PHAGOCYTOSIS OF SENESCENT NEUTROPHILS
BY HUMAN MONOCYTE-DERIVED MACROPHAGES
AND RABBIT INFLAMMATORY MACROPHAGES

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From the time that the inflammatory reaction was first characterized by its cardinal
signs of color, rubor, tumor, et dolor, until the studies on phagocytosis by Metchnikoff,
it was considered that this reaction by the organism was detrimental to its well being.
Metchnikoff (1), however, saw the process of inflammation as a “...salutary reaction
against some injurious influence.” He stated (1) that there were “...two classes of
phenomena in inflammation; first, inflammation properly so-called, i.e., the lesion of
the vessel walls and other disturbances brought about by the irritating cause; second,
repair, consisting in the regeneration of the missing tissues and in the formation of a
scar.” It is the second process, that of repair or resolution of the inflammatory
response, that was the impetus for this investigation.

The ingestion of phagocytes by other phagocytes was first described by Metchnikoff.
In studies in the fins of tadpoles (2), he noted that phagocytes that had perished at
the site of inflammation were “englobed by other phagocytes.” Metchnikoff also
described this phenomenon in an inflammatory response to streptococci: “the strep-
tococci are englobed only by polynuclear leukocytes, and are never taken up by the
macrophages, which, however, carry out the entire work of absorption, and even
englobe the microphages, many of which perish in the struggle with the microbes and
have to be themselves absorbed” (3).

The ingestion of effete neutrophils by incoming macrophages is probably one of
several mechanisms that are operable in the final stages of an inflammatory reaction.
Since Metchnikoff, however, other descriptions of the ingestion of neutrophils by
macrophages have come mainly from the clinical literature. This phenomenon has
been described in various disease states, such as in macrophage granulomas in the
liver after infection with ornithosis virus (4), in marrow macrophages in childhood
chronic benign neutropenia (5), in connective tissue macrophages in histiocytic
medullary reticulosis (6), in splenic macrophages in thrombotic thrombocytopenic
purpura (7), and in splenic Reed-Sternberg cells in Hodgkin’s disease (8).

Heifets et al. (9) reported that resident mouse peritoneal macrophages can ingest
human neutrophils or neutrophil granules that provided the macrophages with
neutrophil peroxidase that was functional in iodination when the macrophages
phagocytized opsonized zymosan. In ultrastructural studies, a few guinea pig perito-

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neal macrophages were found to have engulfed neutrophils in the presence of Staphylococcus aureus and guinea pig serum (10). Neutrophil phagocytosis by macrophages in normal human bone marrow has also been described (11).

In this paper, we show that aged but not fresh neutrophils were recognized and engulfed by mature "inflammatory" macrophages and that freshly isolated monocytes or resident lung macrophages did not have this capability. These results are considered as they relate to the resolution of inflammation.

Materials and Methods

Human Neutrophils and Monocytes. Human peripheral blood monocytes and neutrophils were prepared under sterile conditions by dextran sedimentation and Ficoll-Hypaque centrifugation as previously described (12). Erythrocytes were removed from the erythrocyte-neutrophil layer using an ammonium chloride lysing solution (13), and the neutrophils were washed in Hanks' balanced salt solution (HBSS) containing 0.25% bovine serum albumin (Hanks'-BSA) and standardized to the appropriate concentration.

The monocyte-lymphocyte layer (mononuclear cells) was washed in HBSS containing 20 mM Hepes and 100 U/liter of penicillin-streptomycin (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) (Hanks'-Hepes) and was standardized to 3 X 10^6 cells/ml in Hanks'-Hepes containing 0.1% autologous serum. 1 ml of mononuclear cells was added to 24-well tissue culture plates with 16-mm Diam wells (Costar, Data Packaging, Cambridge, MA) and allowed to adhere for 1 h at 37°C in 5% CO2-95% air. After washing in Hanks'-Hepes, >97% of the adherent cells were esterase-positive, and 90% phagocytized latex. The adherent monocytes were cultured in M 199 (Gibco Laboratories, Grand Island Biological Co.) containing 5% autologous serum, 100 U/liter of penicillin-streptomycin, and 20 mM Hepes, as described previously (14, 15).

