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Biochemical Properties of Polymorphonuclear Neutrophils from Venous Blood and Peritoneal Exudates of Rabbits

KENJI TAKAMORI* AND TATSUHISA YAMASHITA

Laboratory of Physiological Chemistry, School of Medicine, Juntendo University, Hongo, Bunkyo-ku, Tokyo 113, Japan

The biochemical properties of polymorphonuclear neutrophils from blood and peritoneal exudates of rabbits were compared. All enzymes measured showed almost identical activities in both types of cells, except for alkaline phosphodiesterase, the activity of which was seven times higher in peritoneal neutrophils. During phagocytosis, blood and peritoneal β-glucuronidases were released in very similar fashions. Lysozyme, one of the enzymes concerned with killing of bacteria, as well as β-glucuronidase, showed the same releasing pattern in both types of cells, but peroxidase was hardly released. Although superoxide anion generation in peritoneal neutrophils was two times higher than superoxide generation in blood neutrophils, phagocytic and bactericidal activities were almost the same in blood and peritoneal neutrophils. Blood neutrophils were more resistant to hypotonic lysis than were peritoneal neutrophils. These results show that there are no distinct differences in enzymatic and functional properties between blood and peritoneal polymorphonuclear neutrophils, except for alkaline phosphodiesterase activity, superoxide anion production, and osmotic fragility.

Studies on the in vitro function of polymorphonuclear neutrophils (PMNs) have been carried out with cells either from venous blood or from peritoneal exudates. However, PMNs from peritoneal exudates have been used more frequently due to the difficulty in collecting sufficient numbers of granulocytes from blood. In addition, we have studied the function of PMNs by using glycogen-induced peritoneal PMNs (25-27). Recently, it has been reported that PMNs release granule-associated enzymes extracellularly (15, 28, 29) and generate microbicidal agents by the reduction of oxygen (2, 17, 20) when they are stimulated by exposure to the appropriate phagocytic and nonphagocytic stimuli. Since PMNs from glycogen-induced peritoneal exudates are thought to be cells which have infiltrated to the site of inflammation from the circulating blood, it might be expected that peritoneal PMNs would display properties different from those of blood PMNs. Therefore, it was of interest to compare the biochemical properties of blood and peritoneal PMNs. Differences in enzymatic and functional properties between blood and glycogen-induced peritoneal PMNs were studied in the work reported below.

MATERIALS AND METHODS

Preparation of PMNs. PMNs were obtained from venous blood (blood PMNs) and glycogen-induced peritoneal exudates (peritoneal PMNs) of rabbits.

Blood PMNs were isolated by layering venous blood anti-coagulated in acid citrate-dextrose (ratio of blood to acid citrate-dextrose [2.5% sodium citrate, 0.8% citric acid, 1.2% dextrose], 4:1) onto the top of a separation solution composed of 1 volume of 32.8% sodium metrizoate solution (Nyegaard & Co. A/S, Oslo, Norway) and 5 volumes of 6% dextran in 0.9% NaCl. After the sedimentation of erythrocytes, the plasma layer containing leukocytes was pipetted from the separation solution, filtered through three layers of gauze, and centrifuged at 100 × *g* for 10 min at 4°C. Pellets were suspended in about 2 ml of Dulbecco phosphate-buffered saline (PBS) without divalent cations [PBS(−)]. Hemolysis of residual erythrocytes was achieved by exposing the cell suspension to 10 volumes of 0.2% saline for 60 s, followed by adding an equal volume of 1.61% saline. After centrifugation, the cells were washed twice with PBS(−) and finally suspended at the desired concentration in PBS(−) or PBS. Leukocytes (1×10^8 to 2×10^8 cells) were usually obtained from 100 ml of venous blood, and more than 96% of the cells excluded trypan blue. Differential cell counts with Wright-Giemsa stain showed that 93 to 95% of the cells were PMNs.

Peritoneal PMNs were elicited in a fasted rabbit by the intraperitoneal injection of 300 ml of 0.12% glycogen in 0.9% saline. After 12 to 14 h, the animal was killed with ether and perforated, and 300 ml of acid citrate-dextrose was introduced into the peritoneal cavity. The abdomen was kneaded for a few minutes, and the fluid (300 to 400 ml) was withdrawn. The exudates were filtered through three layers of gauze and centrifuged at 100 × *g* for 10 min. Further procedures were carried out in the manner described above for blood PMNs. Leukocytes (4×10^9 to 6×10^9 cells) were usually obtained from a single animal. More than 94% of the cells were viable, judging from the trypan blue exclusion test, and 91 to 94% of the cells were

PMNs. Centrifugation was carried out with a model 05PR-22 Hitachi refrigerated centrifuge and a swinging bucket rotor. All glassware used in this experiment was siliconized.

