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Transendothelial cell diapedesis of neutrophils in inflamed human skin

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Summary. The mode of extravasation of neutrophils (PMNs) in cutaneous inflammation was studied in sequential biopsy specimens taken from human skin. Inflammatory skin reactions were produced by intracutaneous injection of endogeneous mediators of inflammation – $C5a_{des\ arg}$, LTB_4, neutrophil-activating peptide (NAP) and interleukin-1 (IL-1). Within 30 min after injection neutrophils were observed in close contact with endothelial cells of postcapillary venules and, following cytoplasmic engulfment, the cells were found to be transported transcellulary through the endothelial layer. In a total of 20 biopsy specimens taken at various times, cell migration via interendothelial gaps was absent. Instead, the transcellular pathway appeared to be the first and foremost mode of diapedesis. During this migratory process PMNs lacked signs of degranulation and numerous electron-lucent vesicles and secondary lysosomes were found. In addition, coated pits present on leukocyte as well as endothelial-cell membranes were indicative of receptor-mediated endocytotic processes.

Key words: Cell diapedesis – Polymorphonuclear leukocytes – Cutaneous inflammation – Interleukin – Endocytotic processes

Migration of white blood cells across the walls of postcapillary venules represents one of the early events in inflammation. During this process circulating leukocytes become adherent to endothelial cells and subsequently migrate into the surrounding extravascular tissue.

A number of studies have addressed the question by which route neutrophils traverse the endothelium [8, 13, 17, 18, 31]. In 1961 Marchesi presented a detailed description of neutrophils actively migrating through endothelial intercellular junctions [17]. Since then it became widely accepted that during the process of polymorphonuclear leukocyte (PMN) diapedesis endothelial cells (ECs) become separated by wide gaps, thereby allowing neutrophils to pass through [7, 12, 14, 16, 20, 28]. This view was extended by recent in vitro studies showing that monolayered ECs after activation by tumor necrosis factor-α (TNF-α) are able to contract, whereby cocultured PMNs gain the ability to cross the monolayers [19]. Interestingly, in this investigation neutrophils at the site of endothelial cell retraction were found to preferentially attach to EC margins and subsequently seemed to squeeze through rapidly forming intercellular gaps.

By studying sequential biopsy specimens from experimentally induced inflammatory reactions in human skin we now observed a novel mode of PMN diapedesis which is different from previously described forms. In the following a transcellular mode of PMNs traversing the endothelial cell lining will be described. During this transcellular passage endothelial cells are observed to be engulfing neutrophils and subsequently releasing them at the abluminal site.

Materials and methods

Materials

$C5a_{des\ arg}$ was isolated from yeast-activated human serum according to a modification of the method established by Fernandez and Hugli [9] as described by Schröder and Christophers [22]. Monocyte-derived neutrophil-activating peptide (NAP) was prepared from supernatants of bacterial lipopolysaccharide stimulated peripheral blood monocytes according to the purification protocol recently described by Schröder et al. [23]. Interleukin-1α (IL-1α) was kindly provided by Hoffmann-La Roche, Basle, Switzerland. Leukotriene B_4 (LTB_4) was purchased from Paesel, Frankfurt/M., FRG. All reagents were dissolved in sterile pyrogen-free physiologic saline at the following concentrations: $C5a_{des\ arg}$, 1 μg/ml; IL-1α, 200 U/ml; LTB_4, 10 ng/ml; NAP, 1 μg/ml.

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Patients

Ten patients hospitalized for the treatment of facial skin tumors (basal cell carcinoma, squamous cell carcinoma) or varicose legs agreed to participate in this study, giving their written consent after being informed.

All investigations were performed on the flexor aspects of the forearms. The skin sites were not affected by any disease and none of the subjects had taken systemic drugs within at least 2 days preceding the study.

In order to produce inflammatory tissue reactions the following endogenous mediators of inflammation were used: the des arg form of complement split product C5a ($C5a_{des\ arg}$), NAP, IL-1α and LTB_4. In every case 100 µl of the mediator solution was injected intracutaneously into the skin of the forearm. Additionally, as a control 100 µl of sterile pyrogen-free physiological saline was injected intracutaneously into another healthy skin site of each volunteer.

Punch biopsy material was obtained 30 and 60 min after injection of 2% Scandicain without epinephrine as local anesthesia.

