Quantification of Bovine Neutrophil Migration Across Mammary Epithelium in vitro

Y. Lin, J. Cai, J.D. Turner and X. Zhao

ABSTRACT

A microassay system was developed to quantify bovine neutrophil transepithelial migration in vitro. The bovine mammary epithelial cell line (MAC-T) formed a confluent monolayer that served as a biologically meaningful barrier for neutrophil migration. Neutrophils added into the upper compartment of an inverted monolayer were driven to migrate across the epithelium from a basal-to-apical direction by the addition of zymosan activated serum (ZAS) into the lower compartment. The numbers of migrated neutrophils were determined by assaying the neutrophil azurophilic granule marker, myeloperoxidase. Results showed that ZAS stimulated neutrophil migration across the epithelium in a time- and dose-dependent manner. In the presence of 5% ZAS and 2 × 10^6 neutrophils, approximately 2.4 × 10^5 neutrophils migrated across the epithelium in 120 min. The procedures we have developed in this study provide a simple, precisely controlled system to investigate the normal dynamics of bovine neutrophil transepithelial migration and a means to detect and study impaired neutrophil migration.

RÉSUMÉ

Une méthode microscopique a été développée afin de quantifier in vitro la migration trans-épithéliale des neutrophiles bovins. Dans cette épreuve, une lignée cellulaire de cellules mammaires bovines formant une monocouche confluente servant de barrière biologique à la migration des neutrophiles a été utilisée. Des neutrophiles ajoutés au compartiment supérieur d'une monocouche inversée étaient stimulés à traverser la couche épithéliale de la base vers l'apex par l'addition de sérum activé par le zymosan (SAZ) dans le compartiment inférieur. Le nombre de neutrophiles ayant migré était déterminé par évaluation du marqueur des granules azurophiliques des neutrophiles, la myéloperoxidase. Les résultats ont démontré que la migration des neutrophiles à travers l'épithélium a été stimulée par le SAZ en relation directe avec la dose et le temps d'exposition. Lorsque 2 × 10^6 neutrophiles étaient en présence de 5% de SAZ, environ 2,4 × 10^5 neutrophiles avaient migré à travers l'épithélium en 120 min. L'épreuve mise au point dans cette étude fournit un système simple et bien contrôlé permettant d'investiguer la dynamique normale de la migration trans-épithéliale des neutrophiles bovins ainsi qu'un moyen de détecter et d'étudier une migration altérée des neutrophiles.

(Traduit par docteur Serge Messier)

INTRODUCTION

Mastitis continues to cause significant economic losses to the dairy industry, indicating the need for further study of the factors involved in mastitis defense. Neutrophils are one of the major cellular immune components used by the body to defend against invading pathogenic bacteria during mastitis (1). The ability of neutrophils to migrate promptly into milk has long been considered as an important indication of their function and thus the outcome of the disease (2).

Traditionally, neutrophil migration has been evaluated in vitro using an under-agarose system (3,4), and in vivo via experimentally induced mastitis (5). The agarose system, however, can not quantify the rate of neutrophil migration, which is a critical parameter of neutrophil function. Moreover, this system does not present a biologically meaningful barrier for neutrophil migration. On the other hand, the in vivo model has disadvantages such as difficulty identifying the individual factor involved.

To circumvent these problems, we have developed an in vitro model to study bovine neutrophil transepithelial migration. By using this model, the effect of neutrophil migration on transepithelial electrical resistance has been determined (6), and the process of bovine neutrophil transepithelial migration has been demonstrated morphologically (7). The experiments described below were carried out as an extension of previous work in our laboratory. The purpose of this study was to further modify the in vitro system in order to permit neutrophil transepithelial migration from a basal-to-apical direction, a physiologically relevant direction, and measure the rate of neutrophil migration in this in vitro system.

