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Lipopolysaccharide and Granulocyte Colony-Stimulating Factor Delay Neutrophil Apoptosis and Ingestion by Guinea Pig Macrophages

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In previous studies, neutrophil-ingesting macrophages were clearly and easily observed in the peritoneal cavity of guinea pigs after intraperitoneal injection of thioglycolate medium, and phagocytosis of neutrophils by macrophages could be detected in in vitro cultures of peritoneal exudate cells. Using an in vitro system, we examined the effect of bacterial lipopolysaccharide and recombinant human granulocyte colony-stimulating factor on the apoptosis (programmed cell death) of neutrophils and their subsequent ingestion by macrophages. Lipopolysaccharide delayed karyopyknosis and apoptosis of neutrophils, as shown by endogenous endonuclease activity and a high proportion of trypan blue-excluding cells, and subsequent ingestion by autologous macrophages. Granulocyte colony-stimulating factor also delayed neutrophil karyopyknosis and ingestion by macrophages. When a thioglycolate medium was coinjected intraperitoneally with lipopolysaccharide into guinea pigs in the in vivo system, delays in neutrophil disappearance and ingestion by macrophages in the peritoneal cavity were also observed. We suggest that bacterial products and cytokines regulate neutrophil apoptosis and subsequent ingestion by macrophages at inflamed sites.

When inflammation is triggered by microbes or irritants, many neutrophils and macrophages infiltrate the site within several hours. Neutrophils accumulate primarily to phagocytose and kill the microbes or to scavenge the irritants. To accomplish this, neutrophils have about 20 lysosomal enzymes. However, the antimicrobial system may be toxic to host tissue (26, 33). For normal resolution of inflammation, neutrophils must act without disgorging their toxic contents at inflamed sites. In fact, neutrophils disappear from inflammatory sites without expanding tissue injury in the resolution stage of acute inflammation.

The establishment of the concept of apoptosis clarified that neutrophils also die by the process of apoptosis (16, 24, 34). Neutrophil apoptosis occurs in the same way as that of other cell types. Light-microscopic features of neutrophil apoptosis are pyknotic change of nucleus and decreased cell size and number of cytoplasmic granules. These changes take place without loss of membrane integrity, as shown by trypan blue exclusion (24). Endogenous endonuclease activity, which is shown by a ladder pattern of DNA after electrophoresis, is also a characteristic of apoptosis (24).

It is known that macrophages phagocytose senescent or apoptotic neutrophils in vitro (3, 18, 23, 24, 37). The phenomenon is observed in an in vivo experimental model (21) and in clinical materials (9, 13, 20, 27). We reported previously that when guinea pigs were injected intraperitoneally (i.p.) with bacteria or irritants, neutrophil-ingesting macrophages were clearly observed among the peritoneal exudate cells (PECs) (21). The data suggested that almost all infiltrating neutrophils in the peritoneal cavity were phagocytosed by macrophages. Furthermore, we recently reported that neutrophil-ingesting macrophages were clearly observed when inflammatory PECs of guinea pigs were cultured in vitro (37).

In the in vitro system, the mechanism regulating the ability of macrophages to ingest neutrophils could be analyzed more precisely. Ingestion of neutrophils by macrophages can be regulated by factors which affect neutrophil survival or neutrophil apoptosis, including bacterial products, serum components, and cytokines. We reported previously that serum, especially its globulin-rich fraction, suppressed the karyopyknosis of neutrophils and their ingestion by guinea pig macrophages in vitro (37). Among the bacterial products, lipopolysaccharide (LPS) is reported to increase neutrophil survival and suppress apoptosis (11). Granulocyte colony-stimulating factor (G-CSF) is a monokine which is produced by macrophages after stimulation by LPS (17, 19), and it also suppresses the apoptosis of neutrophils (34).

In our present study, we examined the effect of LPS and recombinant human G-CSF (rhG-CSF) on neutrophil apoptosis in in vitro cultures of guinea pig PECs. We report here that LPS and G-CSF delay apoptosis and subsequent ingestion of neutrophils by autologous macrophages. The effect of LPS was also examined in vivo.

MATERIALS AND METHODS

Animals. Female guinea pigs of the outbred Hartley strain weighing 450 to 550 g were purchased from Seiwa Experimental Animal Co. (Oita, Japan) and used in this study.

Reagents. Phenol-extracted *Escherichia coli* (O127:B8) LPS was purchased from Difco Laboratories, Detroit, Mich. rhG-CSF was donated by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). rhG-CSF was dissolved in a solution containing 0.1% human albumin, 0.05% gelatin, and 1% mannitol. The solution alone was used as a control in the rhG-CSF experiment. RPMI 1640 medium was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Thioglycolate medium

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was from Eiken Chemical (Tokyo, Japan). These media were dissolved in pyrogen-free water (Otsuka Pharmaceutical Co., Naruto, Japan).

Preparation of PECs. Guinea pigs were injected i.p. with 10 ml of 3% thioglycolate medium. Fifteen hours after the injection, the guinea pigs were bled by cardiac puncture, and PECs were collected in RPMI 1640 medium containing 10 U of heparin per ml. The number of viable cells was determined by the trypan blue dye (0.1%) exclusion method on a hemacytometer. Differential cell counts were done on Wright-Giemsa-stained smears of PECs. After being washed twice with RPMI 1640 medium by centrifugation, PECs were suspended in the same medium supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Nakalai Tesque, Inc., Kyoto, Japan) and 100 U of penicillin G per ml and 100 μg of streptomycin per ml, and adjusted to a concentration of 6.25×10^6 cells per ml. The cells were processed for the separation of neutrophils and for the in vitro assay for neutrophil-ingesting macrophages.

Separation of neutrophils from PECs. Neutrophils were separated from PECs by density gradient centrifugation. PECs (10^8) in 4 ml of phosphate-buffered saline (PBS) were poured gently onto 3 ml of GP-SMF (Japan Immunoresearch Laboratories Co., Ltd., Takasaki, Japan) in a 15-ml conical tube. After centrifugation at $150 \times g$ for 30 min at room temperature, the supernatant was discarded and the cell pellet was washed twice with RPMI 1640 medium by centrifugation. Neutrophils were suspended in the same medium supplemented with 20 mM HEPES, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml and adjusted to a concentration of 2.5×10^6 cells per ml. Neutrophil preparations contained approximately 0.8% macrophages.

