

Concurrent evolution and resolution in an acute inflammatory model of rat carrageenin-induced pleurisy

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Abstract: Granulocyte apoptosis and subsequent clearance by phagocytes are critical for the resolution of inflammation. However, no studies have addressed how the resolution proceeds in the inflammatory site. We studied the time course of neutrophil apoptosis and the following ingestion by mononuclear leukocytes in rat carrageenin-induced pleurisy, detecting DNA fragmentation by the deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) method, by acridine orange staining, and from the DNA ladder pattern on electrophoresis. Neutrophil accumulation started 3–5 h after carrageenin injection and then maintained a plateau until 24 h. Neutrophils decreased steeply between days 1 and 3. Mononuclear leukocytes started to accumulate at 5 h and reached a peak at day 2. TUNEL-positive bodies and acridine orange-positive bodies first became detectable in the cytoplasm of the mononuclear leukocytes from 24 h and 9 h, respectively. Both methods indicated that mononuclear leukocytes containing fragmented DNA increased rapidly on days 1 and 2 and reached a peak at day 3. The characteristic ladder pattern of neutrophil DNA was observed from 5 h. Tumor necrosis factor α was detectable on the start, and the levels of interleukin-10 and transforming growth factor- β 1 rose together with signs of neutrophil apoptosis and the following ingestion by mononuclear leukocytes. These results indicate that neutrophils start to undergo apoptosis just after the beginning of their accumulation in the inflammation site. Thus, evolution and resolution processes may proceed concurrently in acute inflammation. *J. Leukoc. Biol.* 73: 456–463; 2003.

Key Words: TUNEL · TNF- α · interleukin · TGF- β 1

INTRODUCTION

The evolution of the acute inflammation is characterized by the sequential release of mediators, resulting in plasma exudation and infiltration of neutrophils and mononuclear leukocytes. The recruited leukocytes ingest offending agents and release

further mediators. These responses are, however, self-limiting and are finally resolved. Absorption of accumulated exudate and clearance of recruited leukocytes occur during the resolution of inflammation. Attention has in recent years become increasingly focused on the mechanisms that regulate the resolution of inflammation. However, it is not yet well understood when the resolution starts or how the process proceeds in the course of inflammation. It has been assumed that the resolution starts when proinflammatory responses, namely plasma exudation and leukocyte infiltration, cease. As a result, authors have focused their attention on a rather later phase of the inflammatory models.

Neutrophils are essential components of the inflammatory and immune responses. They form the first line of defense against offending agents. The removal of recruited neutrophils and their potentially histotoxic contents from the inflamed site has particular importance in the resolution of acute inflammation. Macrophages migrate from the inflammatory site to the regional lymph nodes [1, 2]. However, emigration of neutrophils back into the circulation does not occur after acute inflammation. Neutrophils are constitutively programmed to undergo apoptosis, which limits their proinflammatory potential and leads to rapid and specific recognition and subsequent engulfment by macrophages or semiprofessional phagocytes [3, 4]. Apoptosis, or programmed cell death, is an active process involving gene transcription and protein synthesis that leads to an orderly sequence of events. It is characterized biochemically by internucleosomal 180- to 200-bp DNA fragmentation [5] and exposure of phosphatidylserine on the surface of the plasma membrane [6] and morphologically, by cell shrinking, chromatin condensation and fragmentation, plasma-membrane ruffling and blebbing, and the formation of apoptotic bodies [7–9]. Neutrophil apoptosis in inflamed sites was demonstrated in tracheal lavage of infants recovering from respiratory distress syndrome [10], bacterial lipopolysaccharide-induced acute lung inflammation [11], and oleic acid-induced acute lung injury [12]. However, no studies have addressed the question of when neutrophils start to undergo apoptosis or of

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how ingestion of apoptotic neutrophils by macrophages proceeds during the course of inflammation.

Neutrophils have a very short half-life (8–20 h) in the circulation [13]. However, it has been suggested that their survival may be prolonged in the inflammatory site. A role of endothelial transmigration in the regulation of neutrophil apoptosis has been reported. Adhesion molecules on endothelial cells can generate intracellular signals that trigger enhanced survival of neutrophils as they migrate from the bloodstream into tissues [14]. Furthermore, the rate of apoptosis in neutrophil populations *in vitro* can be markedly inhibited by inflammatory mediators, suggesting an extended functional lifespan of neutrophils in inflamed sites (for review, see refs. [15–17]). However, no study has yet addressed whether these regulatory mechanisms are operative *in vivo*.

Carrageenin is sulfated polysaccharides isolated from marine algae [18]. Rat carrageenin-induced pleurisy was originally introduced for quantitative and temporal analyses of the accumulation of inflammatory cells and exudate [19] and is one of the most widely characterized acute inflammatory models. High-rate plasma exudation occurs during the very early phase (1–7 h), and the resulting accumulation of pleural exudate peaks around 19 h [20]. The leukocyte infiltration starts 3–5 h after irritation and is initially dominated by neutrophils, which give way later on to an increasing number of mononuclear leukocytes [20]. Although nuclear factor- κ B and cyclooxygenase-2 play an important role in the evolution of acute inflammation, these molecules are also implicated in the resolution in the late phase of this model [21–23]. In the present study, we investigated the time course of neutrophil apoptosis and the subsequent ingestion by mononuclear leukocytes in rat carrageenin-induced pleurisy to clarify temporal relation between the evolution and the resolution in an acute inflammatory model.

