# A STUDY ON PURELY ISOLATED NEUTROPHILIC GRANULES OF LEUKOCYTES BY CYTOCHEMICAL STAININGS AND BIOCHEMICAL ANALYSES

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In a previous paper, nuclear, mitochondrial, neutrophilic granular and microsomal fractions isolated from leukocyte homogenate were studied by cytochemical stains and biochemical analyses. Next, the reliability in localization and specificity of cytochemical stainings was discussed by comparing the cytochemical findings with the biochemical analyses. Furthermore, the biochemical characteristics of mitochondria and microsomes of leukocytes were compared with those of liver cells.

In this report, the biochemical properties of the pure neutrophilic granular fraction successfully isolated by our improved technique of differential centrifugation of leukocyte homogenates, will be discussed based on comparative studies of the findings of cytochemical staining and of biochemical analyses.

## MATERIALS AND METHODS

Leukocytes were separated from horse blood mixed with an anticoagulant E.D.T.A. (final concentration 0.1%) by the technique previously mentioned.[1] The collected leukocytes consisted of 90% neutrophils and a small amount of lymphocytes, monocytes and eosinophils. Platelets were scarcely seen and only one or two erythrocytes were contained among 100 leukocytes.

Isolation of pure neutrophilic granules was carried out by our technique reported elesewhere. Leukocytes collected were suspended in 0.25 M sucrose solution, homogenized with a Potter-Elvehjem's glass homogenizer and fractionated by the differential centrifugation procedure at 0–5°C.[2] [3]

Identification of the granules contained in each of the fractions was made by two techniques: 1) Identification by coloration of the granules isolated which had been labeled supravitally in the cell body before homogenization. 2) Identification by electron micrograms of each granular fraction.

These techniques revealed that a very small amount of blue granules (mitochondria) stained with pinacyanole supravitally could be seen in the fields of Fraction V by the light microscope and that Fraction V was composed of highly pure neutrophilic granules containing one or no mitochondrion in a field of the electron microgram (10 000×). Fraction IV was a neutrophilic

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granular fraction mixed with mitochondria, and Fractions III and II mitochondrial fractions mixed with neutrophilic granules. Nuclear Fraction was made up of unbroken nuclei, mixed with debris of the neutrophils, and small amounts of mitochondria, neutrophilic granules and unbroken neutrophils. The supernatant contained microsomes, soluble components and a small amount of neutrophilic granules.

*A. Methods of Cytochemical Staining*[4) 5) 6)]

Smear preparation of each Fraction were treated with the following methods.

*Protein Stainings:*

1) The alloxan-Schiff staining reaction and the ninhydrin-Schiff reaction for protein.

2) Chèvremont et Frédéricq's ferricyanide method for SH: Fixed with formalin vapor for 5 minutes. Control preparations were treated previously with a saturated aqueous $HgCl_2$ for 1 hour.

3) Millon's staining reaction (A modification of Bensley and Gersh): Unfixed smear preparations were used.

*Lipid Stainings*:

4) The Sudan Black B staining for lipids: Fixed in formalin-calcium solution for 1 to 2 hours.

5) Baker's acid hematein method.

6) Feulgen's plasmal staining reaction: Fixed by flame.

*Polysaccharide Stainings:*

7) Periodic acid Schiff technique (P.A.S. staining): Fixed in ethanol for 1 minute.

8) The saliva test for glycogen.

9) The hyaluronidase digestion test for hyaluronic acid: Digested by testicular-hyaluronidase (10 000 V.U.C.) in 50 ml of 0.1 M acetate buffer (pH 6) containing 0.15 M sodium chloride, for 1 to 2 hours.

10) The toluidine blue method for metachromasic staining of acid polysaccharides: Stained by 0.05% toluidine blue solution (pH 7.0, 4.1 and 2.5).

*Nucleic Acid Stainings:*

11) Feulgen's nucleal reaction for desoxyribonucleic acid (DNA).

12) The methylgreen pyroninestaining and the ribonuclease digestion test for ribonucleic acid (RNA).

*Staining Reactions for Two Enzymes:*

13) Seligman's neotetrazolium method for succinic dehydrogenase*[7) 8)]: The fresh materials were incubated for 1 hour.