Rabbit Neutrophils and Monocytes. Rabbit neutrophils and monocytes were prepared from freshly drawn peripheral blood by dextran sedimentation and Ficoll-Hypaque centrifugation as previously described (16). Rabbit monocytes were washed and adhered in tissue culture plates as described above.

Rabbit Bronchoalveolar Macrophages. Rabbit inflammatory lung macrophages were induced by infusion of 2.5-5.0 mg of immune complexes of BSA-anti-BSA into the bronchus of one lung (17). 3 d later, lung macrophages were obtained from treated and control rabbits by bronchoalveolar lavage, as described by Myrvik et al. (18). Alveolar macrophages were washed, counted, standardized to 1 X 10^6 cells/ml in Hanks'-Hepes, and 1-ml aliquots were allowed to adhere to tissue culture plates as described above. Greater than 98% of adherent cells were macrophages as determined by Wright's stained smears.

51Cr Labeling of Neutrophils. Human and rabbit neutrophils (10^8 cells/ml) were incubated with 100 μCi of 51Cr (1 mCi/ml; New England Nuclear, Boston, MA) per ml of cells for 1 h at 37°C in a shaking water bath. The [51Cr]neutrophils were washed in Hanks'-Hepes and then either suspended in M199 for phagocytosis or "aged" overnight as described below.

Aging of Human and Rabbit Neutrophils. Neutrophils were aged using two different techniques, both of which gave similar results in all experiments that were performed. In the first method, 2 X 10^6 [51Cr]neutrophils were suspended in 40 ml of 75% autologous serum in M199 and incubated overnight at 37°C in 5% CO2-95% air. In the second method, neutrophils were suspended in 40 ml of a 50:50 (vol/vol) mixture of Hanks'-BSA and 0.34 M sucrose (9) and incubated overnight at 4°C. These storage media were chosen for their ability to allow the neutrophils to age without aggregation and to permit an estimation of the effect of serum on the neutrophils. After aging, neutrophils were centrifuged once, resuspended in M199, recounted, and viability was determined by trypan blue dye exclusion. Neutrophils aged by these techniques were routinely 94-98% viable.

Phagocytosis Assay. Human monocyte-derived macrophages, resident or inflammatory rabbit

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1 Abbreviations used in this paper: BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; Hanks'-BSA, HBSS containing 0.25% BSA; Hanks'-Hepes, HBSS containing 20 mM Hepes and 100 U/liter penicillin-streptomycin.
lung macrophages, and human or rabbit monocytes were overlayed with 1 ml (5 × 10⁶) of fresh or aged [⁵¹Cr]neutrophils. This mixture was incubated for varying periods of time at 37°C in 5% CO₂-95% air. Noningested neutrophils were removed by washing twice in Hanks'-Hepes. For quantitation of radioactivity, macrophage monolayers were lysed in 1 ml of 0.05% Triton X-100 (Fisher Scientific Co., Fairlawn, Nj), and the lyte was counted in a Beckman Gamma 7000 (Beckman Instruments Inc., Fullerton, CA). The number of neutrophils representing 1 cpm was determined by counting an aliquot of 5 × 10⁶ neutrophils, and the number of macrophage-associated neutrophils was then calculated. The number of adherent macrophages was determined from separate wells by treating the monolayers with zapoglobin II (Coulter Electronics Inc., Hialeah, FL) to lyse the external membrane of the cells. Nuclei were then quantified on a Model ZB Coulter Counter (Coulter Electronics, Inc.) (15).

To visualize internalized neutrophils, we took advantage of the fact that adherent monocytes lose their lysosomal myeloperoxidase (MPO) within the first few hours of culture (19). Macrophage monolayers in two wells from each experimental group were fixed in 1.25% glutaraldehyde-1% sucrose in 0.01 M phosphate buffer, pH 7.4, and then stained for MPO using dimethoxybenzidine (0-dianisidine HCl; Sigma Chemical Co., St. Louis, MO) and H₂O₂ (20). Macrophages containing neutrophil-derived MPO showed bright orange/brown inclusions and could easily be scored for ingestion or noningestion.