In vitro culture of neutrophils. The neutrophil suspension was used for the following three assays by culturing 0.25 ml per well on eight-well chamber slides (Lab-Tek 4808; Nunc Inc., Naperville, Ill.) to assess the rate of karyopyknosis, 0.5 ml per well on a 24-well tissue culture plate (Falcon 3047) for the trypan blue exclusion assay, and 4 ml per well on a six-well tissue culture plate (Falcon 3046) for DNA extraction.

Assessment of karyopyknosis and trypan blue exclusion. Inflammatory neutrophils could adhere to the bottom of a chamber slide or a 24-well tissue culture plate (Falcon no. 3047). Karyopyknosis of the neutrophils was assessed by Wright-Giemsa staining of the adhering cells on eight-well chamber slides after washing gently twice with Dulbecco's PBS. Neutrophils in which the nuclei shrank in size and the chromatin condensed to a solid, structureless mass were judged as undergoing karyopyknosis. The number of detached cells in the culture supernatant and the PBS wash was also counted on a hemacytometer. The rate of karyopyknosis of detached cells was also assessed by Wright-Giemsa staining of cell smears on slides. Trypan blue dye exclusion was assessed with an inverted microscope after 0.1 ml of 0.5% trypan blue was added to the neutrophil monolayers.

DNA extraction. DNA was isolated from neutrophils by the following procedure. Adherent neutrophils were scraped off the tissue culture plate with a rubber policeman in proteinase K buffer containing 100 mM Tris-HCl (pH 7.5), 12.5 mM EDTA, 150 mM NaCl, 1% sodium dodecyl sulfate, and 200 μg of proteinase K per ml. The suspension was incubated for 3 h in a water bath at 50°C. The same volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the suspension and then extracted three times. After extraction with chloroform once, DNA was precipitated by adding 2 volumes of 99% ethanol. DNA was dissolved in 5 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). RNase A was then added (100 μg/ml), and incubation at 37°C was continued for 1 h. The treatment with proteinase K and extraction with phenol and chloroform were repeated once, and the samples were then precipitated with ethanol at −20°C. The DNA precipitates were pelleted by centrifugation at $13,000 \times g$ for 10 min, washed with 70% ethanol, air dried for 5 min, resuspended in TE buffer, and stored at 4°C.

DNA electrophoresis. A sample containing 30 μg of DNA was loaded into each lane of a 1% agarose gel. Electrophoresis was carried out at 7.5 V/cm for 25 min in 40 mM Tris-acetate (pH 8.0)–1 mM EDTA (TAE buffer). An *Eco*T14I digest of λ DNA was used as size markers. At the end of the run, the gels were stained with 0.5 μg of ethidium bromide per ml and photographed with a UV transilluminator.

In vitro assay for neutrophil-ingesting macrophages. PEC suspension (200 μl) was mixed with 25 μl of LPS-free RPMI 1640 medium or RPMI 1640 medium containing various amounts of LPS samples and then made up to 250 μl with RPMI 1640 medium. Each mixture was transferred to eight-well chamber slides and incubated at 37°C in a CO_2 incubator for up to 3 days. At intervals during incubation, the culture medium was discarded and the wells were washed gently twice with PBS. After drying, the slides were stained with Wright-Giemsa solution (Muto Pure Chemicals Ltd., Tokyo, Japan). The stained slides were observed under a light microscope, and the percentage of neutrophil-ingesting macrophages among 200 to 500 randomly observed macrophages was determined. Neutrophils in which the nuclei shrank in size and the chromatin condensed to solid, structureless masses (that is, pyknotic change) were examined simultaneously. The percentage of neutrophils showing pyknotic change was calculated after examining 200 neutrophils. The number of detached cells in the culture supernatant and the PBS wash was also counted on a hemacytometer. The percentages of macrophages and karyopyknosis of neutrophils in detached cells were assessed by Wright-Giemsa staining of cell smears on slides.

In vivo assay for neutrophil-ingesting macrophages. Ten milliliters of 3% thioglycolate medium alone or with 10 μg of LPS was injected i.p. into guinea pigs to induce chemical inflammation. PECs were washed out of the peritoneal cavity with 50 ml of RPMI 1640 medium containing 10 U of heparin per ml at intervals after the injection of an irritant. The total number of harvested live cells was determined by the trypan blue dye exclusion method. The cells were washed twice by centrifugation, and guinea pig serum was added to the pellet. Smears of the cells were made on slides, dried, and stained with Wright-Giemsa solution. Neutrophils, macrophages, and neutrophil-ingesting macrophages were counted under an oil immersion objective lens (×1,000).

Presentation of data. The experiments were repeated at least three times with one or two guinea pigs in each experiment. Data are shown as means ± standard deviations. Statistical analysis was carried out by Scheffe's test with the SAS System (SAS Institute, Inc., Cary, N.C.). *P* values of less than 0.05 were taken as significant.

