For in vivo study of the phenomena observed in vitro, PMN (polymorphonuclear leukocyte) extravasation was analysed quantitatively in the microcirculation of the hamster cheek pouch using a video system. Topical application of leukotriene B4 or N-formyl-methionyl-leucyl-phenylalanine increased dose dependently the number of PMNs adhering to the venules. Eighty to 90% of the adhering PMNs disappeared from the vascular lumen into the venular wall within 10-12 min after the adhesion. After PMNs had passed through the endothelial cell layer, they remained in the venular wall for more than 30 min after application of the chemoattractants and appeared in the extravascular space. Thus, the process could be divided into five steps: (1) rolling and (2) adhesion to the endothelium, (3) passage through the endothelial layer (4) remaining in the venular wall, and (5) passage through the basement membrane.

**Key words:** fMLP, LTB4, Neutrophil adhesion, Neutrophil extravasation, Vascular basement membrane

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**Introduction**

Transmigration of polymorphonuclear leukocytes (PMNs) from the circulation to the extravascular space is one of the cardinal signs of the acute inflammatory responses. At the microcirculatory level, PMNs have been described as rolling along the venular wall\(^1\) and then adhering to the inner surface of the endothelial cells.\(^1,2,4\) Studies by electron microscopy have also indicated that PMNs penetrate the vessel wall between endothelial cells.\(^6-10\) However, past observations, made in vivo at the microcirculatory level, of the process of transmigration of PMNs have been mainly rather descriptive, and not quantitative.

The initial report from in vitro experiments that incubation of interleukin-1 with cultured endothelial cells from the human umbilical vein increased their adhesivity for neutrophils stimulated studies on adhesion molecules. Since then, many findings on adhesion molecules of neutrophils and endothelial cells have been accumulated using cultured endothelial cells and isolated neutrophils.\(^1,2,4,9\) Two intercellular adhesion molecules, ICAM-1 (intercellular adhesion molecules-1)\(^1,4\) and ICAM-2\(^1,5\) have been identified on endothelial cells in relation to neutrophil adhesion to these cells.

Simultaneously, studies of patients who show repetitive infection and lack of PMN migration\(^16\) have revealed congenital deficiencies of a family of surface glycoproteins of leukocytes, which have now been identified as adhesion molecules of the integrin superfamily (CD11/CD18).\(^17\)

In spite of the extensive efforts made in examining adhesion molecules in vitro, in order to understand properly the phenomenon of neutrophil extravasation, which occurs in vivo at the microcirculatory level, confirmation in vivo of the in vitro findings is essential. Also, in order to discuss PMN transmigration at the molecular level in vivo, quantitative analyses are required.

We reported a quantitative analysis of the rolling and adhesion of PMNs induced by leukotriene (LT) B4 and N-formyl-methionyl-leucyl-phenylalanine (fMLP) in the microcirculation of the hamster cheek pouch.\(^3\) The behaviour of PMNs in the vascular lumen was quantitated from changes in intensity of images transmitted from a monochrome television (TV) camera to a TV monitor screen, by using a photocell placed on this screen, but the subsequent steps of PMN extravasation remain to be analysed.

The present experiments aim to give a quantitative analysis of the entire process of the transmigration of PMNs from the bloodstream to the perivascular space at microcirculatory levels in the cheek pouch of anaesthetized hamsters.

**Materials and Methods**

**Preparation of hamster cheek pouch:** The hamster cheek pouch preparations were set up as previously described.\(^3,18\) Tracheal intubation ensured spontaneous respiration. The male golden hamsters (Mesocricetus auratus, 120–150 g, 10–16 weeks old) were anaesthetized with pentobarbital (Nembutal\(^®\)),
Abbot Lab, Chicago, IL, USA) (60 mg/kg, i.p.). The cheek pouch was everted, cut longitudinally, and extended. The avascular connective tissue was elaborately dissected away to expose the microvasculature of the mucous layer. The thin mucous membrane tissue was spread in a plastic chamber (9 ml) and superfused at 5 ml/min with warmed Tyrode’s solution, which was maintained at 37°C. Body temperature was maintained with a warming pad positioned directly beneath the hamster and maintained at 37°C.