MATERIALS AND METHODS

MAMMARY EPITHELIAL CELL MONOLAYERS

The bovine mammary epithelial cell line, MAC-T (8) was used and maintained by serial passages in a complete medium consisting of high-glucose

Department of Animal Science, McGill University (Lin, Cai, Zhao); AgroCultures Biotechnologies Inc. (Turner); Ste. Anne de Bellevue, Quebec H9X 3V9.

Reprint requests to Dr. X. Zhao.

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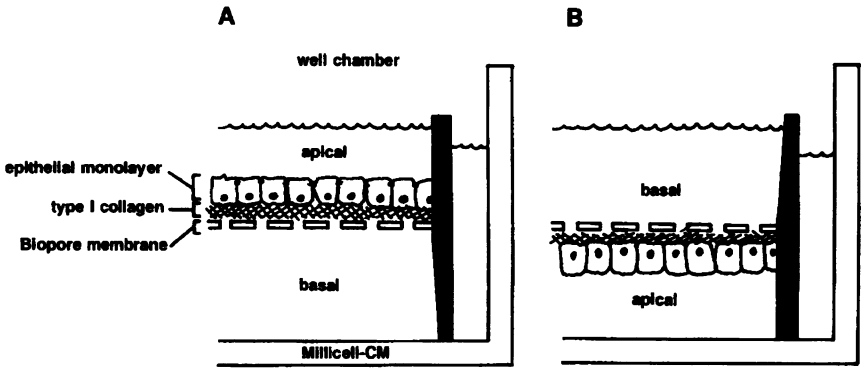

Figure 1. Cross-section of the epithelial monolayer system. The MAC-T cells were cultured at confluence on collagen coated membrane within the cultured chamber (A). Before adding neutrophils, the chamber was placed upside down to create an inverted monolayer (B), thus neutrophils settled on the basal surface of the epithelium.

Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and 50 μg/mL gentamicin (Gibco BRL). Culture chambers (Fig. 1a) were constructed by gluing polycarbonate membrane (5 μm pore-size, Millipore Co., Bedford, Maine) between two insert rings (Millipore Co.) with RTV silicone rubber adhesive sealant (GE Silicones Canada Co., Pickering, Ontario). This kind of sealant does not release trace cytotoxic solvents after setting (9). The membranes were coated with calf tail collagen (6) before MAC-T cells (2×10^5/chamber) were seeded onto the surface of the collagen coated membrane. Chambers were then rested in 6 well culture plates with both apical and basal sides of the monolayer immersed in complete media, and kept in a 37°C incubator with 5% CO_2.

CHEMOATTRACTANT

Zymosan-activated serum (ZAS) was used as a chemoattractant, and prepared by a modified method of Olson (4). Zymosan (Sigma, St. Louis, Montana) was added to newborn calf serum (Gibco BRL) at a concentration of 5 mg of zymosan/mL of serum. The mixture was incubated in a shaking water bath (37°C) for 30 min, then centrifuged (1,500 × g) for 10 min. Aliquots of supernatant (ZAS) were stored at −20°C before used.

NEUTROPHIL ISOLATION

Bovine neutrophils were isolated from whole blood taken from Holstein cows by a modified method of Carlson et al (10). Briefly, neutrophils were isolated via isopycnic centrifugation followed by hypotonic lysis of contaminating erythrocytes. This procedure typically produced a cell fraction containing >95% neutrophils with >99% viability as determined by a differentiating stain (Diff-Quik solution, Baxter Healthcare Corp., McGaw Park, Illinois) and by trypan blue dye exclusion, respectively. Neutrophils (1×10^7 cells/mL) were suspended in Hank's balanced salt solution (HBSS, Gibco BRL) at room temperature before used.

NEUTROPHIL TRANSEPITHELIAL MIGRATION

The culture chambers with epithelial monolayers were rinsed with 37°C HBSS and then placed into new wells containing HBSS. For studying neutrophil migration from a basal-to-apical direction, the chambers were first inverted, thus presenting the basal surface of the monolayer upward (Fig. 1b). Neutrophils (200 μL) were then added to the upper compartment above the monolayer, and forced to transmigrate across the monolayer by the addition of ZAS to the lower compartment. The time-course of neutrophil transepithelial migration was studied by using a fixed concentration of ZAS (5%) for up to 120 min. The dose-response studies were carried out for 120 min with various concentrations of ZAS added to the lower compartment and neutrophils (2×10^6) added to the upper compartment. The culture chambers were incubated at 37°C during the time of transepithelial migration.

