bovine PMNs contain primary or azurophilic granules and secondary or specific granules. Both types of granules contain bactericidal substances such as myeloperoxidase, lysozyme and defensins. The third granule is larger, denser and more numerous than the other two granules, and contains lactoferrin. The mature neutrophils possess a small Golgi apparatus and some mitochondria but very few ribosomes or rough endoplasmic reticulum, thus, they cannot synthesize large quantities of proteins (Tizard, 1992).

2.2. Functionality

The major function of neutrophils is the non-specific phagocytosis and destruction of foreign materials such as invading microorganisms (Nickerson, 1993). This process can be divided into several stages: accumulation within the alveolar lumen, phagocytosis and microbicidal activities.

Neutrophils are triggered to leave the circulation as a result of local environmental changes. Thus in areas of tissue damage or bacterial invasion there is a transient increase in adhesive molecules on the surface of vascular endothelial cells (Bevilacqua et al., 1989). The process of PMN migration from the blood stream into the inflammatory tissues is called diapedesis. This process is stimulated by chemotactic factors produced in inflammatory sites. Therefore, diapedesis is an important prerequisite for PMNs to perform their function.

Once neutrophils have arrived at inflammatory sites of the infected mammary gland, phagocytosis of invading bacteria starts. The first requirement for this process to occur is the recognition and attachment of invading bacteria by neutrophils, which is normally achieved by opsonization of the bacteria, either with complement factors or immunoglobulins (Peterson et al., 1978). A number of different receptors such as FcR receptors and complement receptors have been identified on the surface of neutrophils (Bochsler et al., 1994). The phagocytic process is generally believed to be executed by the action of the cytoskeletal protein actin, which rapidly alters its degree of polymerization on phagocytic stimulation (Stossel, 1988). This process will lead to the formation of pseudopods surrounding bacteria. When the whole bacterium is surrounded, a "phagosome" is formed.

Destruction of the ingested pathogens by neutrophils occurs through two distinct mechanisms: dependent and independent of the presence of oxygen (Clark, 1990). The oxygen-dependent system relies on the activation of a membrane-bound NADPH-oxidase, which will catalyze a one-electron reduction of oxygen with NADPH. This process is usually called respiratory burst and can produce some oxygen species such as hydrogen peroxide and superoxide anion. One important bactericidal pathway is activated by the reaction of superoxide with hydrogen peroxide. This reaction generates hydroxyl radicals and singlet oxygen. Both hydroxyl radical and singlet oxygen are unstable and react with bacterial lipids to form bactericidal hydroperoxides (Tizard, 1992). The oxygen-independent microbicidal system consists of a number of toxic enzymes and proteins in the primary and secondary granules which are released into the newly formed phagosome. Because oxygen radical production and destruction of bacteria by the action of toxic proteins occur in parallel within the same cell, discriminating between these two mechanisms is difficult.

Although neutrophils are capable of phagocytosing a wide variety of pathogens, the phagocytic activity of PMNs in the milk is less efficient compared to those in blood (Daley, 1992). Many studies investigated the factors responsible for this impediment of phagocytic function. Macdonald (1979) has

shown that this dysfunction in milk PMNs is mainly due to their loss of energy stores as a result of diapedesis and phagocytosis, and ingestion of other particles in milk including fat and casein. Therefore, normal milk components are constantly diverting the phagocytic activity of healthy, functional PMNs away from focusing on invading bacteria (Sordillo, 1993). To overcome the interference in the milk environment, immune-modulators such as cytokines are being used to deliberately increase the number and functional activity of neutrophils in milk (Nickerson et al., 1989; Sordillo, 1993).

3. NEUTROPHIL DIAPEDESIS AND MASTITIS

3.1. Diapedesis phenomenon

The accumulation of leukocytes (mostly neutrophils) at the site of injury is an important feature of any inflammation such as mastitis (Persson et al., 1992). Traditionally, diapedesis refers to the movement of leukocytes across the capillary endothelium. However, if inflammation occurs in an organ lined with epithelium, such as the mammary gland, PMNs will continue to migrate across the secretory epithelium (Colgan et al., 1993). Therefore, the diapedesis of bovine neutrophils during mastitis should include the whole process of migration from the bloodstream to the alveolar lumen of the mammary gland.

3.2. Diapedesis mechanism.

It is believed that chemotaxis is an important mechanism responsible for accumulation of PMNs at sites of inflammation (Carroll et al. 1982). Chemotaxis is defined as a direct movement of cells against a concentration gradient of chemotactic factors, or chemoattractants such as bacterial toxins. Diapedesis occurs in response to the stimulation of chemotactic factors in the infected mammary gland. The mechanism by which chemotactic factors interact with the leukocytes and signal them to move is still not fully understood. The initial interaction between chemotactic factors and neutrophils is believed to be receptor mediated (Baggiolini et al. 1993). The binding of the chemoattractants to their receptors leads to a net influx of calcium within the PMNs, which plays a key role in locomotion toward the concentration gradient of chemoattractants (Tizard, 1992).

3.3. Diapedesis Process

In order to reach the site of inflammation, the circulating PMNs must migrate out of the blood vessel and cross subsequent tissue barriers. Using an in vivo model, Harmon and Heald (1982) demonstrated morphologically that the major structural barriers between blood stream and milk include: 1) endothelial cells lining the capillary lumen; 2) basal lamina surrounding the capillaries; 3) periendothelial cells; 4) basal lamina surrounding the alveoli; and 5) a

single epithelial layer lining the mammary alveoli. Their results also suggested that the principal sites of PMN transendothelial migration were interendothelial junctions. After migration across the vessel wall, PMNs arrested temporarily under the basement membrane but soon migrated across the periendothelial cell layer into extravascular connective tissues adjacent to epithelial linings. The PMNs then penetrated epithelial basal lamina and gained access to the epithelial cell layer (Harmon and Heald, 1982).

Alveolar epithelia, the milk secreting cells of the mammary gland, is the final barrier for PMN diapedesis into milk. Although in vivo studies (Frost et al.; Harmon and Heald, 1982; Nickerson and Pankey, 1984) have investigated PMN diapedesis during mastitis, conclusively morphological evidence of PMN transepithelial migration has not been reported. From those studies, several modes of PMN transepithelial migration have been proposed: 1) penetration through individual, degenerated luminal cells; 2) migration between intact epithelial cells; or 3) involvement of both ways.

3.4. Significance of neutrophil diapedesis in mastitis defence

The crucial role of neutrophils in the host defence against bacterial bovine mastitis was demonstrated by Schalm et al. (1976), who turned a chronic Staphylococcus aureus mastitis into a gangrenous disease by pretreating the cow with an anti-bovine leukocyte serum. Subsequently, Hill et al. (1981) established that a rapid recruitment of neutrophils to the udder in response to invading coliform bacteria was of utmost importance for recovery from an infection. Other work also indicated that fatal consequences of udder infections with Escherichia coli were due to a failure in the recruitment of neutrophils to the udder tissue in response to the bacterial challenge (Frost and Brooker, 1986). Recently, cows suffering with recurrent infections have been diagnosed with bovine leukocyte adhesion deficiency (BLAD) (Kehrli et al., 1990). The neutrophils of the BLAD calves were genetically deficient with respect to the CD11/CD18 glycoprotein complex required for neutrophil binding activity to the endothelial cells. This deficiency resulted in the inability of neutrophils to diapedese into infected areas. Therefore, it is evident that both the ability and the rate of neutrophil migration are critical to the outcome of mastitis.

3.5. Diapedesis and epithelial cell damage

As mentioned above, increased SCC due to PMN diapedesis during mastitis results in definite advantages with respect to mastitis resistance. However, a significant negative correlation has also been reported between milk production and SCC (Raubertas et al., 1982; Fetrow et al., 1988). Therefore, there has been a hypothesis that migration of leukocytes from blood into milk directly damages the mammary epithelia and alters cell function, and consequently reduces milk yield (Nickerson and Pankey, 1984; Akers and Thompson, 1987). However, a direct causal relationship between neutrophil diapedesis and the damage of secretory cells has yet to be proven. A recent study suggests that much of the decrease in milk production is due to a systemic effect of the inflammation (Shuster et al., 1991).

3.6. Methodology for studying diapedesis

Traditionally, an "under-agarose" system whereby neutrophils migrate in response to chemical signals placed at specific distances has been used to study neutrophil chemotaxis, such as the determination of chemotactic factors for bovine leukocytes (Carroll et al., 1982); the directional and random migratory response of neutrophils from cows of different ages (Olson, 1990), and the chemotactic properties of bovine mammary macrophages (Politis et al., 1991). In this system, the migration activity of neutrophils is expressed as

a migration index which is derived from dividing the distance moved by cells toward the controlled well by the distance moved by cells toward the chemoattractant (Olson, 1990). However, this system can not quantify the number of migrated neutrophils. Moreover, it can not serve as a biologically meaningful barrier for PMN migration.

Another classical way to study neutrophil chemotaxis is the experimentally-induced mastitis in cows. After infection of the mammary gland with bacteria or endotoxin, the SCC in milk is monitored at intervals during the period of disease (Hill, 1981). The accumulation of PMNs in the mammary gland can also be observed morphologically in mammary tissues obtained from biopsy or sacrificed cows (Harmon and Heald, 1982; Nickerson and Pankey, 1984). Although this system can provide the actual situation for PMN diapedesis, it also has some disadvantages such as the difficulty in observing the process and cellular events of PMN transepithelial migration in real time, the inability to identify the factors involved, and the large cost of using a cow as a model.

Researchers working with human neutrophils (Cramer et al., 1980) have circumvented these problems by using a Madin-Darby canine kidney (MDCK) cell in vitro system. This model is simple and provides a control environment for studying PMN diapedesis across a biologically meaningful barrier (cell

monolayer). Since then, the monolayer cell model has been widely accepted for use in the study of chemotaxis of human neutrophils. For example, using this model, Milks et al. (1986) studied the effect of neutrophil migration on epithelial permeability. Moser et al. (1989) investigated the effect of interleukin-1 on the transendothelial migration of human neutrophils. However, there is as yet no comparable in vitro systems available for studying bovine neutrophils in relation to mastitis.

