Polymorphonuclear Leukocyte Migration through Human Amnion Membrane

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ABSTRACT A new in vitro model has been developed for studying migration of human polymorphonuclear leukocytes (PMN) through living native cellular and matrix barriers. Human amnion membrane consists of a single layer of epithelium bound to a continuous basement membrane interfacing an avascular collagenous stroma. Living amnion was placed in plastic chambers with separate compartments on each side of the membrane. PMN were introduced on the epithelial side of the amnion, and a Millipore filter (Millipore Corp., Bedford, Mass.) was placed against the stromal side. In response to N-formylmethionyl-leucyl-phenylalanine (FMLP) chemoattractant, PMN penetrated the full thickness of the amnion and were collected and counted on the filter. The rate of PMN traversal of the amnion was dependent on the concentration of FMLP (optimal at 10^{-8} M) as well as the slope of the FMLP gradient across the amnion. The route of PMN migration was studied by transmission electron microscopy. PMN first attached to the epithelial surface, then infiltrated between intercellular junctions. PMN migrated around or through tight junction and hemidesmosome attachments. The PMN then penetrated the basement membrane and migrated through the dense collagenous stroma.

The present amnion migration system has characteristics of the in vivo inflammatory state not described in any previous method for monitoring PMN migration in vitro. Prior methods have not used native epithelium, whole basement membrane, or collagenous stroma. PMN penetration of these barriers occurs in the normal inflammatory response and probably involves biochemical mechanisms not required for simple migration through the pores of an artificial filter. The amnion system can be useful for future biochemical and morphological studies of PMN penetration of these barriers and possible repair processes that may follow.

In response to an inflammatory stimulus, polymorphonuclear leukocytes (PMN) adhere to the endothelial cell surface of post capillary venules (1-5), traverse the endothelium through interendothelial cell junctions, and penetrate the basement membrane as they leave the vascular system to accumulate at the site of injury (6).

PMN chemokinesis and chemotaxis are induced by multiple substances (7-9). N-formylmethionyl-leucyl-phenylalanine (FMLP) has been demonstrated to be one of the most potent chemoattractants for phagocytic cells (10, 11). This substance exerts its activity by binding to specific cell receptors on PMN (12-14). FMLP increases PMN adhesiveness to endothelial cells (5, 15, 16) and induces prominent release of the granule-bound enzymes (11, 15, and Gauss-Muller et al., submitted for publication). PMN response to chemotactic factor is markedly dose dependent. An optimal dose range exists for PMN chemotaxis. At doses above the optimal range, PMN homotypic aggregation is increased and migration is reduced. At doses below the optimal range, both aggregation and migration are reduced (11, 17, 20).

The mechanism by which PMN penetrate whole endothelium, basement membrane, collagenous stroma barriers, and eventually an epithelium (21, 22) is poorly understood because this process is difficult to study in vitro. Previous in vitro methods for investigating leukocyte migration and chemotaxis have been restricted to the use of micropore filters (16, 23, 26) or, more recently, epithelial or endothelial monolayers grown on micropore filters (27, 28). These previous systems are not...
optimal for simulating the actual physiological state because they do not include intact cell layers attached to normal continuous basement membrane, interfacing with connective tissue matrix.

The human amnion membrane is avascular and contains all these three basic tissue components (epithelium, basement membrane, and stroma) (29, 30) which can be traversed by leukocytes responding to an inflammatory stimulus. The present report describes a new in vitro amnion model system for studying PMN penetration of human native tissue barriers.

We have quantitated the rate of migration of PMN from the amnion epithelial side, through the basement membrane and the stroma, in the presence of different FMLP gradients. In addition, the histologic route of migration of PMN through the amnion was studied using light microscopy and transmission electron microscopy.

**MATERIALS AND METHODS**

**General Reagents and Supplies**

Materials from the following sources were used: tissue culture media, penicillin and streptomycin, Fungizone (manufactured by Squibb, Princeton, N. J.), HEPES buffer, and phosphate-buffered saline (PBS) were all purchased from Flow Laboratories (McLean, Va.); x-formylmethionyl-leucyl-phenylalanine (FMLP) from Peninsula Laboratories (San Carlos, Calif.); 13-mm Millipore filters (5 μm pore size) from Millipore Corp. (Bedford, Mass.); Ficoll from Pharmacia Fine Chemicals, Inc. (Piscataway, N. J.) and Hypaque from Winthrop Laboratories, New York.

