Malaysian J Pathol 2004; 26(2) : 73 – 87

Ultrastructural characteristics of synovial effusion cells in some arthropathies

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Abstract

Objective: To evaluate the range of activation changes of polymorphonuclear leukocytes (PMN) and the ratio of apoptosis and necrosis in synovial effusions of patients with various arthropathies, and to reveal possible correlations with clinical variants of joint inflammation. *Methods:* Synovial effusions were aspirated from the knee joints of patients with rheumatoid arthritis (RA, 28 cases), and seronegative spondyloarthritides (SSA): Reiter's disease (RD, 9 cases), peripheral form of the ankylosing spondyloarthritis (6 cases) and psoriatic arthritis (6 cases); and primary osteoarthritis (OA, 9 cases). Cytospin preparations were processed for transmission electron microscopy and assessed for the incidence of apoptosis, necrosis, and cytophagocytic cells (CPC) in the synovial fluid (SF). The range of activation changes of the neutrophil granulocytes, the dominating cell population in the arthritic SF, was evaluated. *Results:* In all arthropathies under investigation most of the synovial effusion cells had intact ultrastructure with a certain amount of apoptotic cells dominating over the cells with signs of necrosis, and a few CPC. The highest rate of apoptosis was discovered in the synovial effusions of patients with RA, the lowest in those with OA, while the rate of CPC among the inflammatory joint diseases was the lowest in RA. In RA the current disease activity correlated with the incidence of apoptotic cells and CPC, while the clinical stage was related only to the CPC rate. These data suggest that in RA, despite exposure to the anti-apoptotic signals, apoptosis of the synovial effusion PMN is maintained at a significantly higher level than in non-rheumatoid arthropathies, both inflammatory (SSA) and degenerative (OA), providing elimination of the neutrophils accumulating in the joint cavity and thus stimulating resolution of the joint inflammation.

Key words: synovial effusion, rheumatoid arthritis, Reiter's disease, ankylosing spondyloarthritis, psoriatic arthritis, osteoarthritis, transmission electron microscopy, neutrophil granulocyte, cell death.

INTRODUCTION

A characteristic feature of joint inflammation is the abundance of inflammatory cells in the diseased joint, with the two major components of this infiltrate being macrophages in the synovial tissue and neutrophils in the synovial fluid (SF).[1-4] Under normal conditions SF contains very few cells, the majority being fibroblast-like synoviocytes sloughed off by the synovial intimal lining and rare leukocytes, mainly mononuclear cells. In arthritides the ratio of the synoviocytes and white blood cells in the synovial effusions becomes reversed, with high level of cytosis and the prevalence of the polymorphonuclear leukocytes (PMN) which may comprise up to 80% of total cell count in active phase of the disease.[5,6] Neutrophils are short-lived leukocytes with a key role in the host defense against pathogens; they enter apoptosis spontaneously within 24-48 hours of leaving the bone marrow, although their life span can be extended during inflammatory responses by several proinflammatory cytokines.[7] The neutrophils contribute to different mechanisms of propagation of the synovial inflammation and the progress of joint destruction in arthritides. The PMN recruited in the joint cavity in rheumatoid arthritis (RA) possess potent degradative enzymes and proinflammatory mediators: their elastase, metalloproteinases, including collagenase (MMP-8), are responsible for articular cartilage destruction; prostaglandins and leukotriens provided by them serve as chemoattractants of mononuclear cells; defensins interact with the host immune system mediating acute inflammatory response correlated with the destructive course of the disease; proteases are involved in regulation of cytokine activity and

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pannus formation. The PMN release reactive oxygen intermediates such as superoxide anion (O^{2-}) which are potent mediators of inflammation. The adherence of PMN to the articular cartilage, eventually mediated by immunocomplexes, activates these cells with the subsequent secretion of destructive enzymes and reactive oxygen products resulting in cartilage degradation. For this reason their removal is vital to normal inflammatory resolution.[8-15] Recently some new mechanisms of PMN involvement in the pathogenesis of arthritides were investigated: the physical interaction between SF neutrophils and fibroblast-like synoviocytes which promotes and maintains joint inflammation; and the synthesis of large amounts of class II major histocompatibility complex (MHC) by the neutrophils of rheumatoid synovial effusions displaying a novel interaction of neutrophils with T cells, which is important in terms of the immune arthritides pathology.[16,17] In spite of that, the role of neutrophils in joint destruction remains underestimated.[15]

Synovial effusions are shown to be PMN-predominant in all forms of chronic inflammatory arthritis, irrespective of the possible underlying cause,[8] while in arthritides other than RA, in which joint destruction is milder than in RA with different underlying mechanisms and the considerably lower level of cytosis, the role of neutrophils is less understood.[18,19] The ability to modulate inflammatory PMN infiltration, including apoptosis induction, represents a potentially important mechanism of joint inflammation pathogenesis. Apoptosis as a major route of disposal of extravasated PMN is followed by rapid recognition and intact phagocytosis of PMN by mature tissue macrophages.[8] Decreased rates of SF neutrophil apoptosis amplify inflammatory response in arthritides.[7,20] There are very few papers on the apoptosis of synovial exudate cells in joint diseases;[8,20-24] most of them concern synovial effusions of the patients with RA and OA, and few are done on the ultrastructural level.[25,26] The reported data regarding incidence of apoptosis in synovial effusions, its promotion and inhibition, are controversial.

Some investigators present evidence that SF from a variety of arthritic patients generally promotes neutrophil apoptosis, and the rate of spontaneous apoptosis of the synovial PMN considerably exceeds its rate in the peripheral blood.[21,22,27] Other investigators found a high antiapoptotic activity of SF and a decreased amount of apoptotic PMN in the synovial effusions as a result of the altered sensitivity of SF neutrophils to apoptosis-inducing stimuli in RA.[7,23,28,29] Altered signaling via the Fas receptor and phosphatidylinositol 3-kinase-dependent anti-apoptotic influence of interferon-beta may explain the observed prolongation of the neutrophil lifespan and associated tissue injury at inflammatory sites.[7,22] Thus the fate of neutrophils at sites of inflammation, where these cells are likely exposed to both anti- and proapoptotic influences, needs to be clarified.[24] Detailed analysis of spontaneous PMN apoptosis rate in inflammatory (rheumatoid and non-rheumatoid) and degenerative joint diseases may help to elucidate the persistence of neutrophils in the joint cavity in different arthropathies and its relevance to clinical manifestations of the joint diseases.