In summary, bovine PMNs present in milk serve a protective role against invading pathogens. However, the manner by which PMN diapedese across the mammary epithelia and the relationship between PMN diapedesis and the epithelial damage have not been elucidated by the previous in vivo or in vitro studies. Therefore, this study was conducted to investigated the process and rate of bovine PMN transepithelial migration using an in vitro model.

CHAPTER III. MORPHOLOGICAL OBSERVATIONS OF NEUTROPHIL TRANSEPITHELIAL MIGRATION

INTRODUCTION

One important feature of mastitis is a rapid and massive influx of neutrophils from the bloodstream into the alveolar lumen of the infected mammary gland, thereby increasing the SCC in milk (Nickerson, 1985). Neutrophils are one of the major cellular immune components against invading pathogens, thus, the prompt accumulation of neutrophils in the infected mammary gland is essential for the suppression of bacterial growth and infection. However, two important issues concerning PMN diapedesis remain obscure. How do PMNs diapedese across the alveolar epithelia? Does this process directly damage the secretory epithelial cells? Although several in vivo studies have been conducted to address these questions, the information from these studies is controversial.

For example, by using morphological methods to observe PMN accumulation in mammary tissues obtained from biopsy or from sacrificed cows, Frost et al. (1980) claimed that neutrophils migrated through epithelial lesions but not through intact epithelial surfaces. Similarly, Nickerson and Pankey (1984) proposed that projection through degenerated luminal cells was a predominant mechanism of PMN migration. On the other hand, Harmon and Heald (1982) suggested that

migration between cells was the major mechanism for influx of large number of PMNs during mastitis.

The relation between milk production and PMN infiltration has also been investigated using physiological and biochemical methods. In a study conducted to determine the effects of increased SCC on mammary histology, mammary cell cytology and milk composition, Akers et al. (1987) concluded that leukocytosis during mastitis disrupted epithelia, and might be responsible for decreasing milk production. However, results from another similar study (Shuster et al., 1991) suggested that decrease in milk production during mastitis was mainly due to a systemic effect of the inflammation, rather than the PMN diapedesis itself.

Although in vivo studies have provided some information on the process and related events of PMN transepithelial migration, the complicated condition of the body makes it difficult to observe the diapedesis process and identify the factors involved. To circumvent the disadvantages of studying neutrophil diapedesis in vivo, the present study was conducted in vitro on a mammary epithelial monolayer model, whereby bovine PMN diapedesis can be observed in a controlled environment.

MATERIALS AND METHODS

1. Preparation of Bovine Mammary Epithelial Cell Monolayer

MAC-T3 cell line

The established bovine mammary epithelial cell line MAC-T3 (Huynh et al., 1991) was maintained on tissue culture plastic plates by serial passages in a complete medium consisting of a high glucose Dulbecco's Modified Eagle Medium (DMEM, GIBCO, NY) supplemented with 10% v/v fetal bovine serum (FBS, GIBCO, NY) and 50 μg/ml gentamycin (GIBCO, NY). The cells were cultured in a 5% CO_2 incubator (Model: NU-2700, NUAIRE Inc., MN).

Collagen-coated inserts

Culture inserts (0.45 μm micropore membrane, Millipore Corp., MA) were coated with calf tail collagen. Briefly, a solution of 150 μl collagen matrix (containing 8:1:1 of calf tail collagen, 10 x Weymouth's and 0.34M NaOH) was added on the surface of the micropore membrane within the insert. The inserts were placed at 37°C for 1 hour to let the collagen set, and then sterilized by U.V. light exposure for another hour.

Culture of MAC-T monolayer on the inserts

MAC-T cells were harvested by trypsinization at 80-90%

confluence with 0.25% trypsin containing 0.5 mM ethylenediamine tetraacetic acid (EDTA) in Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO, NY), centrifuged at 500 x g and resuspended at a concentration of 2 x 10^6 cell/ml in the complete medium. MAC-T cells (2 x 10^5/insert) were then seeded onto the collagen-coated inserts. Inserts were suspended in CDMEM in 6-well tissue culture plates (Beckton Dickinson, NJ). The nature of this system allowed the cells to be bathed in CDMEM on both the apical and basal surfaces (Fig. 1). All the culture plates were placed in an incubator (37°C, 5% CO_2, and 100 % humidity).

2. Preparation of Neutrophils

Isolation

Neutrophil isolation was carried out by a modified method of Carlson and Kaneko (1973). Blood was drawn from the caudal vein of Holstein cows of the Macdonald Campus herd with a 20G needle and a 7 ml vacutainer tube (Becton Dickinson, NJ) containing heparin. Blood was diluted 1:1 with Hank's Balanced Salt Solution (HBSS, Gibco, NY) and layered onto a Percoll gradient, d=1.077 g/ml (Pharmacia, Sweden). Following centrifugation in a swing bucket rotor (Centra 8R, IEC Co., MA) for 40 minutes at 1500 x g, the serum, buffy coat and upper portion of the red blood cell layer were discarded.

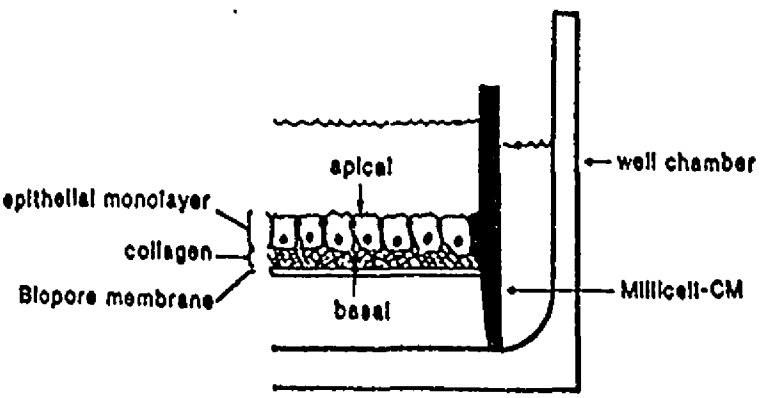

Figure 1

A cross-section of the in vitro mammary epithelial monolayer system. The MAC-T cells are cultivated at confluence on collagen coated micropore membranes within a culture insert (Millipore Corp., Bedford, MA).

Neutrophils, located in the red blood cell pellet, were isolated by hypotonic lysis of red blood cells with Tris-NH_4Cl, (0.017M Tris, Sigma, MO; 0.144M NH_4Cl, Anachemia, NY). Isotonicity of the cell suspension was restored by adding HBSS.

Enumeration and viability

The number of neutrophils recovered during the isolation procedure was determined with a haemacytometer. The neutrophil suspension was then centrifuged at 500 x g for 10 minutes and the cell pellet resuspended in HBSS to a concentration of 1 X 10^7 cells/ml.

The viability of neutrophils was determined by the trypan blue dye exclusion method (Manford, 1979). One hundred μl of 0.4% trypan blue dye was added to 100 μl of the neutrophil suspension. After 5 minutes, both live and dead cells were enumerated as previously described. The viability of PMNs isolated by this procedure is usually >99%.

Purity

Following neutrophil isolation, 100 μl aliquot of the cell suspension was mounted onto glass microscope slides by Cytospin (Shandon Inc., PA) preparation at 1,000 rpm for 10 minutes. Slides were then stained with a Diff Quik stain set (Baxter, IL) to allow differentiation of the blood cell types

present in the suspension. The number of neutrophils was counted with a bright field microscope (Zeiss, Germen). Neutrophil purity estimation was taken as the average of 5 fields. The above-mentioned isolation procedure usually results in a cell fraction containing >95% neutrophils.

3. Staphylococcus aureus

Staphylococcus aureus (Newbould 305, ATCC, Rockville, MD) was used as a chemoattractant in this study. This strain of bacteria was originally isolated from a case of bovine mastitis and has been shown to cause mastitis when inoculated into cow mammary glands (Heald, 1979). The bacteria were cultured in Luria-Bertani liquid media (Bacto-tryptone 10 g/l, Bacto-yeast extract 5 g/l and NaCl 10 g/l, pH 7.5) at 37°C in a shaking water bath for 10 hours. The colony forming unit (cfu) was determined by culturing the ten fold serial dilutions of bacteria onto the LB agar plates. The bacteria were diluted with HBSS to a final concentration of 1×10^7 cfu/ml.

4. Trypan Blue Transfer Assay of Epithelial Integrity

To determine the ability of MAC-T cells to form a monolayer with functional barriers, the transfer of trypan blue across the epithelial monolayer was measured as described by Mangum et al. (1990). Inserts with monolayers were first rinsed twice with DPBS with 0.1% bovine serum albumin (BSA,

Sigma, MO). Two hundred μl of trypan blue (final concentration : 0.2%) in 0.1% BSA-DPBS were then added to the apical surface of the monolayer, and the basal reservoir was filled with 2 ml of 0.1% BSA-DPBS. The rate of trypan blue diffusion was determined at $A_{580\ nm}$ with a spectrophotometer (Spectronic 601, Milton Roy Co., NY). The measurements were made from 6 hours up to 6 days after cells were plated. Samples from the collagen-coated inserts without MAC-T cells were used as controls.

5. Transepithelial Migration of Neutrophils

Inserts with monolayers were washed twice with pre-warmed DPBS, and then placed in new culture plates with DMEM without fetal bovine serum. Neutrophils (1 X 10^6 cells/insert) were placed on the upper compartment of the insert and stimulated to traverse the epithelial monolayer by the addition of Staphylococcus aureus (1 X 10^7 cfu/ml) into the lower compartment of the insert. Inserts were then placed into a 37°C incubator. The PMN transepithelial migration was observed at 10, 30, and 60 minutes after neutrophil addition.