**Neutrophil Granule Preparation.** Neutrophils were suspended to 2 × 10⁹ cells/ml in 0.34 M sucrose and sonicated on ice for 25 s at 40 W with a Sonicator Cell Disruptor (Ultrasonic Instruments International, Westbury, NY). After removal of cell debris by centrifugation at 500 g for 10 min, the supernatant was centrifuged at 9,500 g for 15 min. The granule pellet obtained was resuspended in M199 to give an equivalent whole cell concentration of 5 × 10⁶ cells/ml.

**Electron Microscopy.** Macrophage monolayers that had phagocytosed 8-h aged neutrophils were processed for electron microscopy as described previously (21). Alternatively, an interspersed cationized ferritin procedure was used to label membranes exposed to the extracellular milieu. Cells were fixed in 1.5% glutaraldehyde-1% sucrose in 0.1 M cacodylate buffer, pH 7.3, and then were exposed to cationized ferritin (Miles Laboratories, Inc., Elkhart, IN) for 30 min. Thick and thin sections were cut on an LKB Ultrotome III (LKB Instruments, Inc., Rockville, MD) and examined on a Philips 400T electron microscope (Philips Medical Systems Inc., Shelton, CT) at an accelerating voltage of 60 kV.

**Results**

⁵¹Cr-labeled human neutrophils aged for 16 h in either 75% autologous serum in M199 at 37°C in 5% CO₂-95% air or a 50:50 (vol/vol) mixture of Hanks'-BSA and 0.34 M sucrose at 4°C were found to be phagocytosed by human monocyte-derived macrophages cultured for 7 d. Association of the senescent neutrophils with macrophages was quantified by measurement of the chromium counts and by counting the percentage of macrophages that contained neutrophil-derived myeloperoxidase inclusions. Examples of the patterns of MPO staining are shown in Fig. 1. Macrophage-associated neutrophils were degraded rapidly, and intact neutrophils were only observed early in the reaction (Fig. 1A). Generally, the macrophage-associated neutrophils were detected as round orange/brown globes (Fig. 1B), and/or the MPO was spread throughout the macrophage cytoplasm in a granular pattern (Fig. 1C and D). The staining patterns observed were seen only when macrophages were fed intact neutrophils. When neutrophil granule preparations were fed to macrophages, little MPO was detected, even after 24 h of incubation. To confirm that the macrophages were ingesting intact neutrophils, aged neutrophils were fed to day 7 macrophages for 20 min, fixed in glutaraldehyde, and processed for electron microscopy. As is shown in Fig. 2, monocyte-derived macrophages did indeed ingest intact neutrophils before rapidly digesting them. Post-fixation incubation with cationized ferritin did not reveal
FIG. 1. MPO stain for detection of neutrophils phagocytized by monocyte-derived macrophages. Myeloperoxidase was detected by incubating the macrophage monolayer with dimethoxybenzidine and H$_2$O$_2$ for 20 min. The monolayers were then stained with Harris-modified hematoxylin (Fisher Scientific Co.) for 40 min. (A) a relatively intact neutrophil inside a day-7 macrophage. Note that the nucleus is still partially visible. (B) macrophage with neutrophil inclusions seen as round orange/brown globes. The nucleus has already disappeared. (C and D) macrophages with the neutrophils mostly digested. MPO stain appears in a granular pattern in the macrophage cytoplasm.

penetration of the electron-dense protein marker into the phagolysosomes, which indicated that a true phagocytic event had occurred. Furthermore, it was clear from light microscope observations that macrophages were capable of ingesting several neutrophils at one time.