MATERIALS AND METHODS

Human knee joint SF samples were obtained from patients with confirmed diagnosis at the time of knee surgery or during arthroscopy performed for diagnostic and therapeutic indications. Among them were 28 patients with RA: (19 female and 9 male patients, average age 42.3+/-5.2 years); 9 patients with osteoarthritis (OA, 6 female and 3 male patients, average age 51 years) and 21 patients with seronegative spondyloarthritides (SSA): Reiter's disease (RD, 9 cases: all male between 16 and 62 years old, average age 30 years); peripheral form of ankylosing spondylitis (AS, 6 cases: 5 male and 1 female between 21 and 50 years old, average 34 years) and psoriatic arthritis (PA, 6 cases: 4 female and 2 male patients between 25 and 42, average age 32 years).

Only those patients who had never received corticosteroids in the joint cavity of the examined joint prior to the investigation were included in the quantitative assessment group. Cytospin preparations of the synovial effusions were processed for transmission electron microscopy (TEM) and embedded in Araldit resin. Ultrathin sections were stained with uranyl acetate-lead citrate and viewed under JEM 100S TEM. A differential cell count was performed on the first 100 exudate cells with nuclear profiles encountered in every sample of SF. To evaluate sufficient amounts of cells for statistically significant results we needed to assess between 5 and 10 blocks of tissue from every specimen. The proportion of cytophagocytic cells (CPC), apoptotic and necrotic cells in the synovial effusions was then recorded.

Statistical analysis

Results are expressed as the mean +/- SEM. Statistical comparisons were performed with the percentage discrimination test (apoptotic and cytophagocytic cell rates). Correlation was estimated by Spearman's rank correlation coefficient, using Fisher's transformation where necessary.

RESULTS

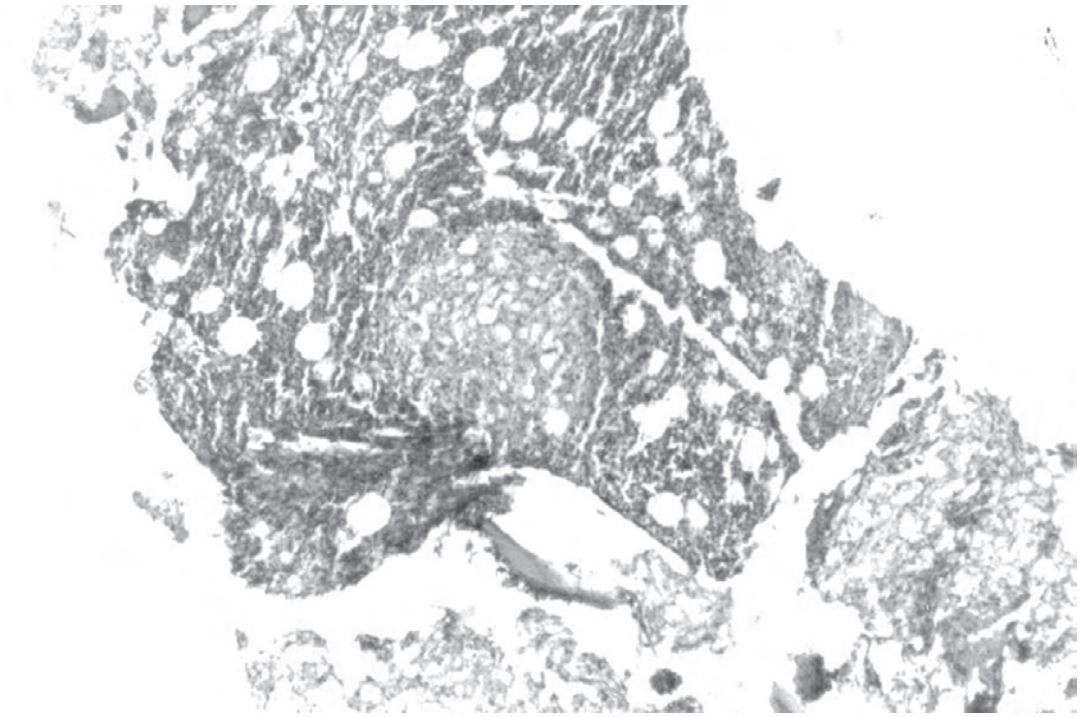
Electron microscopic assessment of cytospin preparations of the synovial effusions demonstrated that in all arthropathies under investigation, both inflammatory and degenerative, most of the cells had intact ultrastructure, while few contained changes consistent with either necrosis or apoptosis. In all arthritides, neutrophils dominated (Fig.1); with other white blood cells, like lymphocytes and monocytes, being much less frequent (Fig.2a). Connective tissue cells like fibroblast-like synoviocytes, macrophages and mast cells were also present in almost every case, with the ultrastructure appearing mostly unchanged (Fig.2b) while few cells displayed irreversible changes (Fig.3a). In all arthropathies, including OA, CPC containing apoptotic cells or nuclei could be identified among the cells of the macrophageal lineage. (Fig.3b). As identification of cell type at the late stages of apoptosis is impeded, the incidence of CPC in the synovial effusion was of particular interest, especially in terms of correlation with their incidence of apoptosis.

Earlier we have shown using ultramorphometric image analysis that polymorphonuclear cells recruited in the joint cavity compared to those in the synovial stromal extravasates gain distinct ultrastructural features of reactive modification, including development of numerous surface specializations, expanded perinuclear cysterna, granule translocation, increased size and volume/numeric density of vacuoles and phagosomes[30] (Fig. 4a). Among the neutrophils of the synovial effusions, a few looked relatively inactive (with smooth external contour, intact evenly distributed granules, without phagosomes, Fig. 4b); others displayed signs of moderate activation without manifestations of cellular lesion (pseudopodia and bleb-like protrusions, redistributed granules many of which contained extracted core, moderate amount of vacuoles and phagosomes, Fig. 5a), and some cells exhibited signs of functional overstrain (most irregular surface, large and numerous vacuoles and phagosomes, expanded perinuclear cysterna, signs of cellular lesions in the cytoplasm, Fig.5b), which is in agreement with the recent classification of activated neutrophilic granulocytes.[31]

Most of the cells containing features characteristic of apoptosis were identified as neutrophils (Fig. 6a). These cells exhibited typical cap- or crescent-shaped chromatin margination and condensation, increased density of the cytoplasm, closely packed organelles with subsequent bleb formation, honey-comb vacuolation of the cytoplasm and formation of apototic bodies often ingested by macrophageal cells. Neutrophils were found apoptotic at the early, advanced and late stages of cell death. Very few cells, both synoviocytes and leukocytes, displayed changes consistent with the cells undergoing necrosis (Fig.3a). Their nucleoplasm and cytoplasm appeared leached out, and cell and organelle membranes were disintegrated. Some apoptotic cells revealed features of secondary necrosis (Fig. 6b). As neutrophils comprise the largest cell population in the synovial effusions of inflamed joints determining the local disease activity, and their fate is essential for the resolution of inflammation, we undertook an assessment of apoptotic cell death rate in synovial effusions in RA, SSA and OA complicated by synovitis. We also estimated the incidence of the CPC as a complimentary index of apoptosis intensity in the synoval effusions.