6. Light and Electron Microscopy

At the end of each incubation, inserts were removed and rinsed 4 times with DPBS. Inserts containing both monolayers and neutrophils were then processed for light and transmission electron microscopy as described by Clermont et al. (1993),

with some modifications. The major procedures were as follows:

1) Prefixation: 2-4 h at 4°C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.05% $CaCl_2$(pH 7.4).
2) Washing: 10 min x 2 - overnight - 10 min x 2, 4°C, using 0.1 M sodium cacodylate with 7% sucrose.
3) Postfixation: 2 h at 4°C with reduced osmium (1:1 mixture of 2% osmium tetroxide and 3% potassium ferrocyanide).
4) Washing: 10 min x 2 with the solution as in step 2.
5) Dehydration: Ethanol series (50% 10 min, 70% 10 min, 90% 10 min and 100% 10 min x 3) and propylene oxide (15 min x 3). The monolayers were detached from the inserts at 70% ethanol stage.
6) Infiltration: Epon mixture + propylene oxide (1:1, 1h; 2:1, overnight and 3:1, overnight), and deaerated Epon mixture (4 h in vacuum).
7) Embedding: The monolayers were cut into the desired size, placed into molds filled with deaerated Epon, and left in an 60°C oven for 48 h.

For transmission electron microscopy, thin sections (60-90 nm thickness) were cut and stained with uranyl acetate (5 min) and lead citrate (2 min), and examined with a transmission electron microscope (Phillps 400, Holland). For light microscopy, thick sections (0.5 μm) were stained with toluidine blue and examined by a bright field microscope

(Zeiss, German)

7. Microscopic Counting of Migrated Neutrophils

Because neutrophils were unable to migrate across the 0.45 μm pore-size filter after migration across epithelial monolayer, they were caught in the collagen gel above the 0.45 μm pore-size filter. Therefore, the PMNs migrated could be counted directly at histological sections with a microscope.

Three separate thick sections stained with toluidine blue were randomly chosen from each monolayer. Neutrophils in the collagen gel area under 1 mm of epithelium were counted with a micrometer. The number of neutrophils was expressed as the average number of neutrophils per mm epithelium.

RESULTS

1. Characterization of Epithelial Monolayers

Light and electron microscopy showed that MAC-T cells, plated in a concentration of 2×10^5 cells/insert, formed a confluent monolayer on the collagen-coated membrane by day 2. Morphologically, these epithelial monolayers were polarized with apical microvilli and displayed junctional complex at the border between apical and basal surfaces (Fig. 2)

The ability of MAC-T cells to form an intact monolayer with a functional barrier was further confirmed by a trypan blue transfer assay. As monolayers reached confluence, the trypan blue transfer decreased, with the $A_{580\ nm}$ values reaching the lowest by day 2. One the other hand, collagen-coated filter provided little obstruction to the dye. The integrity of the monolayer remained at least until day 6 (Fig. 3).

2. Cellular Events of PMN Transepithelial Migration

In order to observe a series of events of neutrophil transepithelial migration, the process of diapedesis was stopped at different times. Neutrophils were observed in various stages of migration.

After plating, neutrophils first adhered to the surface of the epithelium, usually in clusters (Fig. 4). The clusters were found at various points along the epithelial monolayer. As PMNs settled onto the epithelial monolayers, they approached (Fig. 5) and projected pseudopods (Fig. 6a and b) toward the junctional complex located between epithelial cells. For those PMNs projecting pseudopods on the other areas of epithelial cell surface, no penetration of the pseudopods into the epithelial cells was found.

After projecting their pseudopods into the junctional

complex, neutrophils squeezed into and moved between epithelial cells (Fig. 7). In addition, neutrophils were also found to migrate through the same junction one after another (Fig. 8). During this transjunctional migration, neutrophils dramatically elongated, and there was intimate contact between epithelial cells and neutrophils.

Following neutrophil migration, the apical lateral epithelial membranes re-approximated (Fig. 9a) and then the tight junctions re-formed (Fig. 9b). After 60 min, many neutrophils passed the monolayer and were present in the collagen gels (Fig. 10a and b). Morphologically, epithelial damage during the process of PMN transepithelial migration was not evident.

After migration across the epithelial monolayer, neutrophils were caught in the collagen gel above the small pore-size filter (Fig. 10a and b). Approximately 35 neutrophils per mm traversed the epithelium in 60 min under the condition of chemotaxis (DMEM in the upper compartment, chemoattractant in lower compartment). On the other hand, only about 8 neutrophils per mm randomly migrated through the epithelium in 60 min (DMEM in both compartments) (Table 1).

FIGURE 2

Transmission electron micrograph showing a cross section of confluent epithelial monolayer. Epithelium is morphologically polarized with tight junctions (small arrows) at the apical lateral border between adjacent cells and microvilli (large arrows) on its apical surface. Magnification: 11,000x

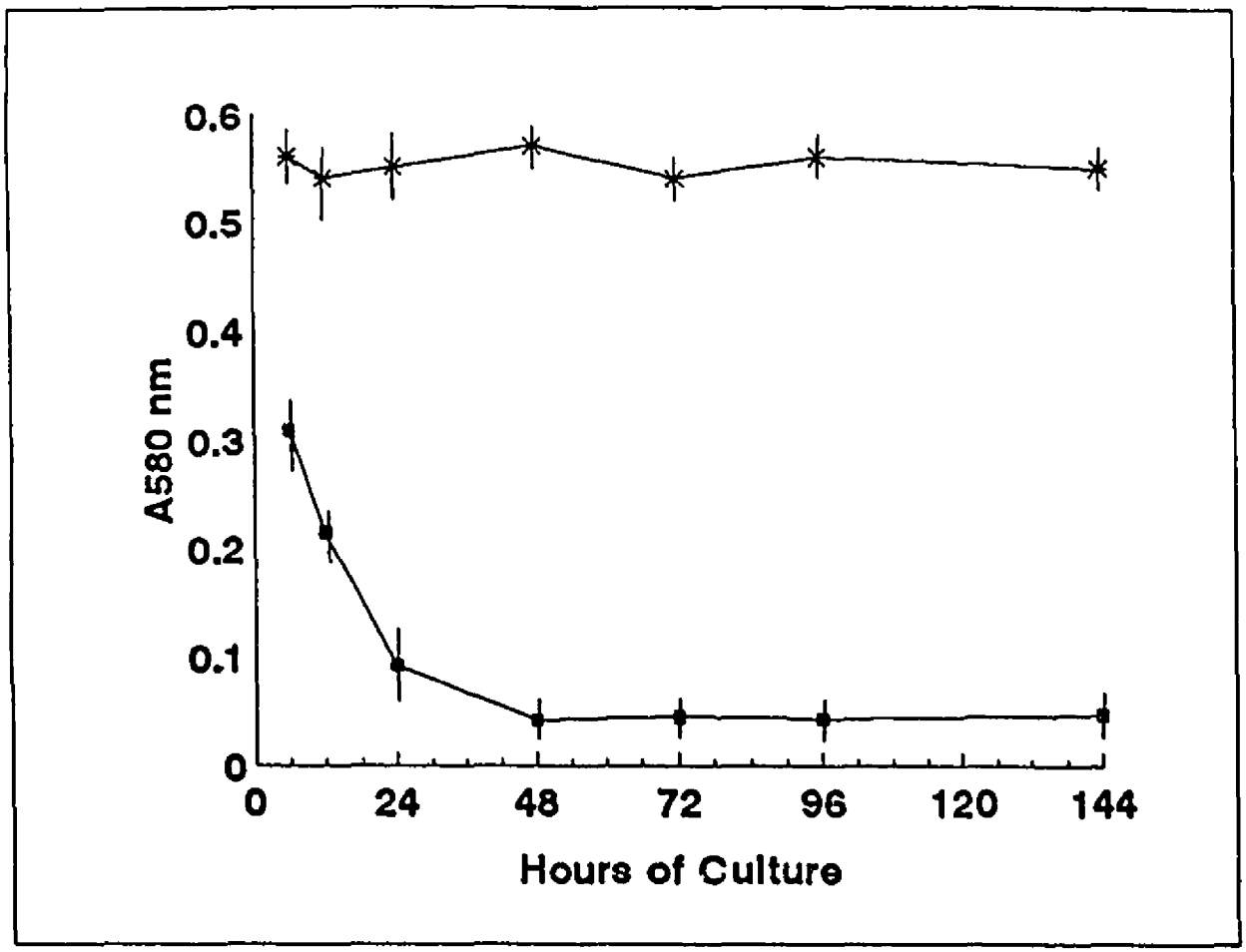

FIGURE 3

The transfer of trypan blue across MAC-T monolayers on collagen-coated filters (■) or collagen-coated filters without MAC-T cells (*). Dye was added to the upper compartment of the insert and the transfer of trypan blue in the lower compartment was measured after 40 min. Each point represents the mean and SEM of 6 independent samples.

FIGURE 4

Photomicrograph showing that many neutrophils are present in clusters at the apical epithelial surface after 10 min incubation. Some neutrophils have passed through the monolayer. Magnification: 150x

FIGURE 5

Transmission electron micrograph of a neutrophil (N) approaching the intercellular space between epithelial cells (E). Magnification: 9200x

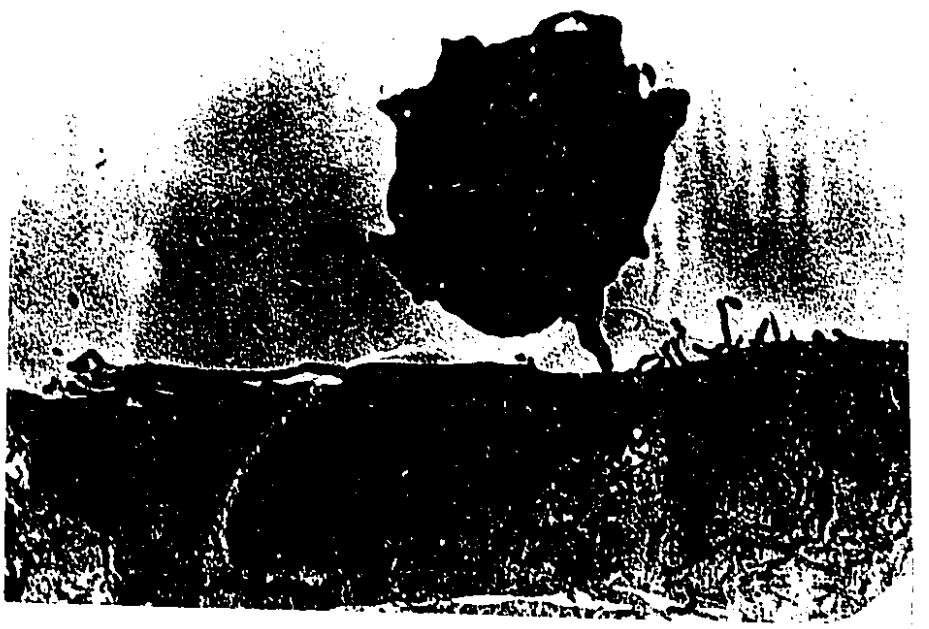

FIGURE 6

Transmission electron micrographs showing a neutrophil (N) projecting a pseudopod directly toward the intercellular space between epithelial cells (E). Magnification: 8200x