Electron microscopy

For transmission electron microscopy all biopsy specimens were immersed in cold (4° C) 5% phosphate buffered glutaraldehyde (pH 7.8) for 2 h, repeatedly rinsed in cold phosphate buffer, and postfixed in 4% phosphate buffered osmic acid for 2 h. After fixation these specimens were dehydrated in graded steps of acetone and then embedded into Araldit (Araldit Cy 212, Sigma). Specimens were sectioned and subsequently contrasted by use of saturated uranyl acetate solution (methanol) and lead

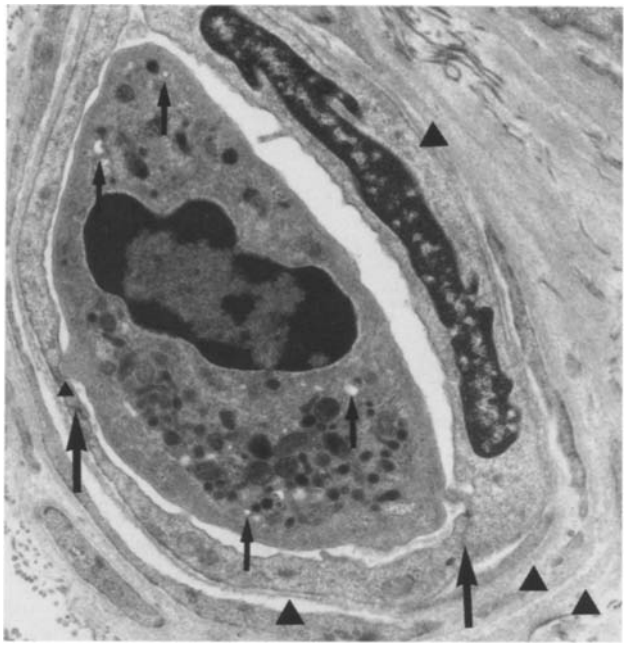

Fig. 1. Early phase of PMN extravasation (Figs. 1 and 2). PMNs in close contact to ECs of a postcapillary venule. Note a tiny cytoplasmic spike (*small arrowhead*) of the PMNs indenting the ECs and the intact intercellular junctions (*large arrows*) next to it, as well as the multilayered basement membrane (*large arrowheads*) of the postcapillary venule; (*small arrows*), endosomes. ×15,600

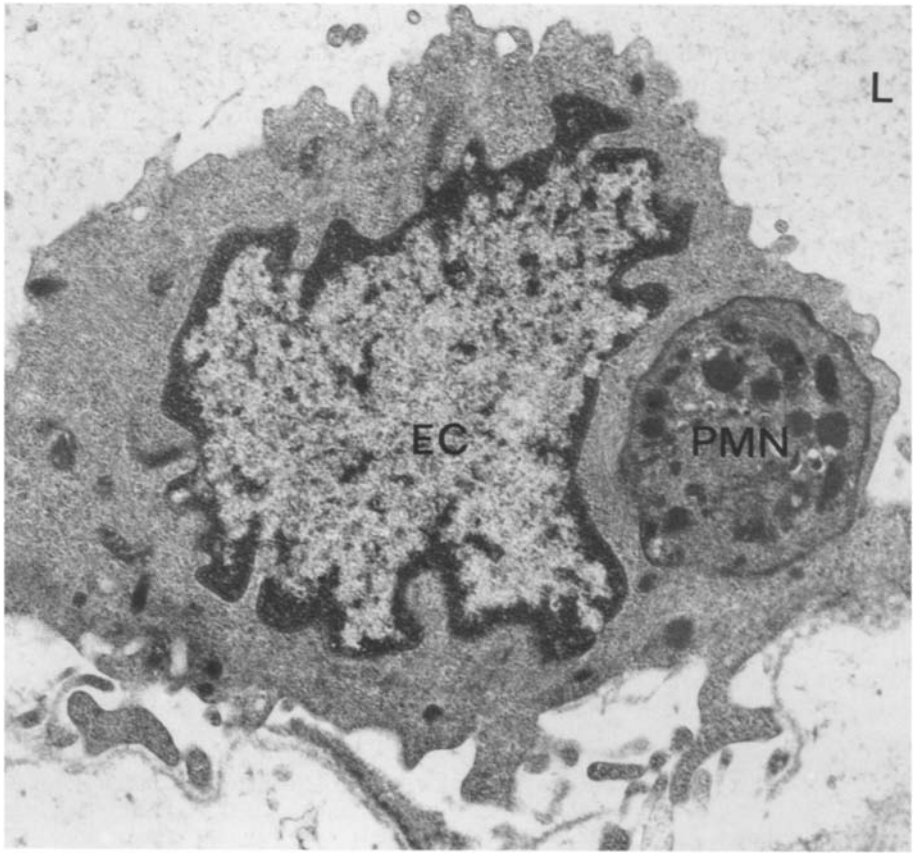

Fig. 2. Single endothelial cell (*EC*) totally engulfing portions of a *PMN*; *L*, vascular lumen. ×22,500