Quantitative assessment showed that in general the share of apoptotic neutrophils was not large. This is presumably because this process is happening very fast, and the apoptotic bodies are immediately taken up by the macrophageal cells.[25,36] The apoptotic index of the exudate cells in different arthropathies is presented in Fig. 7. As is indicated in the diagram, the highest rate of apoptotic neutrophils was discovered in patients with RA, with this rate being significantly higher than in any other arthropathies which did not differ among themselves for this parameter. On the contrary, the incidence of CPC in the synovial effusions proved to be lower in RA than in any of the SSA, the difference between RD and RA being significant. The incidence of CPC did not differ significantly among non-rheumatoid inflammatory arthropathies, while in OA with synovitis they were rare, the difference with inflammatory arthritides being highly significant ($p<0.001$).

We also compared the incidence of CPC and



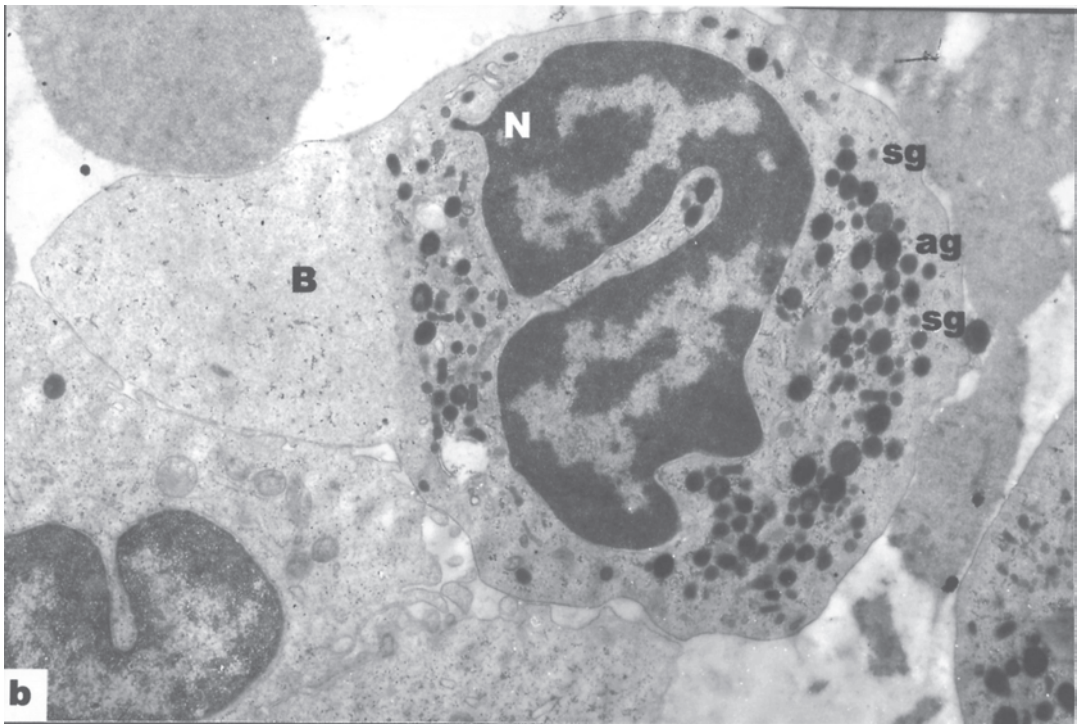


Fig. 1 Electron micrograph of the cytospin preparations of the synovial effusions from the patients with RA (a) and AS (b): a – neutrophil granulocyte with a large phagocytic vacuole (v). N – nucleus, G – Golgi apparatus, ag – azurophilic granules, sg – specific granules, L – lipid droplets. Initial magnification x 6,000. b – neutrophil granulocytes with horseshoe-like nucleus, large bleb (B) without organelles, azurophilic (ag) and specific (sg) granules. Initial magnification x5,000.