MATERIALS AND METHODS

Carrageenin pleurisy

All experiments were performed according to the Guideline for Animal Experimentation of Kitasato University (Kanagawa, Japan). Animals were kept in a controlled environment at a temperature of $21 \pm 1^\circ\text{C}$, humidity between 45% and 55%, and with light from 8:00 to 20:00. Commercial laboratory chow (Clea Rodent Diet CE-2, Nippon Clea, Tokyo, Japan) and water were given *ad libitum*. Pleurisy was induced in male Sprague-Dawley rats (9–10 weeks old, specific pathogen-free), purchased from Nippon SLC (Hamamatsu, Japan), by intrapleural (i.p.) injection of 0.2 ml 2% λ -carrageenin (Sigma Chemical Co., St. Louis, MO) under light ether anesthesia, according to the method described previously [20]. The rats were exsanguinated under ether anesthesia at given times. The pleural exudate was harvested, during which its volume was determined, and it was transferred to a tube containing 1 ml 77 mM EDTA. Then, the pleural cavity was washed twice with 2 ml 10 mM phosphate-buffered saline (PBS; pH 7.2) containing 1 mM EDTA (EDTA–PBS). The exudate and lavage were combined. For cytokine assay, the combined pleural fluid was centrifuged at 1000 *g* for 5 min, and the resulting supernatant was stored at -80°C until assay.

Cell count and classification

The cells in the combined pleural fluid were enumerated using an improved Neubauer cell-counting plate after staining with Turk's solution (Sigma-Aldrich Japan, Tokyo). Cell viability, assessed by the trypan blue exclusion

test, was between 95% and 87% throughout the tested time points. Classification of the cells in the pleural fluid was performed on the cytospin preparation. The cells were spun onto a glass slide coated with poly-L-lysine (Sigma Chemical Co.) by centrifugation with 15 *g* for 5 min at room temperature. The preparation was dried, fixed in 100% methanol for 2–3 s, and stained with Wright-Giemsa solution (Wako Pure Chemicals, Osaka, Japan). Two- to three-hundred cells were classified as neutrophils, mononuclear leukocytes, or others under light microscopy, and their total number in the pleural fluid was calculated.

Deoxyuridine triphosphate (dUTP)-biotin nick-end labeling (TUNEL) assay

The TUNEL method [24] was used for detection of fragmented DNA using a TaKaRa *in situ* apoptosis detection kit (TaKaRa, Kusatsu, Japan), according to the manufacturer's instructions. In brief, the cytospin preparation was air-dried overnight and then fixed with 4% paraformaldehyde in 10 mM phosphate buffer (pH 7.4) at 4°C for 30 min. After washing with the phosphate buffer, endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol for 30 min at room temperature and was then washed. The cell membrane was permeabilized on ice for 4 min with the permeabilization buffer provided by the manufacturer. The preparation was rinsed and labeled with fluorescein isothiocyanate (FITC)-dUTP by terminal deoxynucleotidyl transferase in a humid atmosphere at 37°C for 120 min. After being washed twice, the preparation was incubated with horseradish peroxidase-conjugated anti-FITC antibody for 30 min at 37°C and then with a mixture of 3,3'-diaminobenzidine and H_2O_2 in 0.1 M Tris-HCl (pH 7.5). Counter-staining was conducted using hematoxylin. Two cytospin preparations were made from one exudate sample.

Randomly selected fields were photographed with a digital television camera (DP50, Olympus, Tokyo, Japan) through a light microscope. Two- to three-hundred leukocytes containing TUNEL-positive bodies were separately enumerated on the basis of the size of the bodies on a television monitor: "small", cells containing only bodies smaller than a lobule of the neutrophil nucleus; "medium", cells containing the bodies as large as a lobule of neutrophil nucleus with (or without) bodies smaller than a lobule of neutrophil nucleus; "large", cells containing bodies at least as large as the whole of a neutrophil nucleus. Their number in the pleural fluid was then calculated.

Acridine orange/Hoechst 33342 staining

Acridine orange was used for the detection of single-strand DNA together with the vital nuclear stain Hoechst 33342 for localizing nuclei [25, 26]. The cells in the combined pleural fluid were incubated with 6.5 μM acridine orange (Molecular Probes, Eugene, OR) and 20 μM Hoechst 33342 (Sigma Chemical Co.) for 5 min at room temperature. A part of the sample was mounted on a glass slide and photographed with a digital television camera (DP50, Olympus) through a fluorescence microscope (BX51, Olympus) with excitation at 460–490 nm and emission at 510 nm for acridine orange and with excitation at 330–380 nm and emission at 420 nm for Hoechst 33342. Leukocytes, including bright yellow-green acridine orange-positive bodies, were separately enumerated on the same basis as for the TUNEL assay.