Microscopic observation and recording of leukocyte behaviour: The microvasculature of the hamster cheek pouch was observed with transillumination under a microscope (BHA, Olympus Optical Co. Ltd, Tokyo), using a water immersion lens (x 40) and x 10 eyepieces (Olympus Optical Co. Ltd, Tokyo). Images of the microcirculation were projected into a TV monitor screen via a monochrome TV camera (CTC2600, Ikegami Tsushinki, Tokyo) mounted at the top of a tricorosopic microscope. The leukocyte responses in each experiment were usually recorded with a videotape recorder (VO-5850, Sony Co., Tokyo) and replayed after the experiments. The rolling behaviour, adhesion to the vessel walls, and migration to the extravascular space, of individual leukocytes were clearly visible on the screen. The observation and experimental procedures were performed only once for each animal. The behaviour of individual leukocytes in a post-capillary venule of 13–15 µm diameter were observed over a 110 µm length along a venule. During the 30 min equilibration period, marginating leukocytes could be visualized inside the venules as they rolled along the endothelium at a lower velocity than the bulk flow.

Chemotactic agents: LTB₄ (Paesel, Gmbh and Co, Frankfurt) and fMLP (Peptide Institute, Minoh, Osaka) were used as chemoattractants. Stock solutions of LTB₄ (30 µM in absolute ethanol) or fMLP (1 mM in saline) were kept frozen at −70°C and diluted to appropriate concentrations with Tyrode’s solution immediately before use.

Application of LTB₄ and fMLP: The superfusion of the cheek pouch with Tyrode’s solution was stopped during the experiments. LTB₄ or fMLP solution was applied to the microvasculature at the observation sites with a 50 µl micropipette.

Electron microscopy: Before and 30 min after the application of LTB₄, parts of the thin preparation of the hamster cheek pouch used for examination of the microcirculation were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide. Ultrathin sections were cut on a LKB ultramicrotome and were stained with uranyl acetate and lead citrate, and observed by a JEM-1200EX electron microscope (JEOL Ltd, Tokyo).

Results

Rolling: In the absence of chemoattractants, a few PMNs could be individually visualized as bright white cells against the dark background of the bloodstream, since they rolled slowly on the endothelial wall. Although the number of rolling PMNs was quite variable from preparation to preparation, the majority of the rolling PMNs moved in a jerky fashion, then came to rest suddenly for short, but variable periods of time (3–30 s), and finally returned to the bloodstream (Fig. 1). The behaviour of a PMN moving slowly along the endothelial surface and stopping at the same site for less than one minute was designated as ‘rolling’. Some PMNs rested at the same site for longer periods, but eventually they moved again (Fig. 2).

On the application of a chemoattractant (either LTB₄ or fMLP) the number of rolling PMNs was kept constantly low and was not increased (Fig. 2),
FIG. 2. Changes in the numbers of rolling PMNs after LTB₄ and fMLP application. The abscissa indicates the time lapse after the application of LTB₄ or fMLP (arrowhead, 0). The ordinate indicates the numbers of PMNs counted in a 110 μm length along a venule on the monitor screen, recorded for 50 min after application of LTB₄ or fMLP. Values indicate the means (± SE) obtained from the respective number of animals (n).

whereas the number of PMNs remaining for longer than one minute at the same site was markedly increased dose dependently (Fig. 1). The behaviour of a PMN remaining at the same site for one minute or longer, was designated as ‘adhesion’.