RESULTS

Effect of LPS on karyopyknosis of and trypan blue exclusion by cultured neutrophils. Neutrophils were separated from PECs by density gradient centrifugation. The preparation contained >98% neutrophils, and their viability was also

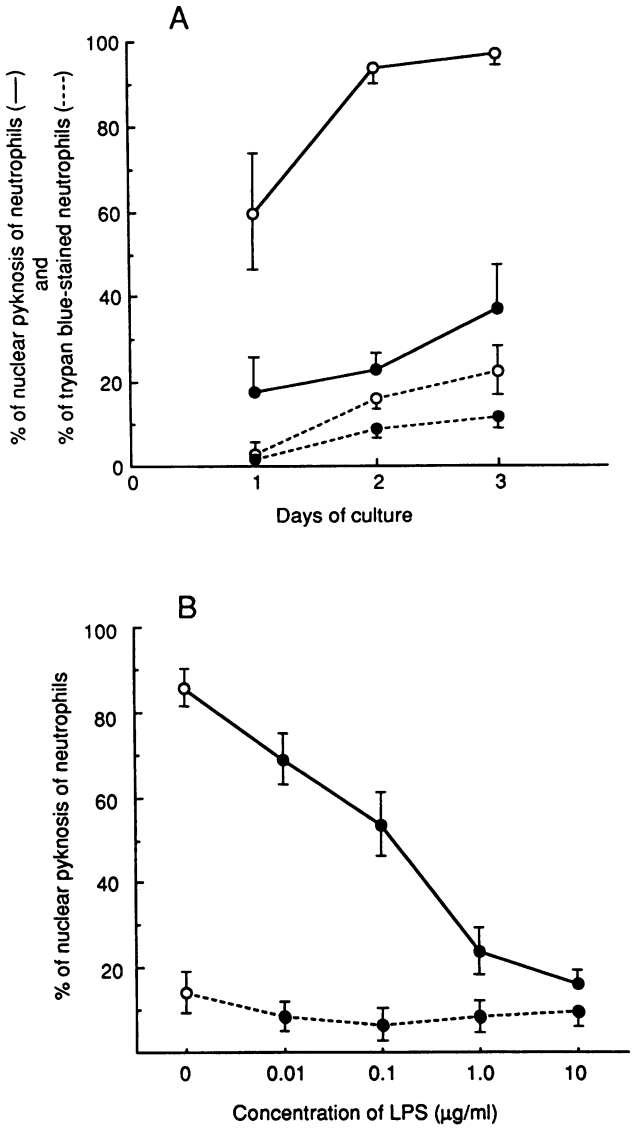

FIG. 1. Effect of LPS on proportion of neutrophil karyopyknosis and trypan blue-positive neutrophils in vitro. (A) Time course of effect of LPS. Separated neutrophils were cultured in RPMI 1640 medium with (●) or without (○) LPS (1 μg/ml). At intervals, the proportions of pyknotic neutrophils were assayed after Wright-Giemsa staining. Proportions of trypan blue-stained cells were assayed in 24-well tissue culture plates. (B) LPS dose-response. Separated neutrophils were cultured in the presence of LPS for 2 days and assayed for karyopyknosis (——) and trypan blue-stained neutrophils (- - - -). The data represent the means ± standard deviations for three guinea pigs.

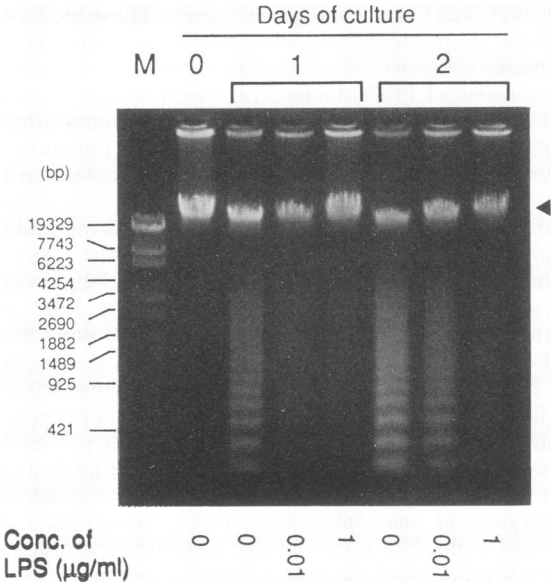

FIG. 2. Electrophoresis of DNA extracted from neutrophils cultured with LPS at various concentrations. DNA was extracted from separated neutrophils, and 30 μg of DNA was electrophoresed in a 1% agarose gel. DNA was detected by UV fluorescence after ethidium bromide staining. Uncleaved high-molecular-weight DNA is shown by an arrowhead. Lane M contains size markers, and DNA sizes are shown on the left (in base pairs).

>98%. Neutrophils were cultured in RPMI 1640 medium with and without LPS in chamber slides or 24-well tissue culture plates, the former for the assay of karyopyknosis and the latter for the trypan blue dye exclusion assay. When neutrophils were cultured without LPS, 60% of the neutrophils became pyknotic on day 1, rising to more than 90% on days 2 and 3 (Fig. 1A). In contrast, 3% of the cells were nonviable (trypan blue positive) on day 1 and 15 to 20% were nonviable on days 2-3. When LPS (1 μg/ml) was added, the rate of karyopyknosis was suppressed, from 17% on day 1 to 37% on day 3. With LPS (1 μg/ml), there were 8% trypan blue-positive cells on day 2 and 11% on day 3. Shown in Fig. 1B is the effect of LPS dose on the percentages of karyopyknosis and trypan blue-positive cells on day 2 of culture. The rate of pyknosis was suppressed as the LPS concentration rose. There were fewer than 10% trypan blue-positive neutrophils at all LPS concentrations examined.

Effect of LPS on neutrophil endonuclease activity. Separated neutrophils were cultured in RPMI 1640 medium with 0, 0.01, or 1 μg of LPS per ml for 1 to 2 days. Extracted DNA (30 μg) was electrophoresed in a 1% agarose gel (Fig. 2). DNA extracted from neutrophils just after lavaging (day 0) was retained as uncleaved high-molecular-weight DNA. On day 1 of culture without LPS, the DNA showed a dense ladder pattern. The ladder pattern became weak when LPS (0.01 or 1 μg/ml) was added to the culture. As the ladder pattern became weaker, the amount of uncleaved high-molecular-weight DNA increased. On day 2 of neutrophil culture, the ladder pattern became clearer, but the pattern became weaker as the LPS concentration became higher. From the size markers, the distance between each rung of the ladder was calculated as 208.0 ± 25.1 bp.

