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Journal of Dermatological Science 24 (2000) 158–165

Journal of Dermatological Science

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# Leukocytoclasis: ultrastructural in situ nick end labeling study in anaphylactoid purpura

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Received 6 December 1999; received in revised form 2 February 2000; accepted 3 February 2000

**Abstract**

In order to characterize leukocytoclasis of polymorphonuclear neutrophils (PMNs), the method of in situ nick end labeling for DNA breakdown was applied on tissue samples from 36 patients with anaphylactoid purpura at ultrastructural, as well as light microscopic, level. Light microscopic immuno-peroxidase technique showed positively labeled PMNs infiltrating in the dermis of 24 cases in which leukocytoclastic vasculitis was fully developed, suggesting that breakdown of DNA strands is triggered in the PMNs. Electron microscopic immuno-gold technique employed in six patients with the fully developed stage of inflammation identified the DNA breaks in the nuclei of PMNs. Ultrastructure of these cells, however, showed that only a minor population ( $\sim 1/60$) of PMNs showed the condensed and marginated nuclei, being compatible with typical apoptotic change. However, the majority of immuno-gold-labeled cells showed relatively intact nuclei without margination of condensed heterochromatin and with disintegrated cytoplasmic organelles and plasma membrane, suggesting that apoptotic cell removal mechanism may be incomplete. The immuno-gold-positive nuclear debris scattering in the tissue is most likely the remnants of unsatisfactory disposal by apoptosis of potentially injurious PMNs, resulting in the vascular and perivascular damage in leukocytoclastic vasculitis in anaphylactoid purpura. 

*Keywords:* Leukocytoclasis; Nuclear debris; Anaphylactoid purpura; Apoptosis; In situ nick end labeling; Polymorphonuclear neutrophils

## 1. Introduction

Apoptosis, which is distinguished from necrosis, is a cell death mechanism involved in normal embryonic development and homeostatic maintenance of normal tissues in highly organized multicellular body [1]. In the skin, apoptotic cells have been shown in lichen planus [2], lupus erythematosus [3] and drug eruption, suggesting a role in removal of damaged cells. In hair follicles, apoptotic cell death is densely shown when the

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0923-1811/00/$ - see front matter 
PII: S0923-1811(00)00092-X

hair cycle changes from anagen to catagen in outer root sheath [4], suggesting a role of elimination of distinct portion of the catagen hair. We previously observed burned tissue in experimental animals and the hair follicle cells undergoing apoptosis were quickly (within 24 h) diminished [5]. In inflammatory tissues apoptotic cell death of leukocytes has been considered as a quick removal of toxic PMNs by phagocytosis of macrophages in the kidney [6].

Leukocytoclastic vasculitis of the skin is a histological hallmark of cutaneous allergic vasculitis such as anaphylactoid purpura. This is characterized by infiltration of polymorphonuclear neutrophils (PMNs), disintegration of PMNs, swelling of endothelial cells and fibrinoid necrosis in dermal small vessels [7]. Deposition of IgA immune-complexes [8,9] and the following activation of complement (C) cascade are suggested to mediate the chemotaxis of the PMNs which contain various pro-inflammatory substances, such as lysosomal enzymes [10,11] and reactive oxygen species [12,13]. The fragmented nuclear debris is thought to be the histopathological evidence of destruction of infiltrated PMNs, and recognized as a diagnostic marker of leukocytoclastic vasculitis [14]. Banno et al. [15] and Barduagni et al. [16] applied an in situ nick end labeling technique [17] and found cleavage of DNA on the tissue sections, suggesting that apoptotic cell death is involved in vasculitis of the skin.

Electron microscopic studies showed that the cells undergoing apoptotic process are characterized by nuclear condensation, chromatin margination and cleavage of nuclei [18], while the cytoplasmic organelles such as mitochondria and lysosomes are relatively preserved. A recent study of leukocytoclastic vasculitis by Shi et al. [19] compared in situ nick end labeled PMNs with ultrastructural alterations. They observed that PMNs infiltrating into the lesions showed characteristic ultrastructural change of apoptosis. A question arises: the observation that a number of PMNs with various degrees of disintegration remain in the dermal tissue in leukocytoclastic vasculitis might be against the concept of quick disposal mechanism of apoptosis to clear potentially harmful PMNs. The present study was, therefore, undertaken to further investigate ultrastructural change of PMNs undergoing leukocytoclasis in anaphylactoid purpura by using in situ nick end labeling technique at both light and electron microscopic levels.