Opsonization of zymosan. Opsonized zymosan was prepared by incubating 1.0 ml of fresh rabbit serum with 8 mg of zymosan per ml at 37°C for 30 min, followed by two washings with PBS.

Enzyme release from PMNs. The release of enzymes from PMNs was examined by incubating 10^7 PMNs per ml in PBS with or without opsonized zymosan (1 mg/ml) for 0, 5, 15, and 30 min in a total volume of 2.0 ml in a shaking water bath (120 excursions per min). After incubation, the tubes were placed in an ice bath and then centrifuged at 1,380 × *g* for 10 min. The activities of β-glucuronidase, lysozyme, peroxidase, and lactate dehydrogenase (LDH) in the supernatant were measured. Total enzyme activities were determined in the presence of 0.1% Triton X-100, using cell suspensions.

Phagocytosis of *Staphylococcus aureus* by PMNs. PMNs (5×10^6 cells per ml) were incubated with *Staphylococcus aureus* (5×10^6 cells per ml) in the presence of 13% autologous serum in a total volume of 4.0 ml with continuous shaking (120 excursions per min) at 37°C. Samples (0.5 ml) of the suspension were removed at 0, 5, 15, 20, and 30 min and added to 1.5 ml of ice-cold PBS to stop phagocytosis. After centrifugation for 10 min at 100 × *g*, the supernatant was diluted serially 10-fold with saline; 0.10-ml amounts of the dilutions were pipetted onto each of two agar plates and immediately spread with a fine wire loop. The plates were incubated at 37°C for 24 h, and then the colonies were counted. On the other hand, the PMNs sedimented at 100 × *g* for 10 min were washed twice with PBS and suspended in 2.0 ml of PBS containing 4% autologous serum. One drop of the cell suspension was placed onto a glass slide and immediately spread, and the slide was dried by airstream flow. Cells on the slide were fixed with methanol and stained with Gram stain. The percentage of cells containing ingested bacteria was determined from counts of at least 400 cells.

Measurement of intracellular killing activity of PMNs. After incubation of PMNs (5×10^6 cells per ml) with the same number of *S. aureus* cells for 15 min under the conditions described above for the phagocytosis experiment, phagocytosis was terminated by placing the tube in an ice-cold bath and shaking it for 1 min. The suspension of bacteria and cells was then centrifuged at 100 × *g* for 10 min at 4°C, and the supernatant was removed. The sedimented PMNs were suspended in ice-cold PBS, washed twice with ice-cold PBS, suspended in 4.0 ml of ice-cold PBS containing 10% autologous serum, and incubated at 37°C. At 0, 15, 30, 60, 90, and 120 min, 0.50 ml of the suspension was removed, added to 0.50 ml of ice-cold PBS to stop intracellular killing, and then centrifuged at 100 × *g* for 10 min at 4°C. After removal of the supernatant, PMNs were lysed by adding 1.0 ml of ice-cold distilled water and then pipetting for 2 min (microscopic observation showed that all PMNs were disintegrated). Serial 10-fold dilutions in 0.9% NaCl were made from the lysed cell suspensions, and 0.10-ml amounts of each diluted sample were pipetted onto agar plates and immediately spread. After incubation of the plates at 37°C for 24 h, colonies were counted.

Measurement of superoxide anion production. Production of superoxide anions by PMNs was measured on the basis of reduction of cytochrome *c* by superoxide anions (16). PMNs (3.3×10^6 cells per ml) were incubated at 37°C with opsonized zymosan (1.3 mg/ml) in the presence of 80 μM ferricytochrome *c* with or without superoxide dismutase (30 μg/ml). At 0, 5, 15, 30, and 60 min, each tube was placed in an ice-cold bath to stop the reaction and then centrifuged at 1,870 × *g* for 10 min. The absorbance of the supernatant at 550 nm was measured by using a Hitachi 203 spectrophotometer, and the value of cytochrome *c* reduction was calculated from the following equation: $E_{550\,nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$, where $E_{550\,nm}$ is the molar extinction coefficient at 550 nm. The amounts of superoxide anions produced by the PMNs were expressed as nanomoles of cytochrome *c* reduced per 10^7 cells.