QUANTITATIVE MEASUREMENT OF NEUTROPHIL MIGRATION

At the end of transepithelial migration, the migrated neutrophils in the lower compartment and neutrophils associated with the cell monolayer were quantified by assaying the neutrophil azurophilic granule marker, myeloperoxidase (MPO) as described by Parkos et al (9), with minor modifications. Briefly, the cultured inserts were washed four times with ice-cold HBSS to remove free neutrophils from the chamber. Myeloperoxidase was released from neutrophils by adding 400 μL of Triton X-100 (0.5%) to the monolayer and the cell pellet was collected from the lower compartment. After 30 min, 100 μL of 100 mM citrated buffer (pH 4.2) was added to each sample. The MPO activity was assayed at 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) and 10 mM H_2O_2 in 100 mM citrated buffer (pH 4.2) for 30 min. Neutrophil equivalents were estimated from a standard curve made with known number of neutrophils. The number of migrated neutrophils was the sum of monolayer-associated neutrophils and neutrophils in the lower compartment.

STATISTICAL ANALYSIS

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

RESULTS

The standard curve of the MPO assay was made for each experiment, and results showed that the concentration of neutrophils from 1-100 × 10^4 cells was correlated with the optical density value ($r^2 = 0.90$) (Fig. 2).

The time-course assay showed that when ZAS was used at a concentration of 5%, the number of migrated neutrophils increased over time, with the most migration occurring during the first 60 min. At 60 min, approximately

2.4×10^5 neutrophils migrated across the monolayers (Fig. 3). This number was about 12% of the total neutrophil added to the upper compartment.

The results of dose response were shown in Figure 4. Compared with the control, as little as 1% ZAS significantly increased migration of neutrophils ($P < 0.01$), with maximum response achieved at 20% of ZAS. Further increase of ZAS to 40% only induced a slight increase in neutrophil migration.

When comparing the efficiency of transepithelial migration of neutrophils from two directions, it was found that in response to 5% ZAS for 120 min, the number of migrated neutrophils from basal-to-apical (B-A) direction was approximately 65% of the number from the apical-to-basal (A-B) direction ($P < 0.05$) (Fig. 5).

DISCUSSION

We have modified our in vitro system to assess neutrophil transmigration across bovine mammary epithelial monolayers in the physiological direction. This work was prompted by analogous research using human intestinal epithelial cell line T84 (9). Research toward understanding neutrophil diapedesis into bovine mammary gland has been hampered by the absence of an in vitro model that closely mimics the ruminant mammary gland. The MAC-T system represents a biologically meaningful barrier with a single cell monolayer model. However, like other studies (11,12,13) for neutrophil transepithelial migration, neutrophil migration was previously studied in our laboratory (6,7) in an apical-to-basal direction, a non-physiological direction. Technical problems arise when neutrophils settle on the bottom of the culture plate due to gravity. This precludes their migration upward in the media against gravity. By inverting the monolayer system, neutrophils are allowed to settle down on and subsequently migrate through the epithelium in a basal-to-apical direction, a physiologically relevant direction. This study demonstrated that neutrophils were able to migrate across the epithelium from an apical-to-basal direction as well as from a basal-to-apical direction. In addition, a difference in the rate of neutrophil migration