Effect of LPS on number of neutrophil-ingesting macrophages relative to karyopyknosis of neutrophils. PECs were cultured in chamber slides with and without LPS. At 1, 2, and 3 days of culture, the number of macrophages ingesting neutrophils and the number of pyknotic neutrophils were counted after Wright-Giemsa staining. Without LPS, the proportion of macrophages ingesting neutrophils was 3% on day 1 and approximately 20% on days 2 and 3. There were 80% pyknotic neutrophils on day 2 and 90% on day 3 (Fig. 3A). A light micrograph of PECs on day 2 of culture without LPS is shown in Fig. 4A. Macrophages ingesting neutrophils are observed among the pyknotic neutrophils. With 1 μg of LPS per ml, the proportion of macrophages ingesting neu-

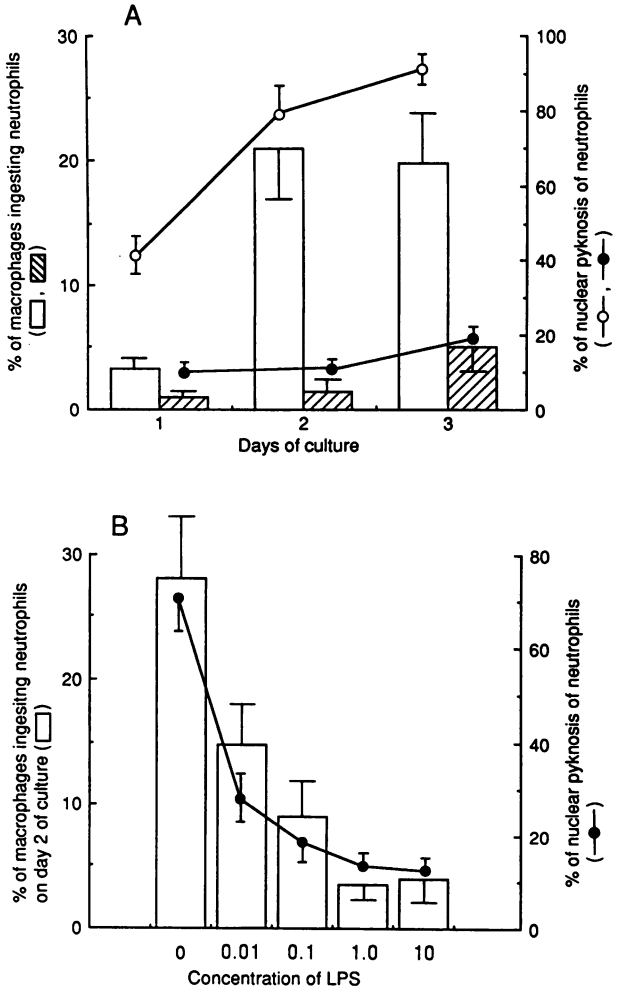

FIG. 3. Effect of LPS on proportion of neutrophil-ingesting macrophages in relation to karyopyknosis of neutrophils. (A) Time course. PECs were cultured in chamber slides, and at intervals, the cells were stained with Wright-Giemsa solution. Macrophages ingesting neutrophils (bars) and neutrophils undergoing karyopyknosis (circles) in the absence (open bars and circles) or presence of LPS (1 μg/ml) (hatched bars and solid circles) were assayed under a light microscope (×1,000). (B) LPS dose-response. PECs were cultured in chamber slides for 2 days, and the proportions of macrophages ingesting neutrophils and neutrophils undergoing karyopyknosis were assayed under a light microscope (×1,000) after Wright-Giemsa staining. The data are the means ± standard deviations for five guinea pigs.

trophils was less than 2% on days 1 and 2 and 5% on day 3, and the rate of pyknotic change of neutrophils was less than 20% for days 1 to 3 (Fig. 3A). A light micrograph of PECs on day 2 of culture with LPS (1 μg/ml) is shown in Fig. 4B. The effect of LPS dose on the proportion of macrophages ingesting neutrophils on day 2 of culture is shown in Fig. 3B. The proportion was suppressed dose-dependently by LPS, parallel to the rate of pyknotic change among the neutrophils.

Effect of G-CSF on proportion of nuclear pyknosis of neutrophils and of macrophages ingesting neutrophils. Separated neutrophils were cultured in RPMI 1640 medium in chamber slides with various concentrations of rhG-CSF. At intervals, neutrophils were stained with Wright-Giemsa solution, and pyknotic change was assayed (Fig. 5A). With more than 2 ng of rhG-CSF per ml, neutrophil pyknosis was significantly suppressed. Next, PECs were cultured with rhG-CSF, and the proportion of macrophages ingesting neutrophils was assayed (Fig. 5B). The appearance of neutrophil-ingesting macrophages was significantly suppressed by the addition of more than 2 ng of rhG-CSF on day 2 and more than 20 ng on day 3.

Assessment of detached cells during culture. The number of detached cells in the chamber slides was assayed with three guinea pigs. During the period of culture, separated neutrophils adhered well to the bottom. In the control culture, 3 to 5% of the cells detached on days 1 and 2, and 6 to 10% detached on day 3. When LPS (0.01 to 1 μg/ml) was added to the culture, the rate was 2 to 4% on days 1 and 2 and 4 to 10% on day 3. When rhG-CSF (2 to 50 ng/ml) was added, the rate was 3 to 6% on day 1, 5 to 10% on day 2, and 6 to 15% on day 3.

The percentages of pyknotic neutrophils were parallel to the data for adherent neutrophils shown in Fig. 1A and 5A. More cells detached in PEC cultures than in neutrophil cultures. In the control culture, rates of cell detachment were 11 to 14% on days 1 and 2 and 20 to 26% on day 3. The presence of LPS did not change the rate. rhG-CSF accelerated the detachment; the rate was 12 to 22% on day 1, 16 to 28% on day 2, and 17 to 30% on day 3. Macrophages constituted less than 10% of total detached cells in all experiments.