Hypotonic lysis of PMNs. The resistance of the plasma membranes of PMNs to hypotonic solutions was checked by measuring the activity of LDH released from PMNs exposed to hypotonic PBS(−) in which only the NaCl concentration was changed. PMNs (2×10^6 cells) were suspended in 0.50 ml of PBS(−) containing various concentration of NaCl (0 to 0.8%) and incubated for 1, 2, 3, 5, and 10 min at 0°C. After incubation, the concentration of NaCl in each incubation mixture was brought to 0.8% by adding 0.50 ml of PBS(−) along with various concentrations of NaCl (1.6 to 0.8%), and the mixtures were centrifuged at 1,870 × *g* for 10 min at 4°C. Then the LDH activity of each supernatant was measured. Total activity was determined by using a sample of cell suspension which had been disrupted by the addition of 0.1% Triton X-100. The release of LDH from the PMNs into the supernatant was expressed as a percentage of the total activity.

Determination of enzyme activity. Enzyme assays were carried out in the presence of 0.1% Triton X-100 unless otherwise stated. Beta-glucuronidase was measured by the procedure of Avila and Convit (1), using phenolphthalein glucuronidate. Peroxidase was assayed by the method of Baggiolini et al. (3). LDH was measured by the method of Bergmeyer and Bernet (6). Determinations of acid and alkaline phosphatase activities were done with two different substrates; phenyl phosphatase activity was determined by the procedure of Kind and King (19), using phenyl phosphate as a substrate, and *p*-nitrophenyl phosphatase was assayed by the method of Bretz and Baggiolini (7). Lysozyme was assayed by incubating a sample with 0.1 mg of *Micrococcus lysodeikticus* per ml in 50 mM sodium phosphate buffer (pH 6.2) containing 0.05 M NaCl in a total volume of 3.0 ml. The increase of transmittance at 540 nm was measured for 5 min at room temperature. With every assay, a calibration curve was made with crystalline egg white lysozyme. Elastase activity was determined by measuring the rate of liberation of *p*-nitrophenol from *N*-tert-butoxycarbonyl-L-alanine *p*-nitrophenyl ester, as described by Dewald et al. (11). Neutral protease activity was determined by the procedure of Dewald et al. (11), using casein as a substrate. Leucine aminopeptidase

activity was measured in the absence of Triton X-100 by the method of Goldberg and Rutenburg (12), using L-leucyl-β-naphthylamide hydrochloride as a substrate. Alkaline phosphodiesterase activity was measured by the method of Touster et al. (23).

RESULTS AND DISCUSSION

Enzyme activities in PMNs. The enzyme activities of blood and peritoneal PMNs are listed in Table 1. These selected enzymes are markers of subcellular fractions and enzymes involved in bactericidal action. There was no difference in activity of LDH (a marker of cytosol) between blood and peritoneal PMNs. The activities of β-glucuronidase, acid phosphatase, elastase, and neutral protease, enzymes which are associated with the azurophilic granules (3–5, 10), showed almost the same values in both types of PMNs. The activity of alkaline phosphatase, a marker enzyme of the specific granules in rabbit PMNs (4), was about 1.5 times higher in peritoneal PMNs than in blood PMNs. We observed no differences in the activities of lysozyme and peroxidase, which are known to be involved in bactericidal action, between blood and peritoneal PMNs. The only distinguishing difference between the two types of PMNs was found in alkaline phosphodiesterase, which is known to be located in the plasma membrane (10), although leucine aminopeptidase, an ectoenzyme of PMNs (21a), exhibited the same activity; peritoneal PMNs showed nearly seven times higher activity than blood PMNs.

Enzyme release from PMNs during phagocytosis. It is well known that PMNs selectively release granule-associated enzymes when exposed to appropriate phagocytic and nonphagocytic stimuli (13, 14, 24). Therefore, we investigated the difference in the release of several enzymes from blood and peritoneal PMNs during phagocytosis. As Fig. 1 shows, the release of β-glucuronidase and lysozyme increased with incubation time with zymosan in both types of PMNs, but the degree of release of lysozyme was higher than that of β-glucuronidase. On the other hand, the release of peroxidase, which is known to play an important role in bactericidal action (8, 20, 21), was not stimulated by zymosan, which is in agreement with the findings of Okamura et al. (22). These results suggest that lysozyme might be involved in both intracellular and extracellular killing of bacteria and that peroxidase acts on only intracellular killing of bacteria.