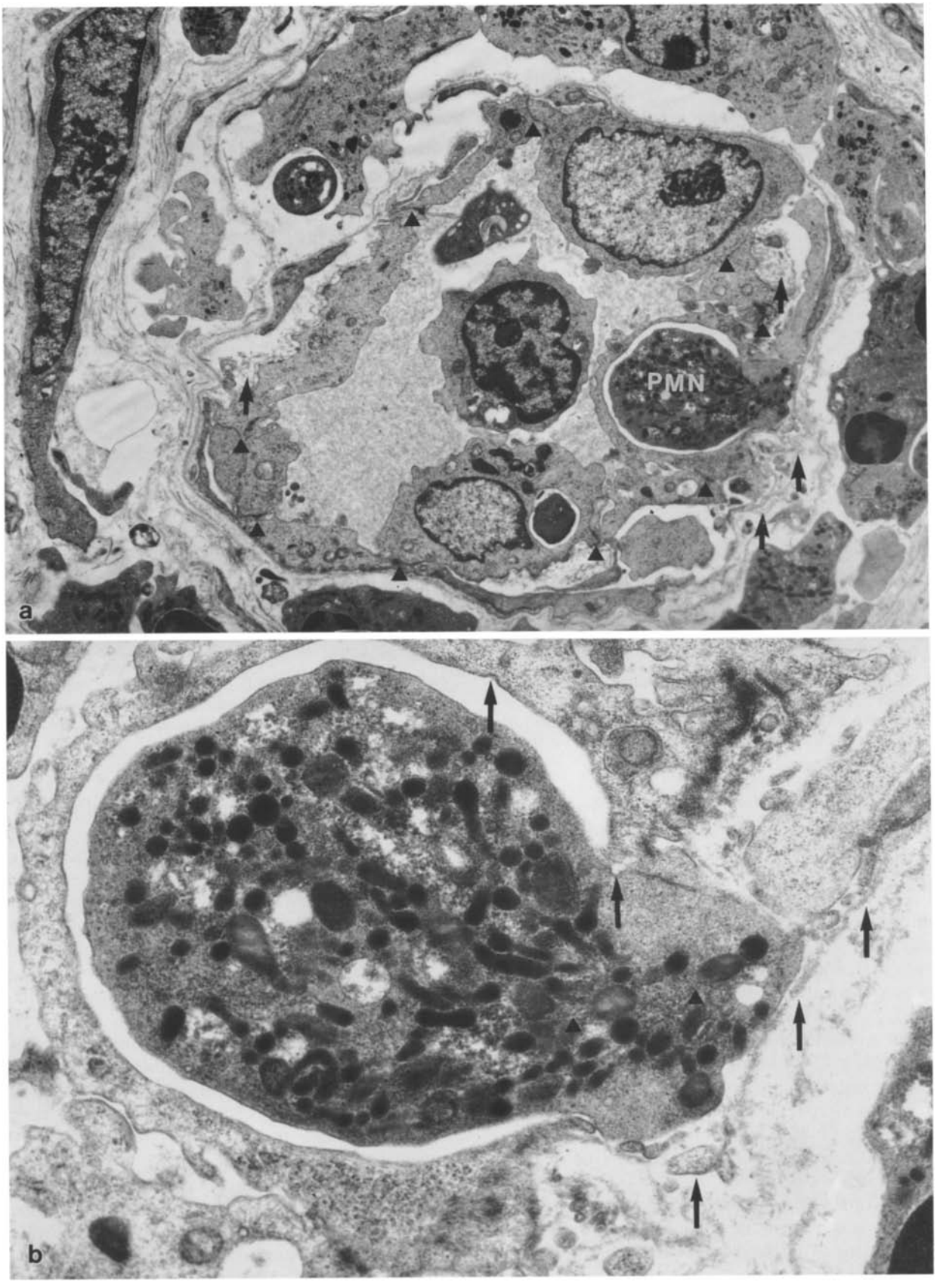

Fig. 3. Late phase of PMN extravasation. **a** PMN leaving an EC vacuole at the abluminal side of the endothelial layer. All of the intercellular junctions (*arrowheads*) between the ECs appear intact. Note the multilayered basement membrane of the postcapillary venule (*arrows*); × 7,100. **b** Same section at a higher magnification (× 27,700). Only the basal lamina (*large arrows*) is separating the PMN from the perivascular tissue. Coated pits (*small arrows*) are present on EC and PMN membranes. PMN granules show a special arrangement and are seen in close association to microtubules (*arrowheads*)