Effect of LPS on proportion of neutrophil-ingesting macrophages in vivo. When guinea pigs were injected i.p. with thioglycolate medium, $\sim 5 \times 10^8$ neutrophils infiltrated the peritoneal cavity on day 1, and the number decreased to $\sim 10^6$ on day 4 (Fig. 6A). The number of macrophages was about 10^8 during this period. The proportion of macrophages ingesting neutrophils reached a peak on day 2, at 20%. When 10 μg of LPS was injected i.p. simultaneously with thioglycolate medium (Fig. 6B), the disappearance of neutrophils was delayed; $\sim 10^7$ neutrophils still remained in the peritoneal cavity on day 4. The proportion of macrophages ingesting neutrophils reached a peak on day 3, that is, a 1-day delay in comparison to the result obtained when thioglycolate medium alone was injected.

DISCUSSION

LPS and G-CSF delayed both karyopyknosis and apoptosis of neutrophils and their subsequent ingestion by macrophages in vitro. The effect of LPS was also observed in vivo; i.p. coinjection of LPS with thioglycolate medium delayed by 1 day the peak of the proportion of neutrophil-ingesting macrophages. More free neutrophils that were not phagocytosed remained in the peritoneal cavity than after a thioglycolate-only injection.

It has been reported that LPS enhances several activities of neutrophils, including adherence (4), enzyme secretion (8), and release of oxygen metabolites (1, 10). Recently, Haslett et al. showed in a preliminary report that LPS inhibited apoptosis of neutrophils (11). We confirmed here that *E. coli* LPS suppressed apoptosis of neutrophils in vitro. The apoptosis was proved by fragmentation of DNA into integer multiples of ~200 bp and by pyknotic change of nuclei without loss of membrane integrity (by trypan blue dye exclusion).

The mechanisms by which LPS suppresses neutrophil apoptosis were not elucidated here. In addition to the direct effect of LPS, cytokines produced by LPS-stimulated neutrophils and contaminating macrophages may play a role in

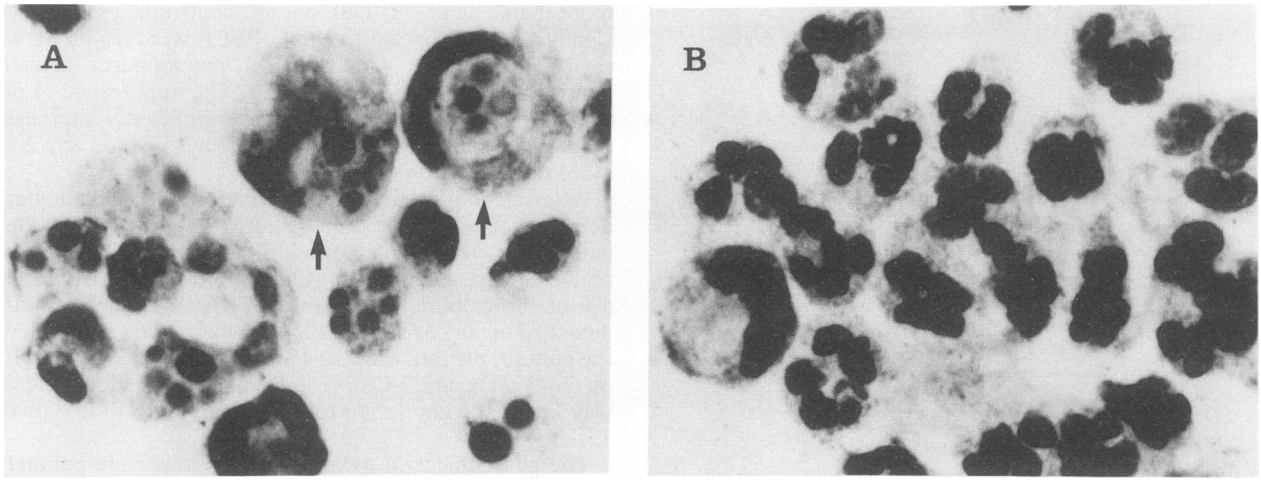

FIG. 4. Light micrographs of macrophage-neutrophil interaction and effect of LPS. (A) PECs were cultured without LPS for 2 days. About 80% of all neutrophils underwent karyopyknosis. Macrophage-ingesting neutrophils are indicated by arrows. (B) PECs were cultured with LPS (1 μg/ml) for 2 days. Only a few neutrophils underwent karyopyknosis.

suppression. Among the cytokines, G-CSF was shown to suppress neutrophil apoptosis (discussed below), but it has been reported that tumor necrosis factor alpha accelerated the apoptosis of human neutrophils (29). In any case, the presence of LPS suppressed the appearance of neutrophil-ingesting macrophages by inhibiting neutrophil apoptosis.

In this study, we showed that rhG-CSF suppressed karyopyknosis of guinea pig neutrophils and subsequent ingestion by autologous macrophages. It has been reported that G-CSF enhanced the survival of human neutrophils (2) and promoted survival of a mouse bone marrow cell line by suppressing apoptosis (34). G-CSF is produced by LPS stimulation from mouse peritoneal cells (17), a simian virus 40-transformed mouse macrophage cell line (19), and human monocytes (30). It has also been reported that the level of G-CSF is elevated in the serum of patients who are infected (32). It is suggested that, in addition to the direct effect of LPS on neutrophils, G-CSF produced from macrophages delayed neutrophil apoptosis and subsequent ingestion by macrophages at inflammatory sites.

Although our data strongly suggest that apoptotic change of neutrophils is the most important factor inducing phagocytosis by autologous macrophages, there are factors that may affect the proportion of neutrophil-ingesting macrophages in both in vitro and in vivo experiments. Because both neutrophils and macrophages adhered to the glass, macrophages need to migrate in order to come in contact with and ingest neutrophils. If LPS and G-CSF have an inhibitory effect on the in vitro migration of macrophages, subsequent ingestion of neutrophils may be suppressed. As far as we know, there is only one study showing a slight inhibitory effect of LPS on the in vitro migration of macrophages obtained from LPS-sensitive mice (31). In our in vitro system, in which the cells are crowded, the inhibition of macrophage migration may have only a minimal effect on the proportion of neutrophil-ingesting macrophages.