DNA ladder

The pleural fluid cells were washed twice with EDTA–PBS and were counted, and small fragments of DNA were extracted by the method of Tanuma et al. [27] with slight modifications. In brief, cells (10^7 cells/sample) were resuspended in 200 μl lysis buffer, composed of 50 mM Tris-HCl buffer (pH 7.5), 20 mM EDTA, and 1% Nonidet P-40 (Sigma-Aldrich Japan) at room temperature for 10 min. The lysate was centrifuged at 600 *g* for 5 min to remove integral nuclei. The resulting supernatant was digested with 0.5 mg/ml proteinase K (Sigma Chemical Co.) and 0.5 mg/ml RNase A (Sigma Chemical Co.) for 30 min at 37°C in the presence of 0.5% sodium dodecyl sulfate (Bio-Rad, Hercules, CA). Then, fragmented DNA was extracted by the method of Ishizawa et al. [28]. The sample was incubated with 6 M sodium iodide containing 13 mM EDTA, 0.5% sodium N-lauroylsarcosinate, 10 $\mu\text{g}/\text{ml}$ glycogen, and 26 mM Tris-HCl buffer (pH 8.0) for 15 min at 60°C . The fragmented DNA was precipitated by the addition of an equal volume of isopropanol and was incubated for 15 min at room temperature. After centrifugation at 20,000 *g* for 15 min, the precipitate was rinsed twice by addition of 1 ml 70% ethanol and

centrifugation at 20,000 *g* for 5 min. The resulting DNA pellet was dissolved in 20 μ l 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and was then electrophoretically separated on 1.5% agarose gel (TaKaRa LO3, TaKaRa) in TAE buffer (40 mM Tris-HCl buffer, pH 8.4, containing 40 mM acetic acid and 1 mM EDTA). λ HindIII (ABgene, Surrey, UK) and ladder markers in multiples of 100 bp (New England BioLabs, Beverly, MA) were used as markers. The gel was stained with 1 μ M ethidium bromide in the TAE buffer for 30 min and was photographed with a charged-coupled device camera (Printgraph, Atto, Tokyo).

In separate experiments, mononuclear leukocytes and neutrophils were separated from each other by elutriation using Lymphoprep™ (specific density, 1.077 g/ml; Axis-Shield PoC AS, Oslo, Norway) and Polymorphprep™ (specific density, 1.113 g/ml; Axis-Shield PoC AS) with centrifugation at 500 *g* for 30 min at room temperature and were analyzed for DNA ladder. Purity of each fraction was $80.8 \pm 3.9\%$ (*n*=8) for mononuclear cells and $90.2 \pm 1.3\%$ (*n*=10) for neutrophils.

Cytokine assay

The frozen, cell-free supernatant of the pleural fluid was thawed, and tumor necrosis factor α (TNF- α), interleukin (IL)-10, and transforming growth factor (TGF)- β 1 were determined using a rat TNF- α enzyme-linked immunosorbent assay (ELISA) kit (Biosource, Camarillo, CA), a rat IL-10 ELISA kit (Biosource), and a human TGF- β 1 ELISA kit (R&D Systems, Minneapolis, MN), respectively. The detection limits were 0.1 ng for TNF- α , 0.2 ng for IL-10, and 2 ng for TGF- β 1.

Data analysis

Results were expressed as the mean \pm SEM from *n* experiments.

RESULTS

Inflammatory reaction

The amount of pleural fluid collected from normal control rats was 0.12 ± 0.02 ml (*n*=3). The total count of leukocytes harvested from the lavage fluid was $8.9 \pm 0.9 \times 10^6$ cells (*n*=3). Neutrophils and mononuclear leukocytes accounted for $1.2 \pm 0.3 \times 10^6$ cells and $4.3 \pm 0.7 \times 10^6$ cells, respectively. The remaining cells included eosinophils ($1.5 \pm 0.6 \times 10^6$ cells), mast cell ($1.2 \pm 0.9 \times 10^6$ cells), and lymphocytes ($0.1 \pm 0.01 \times 10^6$ cells). After the i.pl. injection of carrageenin, pleural exudate was accumulated, reaching a peak of 4.3 ± 0.4 ml (*n*=4) at 19 h and then declined to 0.4 ± 0.06 ml (*n*=9) at day 7 (Fig. 1, upper panel). The leukocyte accumulation in the pleural cavity also started on the injection of carrageenin. The total cell count increased rapidly until day 1, kept a plateau between days 1 and 2 (more than 300×10^6 cells), and then declined. Neutrophils started to accumulate in the very early phase, 3 h after carrageenin injection, and maintained a plateau for 5–24 h ($\sim 200 \times 10^6$ cells). Subsequently, again, they rapidly decreased to $3.5 \pm 1.1 \times 10^6$ (*n*=12) at day 4. In contrast, accumulation of mononuclear leukocytes was slower than that of neutrophils. Mononuclear leukocytes started to accumulate approximately 5 h after carrageenin injection and reached a peak at day 2 ($203 \pm 13 \times 10^6$, *n*=13). The cell count then gradually decreased to $77.8 \pm 8.6 \times 10^6$ (*n*=13) at day 7 (Fig. 1, lower panel).

Morphological observation indicated that the size and number of vacuoles in the cytoplasm of the mononuclear leukocytes gradually increased with time (Fig. 2, upper panel). Furthermore, mononuclear leukocytes often contained deep violet-