## 2. Subjects and methods

### *2.1. Patients and tissue samples*

Paraffin-embedded tissue specimens biopsied from the legs of 36 (male 16, female 20) patients between 4 and 72 (mean 37.3) years old with anaphylactoid purpura treated in Saitama Medical Center Hospital from 1988 to 1998 were collected. The diagnoses were re-confirmed on their clinical and histologic evidences. Those cases possibly induced by medicinal drugs were excluded. In 35 out of a total of 36 cases direct immunofluorescence microscopic method was carried out on fresh-frozen sections [8] to observe tissue deposition of IgA, IgG, IgM, $C_3$ and fibrin.

### *2.2. Light microscopic in situ nick end labeling technique*

The paraffin blocks were serially cut and deparaffinized. A section of each block was stained with hematoxylin and eosin (H&E) and remaining sections were treated in 0.05% bacterial protease (type XXIV, Sigma, St Louis, MO) for 20 min. After washing in 0.01 M phosphate buffered saline (PBS), pH 7.4, they were immersed in 3% $H_2O_2$ in PBS for suppression of intrinsic peroxidase activity. In situ nick end labeling was performed by means of 'ApopTag Plus In Situ Apoptosis Detection Kit-Peroxidase' (Oncor, Gaithersburg, MD) [20,21]. Briefly, sections were incubated in equilibration buffer, followed by working solution of terminal deoxynucleotidyl transferase (TdT) to catalyze binding of digoxigenin-nucleotide [22] to 3′-OH ends of double- or single-stranded DNA at 37°C for 30 min. The sections were washed and reacted with 55 μl of anti-digoxigenin-peroxidase solution. The activity of bound-peroxidase was visualized in 3′,3′-diaminobenzidine as substrate [23]. Counterstain

was done with methyl green. Negative control of the reaction was done by substituting for TdT enzyme solution with distilled water in the reaction step, and positive control for the reaction was performed with the young adult rat mammary glands obtained at the 4th day after weaning, as in the technical instruction of 'ApopTag Plus'.

### 2.3. Electron microscopic in situ nick end labeling technique

For electron microscopic study freshly obtained skin samples with anaphylactoid purpura were immediately fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 4°C. Post-fixation was performed with 1% osmium tetroxide for 1 h at room temperature. Plastic embedded blocks in Epon 812 (TAAB Lab Equipment, Berkshire, UK) were prepared. We selected six plastic embedded blocks that showed acute infiltration of PMNs on the H&E preparations and positive labeling on the light microscopic immunoperoxidase investigation. Ultrathin sections were cut at 80-nm thickness from the selected areas on semi-thin preparations and placed on the collodion coated nickel grids [24]. The ultrathin sections were processed to the etching step with drops of saturated sodium metaperiodate for 2 min over a sheet of plastic film. After washing the sections were reacted with TdT enzyme working solution at 37°C for 60 min in a moist chamber. Reaction was stopped, sections were washed and incubated in 0.1% bovine serum albumin in 0.02 M Tris buffer, pH 7.4, for 5 min, three times, finally with 10% normal sheep serum. Sheep anti-digoxigenin antibody conjugated with colloidal gold (10 nm in diameter, British Bio Cell, Cardiff, UK), diluted to 1/20 with normal sheep serum, was used to label fragmented DNA. After three washes with drops of distilled water, the sections were stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (JEM-1210) at 100 kV.

Table 1
Demographic data on the patients with anaphylactoid purpura (AP)

| | | | |
|---|---|---|---|
| No. of patients | 36 (male 15, female 21) | | |
| Age | 4–77 (mean 38.6) years old | | |
| Duration of AP | 1–120 (mean 18.7) days | | |
| *Location of purpura* | | | |
| Lower limbs | 17 | | |
| Upper and lower limbs | 7 | | |
| Trunk and lower limbs | 5 | | |
| Trunk and all extremities | 7 | | |
| *Complication* | | | |
| Arthralgia | 15 | | |
| Abdominal symptoms | 10 | | |
| Renal involvement | 13 | | |
| *Histopathological findings (biopsy, H&E)* | | | |
| | ++ | + | +/−[a] |
| Neutrophil infiltration | 14 | 19 | 3 |
| Erythrocytes extravasation | 12 | 18 | 6 |
| Lymphocytes infiltration | 1 | 3 | 32 |
| Nuclear debris | 16 | 10 | 10 |
| Fibrinoid degeneration | 14 | 17 | 5 |
| *Direct immunofluorescence study, deposition of:* | | | |
| IgA | 5 | | |
| $C_3$ | 2 | | |
| IgA + $C_3$ | 8 | | |
| Negative | 20 | | |

[a] ++, abundant; +, moderate; +/−, weak.