**Human Amnion Membrane**

Normal term placentas were obtained fresh after delivery. The amnion faces the amniotic fluid compartment and is adherent to the chorion. The amnion was aseptically peeled away from the chorion by blunt dissection and rinsed twice with PBS solution containing 0.02% sodium hypochlorite. Then the membrane was immersed in PBS containing penicillin-streptomycin (PS) (100 IU/ml and 100 μg/ml, respectively). Amnions were finally immersed in Minimal Essential Medium (with Earle's salts) with glutamine (4 mM) and PS and used immediately.

**Amnion Chamber**

Two-compartment chemotaxis chambers (Fig. 1) were constructed in the form of two Lucite rings. The rings measure 3.2 cm outside diameter and 1.2 cm inside diameter. The upper ring is 1.0 cm in height and the lower ring is 0.2 cm in height. Once stretched and clamped between the two halves of the chamber, the amnion divides the chamber into two compartments: upper and lower (Fig. 1). A Millipore filter (5 μm pore size, 2.3 cm diameter, Millipore Corp.) was sandwiched in the chamber so as to be in direct contact with the amnion stromal surface. The chambers are placed in 6-well cluster dishes (Costar, Data Packaging, Cambridge, Mass.). The amnions were verified to be free of leaks as described previously (31).

**Neutrophil Preparation**

Human PMN were isolated from blood of normal healthy volunteers by the Ficoll-Hypaque method, and contaminating erythrocytes were removed by dextran sedimentation and hypotonic lysis. This procedure routinely resulted in a cell fraction containing 90% PMN with 95% viability as determined by trypan blue exclusion and Sudan Black B staining. The PMN were suspended in Hank's balanced salt solution buffered with HEPES (pH 7.4).

**PMN Migration Studies**

FMLP was introduced into the lower compartment. PMN were added to the upper compartment onto the amnion epithelial layer at the concentration of 1 x 10⁶ cells in a total volume of 1.5 ml. The chambers containing the neutrophils were incubated at 37°C (5% CO₂, 95% air). Migration was observed periodically through an inverted microscope. Quantification of the PMN that had traversed the full thickness of the amnion was done by staining the Millipore filter (Millipore Corp.) with standard hematoxylin solution (11). PMN cells trapped within or adherent to the filter were easily identified against the white filter background (Fig. 3C). The whole filter (1.13 cm²) was scanned (magnification, x400) and the total number of PMN were counted on each filter. The means and ranges were recorded for triplicate experiments using PMN obtained from the same donor. PMN were routinely counted on the filter 3 h after their introduction into the upper compartment.

**Electron Microscopy**

Tissues were fixed in 2.5% glutaraldehyde in Sorenson's buffer (pH 7.4) and postfixed in 1% osmium tetroxide. The tissues were dehydrated in a graded series of ethanol and embedded in M Milepore-6. Thin sections were cut on a Sorvall MT2 ultramicrotome (DuPont Instruments, Newtown, Conn.). Sections were stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 1A electron microscope. For studies of amnion epithelial junctions, ruthenium red staining was performed as described by King (30). The ruthenium red solution was added to the upper chamber so that the glycocalyx surface was stained and occluding junctions were identified by failure of the stain to penetrate between cell junctions.

**Amnion Viability**

Amnion epithelial viability was studied by trypan blue staining. Trypan blue (0.01%) was mixed in Gey's buffer, applied to the epithelial surface, and immediately rinsed off. Nonviable cells were identified by uptake of the blue stain in the nucleus. For some experiments, all the amnion epithelial cells were rendered nonviable by alkali treatment with 0.1% ammonium hydroxide in distilled water (15 min, 25°C) followed by extensive washing in Gey's buffer. For other experiments, the epithelium was denuded as described previously (31), leaving an intact basement membrane surface.