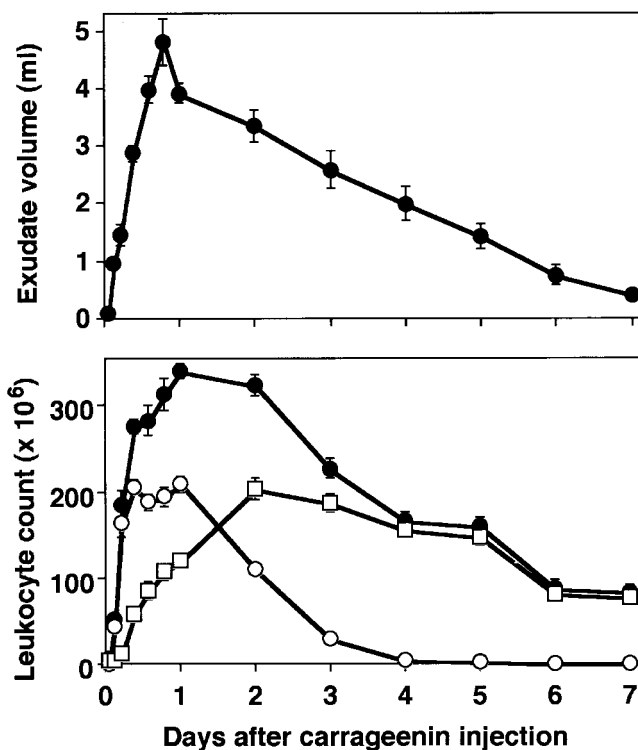


Fig. 1. Changes in the volume of pleural exudate (upper panel) and in the leukocyte count (lower panel). The symbols in the lower panel have the following meanings: closed circles, total leukocytes; open circles, neutrophils; open squares, mononuclear leukocytes. Each value indicates the mean \pm SEM of four to six rats.

colored bodies other than its nucleus stained by the Wright-Giemsa method. However, no morphologically distinguishable neutrophils were observed in the mononuclear leukocyte cytoplasm. In contrast, most of neutrophils did not show obvious morphological changes with time. Although apoptotic bodies were never observed, neutrophils with fragmented nuclei were seen at a frequency of less than 0.01% (Fig. 2, lower panel).

Detection of fragmented DNA by TUNEL assay

Various numbers and sizes of brown-colored TUNEL-positive bodies, which probably correspond to the deep violet-colored bodies stained by the Wright-Giemsa method, were observed in mononuclear leukocytes (Fig. 3). All of the bodies were located in the cytoplasm. Morphologically typical neutrophils with lobular nuclei did not appear TUNEL-positive, with the exception of very rare cases (less than 0.01%). The nuclei of mononuclear leukocytes themselves were never TUNEL-positive.

A significant number of mononuclear leukocytes containing TUNEL-positive bodies were observed from 24 h after carrageenin injection (Fig. 4). Mononuclear leukocytes were separately enumerated according to the size of the TUNEL-positive bodies within them. The mononuclear leukocytes classified as large, medium, or small reached peaks at day 2 ($7.1 \pm 1.7 \times 10^6$ cells, *n*=5), day 3 ($15.5 \pm 8.0 \times 10^6$ cells, *n*=5), and day 5 ($58.1 \pm 11.5 \times 10^6$ cells, *n*=5), respectively. Thus, the size of the bodies decreased with time. The total count of mononuclear

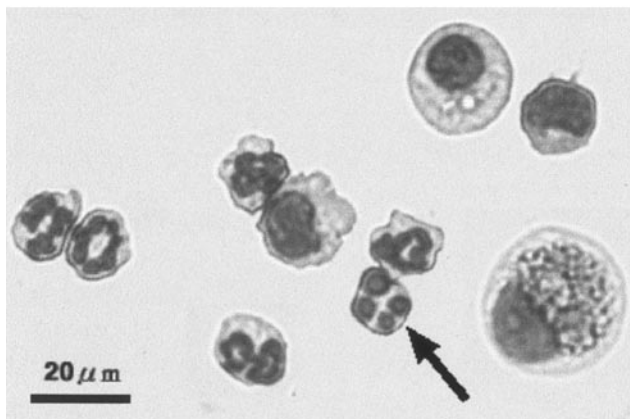
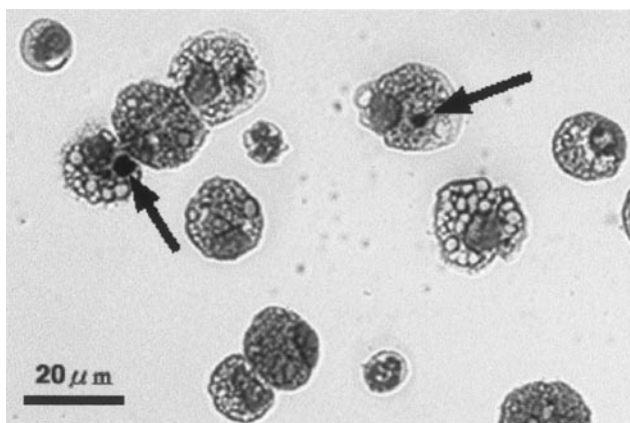


Fig. 2. The exudate cells harvested from day 3 (upper panel) and day 2 of pleurisy stained by the Wright-Giemsa method. Arrows in the upper panel indicate deep violet-colored bodies in cytoplasm of mononuclear leukocytes. An arrow in the lower panel points to a neutrophil with fragmented nuclei observed in a very rare case.

leukocytes containing TUNEL-positive bodies increased rapidly between days 1 and 2, during which the neutrophil count decreased markedly (Fig. 1), reaching a peak at day 3 ($74.4 \pm 7.6 \times 10^6$ cells, $n=5$).

Detection of single-strand DNA with acridine orange

As shown in the upper panel of **Figure 5**, bright yellow-green bodies that varied in number and size and are thought to have corresponded to the TUNEL-positive bodies were observed in mononuclear leukocytes harvested at the third day after carrageenin injection by staining with acridine orange. Similarly to the TUNEL-positive bodies, all of the acridine orange-positive bodies were observed in the cytoplasm. Hardly any bright bodies were observed in neutrophils that had not been ingested by mononuclear leukocytes. Furthermore, the nuclei of mononuclear leukocytes were hardly ever stained bright yellow-green by acridine orange.