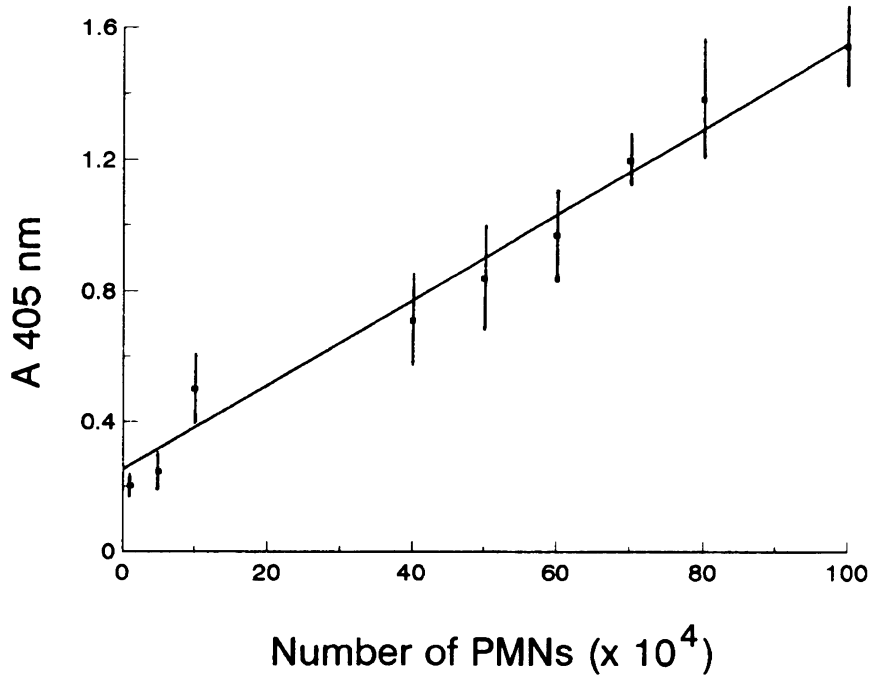

Figure 2. Standard curve of the myeloperoxidase assay. Various concentrations of neutrophils were first treated with 0.5% Triton X-100, and then mixed with a solution containing enzyme substrate and H_2O_2. After 30 min, the colour development was assayed on a microtiter plate reader. Each point represents the mean and SEM of 6 samples from 3 individual experiments.

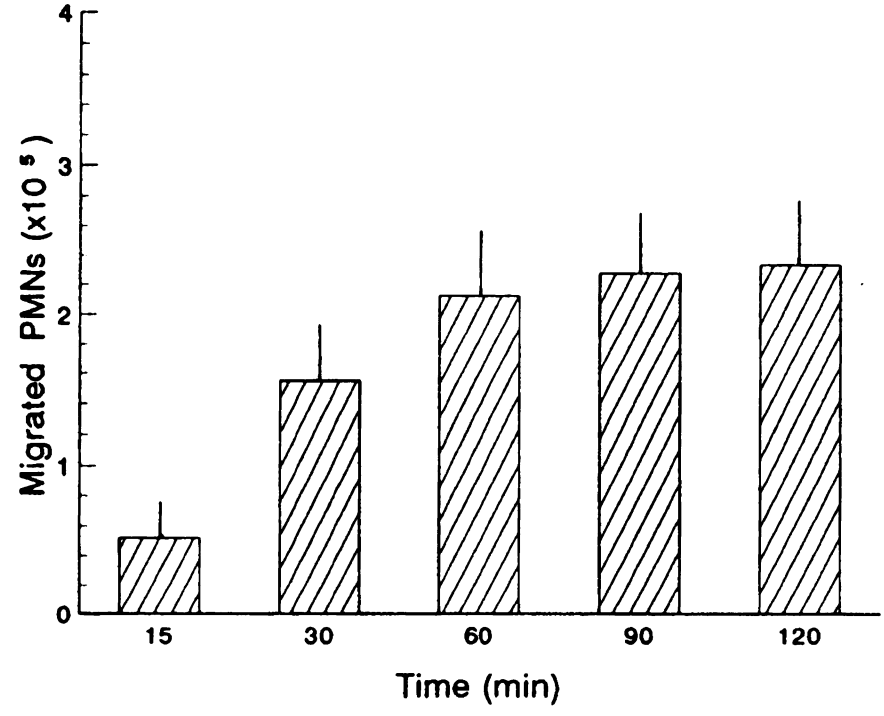

Figure 3. Time-course of neutrophil migration across MAC-T monolayer. Neutrophils were added on the basal surface of the monolayer in the presence of a transepithelial gradient of ZAS (5%). At the time points indicated on the curve, neutrophils that had transmigrated were quantified using the MPO assay.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