## 3. Results

### 3.1. Profiles of the patients

The profiles of the investigated patients with anaphylactoid purpura are summarized in Table 1. Histopathologically, 24 out of 36 cases showed abundant or moderate degree of infiltration of PMNs, extravasation of erythrocytes, fibrinoid degeneration of small vessel walls, and fragmented nuclei of PMNs. We judged these cases as fully-developed stage of typical leukocytoclastic vasculitis. In five cases moderate infiltration of PMNs was observed in the perivascular area with mild or little fibrinoid degeneration, extravasation of erythrocytes, and scanty nuclear dusts. They were judged as early stage of leukocytoclastic vasculitis. In the other seven cases PMNs were only weakly infiltrated, or more lymphohistiocytic cells were observed than PMNs. In these cases the nuclear dust was found in relatively small num-

Table 2
Summary of in situ nick end labeling

| Degree of immuno-labeling | ++ | + | +/−[a] |
|---|---|---|---|
| Light microscopic, (%)[b] | 13 (36) | 11 (31) | 12 (33) |
| Electron microscopic, (%)[c] | 4 (67) | 2 (33) | 0 |

[a] ++, numerous (>60%) of PMN are positively labeled; +, moderate number (>20%) of PMN are labeled; +/−, small number of cells are labeled.
[b] $n = 36$.
[c] $n = 6$.

bers. We judged them as late stage. Direct immunofluorescence technique showed deposition of IgA and/or $C_3$ in 15 (12 from early and fully-developed phase) out of 35 cases examined.

### 3.2. Light microscopic observation

In situ nick end labeling study using light microscopy showed in the normal skin mildly positive cells on anagen cells of hair bulbs and granular cells (not shown) in the epidermis, as reported by Tamada et al. [25]. More intense immuno-peroxidase reaction was clearly demonstrated in the dermal infiltrates in the 24 fully-developed cases (Table 2). In the five cases with the early phase of inflammation immuno-labeling was sparse and weak, while in the remaining seven cases with chronic stage of inflammation the immuno-labeling was relatively weak. In the early to fully-developed stage the immuno-peroxidase labeled cells were distributed mainly in the perivascular area (Fig. 1a) of papillary and upper dermis where the leukocytoclastic vasculitis was identified on the adjacent H&E stained sections. The peroxidase activity-label was co-localized on the nucleus area of PMNs in the inflamed dermal tissue (Fig. 1b). It was of note in the fully-developed stage of leukocytoclastic vasculitis that a number of PMNs remaining in the tissue with various degrees of degradation were positively peroxidase-labeled (Fig. 1c). Lymphocytic cells on the sections from the late stage of inflammation did not show the positive immuno-staining. Endothelial cells and fibroblasts were negative for the present in situ nick end labeling.

We observed in the fully-developed stage that nuclear debris scattering around the vessels similarly showed positive immuno-peroxidase reaction (Fig. 1c), suggesting that even though apoptotic process may be initiated in the PMNs the damaged cells were not clearly removed from the tissue. This led us to further investigate ultrastructural change in positively labeled cells.