A significant number of mononuclear leukocytes containing the acridine orange-positive bodies began to be observed from 9 h after carrageenin injection (**Fig. 6**). Mononuclear leukocytes were separately enumerated according to the size of the bright bodies that they contained, as was done with the TUNEL method. The counts of mononuclear leukocytes classified as

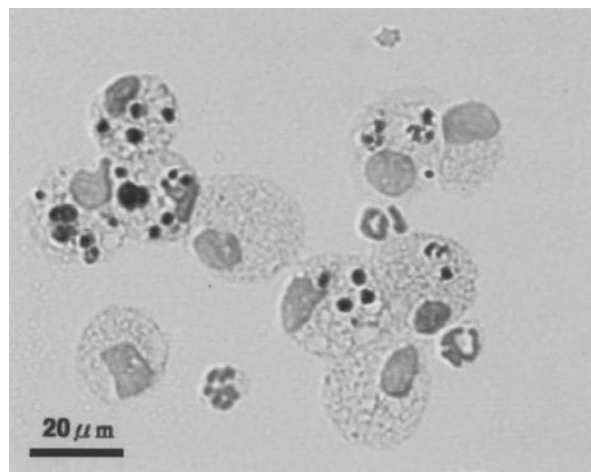


Fig. 3. The exudate cells harvested from day 3 of pleurisy stained by the TUNEL method with counter-staining by hematoxylin. Various numbers and sizes of brown-colored TUNEL-positive bodies were observed in the cytoplasm of mononuclear leukocytes.

large, medium, or small peaked at day 2 ($18.1 \pm 1.7 \times 10^6$ cells, $n=4$), day 3 ($49.2 \pm 7.7 \times 10^6$ cells, $n=4$), and day 4 ($61.2 \pm 5.3 \times 10^6$ cells, $n=4$), respectively. Thus, the sizes of the bodies decreased with time, as with the TUNEL method. The total count of mononuclear leukocytes containing the acridine orange-positive bodies increased rapidly between days 1 and 2 and reached a peak at day 3 ($114.1 \pm 8.7 \times 10^6$ cells, $n=4$). These time courses were very similar to that in the TUNEL method, although the count of cells containing acridine orange-positive bodies was 1.5–2.5 times that of those containing TUNEL-positive bodies throughout the time course.

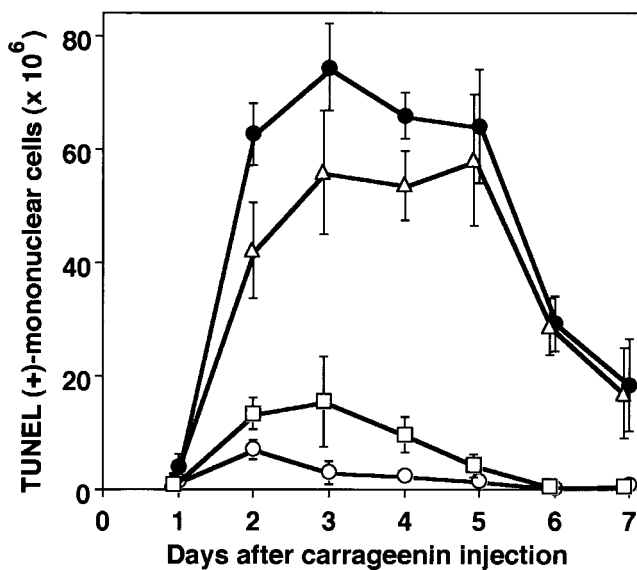


Fig. 4. Changes in the count of mononuclear leukocytes including TUNEL-positive bodies. Closed circles, total; open triangles, cells classified as small; open squares, cells classified as medium; open circles, cells classified as large. (See the Materials and Methods for details of classification.) Each value indicates the mean \pm SEM of four to six rats.

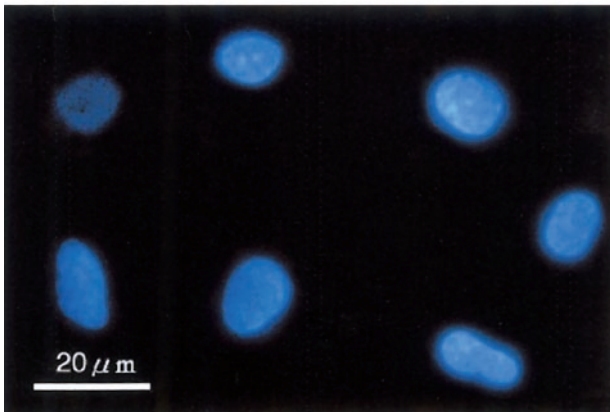
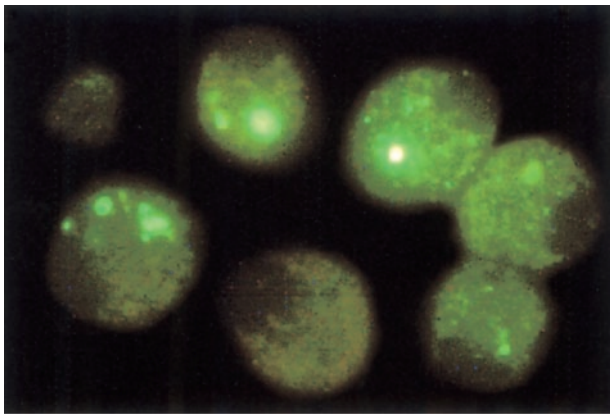


Fig. 5. The exudate cells harvested from day 3 of pleurisy stained by acridine orange (upper panel) and Hoechst 33342 (lower panel). Both panels are of the same field. In the upper panel, various numbers and sizes of bright yellow-green acridine orange-positive bodies were observed in the cytoplasm of mononuclear leukocytes. The nuclei of these cells are located in the relatively dark part of the cytoplasm in the upper panel, as indicated in the lower panel.