The data for karyopyknosis, trypan blue exclusion, and neutrophil-ingesting macrophages were calculated after counting adherent cells. If large numbers of cells are detached, the data cannot reflect the precise proportion of apoptotic neutrophils, but the number of detached cells as a percentage of initial cell numbers during neutrophil culture was less than 15% and the rate of pyknotic neutrophils in detached cells was almost parallel to the rate in adherent cells. This means that the rate of apoptotic change of adherent neutrophils was barely influenced by the presence of less than 15% detached cells.

The number of detached cells increased when PECs were cultured. Because detached macrophages constituted less than 10% of the total detached cells, the data will reflect the precise rate of neutrophil-ingesting macrophages. In the in vivo experiment (Fig. 6), our results were obtained from lavaged PECs, and cells adhering in the peritoneal cavity could not be analyzed by our method. Therefore, even with our results, we cannot avoid the possibility that LPS may modify cell adhesion and subsequent cell recovery by lavage. However, because there was no great difference in the total cell yield and cell composition, it is unlikely that the differences in the time course to appearance of neutrophil-ingesting macrophages (Fig. 6A and B) were caused by the differential effects of cell adhesion on the peritoneum.

There are two processes in the death of neutrophils at inflammatory sites. The first is cell lysis due to membrane damage by chemical injury and bacterial toxins, such as leukocidin. In the case of cell lysis, inflammatory reaction and tissue injury may increase when the cytotoxic granular proteins of neutrophils are released. Another mechanism is apoptosis (programmed cell death). The concept of apoptosis was established by Kerr and colleagues (12, 25). Apoptotic cells or apoptotic bodies are ingested into neighboring intact cells. Apoptotic neutrophils are ingested by macrophages. In this case, an inflammatory reaction does not take place because they are ingested by macrophages, keeping cell membranes intact. Wyllie and his colleagues found that apoptosis is associated with endogenous endonuclease activation, detected by the ladder pattern in DNA electrophoresis (35, 36).

Ingestion of neutrophils by macrophages has been studied both in vitro and in vivo. Among the in vitro studies, Brewer found that neutrophils are ingested by guinea pig macrophages (3). The phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit inflammatory macrophages was reported by Newman et al. (18). Savill et al. reported that apoptosis of neutrophils leads to

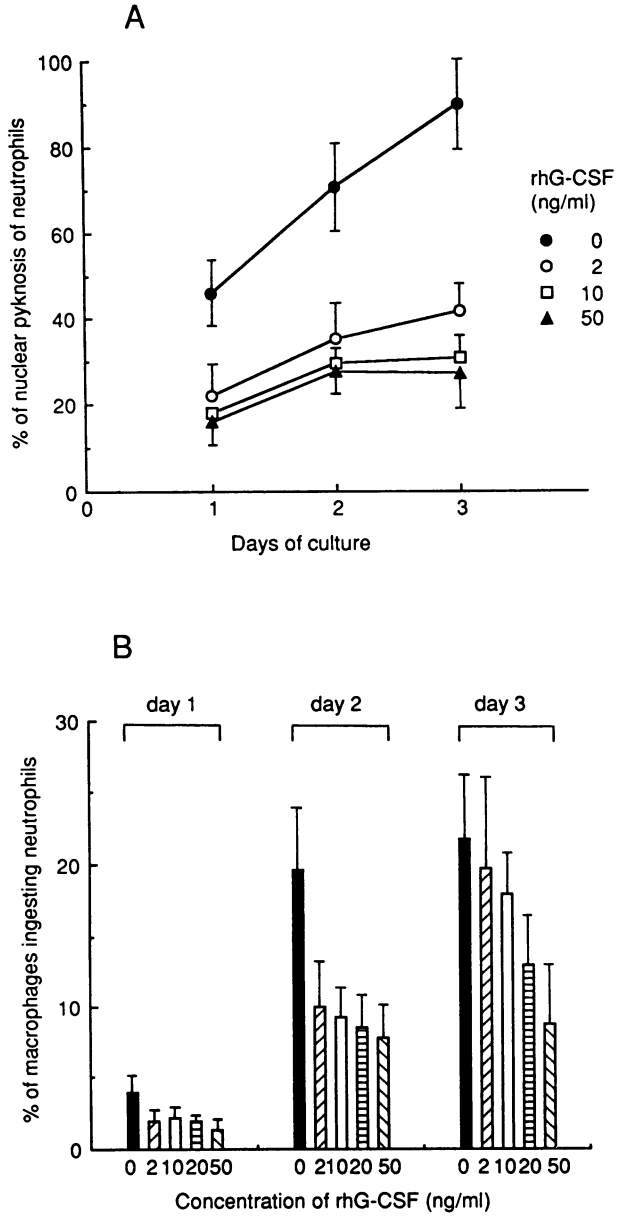

FIG. 5. Effect of rhG-CSF on nuclear pyknosis of neutrophils (A) and ingestion of neutrophils by macrophages (B). (A) Separated neutrophils were cultured with various concentrations of rhG-CSF for up to 3 days. At intervals, the number of pyknotic neutrophils was determined after Wright-Giemsa staining. (B) PECs were cultured in the presence of rhG-CSF, and the percentage of macrophages ingesting neutrophils was assayed after Wright-Giemsa staining. The data are the means ± standard deviations for six guinea pigs.