14) Seligman's 6-bromo -2-naphthyl $\beta$-*D*-glucopyruronoside method for $\beta$-

* According to biochemical analysis by Oda[8') ] the reduction of neotetrazolium by tissue homogenate, using sodium succinate as a substrate, corresponds mainly to the succinoxidase system activity.

glucuronidase[9]: Fixed in formalin vapor for 7 minutes.

*B. Methods of Chemical Analyses*

1) Schneider's fractionation technique of phosphorus compounds[10]: Phosphorus contents were determined by Fiske and Subbarow's method.[11] Nitrogen contents were determined by the micro-Kjeldahl's method.

2) Eelectrophoretic analysis of the soluble high-molecular substances of each fraction: Each fraction was resuspened in a veronal buffer after freezing-thawing, homogenized by a Potter-Elvehjem's homogenizer, and then dialysed in the veronal buffer for 24 hours at 4° C. This material was analysed by means of the schlieren-scanning technique using Shimazu's electrophoresis apparatus, in a veronal buffer (pH 8.45, ionic strength: 0.144).

3) Estimation of glucosamine: Each fraction was washed three times with physiologic saline, frozen-dried, and hydrolized with 4 N-HCl at 70° C for 22 hours. The glucosamine content of this hydrolized product was determined by the method of Elson and Morgan.[12]

4) Estimation of succinoxidase activity: Activities of succinoxidase were determined by Schneider and Potter's method.[13]

## RESULTS

*A. Cytochemical Stainings* (Shown in the Table 1)

*Protein Stainings:*

1) The alloxan-Schiff and ninhydrin-Schiff staining reactions appeared similarly. In the neutrophile leukocyte as a whole cell, the nuclear chromatin was stained moderately and the cytoplasm weakly and homogeneously, with these two staining reactions. In the nuclear Fraction and Fraction I isolated by our technique, nuclei and broken cytoplasmic components were clearly stained. In Fractions II and III particles collected in the fractions were stained weakly, and Fractions IV and V were stained moderately.

2) Chèvremont et Frédéricq's ferricyanide method for SH staining was negative for the neutrophile leukocyte as a whole cell. Fractions IV and V were stained moderately by this method, Fractions I, II and III weakly, and the nuclear Fraction negative. The color tone of the sulfhydril staining of the positive fractions did not decrease despite previous treatment with $HgCl_2$ for 1 hour.

3) Millon's reaction was negative in the neutrophile leukocyte, but weakly positive in Fractions IV and V, and in the nuclear Fraction, and negative in Fractions II and III.

*Lipid Stainings:*

4) The Sudan black B stain stained clearly the neutrophilic granules in the cell. This staining was negative in the nuclear Fraction, positive in the granules of Fractions I, II and III, and strongly positive in Fractions IV and V.

5) Baker's acid hematein method was positive on the intracellular granules of the neutrophils. All the granular fractions isolated from leukocytes were more or less positive; however, Fractions IV and V showed the most intensive

TABLE 1. Cytochemical Stainings of each Fraction of Horse Leukocyte

| Staining ↓ / Fraction → | Nucl. | I | II | III | IV | V |
|---|---|---|---|---|---|---|
| 1) Alloxan-Schiff | + | + | + | + | ++ | ++ |
| 1') Ninhydrin-Schiff | − | + | + | + | ++ | ++ |
| 2) SH groups (Chèvremont et Frédéricq) | − | + | + | + | + | + |
| 3) Millon reaction | + | + | ± | ± | + | + |
| 4) Sudan black B | − | ± | + | + | ++ | ++ |
| 5) Phospholipin (Baker's method) | − | ± | + | + | ++ | ++ |
| 6) Plasmal reaction (Feulgen's) | − | − | − | − | − | − |
| 7) PAS | − | + | ± | + | ++ | ++ |
| 8) Glycogen (Saliva digestion) | − | − | − | − | − | − |
| 9) Hyaluronic acid (hyaluronidase digestion) | − | − | − | − | − | − |
| 10) Metachromasia | − | − | − | − | − | − |
| 11) DNA (Feulgen Nucleal) | +++ | + | − | − | − | − |
| 12) RNA (Pyronine-methyl-green) | − | − | − | − | − | − |
| 13) Succinoxidase (Seligman's method) | − | ± | ++ | +++ | ++ | ± |
| 14) β-Glucuronidase (Seligman's method) | − | ± | + | ++ | + | ± |

staining, therefore, the neutrophilic granule was considered to be strongly positive with this staining reaction.