Adhesion: Figure 3 indicates the changes in the number of adhering PMNs in a 5 min period upon topical application of a chemoattractant. In absence of chemoattractants, the number of adhering PMNs was constantly low. Immediately after the application of LTB₄ or fMLP, adhesion was clearly induced. The number of adhering PMNs was increased dose dependently by the application of either LTB₄ or fMLP, and then the effect slowly waned. The cumulative numbers of adhering PMNs over a period of 50 min after the application of 0.15, 1.5, and 15 pmol/50 μl of LTB₄ were 7.8 ± 3.1 (n = 5), 21.0 ± 3.9 (n = 6), 31.8 ± 6.4 (n = 6) (mean ± SE), respectively and those after 1, 3 and 10 nmol/50 μl of fMLP were 13.3 ± 6.2 (n = 5), 25.7 ± 3.5 (n = 5) and 45.0 ± 6.0 (n = 5), respectively.

FIG. 3. Changes in the numbers of adhering PMNs after LTB₄ and fMLP. The abscissa indicates the time lapse after LTB₄ or fMLP application (arrowhead, 0). The ordinate indicates the numbers of PMNs newly adhering to a 110 μm length along a venule on the monitor screen, recorded for 50 min after LTB₄ or fMLP application. Values indicate the means (± SE) obtained from the respective numbers of animals (n).

Passage through the endothelial cell layer: Observation of PMNs on the videotapes revealed that those PMNs that were adhering to the venular wall had a large area of contact with it (Fig. 4B). This contact area was then seen to diminish as the cells became thinner and ‘taller’ (Fig. 4C), and then to decrease their ‘height’ gradually (Fig. 4D and E), until they finally disappeared from the vascular lumen. The
average time from the onset of adhesion to disappearance from the vascular lumen was about 10 min, irrespective of the dose of LTB₄ or fMLP (Table 1). The shortest time recorded was 3 min and the longest was 25 min.

Figure 5 indicates that 80–90% of the PMNs, which had adhered to the venular wall, disappeared into it irrespective of the dosage of LTB₄ (1.5 to 15 pmol/50 µl) or fMLP (1 to 10 nmol/50 µl), and never returned to the bloodstream.

Presence in venular wall: Subsequent to passage through the endothelial cell layer, PMNs remained for longer than 30 min in the venular wall between the endothelial cell layer and the basement membrane of the pericyte/endothelial cells, as shown in the electron micrograph in Fig. 6. Parts of the vascular wall were seen on the TV monitor screen to become thicker, possibly as a result of the PMNs present in the wall.

### Table 1. Time from adhesion of PMNs to disappearance from the vascular lumen after the application of LTB₄ and fMLP

| Treatment          | Numbers of animals used | Numbers of PMNs observed | Time to disappearance mean ± SE (min) |
|--------------------|-------------------------|---------------------------|--------------------------------------|
| LTB₄ (pmol/50 µl)  |                         |                           |                                      |
| 0.15               | 5                       | 10                        | 9.5 ± 1.9                            |
| 1.5                | 6                       | 30                        | 11.4 ± 1.1                           |
| 15                 | 6                       | 30                        | 10.0 ± 0.9                           |
| fMLP (nmol/50 µl)  |                         |                           |                                      |
| 1                  | 5                       | 10                        | 9.2 ± 2.1                            |
| 3                  | 5                       | 20                        | 10.2 ± 1.0                           |
| 10                 | 5                       | 25                        | 12.0 ± 1.3                           |

**Migration into extravascular spaces:** The number of PMNs in the extravascular space, observed up to 90 min after the application of LTB₄ or fMLP, increased with time (Fig. 7). When observed on the monitor screen during its migration into an extravascular space, a PMN would extend the process through an exit in the vascular wall into the extravascular space, and this was followed by amoeboid movement of the whole cell into the interstitial space.

The time taken for a PMN to enter the extravascular space completely ranged from 11 to 49 min, with a mean time of 25 to 34 min (Table 2).