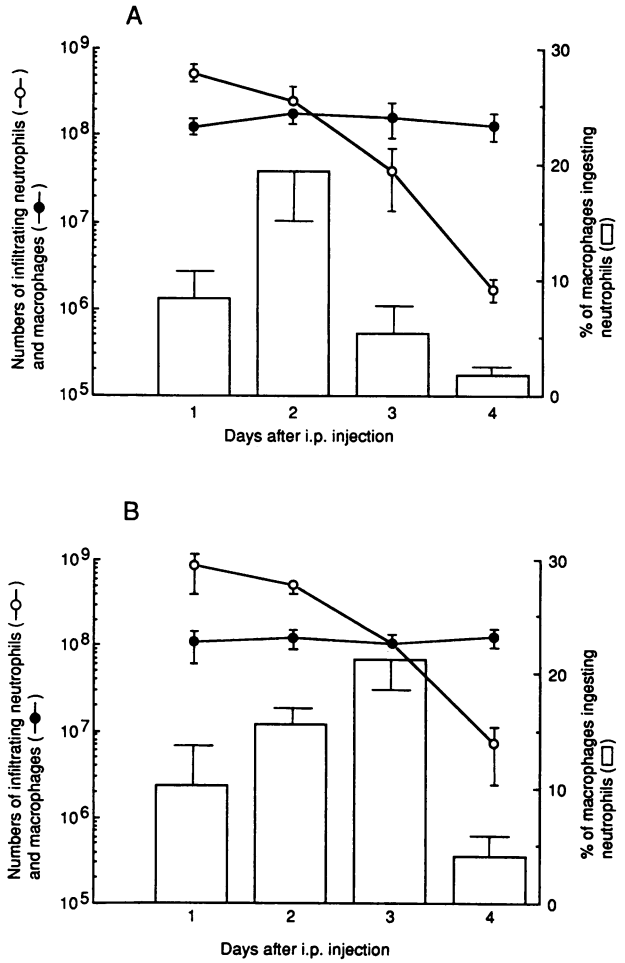

FIG. 6. In vivo effect of LPS on the proportion of neutrophil-ingesting macrophages induced by thioglycolate medium. Guinea pigs were injected i.p. with 10 ml of 3% thioglycolate medium alone (A) or plus 10 μg of LPS (B). At intervals, PECs were harvested, and the numbers of neutrophils and macrophages were counted. The ratios of neutrophil-ingesting macrophages to total peritoneal macrophages (open bars) were calculated. The data are the means ± standard deviations for six guinea pigs.

their ingestion by macrophages (24). Phagocytosis was observed in human bone marrow (6).

An in vivo study was also carried out by us. We reported previously that abundant neutrophil-ingesting macrophages could be clearly detected in an acute inflammation of the guinea pig peritoneal cavity (21). These cells are also found in clinical materials. The joint fluid of Reiter's disease patients contained macrophages that have engulfed neutrophils (20, 27). The cells were also found in bronchoalveolar lavage fluid from an inflamed airway (9) and in the injured corneas of cynomolgus monkeys (13). The above evidence implies that the phenomenon is a very common mechanism for eliminating neutrophils from inflammatory sites.

The mechanisms by which macrophages recognize "aged" neutrophils have also been reported. A diminution in sialic acid contents (5) accompanied by a decreased negative charge (23) may be responsible for this recognition. Savill et al. found that in vitro phagocytosis of neutrophils by human monocytes was inhibited in the presence of vitronectin (22), implying vitronectin-mediated phagocytosis. In our guinea pig models, neither calf fibronectin nor fibronectin attachment peptide could inhibit the appearance of neutrophil-ingesting macrophages (unpublished observation). The difference in the two results is thought to be due to the difference in cell sources, i.e., human peripheral blood versus guinea pig PECs. We reported that in the case of hyperthermia (42°C)-induced ingestion, complement may mediate the phagocytosis (37). A carbohydrate-dependent mechanism may be working in the ingestion of apoptotic

thymocytes by mouse macrophages (7) and in the ingestion of neutrophils by human blood monocytes (23). Further experiments are necessary to elucidate the recognition mechanism in our experimental system.

It has been reported that the apoptosis of macrophages was suppressed by LPS (15) and LPS-associated protein (14). In the case of eosinophils, interleukin-5 delayed aging of the cells, and they escaped from phagocytosis by macrophages (28). These results show that apoptosis of not only neutrophils but also macrophages and eosinophils is regulated by LPS and/or cytokines. Determining the regulation of apoptosis of inflammatory cells will give new insight into the nature of inflammation.

We suggest that neutrophil apoptosis is regulated by bacterial products, serum components, cytokines, etc. Neutrophil apoptosis and subsequent ingestion by macrophages play an important role in the termination of acute inflammation, but participation at chronic inflammatory sites remains to be investigated.