Detection of DNA ladder

As shown in **Figure 7**, a characteristic ladder pattern on electrophoresis was obviously observed with DNA extracted from cells 5 h after carrageenin injection but not in DNA from the peripheral blood leukocytes, pleural lavage cells of normal control rats, or pleural exudate cells harvested 3 h after irritation. The ladder pattern became clearer until ~24 h had passed. However, it again became unclear later, and the amount of small molecular weight DNA less than 180 bp started to increase in step with the detection of TUNEL-positive bodies (Fig. 4).

When mononuclear leukocytes and neutrophils were separately analyzed, the ladder pattern was detectable with neutrophils from 5 h and gradually intensified until 24 h (**Fig. 8**, upper panel). In contrast, the obvious ladder pattern from mononuclear leukocytes was first detected between 7 and 9 h (Fig. 8, lower panel), during which period the acridine orange-positive bodies started to be observed in the cells (Fig. 6).

The level of cytokines in the pleural exudates

The TNF- α level in the lavage fluid from normal control rats was below the detection limit. Its level rose sharply, reaching a peak (27.46 ± 9.99 ng, $n=4$) at 3 h. The level again declined

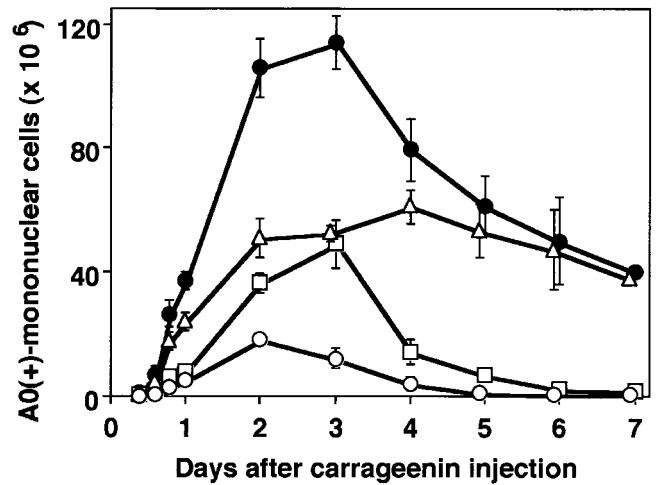


Fig. 6. Changes in the count of mononuclear leukocytes containing acridine orange (AO)-positive bodies. Closed circles, total; open triangles, cells classified as small; open squares, cells classified as medium; open circles, cells classified as large. Each value indicates the mean \pm SEM of four rats.

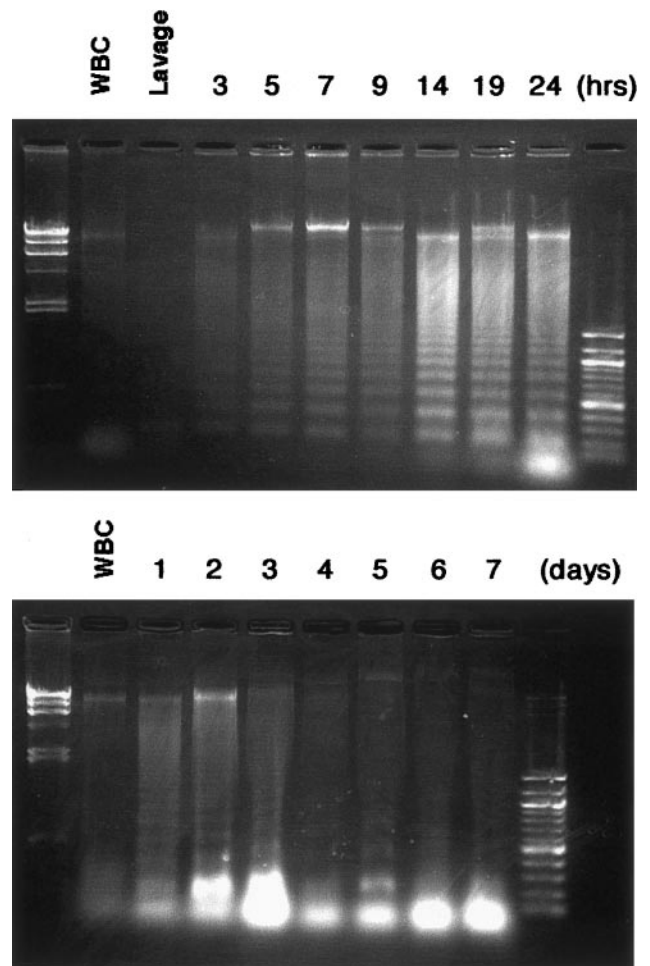


Fig. 7. The ladder pattern of the electrophoresis of DNA extracted from cells harvested at indicated time interval (hrs in the upper panel; days in the lower panel). WBC, Peripheral blood leukocytes of a normal control rat; Lavage, pleural lavage cells of a normal control rat. The left-most lane is λ HindIII marker, and the right-most lane is a ladder marker of a multiple of 100 bp in both panels. Similar results were obtained from two additional sets of experiments.