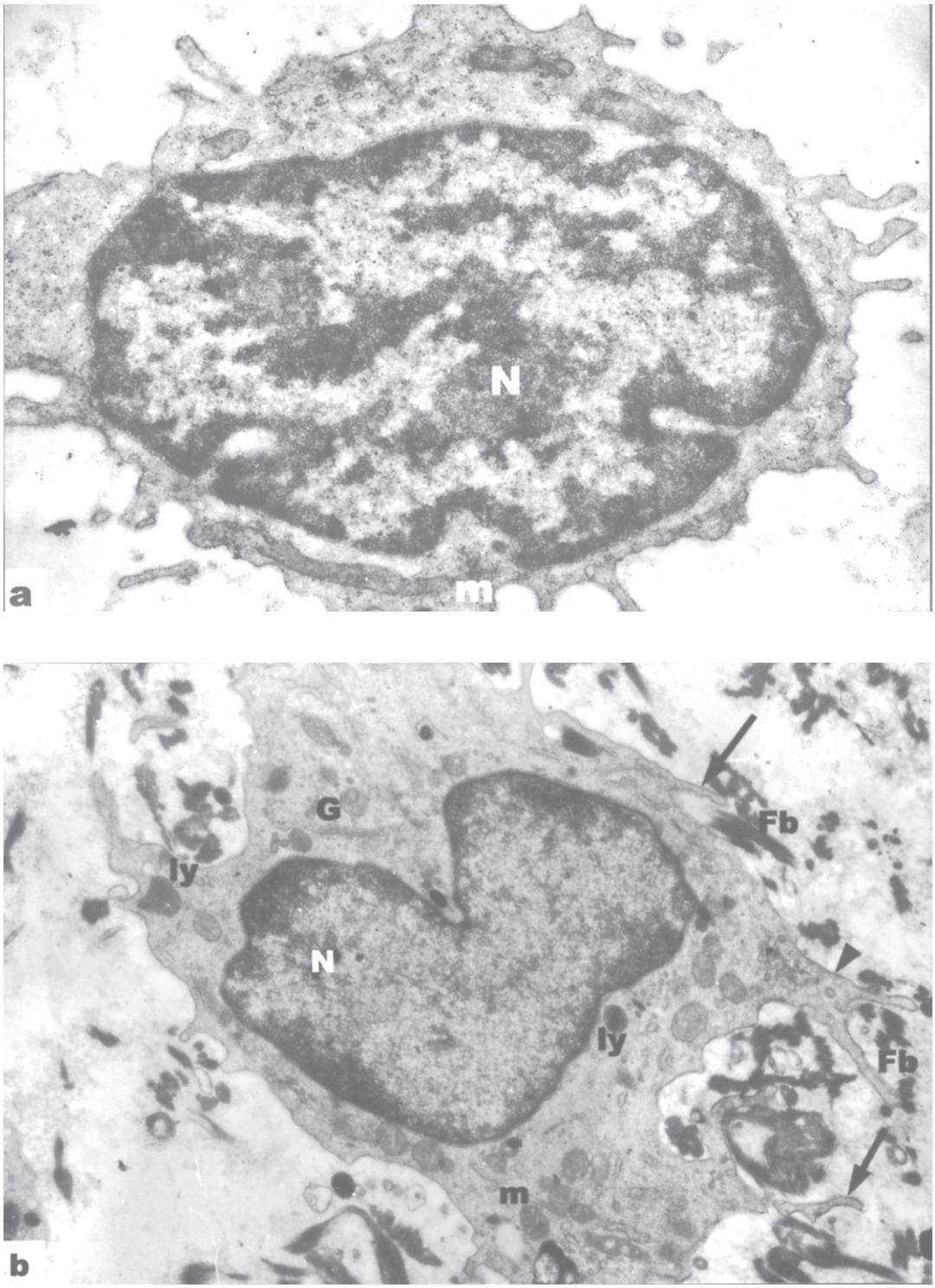


Fig. 2 Electron micrograph of the cytospin preparations of the synovial effusions in the patients with PA (a) and RA (b): a – lymphocyte with large mitochondria (m). N – nucleus. Initial magnification x10,000. b - macrophage of the monocytoid type with many surface membrane protrusions. Filopodia (arrows) and pseudopodia (arrowhead) are seen engulfing fibrin filaments (Fb). Few lysosomes (ly) are visible in the cytoplasm. N – nucleus, m - mitochondria, G – Golgi apparatus. Initial magnification x6,000.

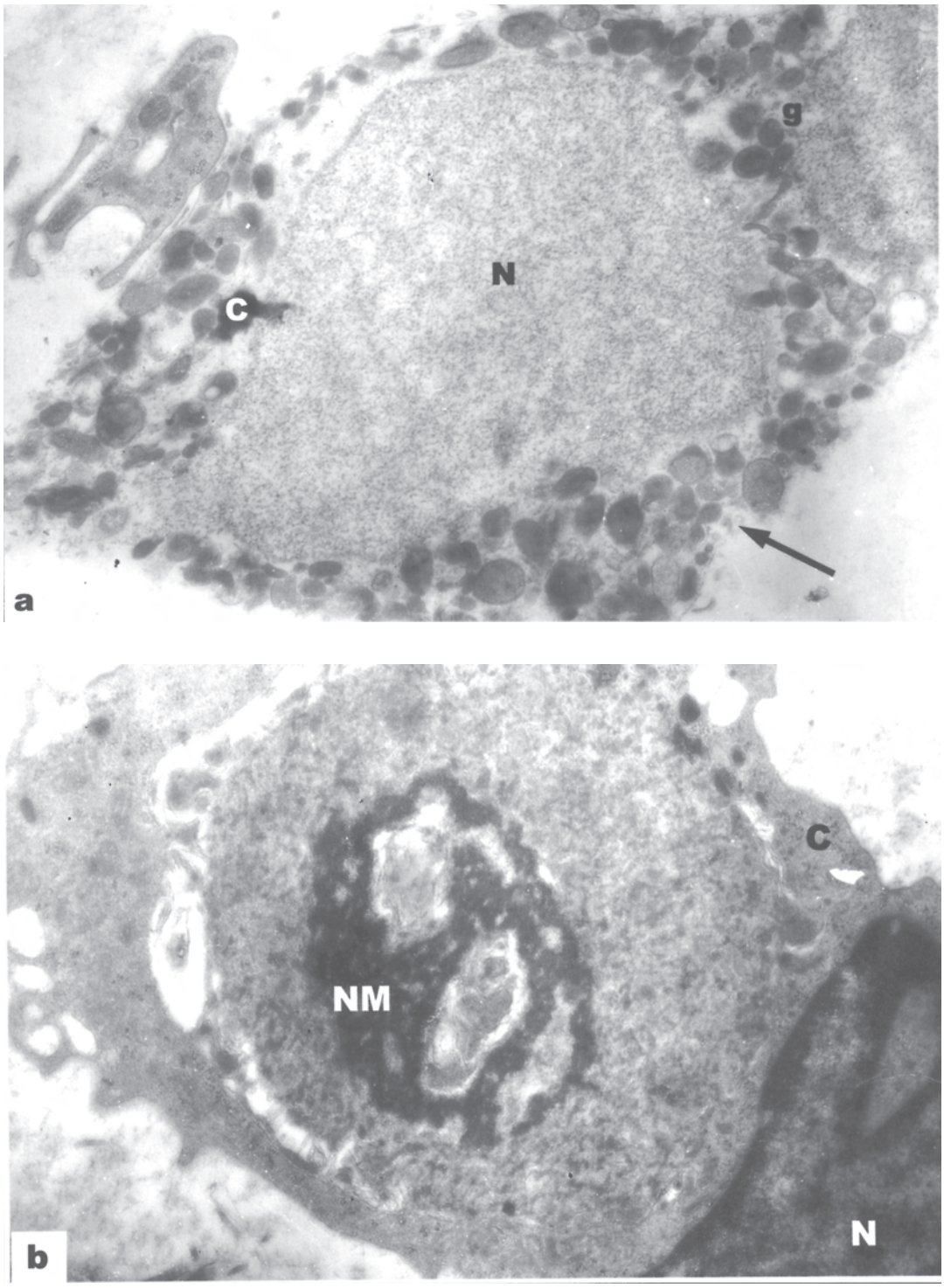


Fig. 3. Electron micrograph of the cytospin preparations of the synovial effusions in the patients with RA (a) and PA (b): a – mast cell exhibiting characteristics of necrosis. Chromatolysis in the nucleus (N) and disintegration of plasma membrane (arrow) are notable, while granules (g) remain intact. Initial magnification x 10,000. b - cytophagocytic mononuclear cell (C) at the initial stage of phagocytosis of an apoptotically changed cell. N – nucleus of the cytophagocytic cell, NM – dense nuclear material of the ingested cell, presumably polymorphonuclear leukocyte. Initial magnification x 10,000.