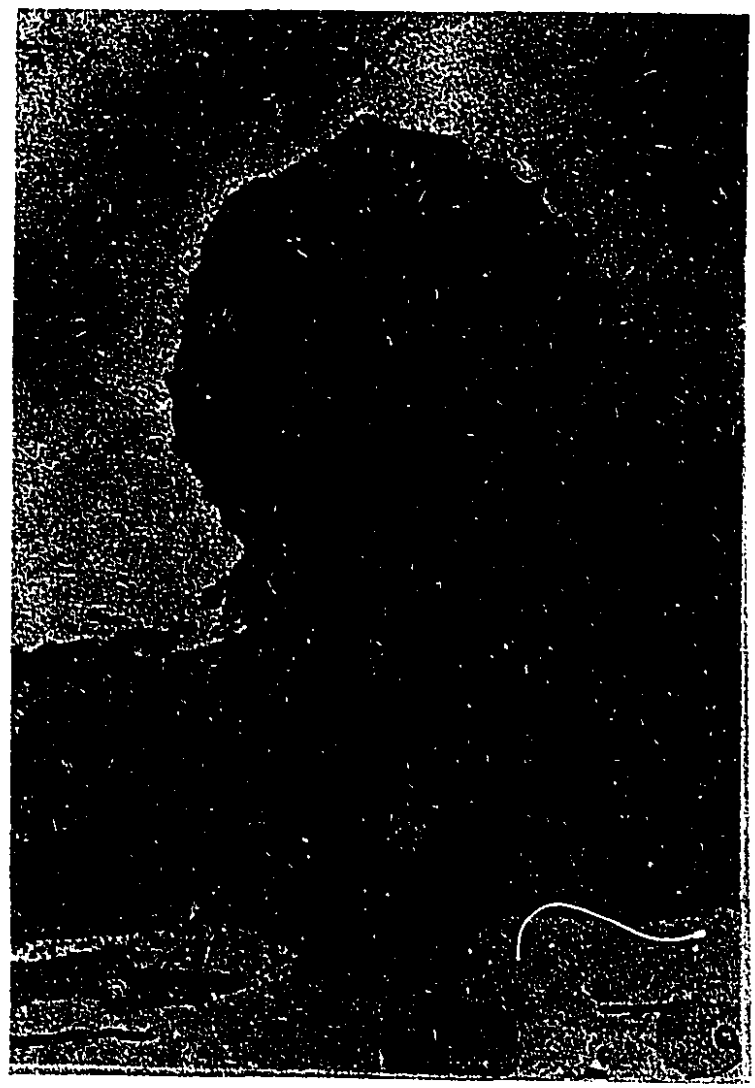

FIGURE 7

Transmission electron micrograph of a neutrophil (N) squeezing into the intercellular space between epithelial cells (E). Magnification: 9200x

FIGURE 8

Transmission electron micrograph showing two neutrophils (N) passing between the epithelial cells (E) one after another. Magnification: 9200x

FIGURE 9a

Transmission electron micrograph showing the re-approximation (arrow) of epithelial membranes after the neutrophil (N) has migrated between epithelial cells (E). Magnification: 9200x

FIGURE 9b

Transmission electron micrograph showing the re-sealed junctions (arrows), after a neutrophil (N) has just passed the epithelial monolayer (E). Magnification: 9200x

FIGURE 10

Transmission electron micrograph showing that after incubation for 60 min, many neutrophils (N) have crossed the monolayer and are present in the collagen layer above the surface of the 0.45 μm filter. Magnification: 5600x

TABLE 1. Numbers of neutrophils that have migrated across the epithelium

Time (min)	Stimulus	Migrated PMNs* (mm)
10	S. aureus	5 ± 1.5
30		19 ± 5.2
60		31 ± 9.4
10	DMEM	1 ± 0.5
30		6 ± 2.5
60		8 ± 3.2

* Mean and SEM of 3 sections of epithelium.

DISCUSSION

Neutrophil diapedesis is a hallmark of bovine mastitis and is critical to the outcome of the disease (Nickerson, 1985). To investigate how neutrophils migrate across the alveolar epithelia into milk, this study adopted an *in vitro* monolayer system in which PMNs were allowed to migrate across the epithelium in a controlled environment. The morphological observations from this study have revealed several important aspects of bovine PMN diapedesis.

Firstly, this study demonstrated morphologically that bovine neutrophils traverse the epithelium **only** between epithelial cells. This result is consistent with those from a study on human PMN migration across a monolayer of Madin-Darby canine kidney (MDCK) epithelial cells (Cramer *et al.*, 1980). Studies using *in vivo* models, however, have not resolved the question how bovine neutrophils migrate across the mammary epithelium. While two studies suggested that bovine neutrophils migrated between as well as through individual epithelial cells, they disagreed on which route was the predominant one (Harmon and Heald, 1982; Nickerson and Pankey, 1984). On the other hand, another study suggested that bovine neutrophils migrated only through individual cells (Frost *et al.*, 1980). In the present study, the penetration of neutrophils through intact epithelial cells was not observed,

suggesting that migration between cells is the predominant mode by which PMNs diapedese into milk during mastitis.

Secondly, the results from the present study indicated that considerable diapedesis activity can occur without morphological damage to epithelial cells. A previous study (Macdonald et al., 1994) also demonstrated that PMN diapedesis caused no significant change of the transepithelial electrical resistance, which implied a nondestructive neutrophil migration mechanism. Although in vivo studies showed that PMN diapedesis into milk was accompanied by epithelial damage (Frost et al., 1980; Nickerson et al., 1984), evidence that links this damage directly to PMN diapedesis activity has not been obtained. Therefore, the epithelial damage during mastitis may occur as a result of a complex action rather than PMN diapedesis itself, including bacterial cell attachment (Gadding et al., 1984), bacterial toxins (Nickerson and Heald, 1981), lysosomal enzymes released from phagocytosing and degenerating neutrophils (Capuco et al., 1986), and the involvement of macrophages and lymphocytes (Sordillo et al., 1989).

Thirdly, this study for the first time clearly showed the series of events of transjunctional migration of bovine PMNs. Since alveolar epithelial cells are joined by a junctional complex (Powell, 1981), it has long been proposed that

neutrophils penetrate these barriers when they pass between cells (Nickerson and Pankey, 1984). However, the previous studies did not provide conclusive morphological evidence of PMN penetration of these junctional complexes. The series of events of PMN transjunctional migration observed in the present study included: adhering to the surface of epithelial cells, approaching and projecting pseudopods toward the intercellular space, squeezing and moving between epithelial cells, and re-sealing of the tight junctions following PMN migration.

Interestingly, in the histological sections examined, the frequency of capturing PMNs in the process of crossing between cells was only approximately 5%, even though many neutrophils were seen to have passed through the epithelium. An image of a PMN penetrating the junctions was even rarer. Similar findings were reported by other in vitro studies of human neutrophils (Nash et al., 1987). There are several possible explanations for these phenomena. Firstly, the transjunctional migration must occur very rapidly, probably within seconds to minutes (Nash et al., 1987). An early study by Hudspeth (1975) indicated that when injured, tight junctions could be repaired within 30 minute. Results from the present study also showed that the resealing of tight junctions was observed in the histological sections of PMN migration at 15 and 30 minute. Secondly, PMN diapedesis across the monolayer is not entirely

at random. Variation in junctional development may exist, which would give PMNs a chance to migrate through the junctions with lower barrier functions. These hypotheses are supported by the present observations that clusters of PMNs were found on some areas of the monolayer but not on others, and that PMNs were shown to cross the epithelia one after another through an invasion site.

The exact mechanism by which PMNs cause the transient dissolution of the junctional complex is still unknown. Powell (1981) suggested that the disruption of epithelial tight junctions was simply due to mechanical force generated by PMNs during their migration. In 1988, Nash et al., investigated the involvement of chemical mediators produced by PMNs in the dissolution of tight junctions. They found that oxygen metabolites were not required for transepithelial migration, and the release of proteases by PMN during transepithelial migration was not the prerequisite for PMNs to cross tight junctions.

As in most other in vitro studies on PMN diapedesis, PMNs were allowed to diapedese the epithelium from the apical-to-basal direction in the present study. Parkos et al. (1991) demonstrated that neutrophil transepithelial migration was similar in both directions (from basal-to-apical or from apical-to-basal) in several qualitative ways (i.e., ability

for migration to proceed, specific chemotactic gradients required, and CD11b/18 dependence). However, a difference was found in quantity of PMN migration from two opposite directions (Colgan et al., 1993).

In the present study, the numbers of migrated PMNs were also monitored by direct microscopic counting of the migrated PMNs. Although these numbers can roughly reflect the rate of PMN migration, they cannot give an accurate quantitative measurement of the total number of migrated neutrophils. Moreover, this method of counting is a time-consuming procedure. Thus, a further study is needed to address this issue.

In summary, the process of bovine PMN diapedesis across the bovine mammary epithelium has been demonstrated in this study by using an in vitro model. Neutrophils traversed the epithelial monolayer by migrating through the paracellular space. The intercellular tight junctions were resealed after PMN migration. Epithelial damage directly caused by PMN diapedesis was not evident. Migration between epithelial cells may represent a major mechanism of PMN diapedesis into milk during mastitis.