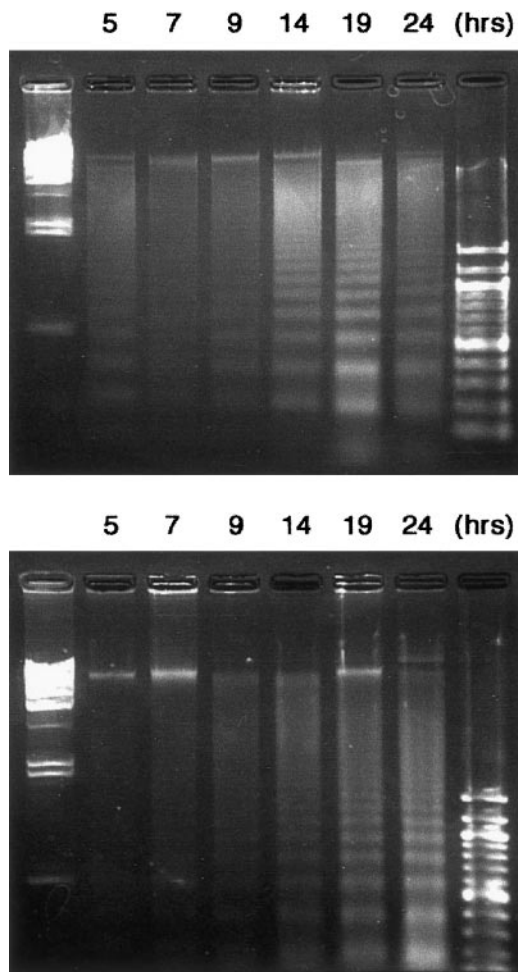


Fig. 8. The ladder pattern of the electrophoresis of DNA extracted from neutrophils (upper panel) and mononuclear leukocytes (lower panel) at the time intervals indicated. Cells were separated by elutriation. The left-most lane is λ *Hind*III marker, and the right-most lane is a ladder marker of a multiple of 100 bp in both panels. Similar results were obtained from two additional sets of experiments.

sharply and fell almost to the lavage level (0.66 ± 0.18 ng, $n=3$) by 14 h (**Fig. 9**, top panel).

The level of IL-10 in the lavage fluid from normal control rats was 0.14 ± 0.04 ($n=4$). It increased on the i.p. injection of carrageenin and peaked at 3.51 ± 1.12 ng ($n=6$) at 7 h. Its level declined by day 2 (**Fig. 9**, middle panel).

The level of TGF- β 1 in the lavage fluid from normal control rats was 1.70 ± 0.06 ng ($n=5$). It immediately started to increase after the injection of carrageenin and remained elevated until day 5 with a peak of 36.62 ± 7.35 ng ($n=5$) on day 3 (**Fig. 9**, bottom panel).

DISCUSSION

The progressive apoptosis of exudate cells and their following clearance was studied in an acute inflammatory model of rat carrageenin-induced pleurisy. The following morphological observations were made: dense bodies, using the Wright-Giemsa method (**Fig. 2**), TUNEL-positive bodies (**Fig. 3**), and acridine

orange-positive bodies (**Fig. 4**) in the cytoplasm of mononuclear leukocytes. Furthermore, the characteristic DNA ladder pattern on electrophoresis was observed with exudate cells (**Figs. 7 and 8**). All these results suggest that the pleural exudate cells undergo apoptosis, and then mononuclear leukocytes ingest the resulting apoptotic cells. It is reported that macrophages release IL-10 and TGF- β 1 when they ingest apoptotic cells but that they release TNF- α , IL-8, and thromboxane after the ingestion of necrotic cells [29]. The present study demonstrated that TNF- α was detectable in the very early phase, and the levels of IL-10 and TGF- β 1 rose in accordance with the detection of DNA ladder or other apoptotic signs. Moreover, it is reported that the thromboxane level rises at a very early stage in rat carrageenin-induced pleurisy [20]. Thus, the mediator milieu in the inflammatory site also suggests the occurrence of apoptosis. However, hardly any neutrophils indicating morphologically typical signs of apoptosis were observed. In addition, mononuclear leukocytes themselves did not show any sign of apoptosis.

The neutrophil count decreased steeply between days 1 and 3, falling below 1% of the peak at day 4. The count of mononuclear leukocytes maintained a plateau on the third day after carrageenin injection and then decreased gradually (**Fig. 1**). TUNEL assay and acridine orange staining indicated that the count of mononuclear leukocytes, including fragmented DNA, increased rapidly during the second day and reached a peak at day 3 (**Figs. 4 and 6**). Furthermore, TNF- α and IL-10,