In the experiments to be presented, monocytes for culture were obtained from one donor, and neutrophils were obtained from a second donor. In this manner, 16-h aged neutrophils were ready to be incubated with macrophages on the 7th d of culture. However, to insure that the experimental results obtained would be applicable to the in vivo situation, it was necessary to show that under the in vitro conditions used, monocyte-derived macrophages could recognize and phagocytize autologous senescent
Fig. 2. Electron micrograph of senescent neutrophils ingested by macrophages. (A) Human monocyte-derived macrophage (day 7) with a relatively intact neutrophil after 20 min incubation with aged granulocytes. Note partially disrupted nucleus. × 15,300. (B) Macrophage with granulocyte debris in phagosomes 1 h after incubation with aged neutrophils. This preparation was incubated with cationized ferritin. The ferritin can be seen on the macrophage plasma membrane but not in the phagolysosome. × 12,000.
neutrophils. The results of such an experiment are shown in Table I. Macrophages from donor M.F. phagocytized autologous aged neutrophils almost as well as heterologous aged neutrophils. Furthermore, macrophages from a second donor (R.M.) did not ingest M.F. neutrophils to any greater degree than M.F. macrophages.

Fig. 3 shows the time-course of macrophage ingestion of senescent neutrophils. Both the number of macrophage-associated neutrophils and the percentage of macrophages capable of phagocytosis increased over a period from 1-5 h. 4 h was chosen as the standard incubation period.

Phagocytosis of senescent neutrophils by day 7 macrophages was dependent on the number of neutrophils added (Fig. 4). The number of macrophage-associated neutro-

| Table I |
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| **Phagocytosis of Senescent Neutrophils by Autologous and Heterologous Macrophages** |
| Macrophage donor | Neutrophil donor | Number macrophage-associated neutrophils/10⁶ macrophages | Macrophages ingesting % |
| M.F. | M.F. | 1.7 | 16 |
| M.F. | C.G. | 2.2 | 14 |
| R.M. | M.F. | 1.9 | 20 |
| R.M. | C.G. | 1.4 | 15 |

* Senescent neutrophils and macrophages were incubated for 4 h at 37°C in 5% CO₂-95% air, and macrophage monolayers were processed as described in Materials and Methods.

Fig. 3. Kinetics of phagocytosis of aged neutrophils by monocyte-derived macrophages. Day-7 macrophages were washed, and 1 ml of aged neutrophils (5 × 10⁶) in M199 was added to the monolayer. Macrophages and neutrophils were incubated at 37°C in a moist atmosphere of 5% CO₂-95% air for varying periods of time, and then the monolayers were processed for quantitation of radioactivity and stained for myeloperoxidase, as described in Materials and Methods.
phils increased linearly and reached a maximum at ~10 × 10^6 added neutrophils. Interestingly, the percent of macrophages phagocytizing peaked upon the addition of 5 × 10^6 polymorphonuclear leukocytes and did not increase even when three times as many neutrophils were added, indicating that the active macrophages were ingesting several neutrophils each.

We have shown previously (14, 15) that monocytes differentiating in vitro acquire many of the characteristics of mature or inflammatory macrophages. Because various functions developed on different days in culture, the stage at which maturing macrophages developed the ability to recognize and phagocytose senescent neutrophils was investigated. Fig. 5 shows that freshly isolated monocytes (day 0) were not capable of phagocytosis of aged neutrophils. After 3 d in culture, ~7% of macrophages had developed the ability to phagocytose senescent neutrophils. Thereafter, the number of macrophage-associated neutrophils and the percent of macrophages ingesting increased in a linear manner through day 7. Thus, the ability to recognize effete neutrophils appeared to be a property of mature macrophages but not peripheral blood monocytes.

We next addressed the question of the length of time the neutrophils must age before they become recognizable by macrophages. As is shown in Fig. 6, freshly isolated neutrophils were not phagocytized by monocyte-derived macrophages. After 4 h of aging, macrophages were able to ingest some neutrophils, with the neutrophils becoming maximally ingested after 12 h.

Although it had previously been determined that neutrophils could be aged in the absence of serum (in Hanks'-BSA and sucrose), the effect of different serum concentrations on the aging of neutrophils was examined for inhibitory or enhancing properties on the subsequent phagocytosis of the neutrophils by macrophages. This was an important consideration because it had been reported previously that aged

![Graph](image-url)
Fig. 5. Phagocytosis of senescent neutrophils by macrophages as a function of time of macrophage maturation. Peripheral blood monocytes were cultured as described in Materials and Methods and then tested for their ability to phagocytize senescent neutrophils after different days in culture. Macrophages were incubated with $5 \times 10^6$ polymorphonuclear leukocytes for 4 h at 37°C in 5% CO$_2$-95% air. The data shown is averaged from three experiments.