CHAPTER IV. QUANTIFICATION OF NEUTROPHIL TRANSEPITHELIAL MIGRATION BY A MYELOPEROXIDASE (MPO) ASSAY

INTRODUCTION

The ability of PMNs to migrate is an important indication of neutrophil function. Traditionally, the ability of PMN migration has been determined *in vitro* mainly by an under-agarose migration assay. In this system, the migration index, which is calculated by dividing the distance moved by cells toward the chemoattractant by the distance moved by cells toward the control well, is used to determine the migration activities of neutrophils (Olson, 1990). However, this system cannot quantify the total number of migrated PMNs which should also be an important parameter reflecting the ability of PMN diapedesis. Moreover, this system does not serve as a biologically meaningful barrier for PMN migration.

By using an *in vivo* mastitis model, the rate of bovine neutrophil diapedesis into milk has been quantified by monitoring the SCC at intervals during the period of mastitis (Hill 1981), or by monitoring the isotope-labelled PMNs from multiple biopsy specimens of mammary tissues (Persson *et al.*, 1992). However, this model has disadvantages such as difficulty in identifying the factors involved, and the high economic cost of using cattle as a model.

Nash et al. (1987) have studied the human PMN migration in a system combining a cell monolayer model with an enzyme assay. In this system, the transepithelial migration of PMNs was quantified by measuring the PMN azurophilic granule marker myeloperoxidase (MPO). However, a similar system has not been reported for quantifying the rate of bovine PMN migration across a biologically meaningful barrier (cell monolayer) in vitro . Therefore, the objective of this study was to develop an in vitro microassay system which can be used to quantify bovine PMN migration across the mammary epithelial monolayer. Furthermore, PMN transepithelial migration from a basal-to-apical direction, a physiologically relevant direction, was emphasized.

MATERIALS AND METHODS

1. Preparation of Culture Chambers

The culture chambers were constructed by gluing a micropore membrane (5 μm pore size, 13 mm diameter, Millipore Co., MA) between two insert rings (12 mm diameter, Millipore Co., MA) with RTV silicone rubber adhesive sealant (GE Silicones Canada Co., Ontario). This kind of rubber does not release trace cytotoxic solvents after setting (Parkos et al., 1991). Before use, the chambers were rinsed first with PBS and then 70% alcohol, and placed under U.V. light for 2 hours for sterilization.

2. MAC-T Cell Monolayers

The collagen-coated membranes and the cell monolayers were prepared using the same methods as described in Chapter III.

3. Chemoattractants

Zymosan-activated serum (ZAS) was used as a chemoattractant in this study, and was prepared by a modified method of Olson (1990). Zymosan (Sigma, MO) was added to newborn calf serum (Gibco, NY) at a concentration of 5 mg of zymosan/ml of serum. The mixture was incubated in a shaking water bath (37°C) for 30 minutes, then centrifuged (1500 x g) for 10 minutes. Aliquots of ZAS were stored (-20°C) until used.

4. Neutrophil Transmigration

The cultured chambers with monolayers were rinsed with 37°C HBSS and then placed into new wells containing HBSS. For studying PMN migration from a basal-to-apical (a physiologically relevant) direction, the cultured chambers were first put up-side down to make an inverted monolayer (Fig. 11). Neutrophils were then added into the upper compartment above the monolayer, and driven to migrate across the monolayer by the addition of ZAS to the lower compartment. The dose-response studies were carried out for 120 min with various concentrations of ZAS (0.1-40 %) added to the lower

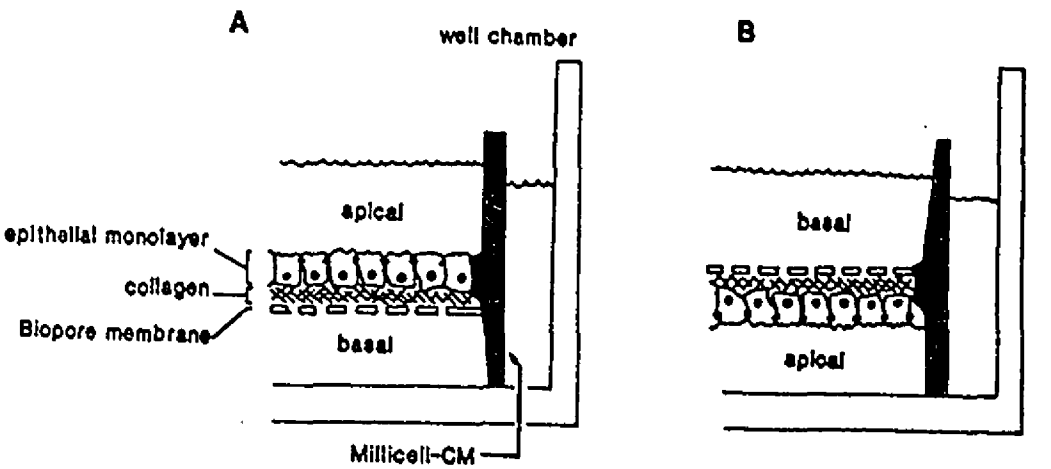

FIGURE 11

A cross-section of the inverted mammary epithelial monolayer system. The MAC-T cells were cultured at confluence on the collagen coated membrane within the cultured chamber (A). Before adding neutrophils, the chamber was put upside down to make an inverted monolayer (B), which allowed the settling down of PMNs on the basal surface of the epithelium.

compartment and PMNs (2 x 10^6/insert) added to the upper compartment. The time courses of the transmigration were studied by using a fixed concentration of ZAS (5%) up to 120 min. The culture chambers were incubated at 37°C during the time of transepithelial migration.

5. Quantitative Measurement of PMN Migration

The migrated PMNs were quantified by assaying the activity of PMN azurophilic granule marker, myeloperoxidase (MPO) as described by Parkos et al. (1991) with some modifications. Briefly, the cultured chambers were washed twice with ice-cold HBSS to remove non-migrated neutrophils. Liquid in the lower compartment was centrifuged and the pellet was collected. Myeloperoxidases were released from PMNs by adding 200 μl of Triton X-100 (0.5%) to the monolayers and the cell pellets from the lower compartment, separately. After 30 minutes, 100 μl of 0.1 M citrated buffer (pH 4.2) was added to each sample. The MPO activity was assayed at $A_{405\ nm}$ on a microtiter plate reader after mixing equal volume of sample and a solution containing 1 mM 2,2'-azino-di-(3-ethyl) dithiazoline sulfonic acid (Sigma, MO) and 10 mM H_2O_2 in 100 mM citrated buffer (pH 4.2) for 30 minutes. Neutrophil equivalents were estimated from a standard curve made with known numbers of neutrophils.

6. Statistical Analysis

Data are presented as the mean ± SEM for each experimental and control group. The effect of different concentrations of ZAS on the number of migrated PMNs was determined by analysis of variance. Statistical differences between the efficiency of migration from the two directions were compared by Student's t test.

RESULTS

The standard curve of the MPO assay was made for each assay, and the results showed that the concentration of PMNs from 0.1-100 x 10^4 cells/ml was correlated with the optical density value ($r = 0.908$), and the minimal number of PMNs detected by the MPO assay was approximately 5 x 10^3 cells. Figure 12 is a typical curve chosen from 10 standard curves.

The time-course assay showed that when ZAS was used at a concentration of 5%, the numbers of migrated PMNs increased with time, with the most migration occurring during the first 60 minute. At 60 minute, approximately 2.4 x 10^5 PMNs migrated across the monolayer (Fig. 13). This number is about 12% of the total neutrophils (2 x 10^6) added to the upper compartment .

The results of the dose response are shown in Figure 14. Compared with the control, as little as 1% ZAS significantly increased migration of PMNs ($P < 0.01$), with the maximum response achieved at 20% ZAS. Further increase of ZAS to 40% only induced a slight increase of PMN migration.

When comparing the efficiency of transepithelial migration of PMN from two directions, it was found that in response to 5% ZAS for 120 min, the number of migrated PMNs from the basal-to-apical (B-A) direction was approximately 1/3 less than the number from the apical-to-basal (A-B) direction ($P < 0.05$) (Fig. 15).

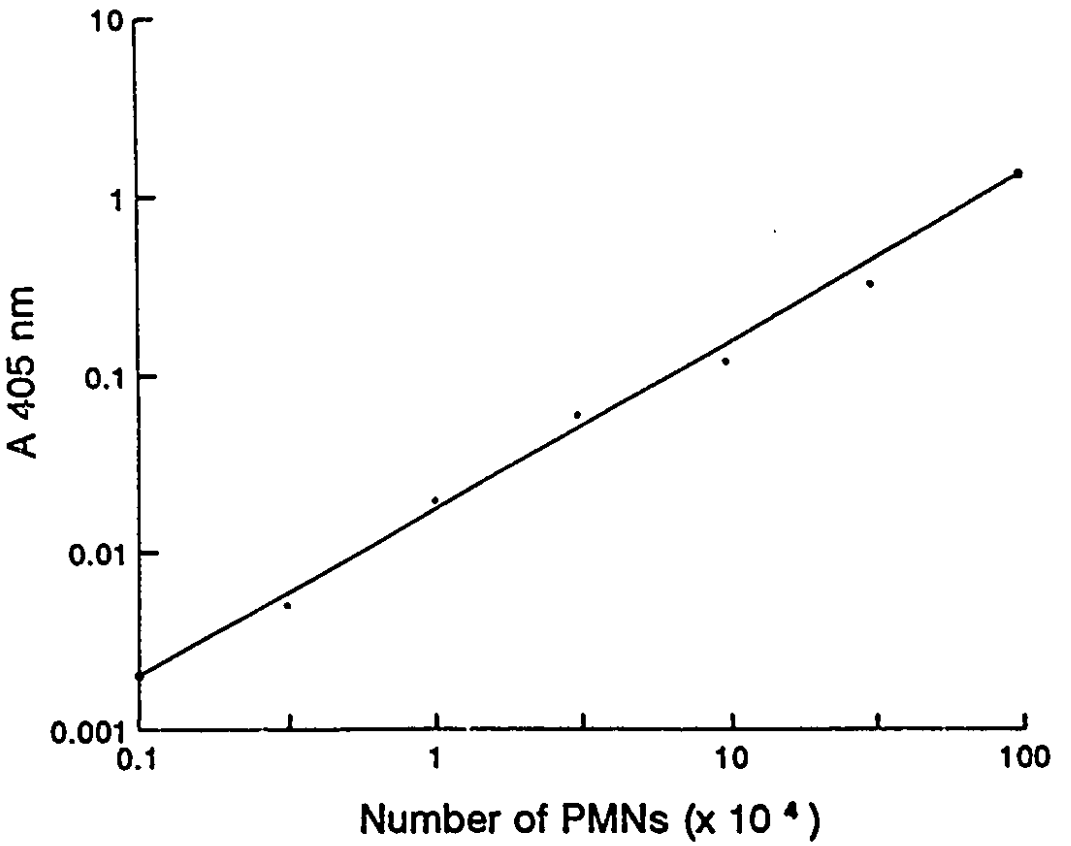

FIGURE 12

A standard curve of the myeloperoxidase assay. Various concentrations of PMNs (0.1-100 x 10^4 cells) were first treated with 0.5% Triton X-100, and then mixed with a solution containing the substrate and H_2O_2. After 30 min, the colour development was assayed on a microtiter plate reader. The results showed a linear standard curve within the range of PMNs used.