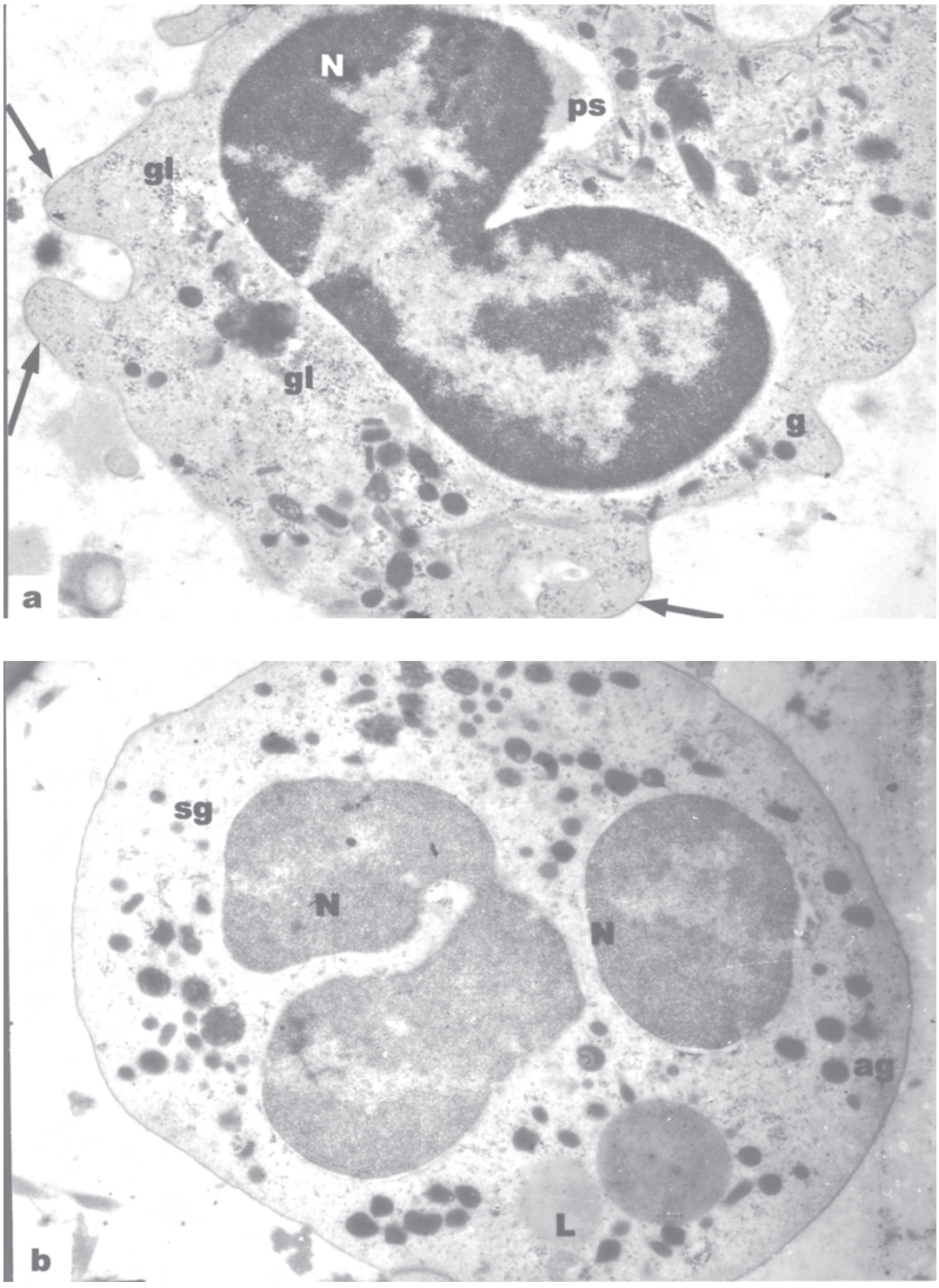


Fig. 4. Electron micrograph of the cytospin preparation of the synovial effusion from the patients with PA (a) and OA (b): a – moderately activated neutrophil granulocyte with surface protrusions (arrows) engulfing solid particles (two arrows), and plasma precipitate (arrow). Perinuclear space (ps) is locally dilated; few granules (g) and glycogen (gl) are seen in the cytoplasm. Initial magnification x 6,000. b – neutrophil granulocyte with a smooth surface, segmented nucleus (N), a few granules both azurophilic (ag) and specific (sg) and lipid droplets (L). Initial magnification x 6,000.