Histological examination of the hamster cheek pouch revealed that the migrating cells were almost exclusively of the PMN type or neutrophils.

### Discussion

The photocell method of quantitative analysis of PMN behaviour, described in the previous paper, is useful for when PMNs are rolling and adhering to the inner wall of the venules. However, the method is of no use after penetration of the endothelial cell layers by the PMNs. In the present experiments, the number of PMNs was counted
Five steps in leukocyte extravasation

with the naked eye directly on the TV monitor screen during repeated replays of videotapes. This was not an elegant technique, but adequate for quantifying the behaviour of PMNs during extravasation.

In the absence of chemoattractants, only a few PMNs were visible rolling or remaining on the inner wall of the venules. The period of time for remaining at the inner wall was 3–30 s, and then the PMNs returned to the bloodstream. Topical application of LTB₄ or fMLP on the surface of the microvasculature did not change the number of rolling PMNs, but resulted in an increase in the number that remained adhering at the same site for at least 60 s (Fig. 1). Thus, the designation of rolling, in which PMNs were stationary for less than 1 min at one site, and that of adhesion, in which they remained at the same site for 1 min or longer, are reasonable. A small number of rolling PMNs were present in the venules before the application of chemoattractants, but this may be attributable to the manipulation of the cheek pouch during the extensive dissection during setting up the preparation and this manipulation may have caused the release of endogenous agents. The rolling behaviour of PMNs may be related to activation of the selectin family.¹⁹

The topical application of a chemoattractant

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**Table 2.** Time from disappearance of PMNs from the vascular lumen to appearance in the perivascular space after LTB₄ and fMLP application

| Treatment  | Numbers of animals used | Numbers of PMNs observed | Time to disappearance mean ± S.E. (min) |
|------------|-------------------------|--------------------------|----------------------------------------|
| LTB₄ (pmol/50 μl) |                         |                          |                                        |
| 0.15       | 5                       | 10                       | 32.4 ± 3.0                             |
| 1.5        | 6                       | 30                       | 25.3 ± 1.4                             |
| 15         | 6                       | 30                       | 31.6 ± 1.9                             |
| fMLP (nmol/50 μl) |                         |                          |                                        |
| 1          | 5                       | 10                       | 29.8 ± 2.7                             |
| 3          | 5                       | 20                       | 34.0 ± 2.0                             |
| 10         | 5                       | 25                       | 31.8 ± 1.9                             |

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FIG. 7. Cumulative numbers of PMNs appearing in the extravascular space after LTB₄ or fMLP application. The abscissa indicates the time lapse after the application of LTB₄ or fMLP. The ordinate indicates the total numbers of PMNs in the perivascular space at given times after the application of LTB₄ (15 pmol/50 μl) or fMLP (10 nmol/50 μl). Values indicate the means (±SE) from five to six animals.
(LTB₄ or fMLP) increased the cumulative numbers of adherent PMNs, in accordance with the concentration used (Fig. 3). This result confirms those of the previous paper.² As the adhering and migrating cells were almost exclusively of the PMN type or neutrophils, as shown by histological examination, it can be concluded that LTB₄ or fMLP induced extravasation of neutrophils.

After in vitro experiments, the potency of LTB₄ was reported to be equipotent to that of C₅a in chemotaxis,²⁰ to that of C₅a des-Arg or fMLP in chemokinesis and chemotaxis,²¹ and weaker than fMLP in release of the lysosomal enzymes from human PMN in the presence of cytochalasin B.²²⁻²⁵

In the present in vivo experiments, the concentration of fMLP for induction of PMN adhesion was 1000 times higher than that of LTB₄. This is in agreement with the previous report.²³ The reasons for the discrepancy between in vitro and in vivo experiments are not known, but hamster PMNs may be less sensitive to fMLP, and, as has already been reported,² the effect of fMLP on adhesion of PMNs to the inner wall of venules is indirect in the hamster, since it is completely inhibited by a 5-lipoxygenase inhibitor. Despite the difference in potency, LTB₄ and fMLP share similar effects on the process of the extravasation of PMNs in the microcirculation of the hamster cheek pouch.