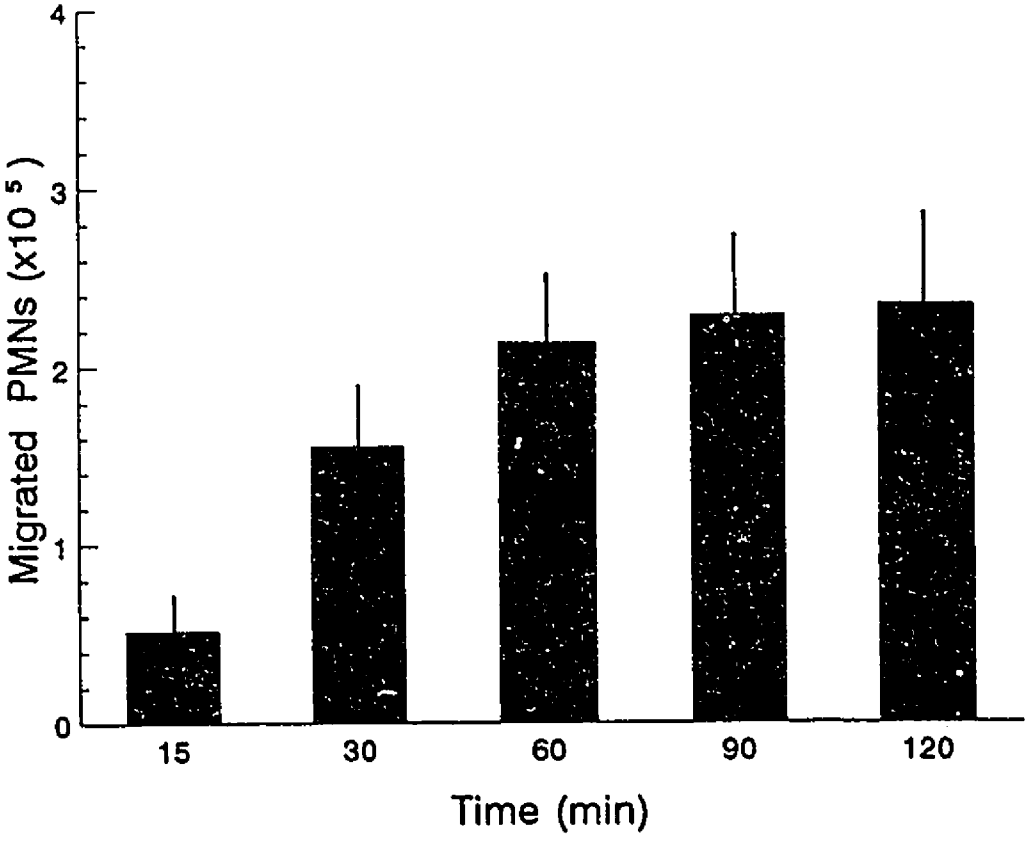

FIGURE 13

Time-course of neutrophil migration across MAC-T monolayer. The PMNs were added on the basal surface of the monolayer in the presence of a transepithelial gradient of ZAS (5%). At the time points indicated on the curve, neutrophils which had transmigrated were quantified using the MPO assay as described in Methods. Data represent mean and SEM of 6 monolayers from 3 individual experiments.

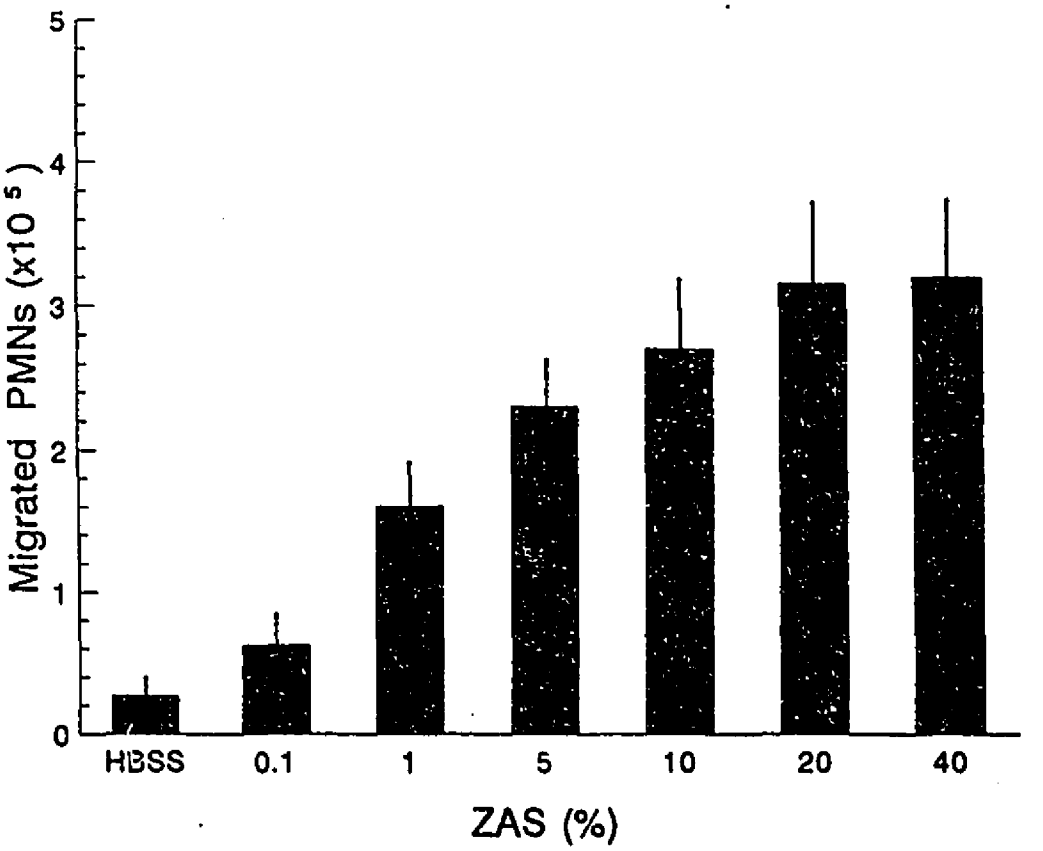

FIGURE 14

Effect of different concentrations of zymosan activated serum (ZAS) on the number of migrated PMNs. Neutrophils were added to the basal surface of the epithelium and driven to diapedese across the monolayer by adding ZAS into the lower compartment. The migrated PMNs were determined by the MPO assay as described in Methods. Results are present as the mean and SEM of 6 monolayers from 3 individual experiments.

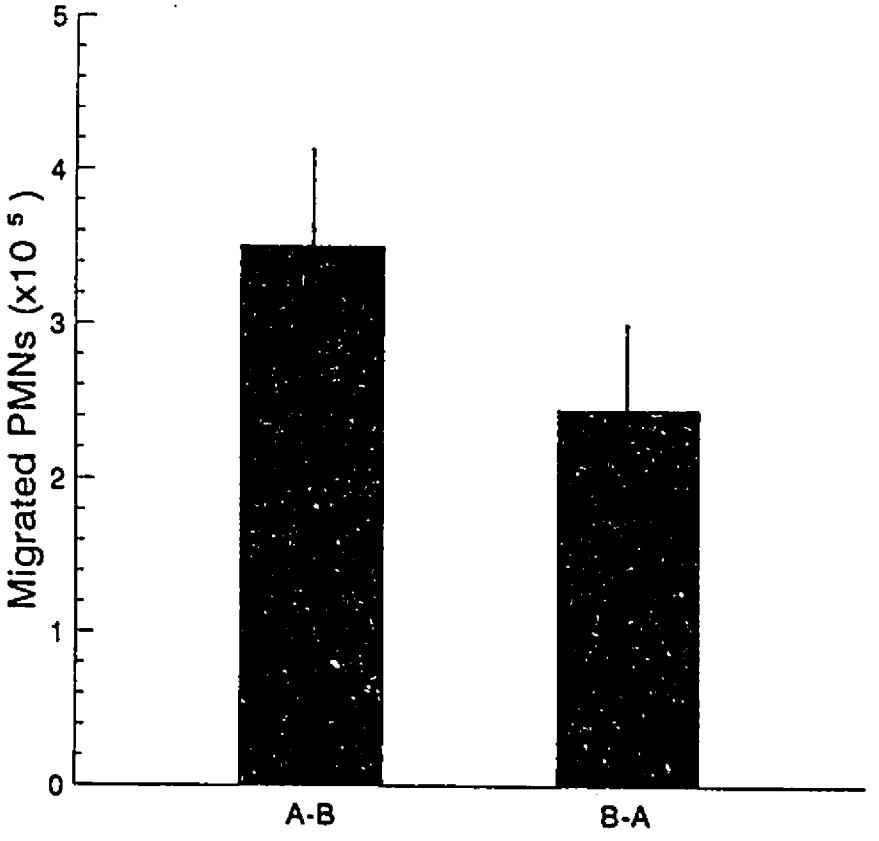

FIGURE 15

Comparison of the efficiency of neutrophil transepithelial migration in the basal-to-apical (B-A) vs. the apical-to-basal (A-B) direction. PMNs were layered onto the basal or apical aspect of the epithelium and induced to transmigrate for 120 min. The migrated PMNs were determined by the MPO assay as described in Methods. Results represent the mean and SEM of 6 monolayers from 2 individual experiments.