Fig. 6. Time-course for aging of human neutrophils. Freshly isolated human neutrophils were labeled with $^{51}$Cr and then either immediately fed to day-7 macrophages or aged for the times shown in 75% autologous serum in M199. At the times shown, $5 \times 10^6$ neutrophils were added to the macrophage monolayer and incubated for 4 h at 37°C in 5% CO$_2$-95% air.

human erythrocytes may be recognized by a natural antibody present in normal serum (22). Neutrophils were aged overnight in concentrations of serum in M199 ranging from 0–75%, washed once, and then incubated with macrophages as described in Materials and Methods. As is shown in Fig. 7, serum was not required for the aging of neutrophils. Furthermore, the presence of different concentrations of serum in the aging mixture had no effect on the subsequent phagocytosis of the neutrophils. Both
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Fig. 7. Effect of serum concentration on the aging of human neutrophils. Peripheral blood neutrophils were aged overnight in the various serum concentrations shown, standardized to $5 \times 10^6$ cells/ml in M199, and 1-ml aliquots were added to day-7 macrophage monolayers for 4 h at 37°C in 5% CO$_2$-95% air.

the number of macrophage-associated neutrophils and the percent of macrophages phagocytizing was similar at all serum concentrations tested.

Because the ability to recognize senescent neutrophils appeared to be a property of mature macrophages, we investigated the ability of rabbit resident lung macrophages to recognize and phagocytize aged rabbit neutrophils. Rabbit $^{51}$Cr-labeled neutrophils were aged in 75% autologous serum in M199 and then were incubated with alveolar macrophages for 4 h at 37°C. Macrophage monolayers were then processed in the same manner as experiments performed with human cells. In preliminary experiments, we found that contrary to expectation, resident alveolar macrophages did not recognize and phagocytize rabbit neutrophils that had been aged up to 48 h. We therefore speculated that either macrophages from the spleen or liver or inflammatory macrophages might be required to perform this scavenger function. To obtain inflammatory lung macrophages, rabbit lungs were instilled with BSA-anti-BSA-immune complexes, and the lungs were lavaged 3 d later. Macrophages obtained from immune complex-treated animals were then tested for their ability to phagocytize 48-h aged neutrophils in comparison with resident alveolar macrophages and rabbit blood monocytes. The results of experiments with four rabbits are shown in Fig. 8. Only inflammatory lung macrophages obtained from immune complex-treated rabbits phagocytized the aged neutrophils; monocytes and resident lung macrophages did not.

Discussion

In this paper, we showed that the ability to phagocytose aged neutrophils was a property of mature inflammatory macrophages but not freshly isolated monocytes or resident rabbit alveolar macrophages. Furthermore, freshly isolated neutrophils were not recognized and phagocytosed, but needed to undergo a time-dependent aging
process before recognition by macrophages occurred. Aged neutrophils were 95–98% viable when added to macrophage monolayers, and, therefore, death of the neutrophil did not appear to be required for phagocytosis. However, it is not known whether the macrophage kills the neutrophil before ingestion or after the neutrophil has been phagocytized.

Phagocytosis of neutrophils by macrophages occurred in a time-dependent manner and was also dependent on the number of neutrophils added. Macrophages first exhibited this scavenger function after 3 d in culture, with the number of macrophage-associated neutrophils and the percent of macrophages phagocytizing these cells increasing in a linear fashion through day 7. The presence of serum was not required for aging, and when present in the aging medium, serum did not appear to have any effect on the subsequent phagocytosis of the neutrophils.

By necessity of the experimental design, these studies were performed with neutrophils and macrophages obtained from different donors. However, macrophages ingested autologous neutrophils just as well as they ingested heterologous neutrophils. Thus, these results are applicable to the situation as it occurs in vivo.