Phagocytic activity of PMNs. If peritoneal PMNs were stimulated, it might be assumed that the phagocytic and bactericidal activities of peritoneal PMNs were more accelerated than the activities of unstimulated PMNs from blood,

TABLE 1. *Enzyme activities of PMNs from blood and peritoneal exudates of rabbits*[a]

PMNs	Activity of:											
	LDH (ΔOD_{340}/min)	β-Glucuronidase (μg of phenolphthalein per h)	Peroxidase (ΔOD_{440}/min)	Acid p-nitrophenyl phosphatase (μg of nitrophenol per h)	Acid phenyl phosphatase (μg of phenol per h)	Lysozyme (μg of egg white lysozyme)	Alkaline p-nitrophenyl phosphatase (μg of nitrophenol per h)	Alkaline phenyl phosphatase (μg of phenol per h)	Elastase ($\Delta OD_{347.5}$/min)	Neutral protease (nmol of L-leucyl-L-leucine per h)	Leucine aminopeptidase (μmol of β-naphthylamine per h)	Alkaline phosphodiesterase (μmol of p-nitrophenol per h)
Blood	2.84 ± 0.30	17.25 ± 1.66	1.12 ± 0.04	154.3 ± 29.3	15.69 ± 6.8	5.74 ± 1.32	220.5 ± 15.8	229.0 ± 6.64	0.107 ± 0.003	65.62 ± 14.67	6.02 ± 0.92	0.100 ± 0.007
Peritoneal	3.17 ± 0.54	16.37 ± 3.45	0.96 ± 0.14	177.0 ± 19.3	10.55 ± 1.86	4.51 ± 1.15	338.0 ± 13.8	335.1 ± 2.76	0.112 ± 0.032	48.65 ± 9.03	4.28 ± 0.997	0.684 ± 0.077

[a] Data are expressed as means ± standard errors of the mean of three or four separate preparations.

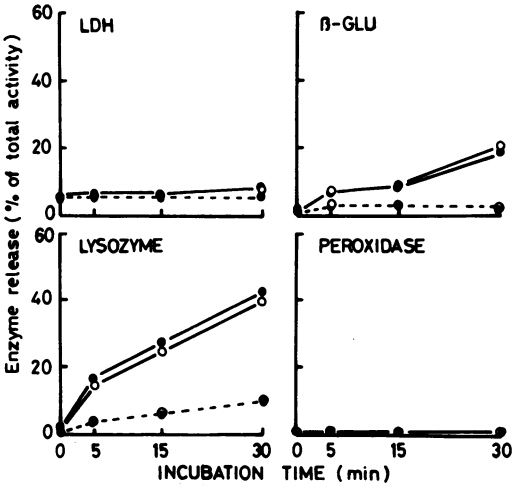

FIG. 1. *Extracellular release of enzymes from neutrophils. PMNs (10^7 cells per ml) were incubated at 37°C with 1 mg of zymosan per ml (solid lines) or without zymosan (dashed lines) for the indicated times. After centrifugation, the enzyme activities of the supernatants were measured. Enzyme release was expressed as a percentage of total activity. Symbols: ○, blood PMNs; ●, peritoneal PMNs. β-GLU, β-Glucuronidase. For details, see text. Data are expressed as means of three separate experiments.*

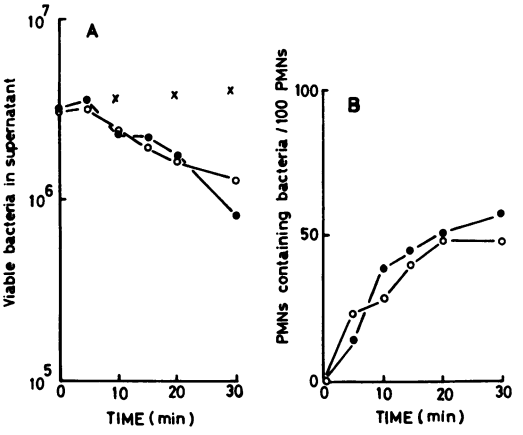

FIG. 2. *Time course of phagocytosis of S. aureus by neutrophils. PMNs (5 × 10^6 cells per ml) were incubated with S. aureus (5 × 10^6 cells per ml) in the presence of 13% autologous serum at 37°C, and then samples of the suspension were centrifuged at the indicated times. (A) Numbers of viable bacteria in the supernatant. (B) Percentages of PMNs containing bacteria. As a control, bacteria alone were incubated in the presence of autologous serum (×). Symbols: ○, blood PMNs; ●, peritoneal PMNs. For details, see text.*