DISCUSSION

The importance of the rate of PMN diapedesis in bovine mastitis has long been recognized (Hill, 1981), however, technical problems for the quantitative measurement of this phenomenon *in vitro* has not been resolved. To address this issue, a microassay system with two important aspects has been developed in the present study.

Firstly, an inverted monolayer system was developed in this study in order to investigate PMN transepithelial migration in a physiologically relevant direction. This inverted system permitted PMNs to adhere to and subsequently migrate across the epithelium in a basal-to-apical direction, a direction which occurs naturally *in vivo*. Traditionally, PMN diapedesis across a cell monolayer system has been predominantly performed (Cramer *et al.*, 1980; Milk *et al.*, 1986) in an apical-to-basal direction, the non-physiological direction. This is mainly due to technical problems such as PMNs settling on the bottom of the cell culture plate due to the gravity, which make them difficult to migrate upward across the epithelial monolayer in a basal-to-apical direction, if an insert is not inverted.

Secondly, this study quantified the rate of PMN migration by combining with an enzyme assay for the azurophilic granule

marker, myeloperoxidase. The principal of the MPO assay is that after treating PMNs with Triton X-100, the myeloperoxidases are released from the azurophilic granules of the PMNs. In the presence of the enzyme's substrate and H_2O_2, the enzyme will catalyze the substrate to produce a soluble end product that can be read spectrophotometrically at $A_{405\ nm}$. The increase in optical density value is thus correlated with the number of neutrophils. A major advantage of this method is that it can quantify the total number of migrated PMNs. Moreover, this method is faster and easier than microscopic counting.

The results showed that in the absence of any chemoattractant, the migrated PMNs usually numbered less than 3×10^4 cells. However, the presence of ZAS greatly stimulated PMN transepithelial migration, and this effect was time- and dose-dependent. However, within the range of ZAS concentrations (0.1 to 40%), and of incubation times (15 to 120 min), the maximal number of migrated PMNs was less than 20% of the total PMNs applied. This phenomenon is consistent with the morphological observations described in the previous chapter. This result is also similar to those from studies with human PMN transepithelial migration (Parkos et al., 1991; Colgan et al., 1993). There are several possibilities for this phenomenon. Firstly, a functional variation may always exist among a population of neutrophils (Jain et al., 1991).

Because of the tightness of the junctional complex, neutrophils must not only be able to migrate but also be able to open tight junctions in order to cross the epithelium. Secondly, an inactivating or inhibiting mechanism from neutrophils and/or epithelial cells may exist. In fact, a variety of agents capable of interfering with PMN chemotaxis have been reported (Goetzl, 1975), and some of these agents are believed to be potentially operative as inflammatory control mechanisms in vivo. For example, Clark and Szot (1982) demonstrated that neutrophils which have already been recruited to the inflammatory site may contribute to the termination of inflammation by oxidation of chemotactic factors. In addition, a local alteration of the tissue, making it unresponsive to some stimulus within a certain time after an inflammatory challenge, has also been described (Colditz and Movat, 1984). However, whether these same mechanisms operate in our in vitro system is not clear.

This study demonstrated that PMNs could migrate across the epithelium from an apical-to-basal direction, as many previous in vitro studies have shown, and from a basal-to-apical direction, as occurs in vivo. However, there was a difference between the PMN migrating efficiency from the two directions. The reason for this phenomenon is unclear. It is suspected that the nature of the surface to which the PMNs first attach would have an important effect. In an apical-

basal system, neutrophils first attach to the epithelial cells, which may be more favourable to PMN adherence and subsequent migration than in the basal-apical system where PMNs first attach to a physical barrier (filter membrane).

In summary, bovine neutrophils can migrate across the MAC-T epithelial monolayer in a physiologically relevant direction in this inverted monolayer system, and the rate of PMN diapedesis can be quantified by the MPO assay. The transepithelial migration of bovine PMN was greatly stimulated by the presence of ZAS, and the effect of ZAS was both time- and dose-dependent. This study provides a microassay system by which a large number of parallel studies for neutrophil diapedesis and related issues can be performed in a controlled environment.

CHAPTER V. GENERAL DISCUSSION AND CONCLUSIONS

1. General Discussion

A major advantage of the model developed in the present study is that it provides a controlled environment in which bovine neutrophils migrate across the epithelial cell barriers can be studied. This model can be used to study the interactions between various types of leukocytes and epithelia. Moreover, this system permits a large number of parallel experiments to be performed. In particular, such a system can be used to study:

(1) Identification of chemoattractants which play major roles in the accumulation of neutrophils during mastitis

Chemotaxis is a complex process and many chemoattractants have been described for human neutrophils. However, the major chemotactic factors which play crucial roles in the accumulation of bovine neutrophils during mastitis have not been clearly identified. It seems that the milk in mastitic cows must resemble inflammatory exudate and contain chemoattractants similar to those described in other systems. Therefore, the identification of chemotactic factors within the infected mammary gland is important.

(2) Interaction among macrophages, lymphocytes and neutrophils during the neutrophil diapedesis process.

During mastitis, not only PMNs but also monocytes and lymphocytes diapedese toward local inflammation sites. There is no information so far about the differences in the ability of these cells to diapedese into milk and their interaction during diapedesis in mastitis. With this in vitro system, it is possible to compare the diapedesis patterns of PMNs, monocytes and macrophages alone, or in combination.

(3) Regulation of the neutrophil diapedesis process and the related molecular bases.

Recent studies suggest that PMN diapedesis in vivo can be regulated by different factors, especially cytokines. However, the complex nature of the body makes it impossible to identify the roles of these factors and their relationships. In this system, the regulation of PMN diapedesis can be investigated under a controlled environment.

i. The individual or combined effects of exogenous cytokines such as IL-1,IL-2, TNF and GM-CSF on PMN diapedesis. The migration of PMN can be quantified by the MPO assay.

ii. Endotoxin induction of cytokine activity. Recent studies suggest that epithelia, and PMNs themselves may also contribute to PMN diapedesis by secreting cytokines such as

TNF and IL-8. Thus, the production of cytokines from epithelial cells and/or PMNs after endotoxin stimulation can be studied in this in vitro system.

iii. Expression of leukocyte adhesion molecules(LAM) and their contribution to the migration of PMNs. Studies of the transmigration of human neutrophils have demonstrated that the expression of a number of leukocyte adhesion molecules is required for PMN transmigration. These molecules can be expressed under the effects of a variety of stimuli such as endotoxin and cytokines, by endothelial cells, epithelial cells and leukocytes themselves. However, there is little information on the bovine system.

(4) Potential factors and mechanisms responsible for the epithelial damage.

Although there is increasing evidence to support the hypothesis that epithelial damage during mastitis is due to complex factors and mechanisms, it is difficult to identify all these factors and their interactions in vivo. By using this in vitro model, it is possible to investigate the individual or combined effects of a variety of potential factors, such as bacterial toxins, bacterial attachment and lysosomes from phagocytosing neutrophils and the involvement of monocytes on the integrity of the epithelium.

2. CONCLUSIONS

The process and rate of bovine PMN transepithelial migration *in vitro* has been studied using the model and techniques developed in this project. From the results obtained, I would like to draw the following conclusions:

(1) The MAC-T cell is able to form a confluent and polarized monolayer with tight junctions and can serve as a biologically meaningful barrier for transepithelial migration of bovine PMNs.

(2) The bovine PMNs cross the mammary epithelium by migrating between intact epithelial cells. In doing this, the neutrophils traverse the junctional complexes between epithelial cells.

(3) The intercellular tight junctions of the epithelial cells have the ability to re-form rapidly after PMN migration.

(4) Morphologically, epithelial damage directly caused by neutrophil transepithelial migration (1×10^6 cells/insert, in a period of 60 min) is not evident.

(5) Neutrophils can, to some extent, migrate across the epithelial monolayer without the addition of any chemoattractants. However, the presence of chemotactic

factors greatly stimulates the migration of neutrophils.

(6) The rate of PMN transepithelial migration can be quantified by a myeloperoxidase assay. The stimulatory effect of zymosan activated serum on PMN migration is time- and dose-dependent.

(7) Neutrophils can migrate across the epithelium in a basal-to-apical direction, as well as in an apical-to-basal direction, and there is a quantitative difference in PMN migration in these two directions.