It has also been found that the MPO obtained from ingested neutrophils disappears within 24 h of placing the macrophages back into culture. Thus, it is unlikely that the macrophages can use the neutrophil-derived myeloperoxidase to enhance its microbicidal or tumorcidal capacity in a permanent manner. Whether or not the macrophages can use the MPO to enhance their killing ability in the short term is not known, and this possibility is currently under investigation. In this regard, Heifets et al. (9) reported that unelicited mouse peritoneal macrophages exhibited high levels of iodinating activity after they had ingested effete human neutrophils or granule
preparations along with zymosan particles. They also found that the iodinating activity declined fairly rapidly, despite the persistence of significant levels of intracellular MPO detected by direct assay from Triton X-100 lysed monolayers.

The mechanism(s) by which macrophages recognize effete neutrophils as nonself is not known. This question may be considered from two points of view: (a) changes that occur in the neutrophils and (b) changes that occur during the differentiation of monocytes into macrophages. In either case, the recognition mechanisms would be applicable to events occurring in an inflammatory reaction and to the removal of effete cells from the circulation.

In a generalized inflammatory reaction, there is a rapid influx of neutrophils into the site of bacterial invasion, and these neutrophils remain the predominant cell type during the first several hours. By 24–48 h, the neutrophils have largely disappeared and have been replaced by mononuclear cells. Incoming monocytes may develop into inflammatory macrophages that are capable of removing remaining bacteria, cell debris, and effete neutrophils.

Macrophages of the reticuloendothelial system play a major role in the removal of effete erythrocytes and neutrophils from the circulation. As these circulating cells age, they reach a point where they are recognized as nonself by the reticuloendothelial system macrophages and are phagocytosed and digested. The ability of human blood monocytes to ingest aged human erythrocytes has been studied by Kay (22, 23) as a model of recognition in cellular senescence. Her investigations have suggested that as the erythrocytes age, they lose membrane sialic acid, and the erythrocytes are then recognized and opsonized by an IgG autoantibody found in the serum. She has also reported that aged neutrophils can be recognized by the same autoantibody (24). The system described in this communication is different from that of Kay in that monocytes were not capable of phagocytosis of senescent neutrophils, and the presence or absence of serum during neutrophil aging had no effect on subsequent phagocytosis, suggesting that an autoantibody probably was not involved in macrophage recognition of senescent neutrophils.

Experiments performed with rabbit alveolar macrophages revealed that inflammatory lung macrophages but not resident lung macrophages were capable of recognizing and phagocytizing senescent neutrophils. Thus, the monocyte-derived macrophages obtained by in vitro culture exhibit one of the properties of inflammatory macrophages. The system described in this paper provides an excellent model for studying the mechanism of recognition of effete neutrophils by macrophages. The elucidation of such mechanisms is currently under investigation.

Summary

An in vitro system to investigate the ability of macrophages to recognize and ingest senescent polymorphonuclear neutrophils has been used that uses chromium-labeled neutrophils and staining for myeloperoxidase (MPO). Human monocyte-derived macrophages obtained from in vitro cultures were able to recognize “aged” but not freshly isolated $^{51}$Cr-labeled human neutrophils and ingest them. Freshly isolated monocytes did not exhibit this property. Because the aged neutrophils were >95% viable, death did not appear to be a prerequisite for recognition and ingestion. Serum was not required for the aging of the neutrophils, and when serum was used, different concentrations did not appear to effect the aging process; that is, neutrophils aged in
different concentrations of serum were ingested equally. Phagocytosis of senescent neutrophils by macrophages occurred in a time-dependent manner and was also dependent on the number of neutrophils added. Monocyte-derived macrophages first exhibited the ability to phagocytose senescent neutrophils on the 3rd d of culture, with the percentage of active macrophages increasing through day 7. In experiments with rabbit mononuclear phagocytes, immune complex-induced inflammatory macrophages from the lung but not resident bronchoalveolar macrophages or peripheral blood monocytes were found to be capable of recognition and ingestion of senescent rabbit neutrophils. These data suggest that the monocyte maturation process, akin to that seen during inflammation, is necessary in vitro before macrophages recognize and remove senescent neutrophils.

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