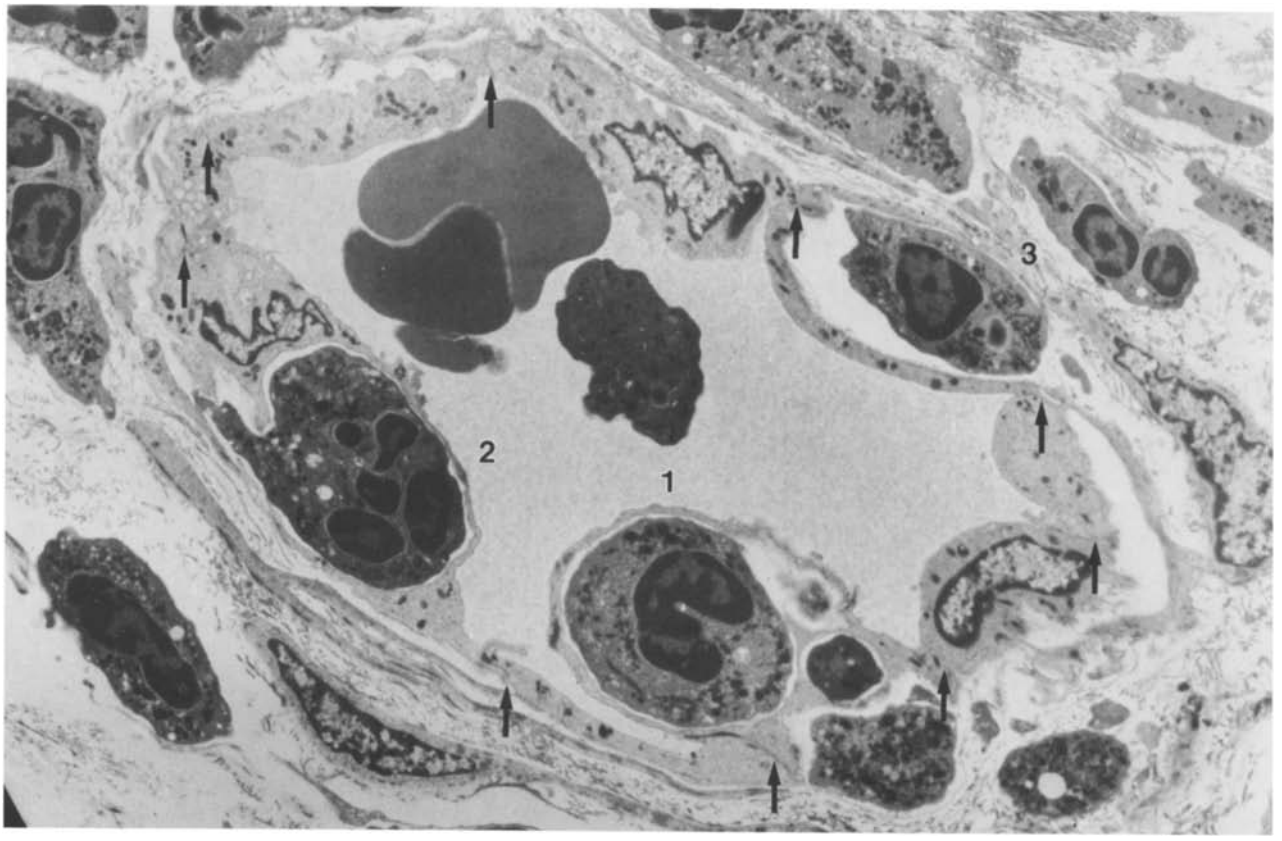

Fig. 4. Section through a cutaneous postcapillary venule showing different stages of transcellular extravasation of PMN: fully engulfed within an EC vacuole (*1*) or outside the vacuole (*2*). Another PMN (*3*) has almost reached the perivascular space outside the basal lamina while major parts of the cell are still surrounded by thin processes. In this cross-section all intercellular junctions (*arrows*) between the ECs appear intact. ×6,400

acetate solution (4% in distilled water, pH 12). Tissue sections were examined with a TEM 201C electron microscope (Philips).