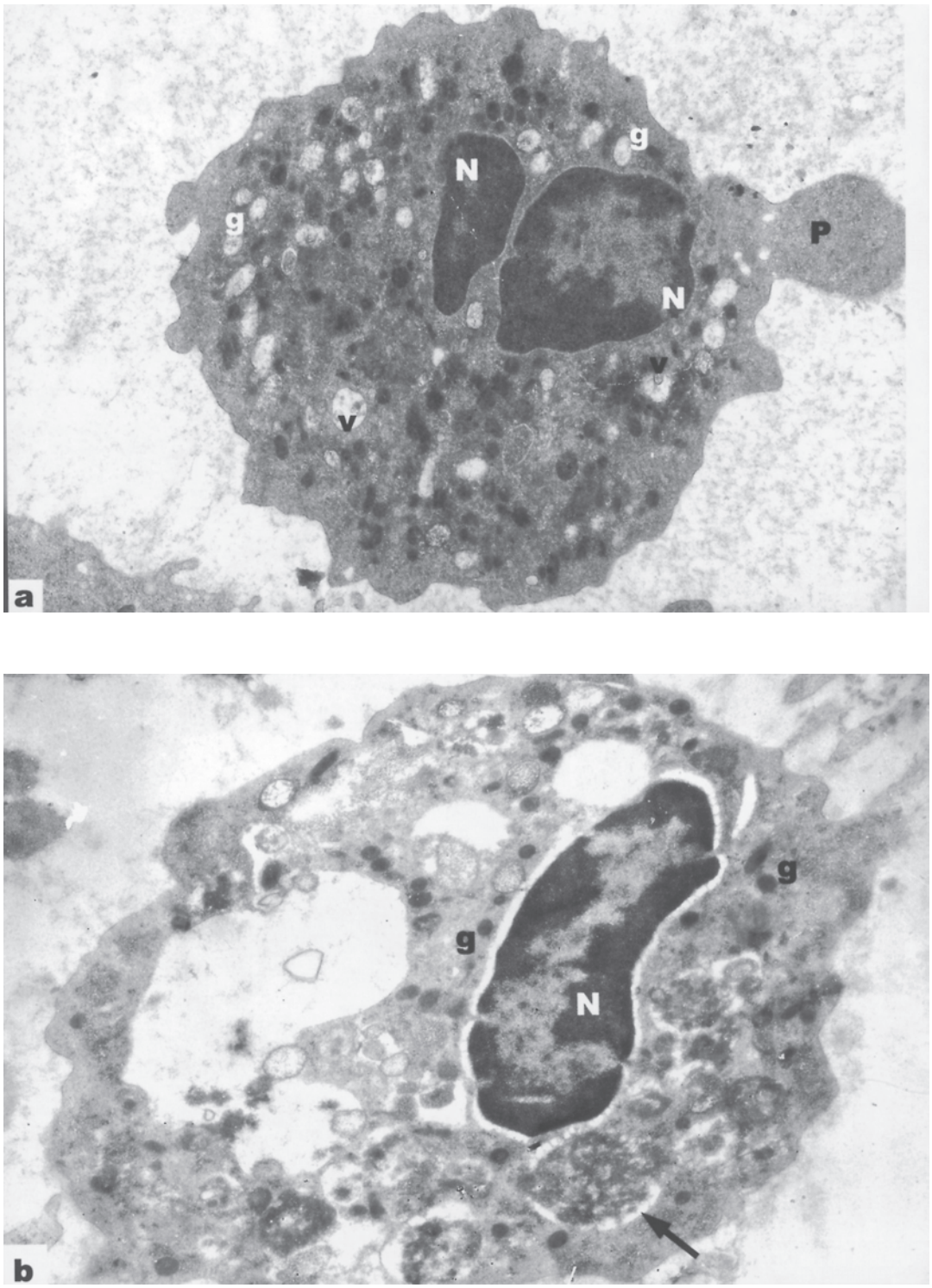


Fig. 5. Electron micrograph of the cytospin preparations of the synovial effusions from the patients with AS (a) and RA (b): a – neutrophil granulocyte with a large pseudopodia (P), multiple granules with extracted core (g) and phagocytic vacuoles (v). N – segments of the nucleus. Initial magnification x5,000. b – neutrophilic granulocytes with manifestation of functional overstrain. Granules (g) in the cytoplasm are rare; loci of the cytolysis are readily visible (arrows). N - nucleus. Initial magnification x8,000.

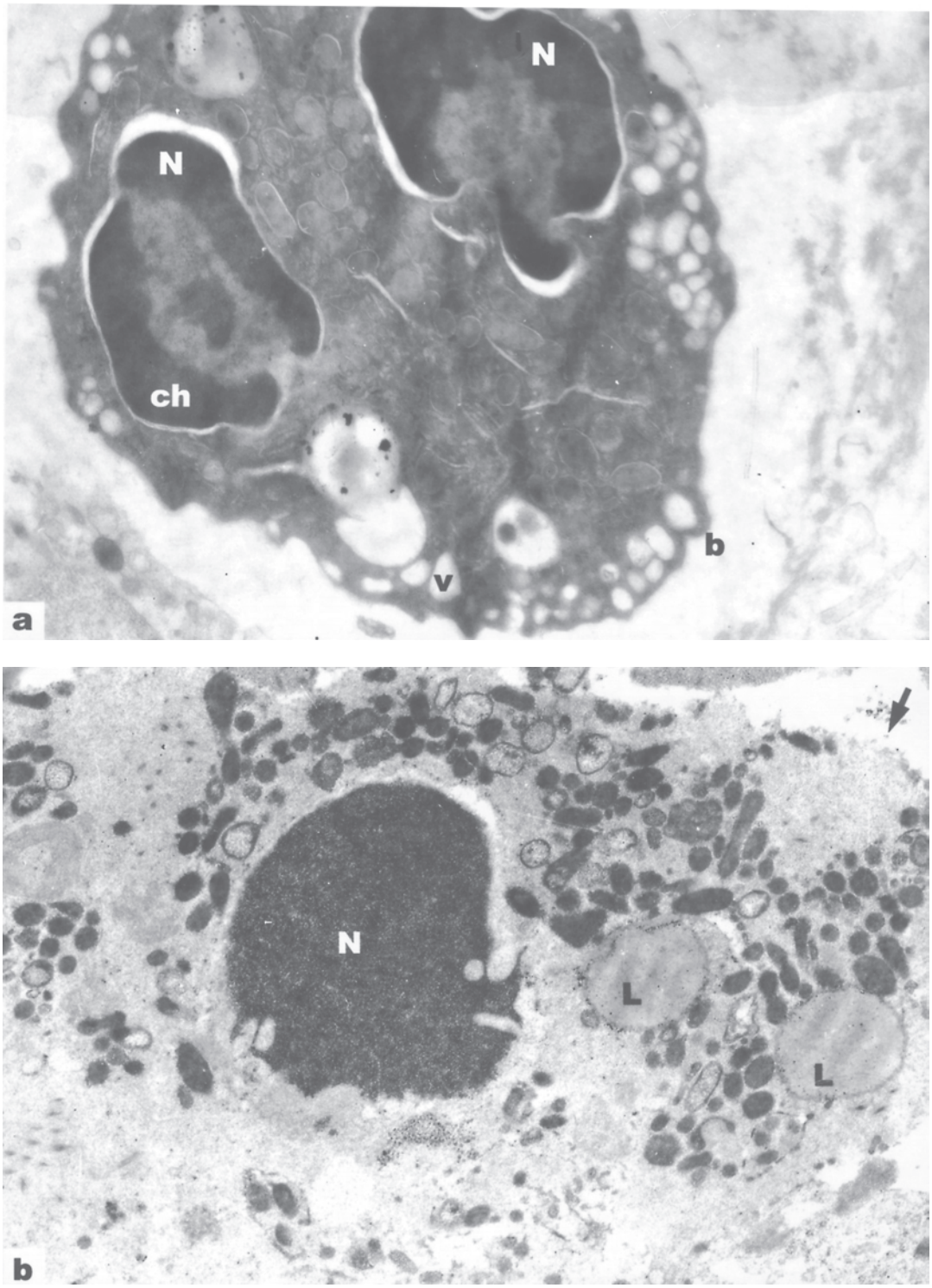


Fig. 6. Electron micrograph of the cytospin preparations of the synovial effusions in the patients with RD (a) and AS (b): a – neutrophil granulocyte in the advanced stage of apoptosis. The cell is reduced in volume, chromatin (ch) is marginated and heavily condensed in both segments of the nucleus (N). Dense cytoplasm is overcrowded by the organelles. The initial stage of honeycomb-like vacuolization of the peripheral part of the cell is visible; v – vacuole, b – bleb. Initial magnification x 10,000. b – necrotic neutrophil granulocyte exhibiting picnotic nucleus (N) with indented borders, disintegrated cell membrane (arrow), depletion of glycogen in the cytoplasm. L – lipid droplets. Initial magnification x8,000.

apoptotic neutrophils in RA patients with different clinical characteristics. It was shown that the presence of the rheumatoid factor in the synovial fluid, systemic manifestations of the disease and the profile of the joint damage progression (mild or severe) did not affect any of the parameters. On the contrary, both apoptotic and cytophagocytic cell rates were related to the general disease activity: both were significantly higher in low disease activity (Stoke index) compared to high activity (Fig. 8). Regarding the incidence of the CPC, it was inversely related to the destructive course of the disease. In erosive RA, CPC were significantly less frequent than in the non-erosive joint disease (Fig. 8), while the apoptotic index did not depend on the course RA.