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REFERENCES

1. **Aida, Y., and M. J. Pabst.** 1991. Neutrophil responses to lipopolysaccharide: effect of adherence on triggering and priming of the respiratory burst. J. Immunol. **146:**1271–1276.
2. **Begley, C. G., A. F. Lopez, N. A. Nicola, D. J. Warren, M. A. Vadas, C. J. Sanderson, and D. Metcalf.** 1986. Purified colony-stimulating factors enhance the survival of human neutrophils and eosinophils in vitro: a rapid and sensitive microassay for colony-stimulating factors. Blood **68:**162–166.
3. **Brewer, D. B.** 1964. Electron-microscope observations on the phagocytosis of neutrophil polymorphonuclear leucocytes by macrophages. J. Pathol. Bacteriol. **88:**307–309.
4. **Dahinden, C., and J. Fehr.** 1983. Granulocyte activation by endotoxin. II. Role of granulocyte adherence, aggregation, and effect of cytochalasin B, and comparison with formylated chemotactic peptide-induced stimulation. J. Immunol. **130:**863–868.
5. **Danon, D., Y. Marikovsky, and E. Skutelsky.** 1971. Red cell structure and metabolism, p. 23–38. Academic Press, New York.
6. **Dresch, C., G. Flandrin, and J. Berton-Gorius.** 1980. Phagocytosis of neutrophil polymorphonuclears by macrophages in human bone marrow: importance in granulopoiesis. J. Clin. Pathol. **33:**1110–1113.
7. **Duvall, E., A. H. Wyllie, and R. G. Morris.** 1985. Macrophage recognition of cells undergoing programmed cell death (apoptosis). Immunology **56:**351–358.
8. **Fittschen, C., R. A. Sandhaus, G. C. Worthen, and P. M. Henson.** 1988. Bacterial lipopolysaccharide enhances chemoattractant-induced elastase secretion by human neutrophils. J. Leukocyte Biol. **43:**547–556.
9. **Grigg, L. M., J. S. Savill, C. Sarraf, C. Haslett, and M. Silverman.** 1991. Neutrophil apoptosis and clearance from neonatal lungs. Lancet **338:**720–722.
10. **Guthrie, L. A., L. C. Mcphail, P. M. Henson, and R. B. Johnston, Jr.** 1984. Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide: evidence for increased activity of the superoxide-producing enzyme. J. Exp. Med. **160:**1656–1671.
11. **Haslett, C., A. Lee, J. S. Savill, L. Meagher, and M. K. Whyte.** 1991. Apoptosis (programmed cell death) and functional changes in aging neutrophils: modulation by inflammatory mediators. Chest **99:**6S.
12. **Kerr, J. F. R., A. H. Wyllie, and A. R. Currie.** 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer **26:**239–257.
13. **Madigan, M. C., P. L. Penfold, B. A. Holden, and F. A. Billson.** 1990. Ultrastructural features of contact lens-induced deep corneal neovascularization and associated stromal leukocytes. Cornea **9:**144–151.
14. **Mangan, D. F., S. M. Wahl, B. M. Sultzer, and S. E. Mergenhagen.** 1992. Stimulation of human monocytes by endotoxin-associated protein: inhibition of programmed cell death (apoptosis) and potential significance in adjuvanticity. Infect. Immun. **60:**1684–1686.
15. **Mangan, D. F., G. R. Welch, and S. M. Wahl.** 1991. Lipopolysaccharide, tumor necrosis factor-α, and IL-1β prevent programmed cell death (apoptosis) in human peripheral blood monocytes. J. Immunol. **146:**1541–1546.
16. **Martin, S. J., J. G. Bradley, and T. G. Cotter.** 1990. HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. Clin. Exp. Immunol. **79:**448–453.
17. **Metcalf, D., and N. A. Nicola.** 1985. Synthesis by mouse peritoneal cells of G-CSF, the differentiation inducer for myeloid leukemia cells: stimulation by endotoxin, M-CSF and multi-CSF. Leukemia Res. **9:**35–50.
18. **Newman, S. L., J. E. Henson, and P. M. Henson.** 1982. Phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit inflammatory macrophages. J. Exp. Med. **156:**430–442.
19. **Ohki, K., A. Nagayama, and S. Nagata.** 1991. Constitutive production of granulocyte colony-stimulating factor by hybrids of a SV40-transformed mouse macrophage and a renal adenocarcinoma cell line. Growth Factors **5:**183–189.
20. **Pekin, T. J., Jr., T. I. Malinin, and N. J. Zvaifler.** 1967. Unusual synovial fluid findings in Reiter's syndrome. Ann. Intern. Med. **66:**677–684.
21. **Sanui, H., S. Yoshida, K. Nomoto, R. Ohhara, and Y. Adachi.** 1982. Peritoneal macrophages which phagocytose autologous polymorphonuclear leucocytes in guinea-pigs. I. Induction by irritants and microorganisms and inhibition by colchicine. Br. J. Exp. Pathol. **63:**278–284.
22. **Savill, J., I. Dransfield, N. Hogg, and C. Haslett.** 1990. Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature (London) **343:**170–173.
23. **Savill, J. S., P. M. Henson, and C. Haslett.** 1989. Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge-sensitive" recognition mechanism. J. Clin. Invest. **84:**1518–1527.
24. **Savill, J. S., A. H. Wyllie, J. E. Henson, M. J. Walport, P. M. Henson, and C. Haslett.** 1989. Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. J. Clin. Invest. **83:**865–875.
25. **Searle, J., T. A. Lawson, P. J. Abbott, B. Harmon, and J. F. R. Kerr.** 1975. An electron-microscope study of the mode of cell death induced by cancer-chemotherapeutic agents in populations of proliferating normal and neoplastic cells. J. Pathol. **116:**129–138.
26. **Simpson, D. M., and R. Ross.** 1972. The neutrophil leukocyte in wound repair. A study with antineutrophil serum. J. Clin. Invest. **51:**2009–2023.
27. **Spriggs, A. I., M. M. Boddington, and A. G. Mowat.** 1978. Joint fluid cytology in Reiter's disease. Ann. Rheum. Dis. **37:**557–560.
28. **Stern, M., L. Meagher, J. Savill, and C. Haslett.** 1992. Apoptosis in human eosinophils: programmed cell death in the eosinophil leads to phagocytosis by macrophages and is modulated by IL-5. J. Immunol. **148:**3543–3549.
29. **Takeda, Y., H. Watanabe, S. Yonehara, T. Yamashita, S. Saito, and F. Sendo.** 1992. Tumor necrosis factor-α promptly accelerates neutrophil apoptosis. Proc. Jpn. Soc. Immunol. **22:**436.
30. **Vellenga, E., A. Rambaldi, T. J. Ernst, D. Ostapovicz, and J. D. Griffin.** 1988. Independent regulation of M-CSF and G-CSF gene expression in human monocytes. Blood **71:**1529–1532.
31. **Verghese, M. W., and R. Snyderman.** 1982. Endotoxin (LPS) stimulates in vitro migration of macrophages from LPS-resistant mice but not from LPS-sensitive mice. J. Immunol. **128:**608–613.
32. **Watari, K., S. Asano, N. Shirafuji, H. Kodo, K. Ozawa, F. Takaku, and S. Kamachi.** 1989. Serum granulocyte colony-stimulating factor levels in healthy volunteers and patients with

various disorders as estimated by enzyme immunoassay. Blood **73:**117–122.

33. **Weiss, S. J.** 1989. Tissue destruction by neutrophils. N. Engl. J. Med. **320:**365–376.
34. **Williams, G. T., C. A. Smith, E. Spooncer, T. M. Dexter, and D. R. Taylor.** 1990. Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature (London) **343:**76–79.
35. **Wyllie, A. H.** 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature (London) **284:**555–556.
36. **Wyllie, A. H., R. G. Morris, A. L. Smith, and D. Dunlop.** 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J. Pathol. **142:**67–77.
37. **Yamamoto, C., S. Yoshida, and Y. Mizuguchi.** 1992. Phagocytosis of polymorphonuclear leukocytes by guinea pig peritoneal macrophages: effects of serum and temperature in vitro. FEMS Microbiol. Immunol. **105:**211–218.