Results

Volunteers injected intradermally with $C5a_{des\ arg}$ developed immediate wheal and flare reactions which were associated with slight pruritus. A maximal reaction was observed about 10 min after the injection of $C5a_{des\ arg}$. Injection of LTB_4 led to slightly raised nonpruritic erythematous indurations which developed within 15 to 30 min. NAP and IL-1α led to faint flares only which developed immediately after injection and disappeared within the first 15 min. The morphological findings which will be described in the following occurred independently of the kind of mediator injected.

Microscopically moderate to heavy leukocyte extravasation was present in all biopsy material. The individual features of this process varied with time and extent; however, they showed a remarkable uniformity with regard to the migratory route and the cellular characteristics of neutrophils and endothelial cells. Therefore, these changes appeared to be uniform features and will be described together.

In 30-min specimens PMNs were seen located in close proximity to ECs of postcapillary venules, which are characterized by a multilayered basement membrane (Figs. 1 and 3a), and parts of the plasma membranes of both cells, ECs and PMNs, were flattened and aligned in parallel (Fig. 1). A constant space of approximately 20 nm was present between the streightened contours of both cell types. In contrast to this, neighboring ECs of postcapillary venules not having contact to PMNs showed different features. These endothelial cells in the absence of luminal PMNs showed irregularly shaped protrusions at their luminal sides and numerous electron-lucent subplasmalemmal vesicles.

The cytoplasmic contents of adhering PMNs (primary and secondary granules) appeared not to be altered and apart from these granules there were numerous electron-lucent vesicles and secondary lysosomes located within the PMNs cytoplasm (Fig. 1). In addition, coated pits could occasionally be detected randomly distributed in the plasma mem-

brane of PMNs and, as demonstrated on Fig. 3b, on the endothelial cell membrane.

In several sections obtained 30 min after stimulus injection, PMNs were noted to form tiny cytoplasmic protrusions indenting into the luminal endothelial plasma membrane (Fig. 1). Occasionally a cytoplasmic bulb could be observed forming a fold within the otherwise smooth EC membrane. Specimens taken 60 min after injection revealed that considerable portions of the adhering PMNs were surrounded by endothelial plasma membranes, thus giving the appearance of PMNs lying in large vacuoles formed by the EC plasma membranes (Fig. 2). The plasma membranes of the engulfing ECs as well as the lock-in neutrophil always appeared to be intact, clearly separating the cytoplasm of both cell types from each other.

Further biopsy material taken at this point in time showed PMNs intracellularly located at the abluminal site of the endothelium. Sometimes it was only the basal lamina which separated these PMNs from the perivascular tissue (Figs. 3 and 4).

During the process of PMNs extravasation cellular organelles, e.g., the primary and secondary granules, increasingly showed a special arrangement, and also parts of the cytoskeleton, i.e., microtubules became visible (Fig. 3b). In neither case could ultrastructural signs of cell damage be observed in PMNs or ECs. Moreover, discontinuities of the endothelial cell membrane were never seen, and, more importantly, the junctions between adjacent endothelial cells always appeared unseparated and intact (Figs. 3 and 4).

Discussion

The observations reported here show that in human skin after intracutaneous injection of defined proinflammatory mediators, circulating neutrophils come in contact with the endothelium of postcapillary venules and subsequently migrate, via a transcellular route, into the extravascular surroundings. The principal cells observed in this process, PMNs and ECs, both show features of cellular activation including the formation of membrane protrusions and coated pits, as well as electron-lucent vesicles of varying sizes.

Whereas these morphological findings demonstrate the proinflammatory potential of the mediators used, the clinical symptoms of the inflammatory response were moderate and rapidly fading. In addition, there was an absence of cell damage in both ECs and PMNs, and extravasation was limited only to a few cells per cross-sectioned vessel. Therefore, the experimental conditions chosen reflect a moderate distinctive inflammatory episode of short duration.

It is interesting to note that whenever smooth surfaced circulating PMNs came into contact with luminal endothelial cell sites, a great number of electron-lucent granules (endosomes) as well as secondary lysosomes became apparent in the cytoplasm of PMNs. These two organelles are known to be involved in endocytotic processes following exposure to soluble ligands [21, 24, 25]. In addition, the occurrence of coated pits on the surface of PMNs indicates endocytotic uptake of soluble stimuli via receptor-mediated endocytosis [1, 11]. Whether these changes which are indicative for metabolic activation are directly or, via the generation of additional mediators, indirectly elicited in PMNs cannot be answered at present.