Correlational analysis revealed the presence of a strong positive and significant correlation between the apoptotic index and the incidence of CPC in the SF and a moderate negative significant correlation between the apoptotic index and the SF neutrophil count in RA (Fig. 9).

It was also shown that in RA there was a moderate negative significant correlation (Fig. 10) between the nuclear segmentation rate and apoptotic cell death rate. In SSA this correlation became strong and highly significant, while in OA no meaningful result was yielded.

DISCUSSION

The present investigation provides data on the incidence of apoptosis of neutrophilic granulocytes in the synovial effusions of patients with arthropathies. In chronic arthritides, neutrophils, in contrast to lymphocytes, may hardly re-enter once having left the microcirculatory bed. They are instead being accumulated at the inflammatory site and activated afterwards. Therefore exudation of neutrophils requires subsequent elimination, which is essential for the resolution of inflammation, for when they are recruited in the joint cavity and activated, the neutrophils secrete strong chemotaxic factors, powerful oxidative and proteolytic lysosomal enzymes, proinflammatory cytokines and reactive oxygen intermediates, which may amplify inflammation in the joints and directly or by implication affect articular cartilage resulting in joint destruction.[10,11,14,15,25,32] Earlier it was shown that peripheral blood neutrophils in RA remain intact

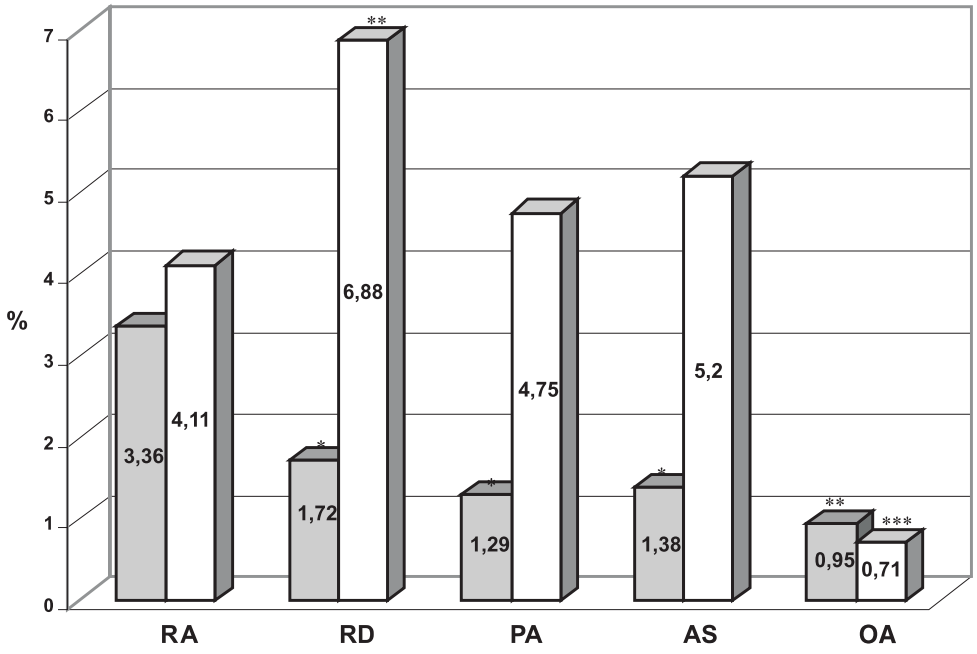


Fig.7. Apoptotic and cytophagocytic cell rate in arthropathies.
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$
cytophagocytic cell rate
apoptotic index

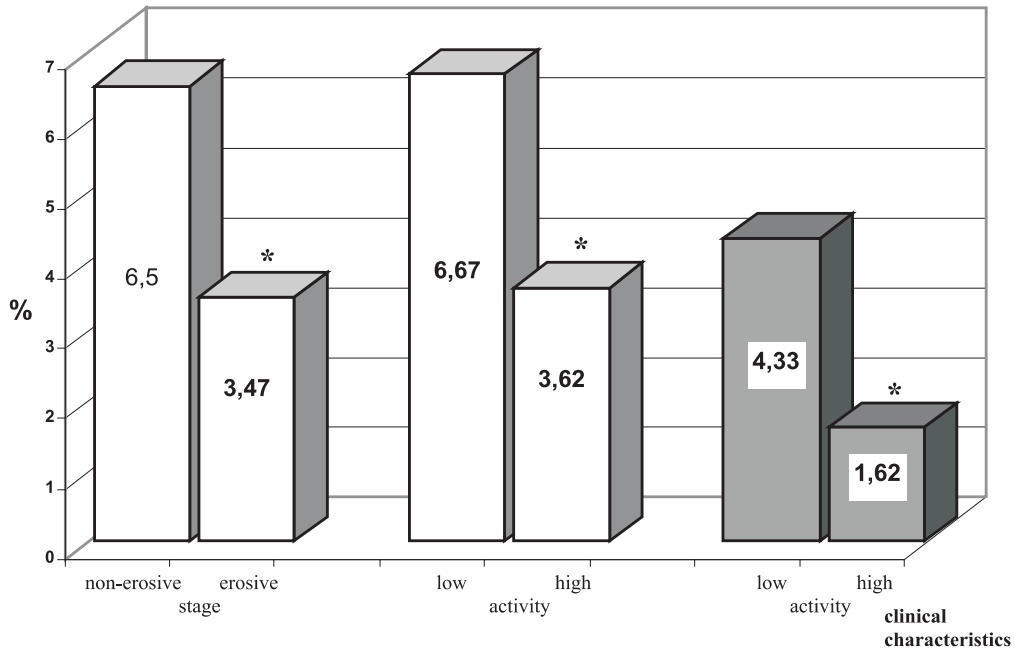


Fig.8. Apoptotic and cytophagocytic cell rate in different clinical variants of rheumatoid arthritis.

* $p < 0.05$

cytophagocytic cell rate

apoptotic index

unless they leave the microcirculation and invade synovial stroma with subsequent priming in the joint cavity in association with which they undergo marked ultrastructural and functional changes, including altered sensitivity to apoptosis-inducing stimuli.[10,23,30,33] As regulation of elimination of these cells from the joint cavity is important for the management of the course of inflammation, evaluation of PMN apoptosis in SF gains special importance.