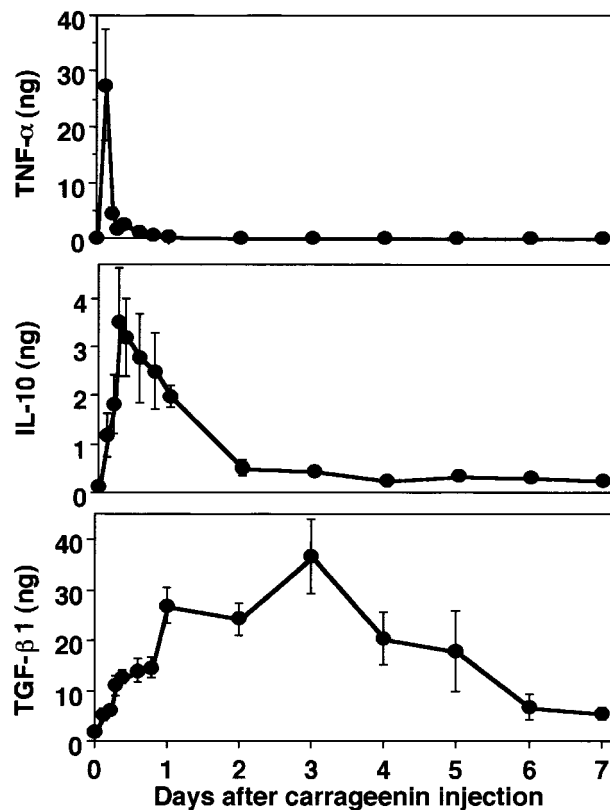


Fig. 9. Changes in the levels of TNF- α (top panel), IL-10 (middle panel), and TGF- β 1 (bottom panel). Each value indicates the total amount of cytokines detected in the individual pleural exudate, showing with the mean \pm SEM of four to eight rats.

which have the ability to promote neutrophil apoptosis [30, 31], were detectable in the early phase of this model (Fig. 9). TGF- β 1, which inhibits production of proinflammatory cytokines [32] and neutrophil apoptosis [33], was detectable in a rather late phase. Thus, phenomena indicating apoptosis paralleled the decrease of the neutrophil count. Moreover, numerically, neutrophils are obviously the largest cell population, and the DNA ladder pattern was detected only in neutrophils, until fragmented DNA was detected in the cytoplasm of mononuclear leukocytes (Figs. 6 and 8). All these results, including the mediator milieu in the inflammatory site, suggest the occurrence of neutrophil apoptosis.

Generally, DNA fragmentation of apoptotic cells proceeds through the action of their own caspase-dependent DNase. In fact, the DNA ladder pattern was detected only in neutrophils in the early phase. The ladder pattern in mononuclear leukocytes was detected in accordance with the appearance of acridine orange-positive cells (Figs. 6 and 8). Most of the fragmented DNA was degraded to a mononucleosomal unit by 24 h (Fig. 7), at which point TUNEL-positive bodies started to be detected (Fig. 4). Furthermore, when mononuclear leukocytes were separately enumerated, according to the size of the TUNEL-positive bodies or acridine orange-positive bodies that they contained, their size gradually decreased with time (Figs. 4 and 6). These results agree with a report indicating the progression of DNA fragmentation after ingestion by lysosomal DNase of phagocytes [34]. There was a difference in the time at which detection of DNA fragmentation occurred with each method: with a TUNEL assay, from 24 h; with acridine orange staining, from 9 h; and with a DNA ladder, from 5 h. These differences may have been caused by the differences in their detection limits, a part of which may depend on the experimental procedure we used. The DNA ladder method is the most sensitive for the detection of fragmented DNA in our experimental setting. The detection limit differences may also explain the discrepancy in the counts of mononuclear leukocytes between the TUNEL assay and the acridine orange-staining method at each observation time point. As demonstrated in the present study, hardly any morphologically apoptotic neutrophils were detectable. All these results suggest that apoptotic neutrophils may be promptly ingested by mononuclear leukocytes. Newman et al. [3] suggested on the basis of in vitro experiments that the uptake and degradation processes are so rapid as to render it difficult to make confident judgments of the morphology of the majority of ingested neutrophils. Phosphatidylserine is localized in the inner leaflet of the plasma membrane bilayer in normal cells. However, the lipid appears in the outer leaflet of the plasma membrane in apoptotic cells, because of a failure to keep membrane integrity [6]. The exposure of phosphatidylserine is widely used for detecting apoptotic cells in the early phase by specific binding with annexin V [35].

The lifespan of neutrophils is very short: 8–20 h in peripheral blood [13]. It has been shown in vitro that this lifespan can be significantly prolonged and its apoptosis significantly delayed by endothelial transmigration [14] or by proinflammatory mediators, certain cytokines, and glucocorticoids [15–17]. However, as demonstrated in the present study, the DNA ladder started to be detectable 5 h after the onset of inflam-

mation, at which time neutrophil accumulation had just started. Furthermore, half of the recruited neutrophils had disappeared from the inflamed site by the end of day 2 (Fig. 1). Therefore, the present results suggest that the lifespan of neutrophils may not be prolonged by much in this acute inflammatory model, although the neutrophil count in the inflammatory site may be determined by the balance between mobilization from the peripheral blood and clearance after apoptosis.

Taken together, these results suggest that neutrophil apoptosis may start at 5 h at the latest, at which time plasma exudation is still high [20], and neutrophils have just started to accumulate in this acute inflammatory model (Fig. 1). Therefore, the evolution and resolution in acute inflammation may not be easily separable but rather, are overlapping processes. Dysregulated granulocyte apoptosis and thus the impaired resolution process have been implicated in the pathogenesis of chronic inflammatory diseases such as adult respiratory distress syndrome [36], idiopathic pulmonary fibrosis [37], ulcerative colitis [38], rheumatoid arthritis, other autoimmune diseases [39, 40], and asthma and other allergic diseases [41]. If this is correct, elucidation of the mechanisms by which acute inflammation normally resolves is likely to provide new insights into the pathogenesis of the persistent inflammatory states that characterize chronic inflammatory diseases and generate new therapeutic targets.

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