REFERENCES

Akers R.M., Thompson W. 1987. Effect of induced leucocyte migration on mammary cell morphology and milk component biosynthesis. J Dairy Sci 70:1685

Baggiolini M., Boulay F., Badwey J.A., Curnutte J.T. 1993. Activation of neutrophil leukocytes: chemoattractant receptors and respiratory burst. The FASEB J 7:1004

Bainton D.F. 1988. Phagocytic cells: Developmental biology of neutrophils and eosinophils. In: Inflammation-Basic principles and clinical correlates. Ed. by Gallin et al., Raven Press Ltd., NY. p.265-280

Bevilacqua M.P., Stengelin S., Gimbrone Jr. M.A., Seed B. 1989. Endothelial leukocyte adhesion molecule I: An inducible receptor for the neutrophils related to complement regulatory proteins and lectins. Science 243:1160

Blosser T.H. 1979. Economic losses from the national research program on mastitis in the United States. J Dairy Sci 62:119

Bochsler P.N., Neilsen N.R., Slauson D.O. 1994. Transendothelial migration of neonatal and adult bovine neutrophils in vitro. J Leukocyte Bio 55:43

Bramley A.J., Dodd F.H. 1984. Reviews of the progress of dairy science: Mastitis control-progress and prospect. J Dairy Res 51:481

Capuco A.V., Paape M.J., Nickerson S.C. 1986. In vitro study of polymorphonuclear leukocyte damage to mammary tissues of lactating cows. Am J Vet Res 47:663

Carlson G.P., Kaneko J.J. 1973. Isolation of leukocytes from bovine peripheral blood. Pro Soc Exp Biol Med 142:853

Carroll E.J., Mueller R., Panico L. 1982. Chemotactic factors for bovine leukocytes. Am J Vet Res 43:1661

Clark R.A. 1990. The human neutrophil respiratory burst oxidase. J Infect Dis 161:1140

Clermont Y., Xia L., Rambourg A., Turner J.D. 1993. Transport of casein submicelles and formation of secretion granules in the Golgi apparatus of epithelial cells of the lactating mammary gland of the rat. Anat Rec 235:363

Colgan S.P., Parkos C.A., Delp C., et al. 1993. Neutrophil migration across cultured intestinal epithelial monolayers is modulated by epithelial exposure to IFN-r in a highly

polarized fashion. J Cell Biol 120:785

Cramer E.B., Milks L.C., Ojakian G.K. 1980. Transepithelial migration of human neutrophils: An in vitro model system. Proc Natl Acad Sci USA 77:4069

Daley M.J., Oldham E.R., Williams T.J., et al. 1991. Quantitative and qualitative properties of host polymorphonuclear cells during experimentally induced Staphylococcus aureus mastitis in cows. Am J Vet Res 52:474

Daley M., Hayes P. 1992. Mastitis: why is it so hard to cure? Cornell Vet 82:1

Eberhart R.J., Harmon R.J., Jasper D.E., Natzke R.P., et al. 1987. Introduction-the mastitis problem. In: Current concepts of bovine mastitis. Natl. Mastitis Counc., Inc., Arlington, VA. p. 6-8

Fetrow J., Anderson K., Sexton S., Butcher K. 1988. Herd composite somatic cell counts: average linear score and weighted average somatic cell count score and milk production. J Dairy Sci 71:257

Frost A.J., Hill A.W., Brooker B.E. 1980. The early pathogenesis of bovine mastitis due to Escherichia coli. Proc Royal Soc London, B209:413

Frost A.J., Brooker B.E. 1986. Hyperacute Escherichia coli mastitis of cattle in the immediate post-partum period. Aust Vet J. 63:327

Gill R., Howard W.H. 1990. Economics of mastitis control. J Dairy Sci 73:3340

Gudding R., Mcdonald J.S., Cheville N.F. 1984. Pathogenesis of Staphylococcus aureus mastitis: bacteriologic, histologic and ultrastructural pathologic findings. Am J Vet Res 45:2525

Harmon R.J., Heald C.W. 1982. Migration of polymorphonuclear leukocytes into the bovine mammary gland during experimentally induced Staphylococcus aureus mastitis. Am J Vet Res 43:992

Heald C.W. 1979. Morphological study of experimentally induced Staphylococcus bovis mastitis in the cow. Am J Vet Res 40:1294

Hill A.W. 1981. Factors influencing the outcome of Escherichia coli mastitis in the dairy cow. Res Vet Sci 31:107

Hudspeth A.J. 1975. Establishment of tight junctions between epithelial cells. Proc. Natl. Acad. Sci. USA 72:2711

Huynh H.T., Robitaille G., Turner J.D. 1991. Establishment of bovine mammary epithelial cells (MAC-T): An in vitro model for bovine lactation. Exp Cell Res 197:191

Jain N.C., Schalm O.W., Carroll E.J., Lasmanis J. 1968. Experimental mastitis in leukopenic cows: immunologically induced neutropenia and response to intramammary inoculation of Aerobacter aerogenes. Am J Vet Res 29:2089

Jain N.C. 1979. Common mammary pathogens and factors in infection and mastitis. J Dairy Sci 62:128

Jain N.C., Paape M.J., Berning L. et al. 1991. Functional competence and monoclonal antibody reactivity of neutrophils from cows injected with Escherichia coli endotoxin. Comparative Haematology International 1:10

Kehrli M.E., Jr., Schmalstieg F.C., Anderson M.J. et al. 1990. Molecular definition of the bovine granulocytopathy syndrome: identification of deficiency of the Mac-1 (CD11b/CD18) glycoprotein. Am J Vet Res 51:1826

Kehrli M., Shuster D. 1994. Factors affecting milk somatic cells and their role in health of the bovine mammary gland. J Dairy Sci 77:619

Macdonald E.A., Xia L., Monardes H., Turner J.D. 1994. Neutrophil function in vitro: Diapedesis and phagocytosis. J Diary Sci 77:628

Manford K.P.Jr. 1979. Measurement of growth and viability of cells in culture. Methods in Enzymology LVIII;152

Mangum J.B., Everitt J.I., Bonner J.C. 1990. Co-culture of primary pulmonary cells to model alveolar injury and translocation of proteins. In Vitro Cell Dev Biol 26:1135

Mcdonald J.S. 1979. Bovine mastitis: Introductory remarks. J Dairy Sci 62:117

Milks L.C., Conyers G.P., Cramer E.B. 1986. The effect of neutrophil migration on epithelial permeability. J Cell Biol 103:2729

Nash S., Stafford J., Madara J.L. 1987. Effects of polymorphonuclear leukocyte transmigration on the barrier function of cultured intestinal epithelial monolayers. J Clin Invest 80:1104

Nash S., Stafford J., Madara J.L. 1988. The selective and superoxide-independent disruption of intestinal epithelial tight junctions during leukocyte transmigration. Lab

Investigation 59:531

Nickerson S.C., Heald C.W. 1981. Histopathologic response of the bovine mammary gland to experimental induced Staphylococcus aureus infection. Am J Vet Res 42:1351

Nickerson S.C., Pankey J.W. 1984. Neutrophil migration through teat end tissues of bovine mammary quarters experimentally challenged with Staphylococcus aureus. J Dairy Sci 67:827

Nickerson S.C. 1985. Immune mechanisms of the bovine udder: An overview. JAVMA 187:41

Nickerson S.C., Owens W.E., Watts J.L. 1989. Local immunostimulation of bovine mammary glands with interleukin-2. J Dairy Sci 72:1764

Nickerson S.C. 1993. Vaccination programs for preventing and controlling mastitis. In: Natl Mastitis Council 1993 Regional Meeting Proceedings. p. 49-50

Nickerson S.C. 1993. The cow's response to mastitis. In: Natl Mastitis Council 1993 Regional Meeting Proceedings. p. 1-15

Nuijens P. 1993. A strategy to increase resistance in dairy cows: expression of human lactoferrin in the milk of transgenic cows. IDF Mastitis News 134:16

Olson D.P. 1990. In vitro migration responses of neutrophils from cows and calves. Am J Vet Res 51:973

Paape M.J., Persson R.E., Schultze W.D. 1978. Variation among cows in the ability of milk to support phagocytosis and in the ability of polymorphonuclear leukocytes to phagocytose Staphylococcus arueus. Am J Vet Res 39:1907

Paape M.J., Wergin W.P., Guidry A.J. 1979. Leukocytes-Second line of defense against invading mastitis pathogens. J Dairy Sci 62:135

Parkos C.A., Delp C., Arnaout M.A. 1991. Neutrophil migration across a cultured intestinal epithelium. J Clin Invest 88:1605

Persson K., Sandgren C.H., Rodriguez-Martinez H. 1992. Studies of endotoxin-induced neutrophil migration in bovine teat tissues, using indium-111-labelled neutrophils and biopsies. Am J Vet Res 53:2235

Peterson P.K., Wilkinson B.J., Kim Y., Schmeling D., et al. 1978. Influence of encapsulation on Staphylococcal opsonization and phagocytosis by human polymorphonuclear leukocytes. Infect & Immun 19:943

Politis I., Zhao X., McBride B.W., Burton J.H. 1991. Examination of chemotactic properties of bovine mammary macrophages. Can J Vet Res 55:321

Politis I., Zhao X., McBride B.W., Burton J.H. 1991. Effect of bovine skim milk and whey on monocyte function. J Dairy Sci 74:2467

Powell D.W. 1981. Barrier function of epithelia. Am J Physiol 241:G275

Radostitis O.M. 1994. Mastitis control in dairy herds, In: Herd Health: Food Animal Production Medicine, 2nd edition, ed by Rostitis et al., W.B. Saunder Co., p. 229-270

Raubertas R.F., Shook G.E. 1982. Relationship between lactation measures of somatic cell concentration and milk yield. J Dairy Sci 65:419

Sandgren C.H. 1991. The neutrophil in the bovine udder, friend or foe?a Ph.D thesis. Swedish University of Agricultural Sciences, Uppsala, Sweden.

Schalm O.W., Lasmanis J., Carroll E.J. 1964. Effects of pre-existing leucocytosis on experimental coliform mastitis in cattle. Am J Vet Res 25:83

Schalm O.W., Lasmanis J., Jain N.C. 1976. Conversion of chronic Staphylococcal mastitis to acute gangrenous mastitis after neutropenia in blood and bone marrow produced by an equine anti-bovine leukocyte serum. Am J Vet Res 37:883

Schultze W.D., Paape M.J. 1984. Effect on outcome of intramammary challenge exposure with Staphylococcus arueus on somatic cell concentration and presence of intramammary device. Am J Vet Res 45:420

Shuster D.E., Harmon R.J., Jackson J.A., Hemken R.W. 1991. Suppression of milk production during endotoxin-induced mastitis. J Dairy Sci 74:3763

Sordillo L.M., Nickerson S.C., Akers R.M. 1989. Pathology of Staphylococcus aureus mastitis during lactogenesis: relationships with bovine mammary structure and function. J Dairy Sci 72:228

Sordillo L.M. 1993. Increasing udder immunity with cytokines. Natl Mastitis Council 1993 Regional Meeting Proceedings. p.56-63

Tizard I.P. 1988. Immunology: An introduction. Saunders College Publishing, p. 47-48

Tizard I.R. 1992. Veterinary Immunology: An introduction. W.B. Saunders Co., p. 17-27