as reported in macrophages (9, 18). Therefore, the phagocytic activities of both types of PMNs were examined by using two different methods. The first method involved determination of *S. aureus* colony formation in the supernatant after incubation with PMNs. As Fig. 2A shows, the number of viable bacteria in the supernatant decreased with increasing incubation time similarly in both types of PMNs. However, since it is known that bacteria are killed extracellularly by bactericidal factors which are released from PMNs (22), such as reactive oxygen metabolites and lysosomal enzyme, a question arose as to whether the above-described results indicated true ingestion of bacteria by PMNs. Therefore, a second method was used; this involved counting the cells containing bacteria. As Fig. 2B shows, the percentage of cells ingesting bacteria increased with increasing incubation time, suggesting that the decrease in viable bacteria in the supernatant observed with the first method depended upon phagocytosis of bacteria by PMNs.

Production of superoxide anions by PMNs. Figure 3 shows the time course of production of superoxide anions from both types of PMNs. Superoxide anion generation in the presence of zymosan increased linearly with increasing incubation time up to 15 min and reached a plateau level in 30 min; values per 10^7 cells were 120 nmol in peritoneal PMNs and 70 nmol in

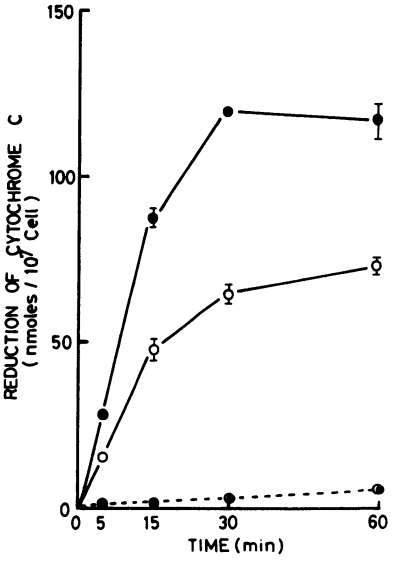

FIG. 3. *Time course of generation of superoxide anions by neutrophils. PMNs (3.3 × 10^6 cells per ml) were incubated with zymosan (1.3 mg/ml) in the presence of 80 μM cytochrome c with or without superoxide dismutase (30 μg/ml) at 37°C. At the indicated times, the production of superoxide anions (solid lines) was measured as described in the text. Generation of superoxide anions by PMNs without zymosan is indicated by the dotted line. Symbols: ○, blood PMNs; ●, peritoneal PMNs. Data are expressed as means ± standard errors of the mean of four separate experiments.*

blood PMNs. It is of interest that peritoneal PMNs exhibited higher superoxide anion generation, although they had the same phagocytic activity as blood PMNs. In the absence of zymosan, both types of PMNs hardly produced superoxide anions. Therefore, these results seem to indicate that the superoxide anion-forming system in peritoneal PMNs is more easily activated by stimuli than that in blood PMNs.

Bactericidal activity of PMNs. *S. aureus* colony formation after incubation with PMNs was examined as an index of the bactericidal activity of PMNs. If the bacteria were killed in PMNs with superoxide anions generated during phagocytosis, there should have been a marked difference in bactericidal activities between blood and peritoneal PMNs, because measured superoxide anions were twice as high in peritoneal PMNs as in blood PMNs (Fig. 3). As Fig. 4 shows, however, little difference in the killing rate of bacteria was observed between the two types of PMNs, indicating that there is no parallel relationship between the amount of superoxide anions released and the number of bacteria killed by PMNs under our conditions. Therefore, we may conclude from these findings that the amounts of superoxide anions released extracellularly from PMNs might not directly reflect the killing activity of *S. aureus* in cells. The above-described lack of parallelism might be based on the difference between the rates of extracellular release of superoxide anions produced in blood and peritoneal PMNs, although both types of PMNs produced similar amounts of superoxide anions.