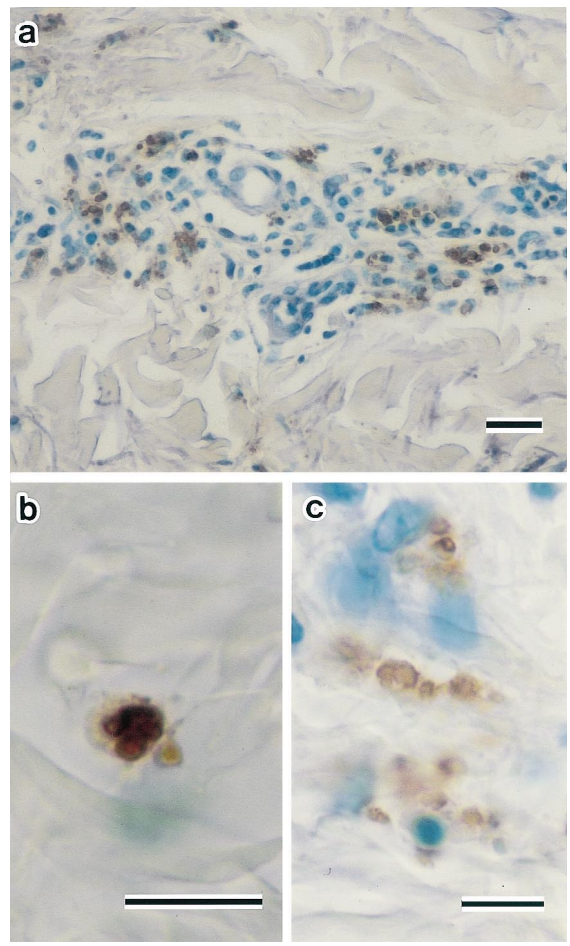


Fig. 1. Light microscopic in situ nick end labeling in anaphylactoid purpura. (a) Significant immuno-peroxidase positive reaction (dark brown) observed with PMNs infiltrating around the vessels of upper dermis in the lesion of anaphylactoid purpura. Counter nuclear stain was done with methyl green. Bar: 40 μm. (b) High power magnification showing positively labeled nuclei of PMNs. Bar: 20 μm. (c) Positively labeled PMNs undergoing various degree of degradation. Bar: 20 μm.

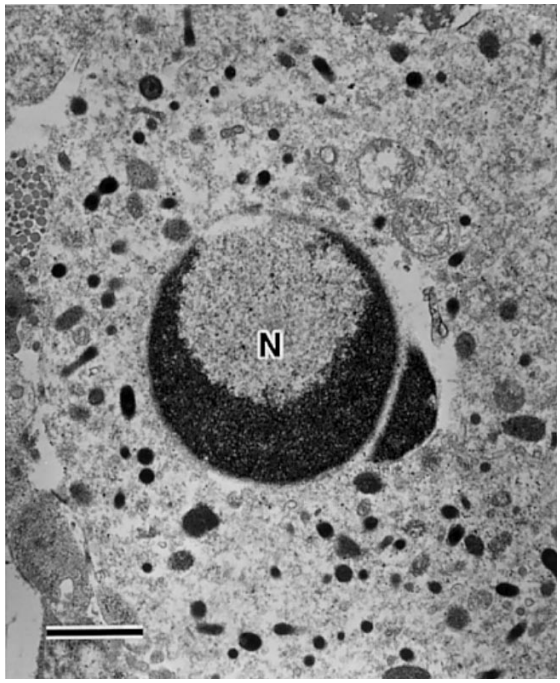


Fig. 2. Electron micrograph of an apoptotic PMN. Heterochromatin margination is clearly observed in the nucleus (N). The cytoplasmic organelles and plasma membrane show only limited disintegration. Bar: 1 μm.

### 3.3. Electron microscopic observation

We found by conventional transmission electron microscopic observation that a PMN showed margination of heterochromatin simulating crescent shape with a low degree of lytic change in the cytoplasm (Fig. 2) suggesting that apoptotic cell death process was taking place in the dermal tissue, being compatible with the findings by Shi et al. [19]. However, the cells undergoing apoptotic change were observed only in a minor population, approximately one cell out of 60 PMNs. Electron microscopic in situ nick end labeling technique employed on such ultrathin sections demonstrated gold particles precipitating on the nucleus of majority of PMNs adjacent to the small vessels. The ultrastructure of the immuno-gold-labeled PMNs showed partial condensation of heterochromatin in the nucleus (Fig. 3), as well as vacuolization of primary granules, decreasing number of secondary granules, diminishment of cytoplasmic glycogen particles and partial discontinuation of the plasma membrane. PMNs with more disintegrated ultrastructure were admixed (Fig. 4a) with more densely positive immuno-gold precipitation on the nuclear area (Fig. 4b). These cells showed extensive vacuolization in the cytoplasm and interruption of the plasma membrane. The cell was round and swollen in shape, without perceivable shrinkage. The in situ nick end labeled nuclei were still relatively preserved in their morphology but only with partial fragmentation. Budding of bleb out of nucleus was unidentified in these cells. The findings in most PMNs are incompatible with typical ultrastructural change of apoptotic cell death.