According to some recent publications, spontaneous and immune complex-triggered neutrophil apoptosis is reduced in the SF of patients with RA, its microenvironment being a proinflammatory milieu responsible for the in loco persistence of activated and long-surviving neutrophils with adenosine playing a crucial role in the inhibition of apoptosis.[24,28] On the contrary, other investigators have demonstrated that synovial effusions contain factors capable of directly or indirectly promoting neutrophil apoptosis and are normally powerful enough to overcome the apoptosis inhibiting effects of cytokines.[21,22]

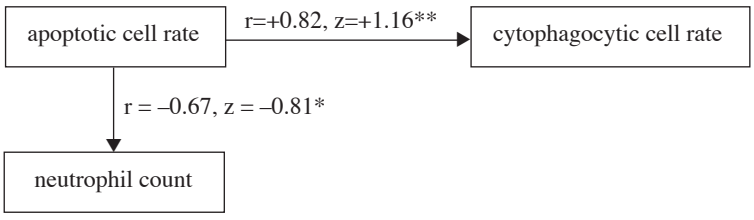


Fig.9. Correlation between apoptotic and cytophagocytic cell rate and neutrophil count in the synovial fluid of the patients with rheumatoid arthritis.
r – Spearman's rank correlation coefficient
z – Fisher's transformation coefficient
* $p < 0.05$
** $p < 0.01$

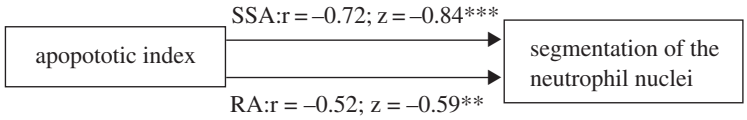


Fig.10. Correlation between apoptotic cell rate and segmentation of neutrophil nuclei in the synovial fluid of the patients with inflammatory arthropathies.
r – Spearman's rank correlation coefficient
z – Fisher's transformation coefficient
** $p < 0.01$
*** $p < 0.001$

In a recent discussion in Rheumatology (Oxford)[27,29] this discrepancy was explained by the diverse methodological approaches, variability of individual responses and differences in the activity/phase of the disease in the patients examined. Having a sufficient number of samples of SF from RA patients with different current disease activity, we undertook a comparative assessment of the incidence of apoptotic neutrophils in patients with low and high current disease activity. We also compared the incidence of apoptosis in the SF of patients with RA and other inflammatory arthropathies (RD, AS, PA) in which joint destruction is usually milder than in RA. As a result it was demonstrated that in RA apoptotic index of synovial effusion cells was significantly higher than in any other arthropathy under investigation, both inflammatory and degenerative. These data indicate that although rates of apoptosis of the SF neutrophils "are being continually modulated by death and survival signals,"[22] this rate is maintained at a level higher in RA than in any other inflammatory arthropathy under investigation, thus making for the resolution of the inflammatory process. Moreover, within the group of patients with RA low disease activity was associated with higher incidence of apoptotic neutrophils than in those with high activity, suggesting that the balance between the pro-apoptotic and anti-apoptotic stimuli establishes a certain apoptotic rate which will further determine the intensity and duration of joint inflammation.

Earlier we demonstrated that apoptosis of the synoviocytes in the synovial membrane was related to the clinical characteristics of RA such as progression profile and radiographic stage of joint damage.[34] This fact evidences the presence of different pathogenetic mechanisms underlying inflammatory and destructive changes in joint tissues. Analysis has shown that there is a moderate negative correlation between neutrophil apoptotic index on one side and total count of neutrophils in the synovial effusions on the other. This is in agreement with the data of other investigators[8] and accorded with an presumption that one would expect accelerated neutrophil apoptosis in resolving fluids and inhibition in persistently active fluids.[27]

Apoptosis rate is related to the age of the synovial effusion storage in vitro.[8] Regarding the in vivo conditions there are several methods of assessment of the synovial exudate age: total WBC count (maximal at the acute phase), number of changed cells (minimal in fresh exudate), and the number of nuclear segments in the neutrophils, the latter being the most convincing parameter as hypersegmented nuclei are one of the significant indications of the aged effusion. We carried out a correlation analysis of the apoptotic index and nuclear segmentation in the synovial effusions. It was shown that in RA there is a moderate negative correlation between the nuclear segmentation rate and apoptotic cell death rate, while in SSA this correlation became strong. This result enables us to conclude that in general the incidence of apoptosis of the synovial effusion cells is related to the age of the exudate, though the conditions of microenvironment (immune status, cytokine profile) amend this correlation. Therefore in spite of the increased level of apoptosis in RA, the lifespan of the neutrophils is extended and the persistence of neutrophils in the joint cavity promotes further cartilage destruction. Onset of apoptosis in many cell types, including the neutrophil granulocytes, leads to recognition and ingestion by macrophages, a key regulatory step in the clearance of inflammatory cells from inflamed sites.[35]

As identification of the dying cells at the late stages of cell death is impeded, the incidence of CPC in the synovial exudate was also taken into account as a complimentary index of apoptosis intensity.[8,25] CPC were described in the SF of patients mainly with SSA and rarely with RA (8,36). Our investigation confirmed the presence

of the highest amount of CPC in RD as discovered by other investigators.[8,36] Though in all examined inflammatory arthropathies the CPC rate was higher than the apoptotic cell rate, the incidence of CPC in the synovial effusions of patients with RA was lower than in any other inflammatory arthropathy while the apoptotic index was accordingly significantly higher, being in agreement with other investigations presuming partial inhibition of recognition of apoptotic bodies by macrophageal cells.[25]

The CPC rate in RA synovial effusions was found to be related to the current general and local disease activity in contrast to the data of some other investigators.[36] This discrepancy might be explained by the different methodological approaches among which ultrastructural examination provides stronger evidence of apoptotic cell death and their engulfment by the macrophageal cells.[37,38] Besides relevance to disease activity, the incidence of CPC was also associated with the course of the disease, being significantly higher in the erosive RA compared to the non-erosive forms. Thus there is a distinct connection between the apoptosis rate among the trigger cells in the synovial tissues and clinical characteristics of RA: the higher incidence of apoptotic and cytophagocyting cells is, the milder the clinical profile of the disease (non-erosive stage, low current disease activity). For this reason, regulation of apoptosis in the synovial tissues represents an important mechanism of joint inflammation control.

Both apoptotic neutrophils and CPC could be found in the SF of OA patients with synovitis in much smaller amounts. Though there are genetic preconditions for the inhibition of apoptosis of effector cells in joint tissues in immune arthritides,[39,40] and neutrophils of synovial effusions are exposed to proinflammatory mediators endowed with either anti-apoptotic or pro-apoptotic properties,[29] spontaneous apoptosis is maintained at certain levels providing elimination of neutrophils from the joint cavity in arthropathies and thus stimulating resolution of the joint inflammation. In RA there are provisions for the promotion of apoptosis, and if this mechanism is adequately triggered the intensity of inflammation may be significantly reduced.

Thus the involvement of neutrophils in joint inflammation is an important factor of homeostasis, which is implicated in host defence and responsible for the clinically significant complications of the joint inflammatory process. It may be utilized as a prophylactic and therapeutic target.

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