**Figure 2** Morphology of normal amnion. (A) Transmission electron micrograph of amnion epithelial intercellular junctions. X6,000. The epithelial cells (E) are interlocked by desmosomes (J). MV, microvilli of epithelial surface; BM, basement membrane; ST, collagenous stroma. (B) Periodic acid-Schiff stain of normal amnion. The continuous basement membrane (BM) is stained. EP, epithelium; ST, collagenous stroma; F, fibroblasts. X200. (C and D) Ruthenium red incubation with amnion epithelium, high (X 6,000) and low (X2,000) magnification. The glyocalyx (GC) stains intensely. Occluding type junctions appear to be present because the stain does not penetrate the junctions (arrows).
RESULTS

Morphology of Human Amnion

The isolated whole amnion is transparent and <0.5 mm in thickness. Histologically, the human amnion membrane consists of a single layer of cuboidal to low columnar epithelial cells and basement membrane resting on nonvascular stroma (Fig. 2B). Each epithelial cell is firmly interlocked to its neighbor by numerous desmosomes (Fig. 2). The epithelium exhibits surface microvilli. Ruthenium red staining demonstrated a prominent surface glycocalyx. The ruthenium red dye failed to completely penetrate between the epithelial junctions (Fig. 2). The epithelium is bound to the continuous basement membrane by numerous hemi-desmosomes. The viability of amnion epithelial cells was carefully examined by trypan blue dye exclusion before each experiment. The amnion epithelial cell viability was consistently >95%. Approximately 50-60% of the amnion preparations showed complete absence of nonviable cells. When nonviable cells were identified, they were usually sparsely and focally isolated in one area of the surface. After brief ammonium hydroxide treatment, 100% of the epithelial cells were rendered nonviable. The nonvascular stroma shows the greatest density at its interface with the basement membrane. By electron microscopy, the stroma contains collagen fibers and occasional fibroblasts (Fig. 2).

PMN Migration Through the Amnion

PMN, when placed on the amnion epithelial surface, penetrated through the full thickness of the amnion in response to the chemotactic stimulus exerted by FMLP. FMLP concentrations ranging from 10^-5 M to 10^-11 M were applied to the bottom compartment of the amnion chamber and compared to controls, without chemoattractant.

The filters (5 μm pore size) placed in contact with the amnion stromal side trapped the PMN that traversed the whole thickness of amnion within the 3 h of experimentation (Fig. 3). The numbers of PMN that have traversed the amnion to enter the filters are shown in Fig. 4 for different concentrations of FMLP.

Quantitation of the FMLP dose-response indicated that an optimal rate of migration occurred at 10^-9 M and 10^-8 M concentration of FMLP applied to the bottom compartment (Fig. 4). At FMLP 10^-9 M, the PMN were uniformly distributed as single cells and small aggregates (2-3 cells) at various stages of penetration within the epithelium (Fig. 3B). PMN migration at this concentration was twofold greater than spontaneous migration as detected without the addition of FMLP. At higher concentrations of FMLP (10^-6 M, 10^-5 M), the PMN were noted to aggregate into large clumps (>50 cells) and to remain on the epithelial surface (Fig. 3A). These clumps showed a tight adherence to the epithelial layer. In this situation (FMLP 10^-5 to 10^-6 M), the directed chemotaxis was even less than random migration (Fig. 4). At FMLP 10^-10 M, adherence of PMN was minimal or not different from the controls. Directed migration at these low concentrations (10^-10 to 10^-11 M) showed only a threefold increase over the controls. Thus, a balance exists between FMLP-induced aggregation and migration for PMN penetration of the amnion barriers. At a concentration of FMLP optimal for chemotaxis, aggregation on the epithelial surface is minimal. At greater than optimal concentrations of FMLP, PMN homotypic aggregation was pronounced and migration was markedly reduced. At sub-optimal concentrations of FMLP, PMN adherence to the epithelium was poor.
Tritium-tagged FMLP ([3H]FMLP) was applied to the lower compartment of the amnion chamber. The time-course of diffusion of [3H]FMLP into the upper compartment showed a rapid rise followed by a plateau as equilibrium was reached (Fig. 5). The kinetics were such that 50% of the equilibrium value was reached by 4.5 h. The shape of the diffusion curve was not altered by more than one standard deviation when the initial concentration of [3H]FMLP was varied between $10^{-6}$ and $10^{-11}$ M.