We also found irregularly shaped electron dense deposits with immuno-gold precipitates (Fig. 5) in the dermal connective tissue. The electron density of these deposits was identical to that of heterochromatin, suggesting that they are the nuclear

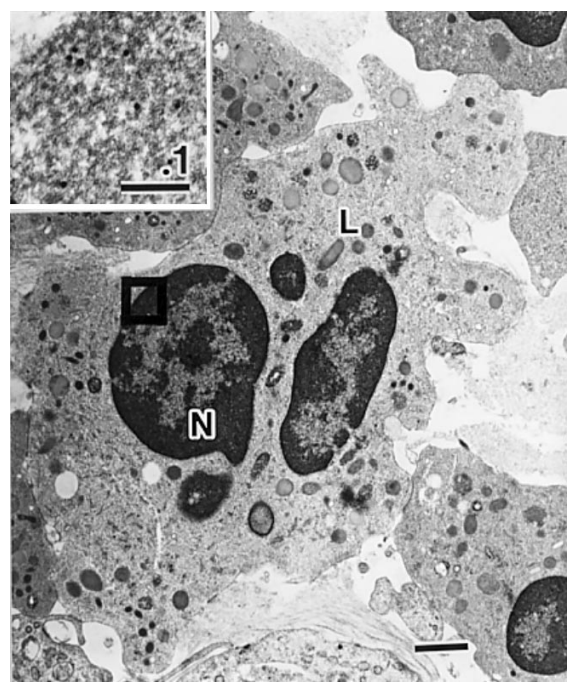


Fig. 3. Electron microscopic in situ nick end labeling. PMN (L) in early degradation stage of leukocytoclasis showing immuno-gold labeled (inset) nuclei (N) which are relatively intact in the ultrastructure, while cytoplasmic organelles demonstrated early degradation process. Bar: 1 μm and 0.1 μm (inset).

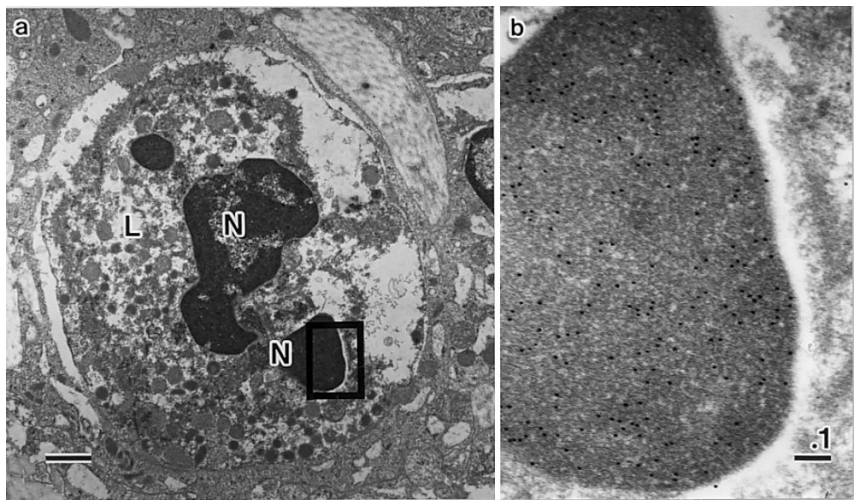


Fig. 4. Electron microscopic in situ nick end labeling. (a) PMN (L) in late stage of leukocytoclastic degradation showing extensive cytoplasmic degradation and beginning of fragmentation of the nuclei (N). Bar: 1 μm. (b) Dense immuno-gold labeling in the nuclear area. Bar: 0.1 μm.

debris derived from the nuclei of infiltrated cells. They were with interrupted membrane structure and lysed cytoplasmic component in the boundary.

## 4. Discussion

In situ nick end labeling technique has been widely used in order to investigate apoptotic cells in various tissues [5,26,27]. The method is basing on catalytic activity of TdT to 3′-OH ends concentrated in apoptotic bodies. This technique principally stains apoptotic cells, but it also detects all DNA breaks [28–30]. Inoki et al. [24] suggested that either ultrastructural study or electrophoretic investigation of extracted DNA is necessary in addition to light microscopic in situ nick end labeling.