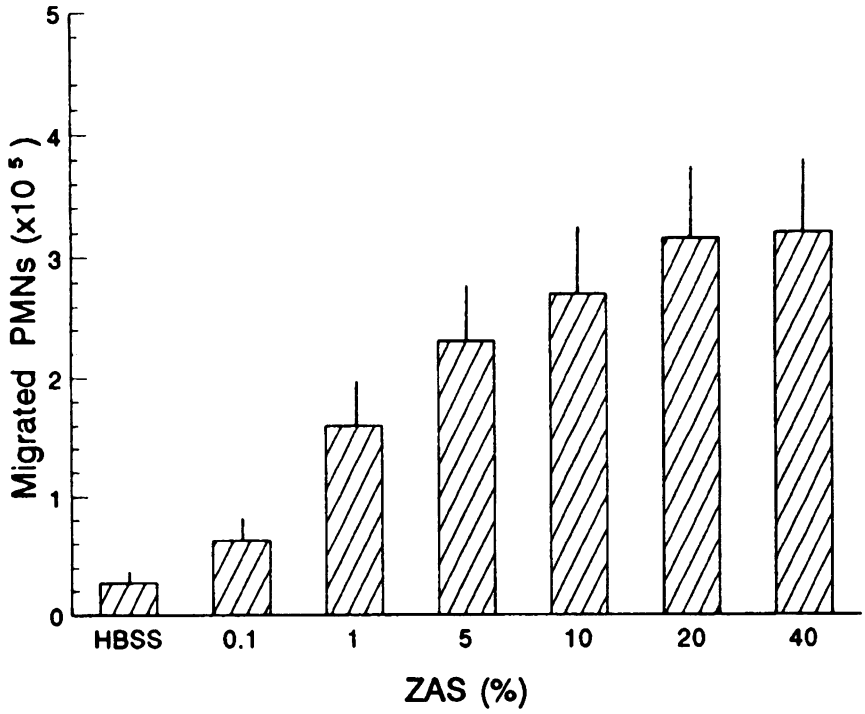

Figure 4. Effect of different concentrations of zymosan activated serum (ZAS) on neutrophil migration. Neutrophils were added to the basal surface of the epithelium and driven to migrate across the monolayer by adding ZAS into the lower compartment. The migrated neutrophils were determined by the MPO assay. Data represent mean and SEM of 6 monolayers from 3 individual experiments.