To study the effect of the FMLP gradient on migration, PMN were applied to the epithelial surface in the upper compartment at various times after application of FMLP in the lower chamber. The number of PMN reaching the filter was measured after 3 h. The most rapid rate of PMN migration occurred when these cells were applied at a time when the [3H]FMLP gradient exhibited the steepest slope (time 0 min, Fig. 5). The rate of migration decreased in proportion to the rate of change of the slope of the FMLP concentration gradient. When FMLP equilibrium across the amnion was reached (at 17 h, Fig. 5), PMN migration to the bottom compartment occurred at a rate less than spontaneous random migration.

To study the effect of amnion epithelial viability on PMN migration, experiments were conducted with 100% viable epithelium, 100% nonviable epithelium or denuded epithelium with the basement membrane surface exposed. For these experiments the lower chamber contained either no FMLP or FMLP at $10^{-8}$ M. The results are shown in Table I. The PMN migrated through all three types of amnion preparations.

The most rapid migration occurred with the living epithelium. Migration through the dead or denuded epithelium was slightly slower. In the presence of FMLP, the PMN were observed to attach to the dead epithelial surface.

![Figure 5](image_url)

**FIGURE 5** Effect of FMLP diffusion gradient across amnion on PMN chemotaxis. The diffusion of [3H]FMLP was measured from the lower to the upper compartments ($\bar{X} \pm SD$). PMN were applied to the amnion epithelial surface at different times during the development of the gradient. The total number ($\bar{X}$ of the range $\pm$ range) of PMN cells traversing the amnion barrier and entering the filter was counted 3 h later.

| Amnion preparation | Number of PMN penetrating the full thickness of the amnion* |
|--------------------|--------------------------------------------------------|
| Living epithelium  | 1409 ± 340                                             |
| Nonviable epithelium | 467 ± 44                                            |
| Denuded epithelium | 620 ± 121                                              |

* Number per filter, mean of the range $\pm$ range.

**Ultrastructural Studies of PMN Migration Route**

PMN placed on the epithelial layer of human amnion aggregated and adhered over intercellular junctions (Fig. 6). After extending pseudopods between adjacent epithelial cells, the PMN seemed to migrate in groups, around or through desmosomes connections, and establishing, in some cases, an intimate contact with epithelial cells. A focal dissolution of the basement membrane was observed at the front of PMN contact (Fig. 7). After traversing the basement membrane, the PMN invaded the dense and loose collagenous stroma (Fig. 8).

**DISCUSSION**

A pathognomonic feature of acute inflammation is the emigration of PMN out of the blood vessels towards the site of injury (6, 7). PMN emigration, involves chemotactic events and complex interactions with cellular and matrix components. The actual mechanism of PMN penetration through natural tissue barriers such as basement membrane and native collagenous stroma is poorly understood.

This report describes a new system for studying in vitro PMN penetration of human amnion epithelium, basement membrane, and collagenous stroma, in response to chemotactic stimulus exerted by FMLP. Previous investigators have studied PMN migration through epithelium or endothelium cultured on micropore filters (27, 28). No previous in vitro system has been reported for quantitating PMN penetration of epithelium attached to its native continuous basement membrane and underlying stroma. The optimal concentration of FMLP ($10^{-8}$ M-$10^{-9}$ M) that induced migration of PMN through the full thickness of the human amnion barrier was similar to that described by other authors to produce maximal chemotaxis through porous filters (11, 20, 32).

A sharply restricted range of FMLP concentration was optimal for PMN migration through the amnion (Fig. 4). A restricted range of optimal FMLP concentrations is also observed for filter systems (10, 11, 32). The optimal concentration of FMLP ($10^{-8}$ M) induced a twelvefold increase in migration compared to random migration. At higher molarities of FMLP ($10^{-5}$ M, $10^{-6}$ M), the PMN aggregated and adhered tightly to the epithelial surface in large clumps and subsequent migration through the amnion did not occur (Figs. 3 and 4).