The fact that no such signs were seen in circulating PMNs gives rise to the suggestion that following close physical contact between ECs and PMNs metabolic changes are induced within the contacting neutrophils.

Recent in vitro studies revealed that chemotactic peptides (e.g., FMLP, C5a) and lipid mediators (PAF, LTB_4) are able to enhance adherence of human neutrophils to cultured human endothelial cells [29]. Furthermore, this enhancement was found to depend on the expression of a family of adhesion molecules expressed on the PMN cell surface [34, 35]. On the other hand, detailed work on the effect of various cytokines revealed that cytokines (e.g., IL-1, tumor necrosis factor) may stimulate the adhesion of circulating PMNs, and this effect is exerted by acting on the endothelial layer [2–4, 19].

Recent work from various laboratories has shown that intracutaneous injection of endogenous proinflammatory mediators (e.g., C5a [27, 32], LTB_4 [5, 6, 30]), and PAF [15] causes neutrophils to accumulate around small blood vessels. In these studies, no detailed descriptions were given concerning the route by which neutrophils migrate across the endothelium. Therefore, it was surprising to observe PMNs traversing the endothelial barrier of postcapillary venules in a transcellular manner. This process appears to be remarkable in that at an early stage, PMNs in contact with the endothelial cell membrane become surrounded by endothelial cell protrusions followed by total engulfment of PMNs. Interestingly, these events are often found to take place in close vicinity to the zone of interendothelial cell junctions, and, even more importantly, these junctions between adjacent ECs remained intact during all these steps of PMN extravasation (Fig. 4).

Although this phenomenon appeared to be consistently present in all of our specimens, it could be influenced by the fact that identification of intercellular junctions is strictly based upon the presence of intact, i.e., closed junctions. As pointed out by Hammersen and Hammersen [13] structural criteria for the existence of open junctions would be the pres-

ence of halved adhesive devices along the plasma membranes. As postcapillary venules are showing only poorly developed intercellular junctions, these structures may hardly be recognizable by electron microscopy [13]. In consequence, an interendothelial mode of cell migration cannot entirely be excluded. However, our present findings indicate that under the conditions chosen the transcellular route indeed appears to be the predominant mode of neutrophil diapedesis.

Up to now the ability of neutrophils to use a transcellular route of extravasation has rarely been considered. In fact in vitro observations by a number of authors unanimously point towards an intercellular route of neutrophil migration [2, 10, 19, 33]. In these studies activated PMNs were layered over cultured endothelial cell monolayers and neutrophils were consistently observed crossing the endothelial layer at the site of intercellular junctions. In contrast to the in vitro pattern, few reports indicate that in vivo, under certain conditions, PMNs are able to pass transcellularly through the endothelium [8, 13, 18, 31].

In studying the effects of arachidonic acid derivatives after topical and intravenous application in laboratory animals, Hammersen and Hammersen [13] observed PMNs migrating transcellularly through the endothelium of cutaneous postcapillary venules. These authors concluded that transcellular migration should be considered as a possible route of leukocyte diapedesis. Faustmann and Dermietzel [8] applied α-bungarotoxin topically to medullae oblongatae of adult cats and found that transcellular migration of PMNs across the endothelial barrier takes place in these animals. Furthermore, in a study conducted by Welsch and Caesar [31], it was shown that topical application of croton oil to the tongue of frogs resulted in massive emigration of PMNs and that most of these cells used the transcellular route.

These results together with our observations made in human skin suggest that in the process of PMN extravasation endothelium no longer should be regarded as a passive barrier [14]. Instead the active role endothelial cells play in cell extravasation suggests that the endothelial layer may even participate in selecting which cell will be permitted to egress. Regulation of such cell specificity was suggested by Tavassoli to take place in the bone marrow where, after stimulation, leukocytes are noted to traverse the bone marrow blood barrier transcellularly [26].

In conclusion, our investigations show that in human skin during a defined inflammatory episode, adherent PMNs are able to become transcellularly transported through the endothelial barrier of postcapillary venules. This happens irrespective of the type of mediator used. Therefore, the transcellular route may be considered as a common pathway of leukocyte extravasation during an inflammatory response in man.

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