Resistance of PMNs to hypotonic solutions. In preliminary experiments we observed that PMNs from glycogen-induced peritoneal exudates were less stable to storage, washing, and homogenization than PMNs from venous blood. Therefore, the resistance of the plasma membranes of PMNs to hypotonic solutions was investigated by measuring the appearance of LDH activity in media from PMNs exposed to hypotonic solutions. Release of LDH (an index of cytoplasmic leakage) increased with decreasing tonicity of the solution and with increasing incubation time (Fig. 5). In blood PMNs the release of LDH was not observed above 0.3% NaCl but it increased linearly with decreasing NaCl concentrations in the solution from 0.3 to 0%. On the other hand, LDH release from peri-

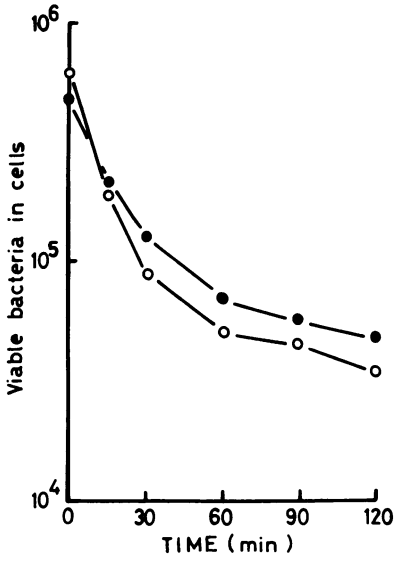

FIG. 4. *Kinetics of intracellular killing of S. aureus by neutrophils. PMNs (5 × 10^6 cells per ml) were incubated with the same number of S. aureus cells in the presence of 13% autologous serum for 15 min at 37°C. After further incubations for the indicated times, the numbers of viable bacteria in the PMNs were measured by the colony formation method described in the text. Symbols: ○, blood PMNs; ●, peritoneal PMNs. For other details, see text.*

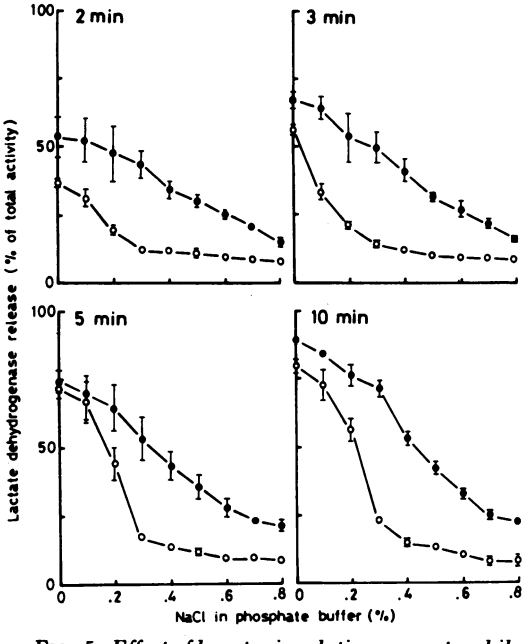

FIG. 5. *Effect of hypotonic solution on neutrophils. PMNs (2 × 10^6 cells) were exposed to the PBS(−) containing the indicated concentrations of NaCl at 0°C for 2, 3, 5, or 10 min. After centrifugation, the LDH activities of the supernatants were measured. Release of LDH is expressed as a percentage of the total activity which was determined with a Triton X-100-lysed cell suspension. Symbols: ○, blood PMNs; ●, peritoneal PMNs. Data are expressed as means ± standard errors of the mean of three separate experiments.*

toneal PMNs increased almost linearly with decreasing tonicity of the solution from 0.8 to 0% without the critical concentration (0.3%) observed in blood PMNs, suggesting the possibility that the plasma membranes of peritoneal PMNs are more fragile than those of blood PMNs to hypotonic treatment.

No distinct differences were found in the biochemical properties of blood and peritoneal PMNs, except for alkaline phosphodiesterase activity and superoxide anion production, whose functional sites are known to be located on the plasma membrane (21). A difference was also observed in the stabilities of the plasma membranes of the two types of PMNs. These findings lead us to conclude that there are no essential differences in enzymatic and functional properties between PMNs from blood and PMNs from peritoneal exudates, although some components of the plasma membranes might be different from each other. Studies of the plasma membranes are now in progress.

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