These findings confirm the PMN motility studies of O’Flaherty and Ward (19) and of Fehr and Dahinden (20) who showed that excessive cellular adhesion impedes cellular move-
ment and induces local cell trapping (33). The kinetics of the chemotaxis gradient formation observed in the amnion showed a rapid exponential rise over a 4-h period, followed by a plateau as equilibrium was reached. The relatively slow rate of diffusion of the tripeptide through the amnion was presumably the result of the multiple barriers to diffusion formed by the collagenous stroma, organized into a dense gel, the continuous basement membrane, and the interactions with the viable epithelial cells.

The maximum rate of PMN migration through the amnion occurred when the FMLP gradient showed the steepest slope (Fig. 5). This is in agreement with the in vitro studies of Zigmond (34) and Zigmond and Sullivan (35) who showed that PMN migration depended not only on the concentration
of chemoattractant but also on the slope of the gradient. However, the reduction in the rate of PMN migration applied 5 h after the FMLP was introduced may have been due to factors other than the slope of the FMLP concentration gradient. The route of PMN migration through the amnion is identical to that found for PMN migration during acute inflammatory response in experimental animals in vivo.

Marchesi studied the time-course of acute inflammation in rat mesentery venules (1, 2). Phillips and Mahler reported leukocyte migration through the rabbit vaginal epithelium (21). Shaw studied lung inflammation in rabbits by intratracheal injection of chemotactic fragments from zymogen-activated serum (36). These investigators all found that the inflammatory cells first adhered to the endothelial surface, then penetrated the intercellular junctions. After being held up at the basement membrane for a time period, the leukocytes were then noted to rapidly pass through local zones of basement membrane dissolution.

The mode of interaction of the PMN cells with the cell surface and intercellular junctions of the amnion epithelium is very similar to that reported by Cramer et al. (27) for canine epithelial cells grown on a Millipore filter (Millipore Corp.). Thus the normal epithelium anchored to its native basement membrane in the present system is not appreciably different in its interactions with PMN compared to a cultured monolayer of epithelium. Ruthenium red staining appeared to indicate the presence of occluding junctions between the amnion epithelium, thus confirming the results of King (30).

In the present amnion system the basement membrane is continuous and impermeable to carbon particles or labeled proteins >60,000 daltons (31). Local dissolution of the basement membrane may be induced by the PMN and is associated with cytoplasmic processes (Fig. 7). The mechanism of dissolution may be enzymatic (37, 38). However, the present results do not rule out a simple mechanical disruption of the basement membrane by PMN.
Occasional nonviable epithelial cells were noted on the amnion surface. This raised the possibility that PMN were migrating only through regions of dead epithelial cells. Therefore experiments were conducted with killed or denuded epithelium. If PMN migrated only through dead epithelium, it would be expected that a marked enhancement of migration would occur when the epithelium was killed or denuded as shown in Table I. This was not the case. The living epithelium was traversed slightly faster than the other two preparations. These data would seem to exclude the possibility that PMN migrate only next to dead epithelial cells. Further evidence in favor of this conclusion is the finding that PMN migrate uniformly over the entire amnion surface. PMN are also uniformly distributed on the filter (Fig. 3). If PMN were migrating preferentially in focal zones of dead epithelium, they would be found on the filter only in focal clusters directly underneath the zones of dead epithelium. Finally, electron micrographs of amnion epithelial cells adjacent to migrating PMN do not show characteristics of dead cells.

The PMN studied here migrated within 3 h through the dense collagenous stroma of the amnion in response to the FMLP attractant. Studies with colloidal carbon particles applied to the stroma surface indicate no preformed passageways in the stroma thickness. Therefore we conclude that the PMN actively disrupt the stroma to produce a migration tunnel.

In conclusion, the PMN penetration of connective tissue barriers, which occurs in the normal inflammatory response, probably involves certain degradative mechanisms not required for simple migration through the open pores of a nonbiological matrix. The present system offers a new approach for elucidating the mechanism of such penetration and also for studying the repair processes that may follow the PMN migration. We
have also found the amnion system to be useful for quantitation of tumor cell invasiveness (39).

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