Adhesion of PMNs was observed only in the venules. The increased adhesion of leukocytes is primarily due to elevated adhesive forces and not the result of decrease in dispersive forces, e.g., wall shear stress.²⁶ Induction of PMN adhesion to the venular endothelium was attributable to changes in the membrane of the PMNs and not to that of the endothelial cells, as was indicated in the previous paper,² in which selective application of a minute amount of LTB₄ with a glass capillary pipette close to the capillary wall induced adhesion in the venules downstream, whereas injection close to the venular wall did not cause the adhesion of PMNs to it, but induced adhesion in the larger venules further downstream. High-affinity receptors for fMLP are reported to be present on the surfaces of leukocytes²⁷ and of cultured endothelial cells.²⁸ Thus, as indicated by in vitro experiments, Mac-1 (CD11b/CD18) and/or LAF-1 (CD11a/CD18) may be adhesion molecules of neutrophils, which are activated by LTB₄ or fMLP and bound to the counter-receptor ICAM-1 on the surface of endothelial cells.¹² In our preliminary experiments in vivo, this adhesion in the hamster check pouch was blocked by an antibody against rat CD18 or ICAM-1 (data to be published).

The present quantitative study revealed that almost all (80-90%) of the PMNs that had adhered to the venular walls, disappeared from the vascular lumen through the endothelial cell layer and did not return to the bloodstream. Thus, the adhesion of PMNs to the endothelial cells is the pivotal step in the process of PMN extravasation.

The disappearance of a PMN from the vascular lumen into the venular wall took about 10 min. This movement of PMNs must require detachment or inactivation of Mac-1 and LFA-1 with crosstalk to other adhesion molecules. The disappearance of PMNs from the vascular lumen probably takes place by passage through the endothelial cell junctions, as indicated by electron micrographs.⁶⁻¹⁰

PMNs that had passed through the endothelial cell layer did not immediately appear in the perivascular space, but remained in the spaces between the endothelial cells and the basement membrane of the pericytes/endothelial cells. This observation confirmed the findings of previous reports.⁵,¹⁰,²⁹ An important finding of the present study was that nearly 30 min elapsed before the appearance of PMNs in the extravascular space from disappearance from the vascular lumen (Table 2). PMNs may split the basement membrane with collagenase/gelatinase, since the last step was inhibited by a selective inhibitor of metalloproteinases (data published separately). Thus, 30 min may be the time required for PMNs to prepare metalloproteinases for action. Furthermore, this last step of the passage of PMNs through the basement membrane was inhibited by pretreatment of the animals with dexamethasone.⁵⁰,⁶¹

The results presented above indicate that PMN extravasation by chemoattractants is not a simple phenomenon, but a serial process in which a variety of adhesion molecules on the surface of PMNs, endothelial cells and other components of the venular wall may be involved. The process can be divided into five steps: (1) rolling on the endothelium, (2) adhesion to the endothelium, (3) passage through the endothelial layer, (4) presence between the endothelial cells and the basement membrane within the venular wall, and (5) passage through the basement membrane into the extravascular space. Quantitative analysis of the extravasation of PMNs is necessary to clarify precisely the underlying mechanism, particularly at the molecular level. Individual steps may be targeted for development of new drugs, which inhibit PMN migration.

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ACKNOWLEDGEMENTS. We wish to thank Mrs C. Shima, Mrs M. Takagi and Miss M. Takahara for their technical assistance. We also are obliged to Mr O. Katsumata for his skilful technical assistance in electron microscopy. This study was partly supported by a Grant-in-aid from the Ministry of Education, Science and Culture (♯01770137) and by the Uehara Memorial Foundation.

Received 14 September 1992; accepted 24 September 1992