The present data at the light microscopic level showing DNA breakdown in infiltrating PMNs in the fully-developed stage of anaphylactoid purpura are consistent with a similar in situ nick end labeling study by Banno et al. [15]. They found that PMNs perivascularly infiltrating into the skin lesions of anaphylactoid purpura are strongly stained with this method. Barduagni et al. [16] compared leukocytoclastic vasculitis and lymphocytic vasculitis and demonstrated nick end labeled cells in both types of vasculitis. Since in our unpublished study of purulent conditions

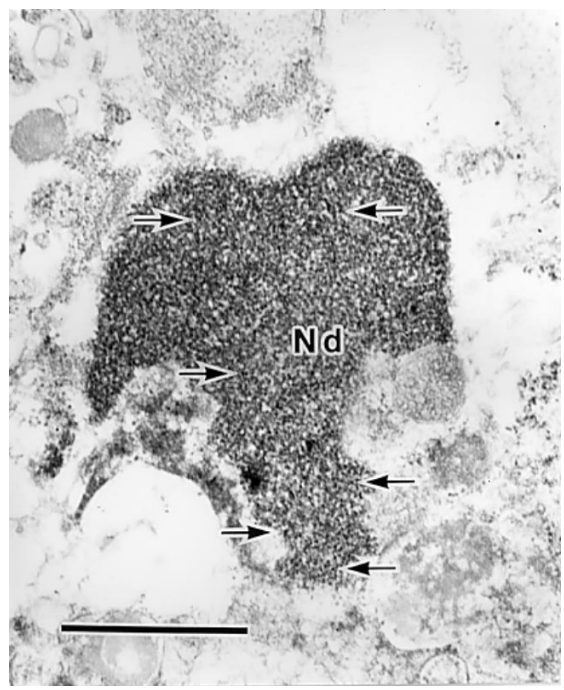


Fig. 5. Electron microscopic in situ nick end labeling showing the nuclear debris (Nd) co-existing with immuno-gold labeling (arrows). Bar: 1 μm.

such as folliculitis and ruptured epidermal cysts PMNs were totally negative by the same immunoperoxidase staining, the DNA breakdown of PMNs shown in the present study, as well as previous investigations [15,16,19], is a rather specific observation to leukocytoclasis. However, our findings at the ultrastructure level disclosed that typical apoptotic cells were only small portion of PMNs while the most of the rest are undergoing on one hand partial degradation of DNA, and on the other hand more dominant lytic change in the cytoplasm at the same time. Immuno-gold-labeled nuclear debris was rather with disintegrating plasma membrane and cytoplasmic organelles in the surroundings. The ultrastructural change may be compatible with an interpretation that apoptotic process was first triggered to lead into DNA degradation, but later interrupted by a presently unknown factor. This may be the reason to account for the PMNs with various degrees of destruction staying in the inflamed dermis.

PMNs contain a variety of proteolytic enzymes such as neutrophil elastase and cathepsins B, D and G [10,11] and reactive oxygen species [12,13] that are potentially harmful for normal tissues. Although they are required to protect human bodies against invading microorganisms, the host needs safe disposal pathway of their toxic contents. Savill et al. in 1992 [31] and Hughes et al. in 1997 [32] investigated kinetics of neutrophils in the kidney and their results were in agreement with the view that apoptosis play a role in a part of the mechanism of safe clearance of PMNs in experimental glomerulonephritis. In peritoneal exudate of guinea pigs apoptosis of PMNs results in ingestion of them by autologous macrophages [33]. In the present study we observed breakdown of DNA in the nuclei of disintegrating PMNs which are remain abundant in the dermal tissue. Our interpretation of this fact is that apoptosis of neutrophils is taking place as a part of resolution mechanism of inflammation but the apoptotic pathway is incomplete and unfinished due to an unknown interrupting factor, leading to vascular and perivascular damage in the dermal tissue, although this assumption is to be further investigated. The authors are now examining the possibility of reproducing apoptosis of PMNs in culture to find out what factors are needed to initiate, interrupt, and finish the apoptotic pathway of PMNs, which will be reported separately.

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