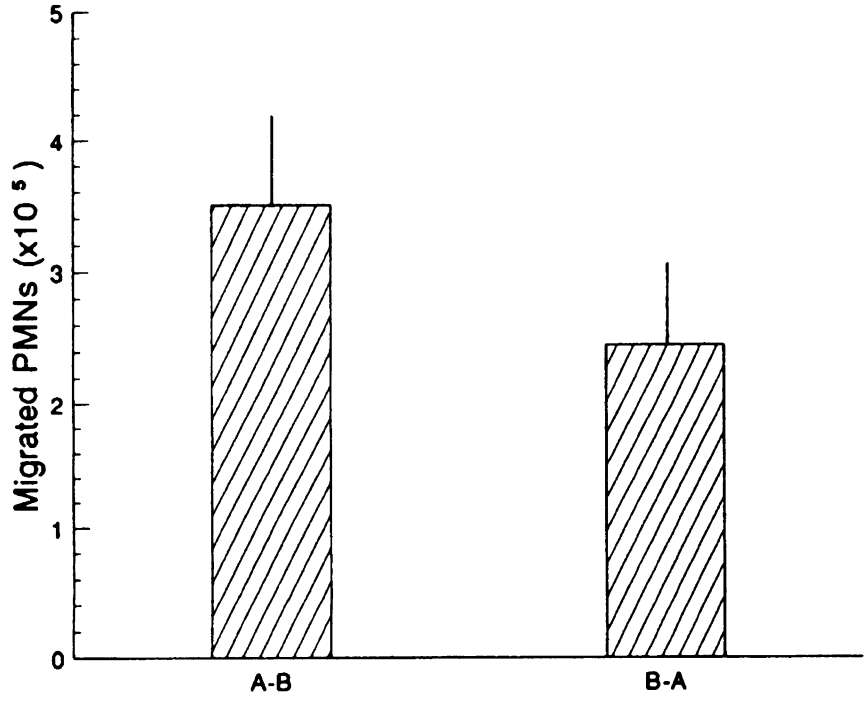

Figure 5. Comparison of the efficiency of neutrophil transepithelial migration in the basal-to-apical (B-A) versus the apical-to-basal (A-B) direction. Neutrophils were added on the basal or apical surface of the epithelium and induced to transmigrate for 120 min. The migrated neutrophils were determined by the MPO assay. Results represent the mean and SEM of 6 monolayers from 3 individual experiments.

from these two directions was noted. The reason for this difference may be due to the initial contact of neutrophils with the insert membrane, instead of the cell monolayer, in the basal-to-apical direction. By using an inverted monolayer system to study human neutrophil migration across an intestinal epithelium, Parks et al (9) confirmed that neutrophil transepithelial migration was similar in both directions in several qualitative ways (i.e., ability of transmigration to proceed, specific chemotactic gradients required, and CD11b/18 dependence). Nevertheless, they observed that neutrophil transmigration in the physiological direction was 5–20 times more efficient than in the routinely studied opposite direction (9).

An additional advance came in the use of a microassay to enumerate neutrophils. Previously, cell numbers were evaluated mainly by direct hemocytometer counting (11). This study employed an enzyme assay (9) for myeloperoxidase that is found in large amounts in neutrophil's azurophilic granules (14). The principle of the MPO assay is that after treating neutrophils with Triton X-100, the myeloperoxidase is released from the azurophilic granules of the neutrophils. 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 405 nm. The increase in optical density value was well correlated with the number of neutrophils within the range of neutrophil number used in this study. The major advantage of this method is that it can quantify the total number of migrated neutrophils. Moreover, this method would be faster and easier than the microscopic counting in histological sections (11).

Zymosan activated serum was chosen as a chemoattractant in the present study because it is proven to be a potent chemotactic factor for bovine neutrophils using standard agarose method (3,4). Using our cell monolayer model, we also found that the ZAS greatly stimulated the transepithelial migration of neutrophils, and this effect was time-and dose-dependent. The results showed that in the absence of any chemoattractant, the number of migrated neutrophils was less than 3×10^4 cells. The presence of

ZAS greatly stimulated neutrophil transmigration. However, within the range of ZAS concentration (0.1 to 40%), and incubation time (15 to 120 min), the maximum number of migrated neutrophils was less than 20% of the total neutrophils applied. There are several possibilities for this phenomenon. Firstly, a functional variation always exists among a population of neutrophils (16). Because of the tightness of the junctional complex, neutrophils are needed not only to migrate but also to open tight junctions in order to cross the epithelium (12). Therefore, it is possible that only those neutrophils having higher activity can open the tight junctions and subsequently migrate across 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 neutrophil chemotaxis have been reported (17), and some of these agents are believed to be potentially operative as inflammatory control mechanisms in vivo (18,19). For example, Clark and Szot (14) demonstrated that neutrophils which have already 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 the same stimulus within a certain time following an inflammatory challenge, has also been described (18).

In summary, an inverted epithelial monolayer system has been developed to study bovine neutrophil transepithelial migration in a physiologically relevant direction. The total number of migrated neutrophils can be quantified by myeloperoxidase assay. This microassay system permits a large number of parallel studies for neutrophil transepithelial migration and related issues in a controlled environment.

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