*Polysaccharide Staining:*

7) The periodic acid Schiff staining of neutrophils was negative in the nucleus, but diffusely positive in the cytoplasm and furthermore P.A.S.-positive coarse deposits appeared in the cytoplasm, although these deposits were not located in the neutrophilic granulation. Some of these coarse deposits were removed after salivary digestion. P.A.S. staining was negative in the nucleus, weakly positive in Fraction II and III, and positive in the granules of Fractions IV and V.

8) After salivary digestion, P.A.S. positive materials remained in all the granular fractions. Therefore, it was concluded that all the granular fractions did not contain glycogen.

9) After testicular-hyaluronidase digestion for 1 hour, stainings with P.A.S., peroxidase and Giemsa methods of each fraction did not change.

10) The neutrophile leukocyte and all the granular fractions isolated did not show any metachromasia with 0.05% toluidine blue solution at pH 7.0, 4.1 and 2.5.

From the findings of 9) and 10), the neutrophilic granules were considered

to contain no hyaluronic acid.

*Nucleic Acid Stainings:*

11) Feulgen's nucleal staining reaction was positive only in the nuclei of the neutrophils. This staining was positive in the nuclei of the nuclear Fraction, and also positive in the nuclear fragments of Fraction I. Occasionally, nuclear reaction-positive materials appeared in Fraction II. This staining was completely negative in Fractions III, IV and V.

12) Pyronine staining for RNA was almost negative in the polymorphonuclear leukocyte. Methylgreen staining appeared in the nuclear Fraction and in Fraction I. In Fractions II, III, IV and V pyronine staining and methylgreen staining were both negative.

*Stainings for Two Enzymes:*

13) Seligman's neotetrazolium method for succinoxidase stained strongly the intracellular mitochondria of neutrophils. The staining was negative in the nuclear Fraction, weakly positive in Fraction I, strongly positive in Fractions II, III and IV, and almost negative in Fraction V. Diformasan (reduced product of neotetrazolium) was deposited in rod or granular form in the staining-positive fractions. The neutrophilic granules were not stained completely. This reaction was completely inhibited by sodium malonate.

14) Seligman's 6-bromo-2-naphthyl-$\beta$-glucopyruronoside method for $\beta$-glucuronidase was very weakly positive in the neutrophile leukocytes and the localization of this staining was obscure. This staining was negative in the nuclear Fraction, weakly positive in Fraction I, strongly positive in Fractions II, III and IV, but weakly positive in Fraction V. In the staining-positive fractions, all areas of the fraction were diffusely stained, and no strongly stained granules were recognized.

*B. Biochemical Analyses*

*1) Results of Chemical Analysis of Each Fraction by Schneider's Method:* (Shown in Table 2)

The total phosphorus content of the pure neutrophilic granular Fraction consisted of 20.88% acid soluble P., 50.12% lipid P., 10.49% nucleic acid P. and 18.57% protein P. From this finding, phospholipin may be considered to be an important component of the neutrophilic granules. According to the content of nucleic acid P. in the neutrophilic granular Fraction, this Fraction may not be rich in nucleic acid.

The total nitrogen of the neutrophilic granular Fraction consisted of 7.17% acid soluble N., 31.29% lipid N., 21.12% nucleic acid N., and 40.42% protein N. Therefore, protein is a component of the neutrophilic granules.

*2) Electrophoretic Analyses of the Soluble High-Molecular Substances of Each Fraction* (Shown in Photograph 1 and Table 3)

In the electrophoretic patterns of the neutrophilic granular Fraction, four peaks appeared and mobilities of these peaks were calculated as follows: 4.4, 7.7, 9.4 and 16.3 ($\times 10^{-5}$ $cm^2$/volt sec.). The first and second peaks were high.

TABLE 2. Chemical Analyses of Phosphorus and Nitrogen Compounds of the Horse Leukocyte Fraction by the Method of Schneider

| | Acid soluble P (γ) | Lipids P (γ) | Nucleic acid P (γ) | Protein P (γ) |
|---|---|---|---|---|
| Nucl. | 937 | 2100 | 49500 | 1050 |
| I | 420 | 480 | 225 | 125 |
| II | 270 | 255 | 220 | 80 |
| III | 120 | 375 | 150 | 70 |
| IV | 150 | 459 | 375 | 110 |
| V | 225 | 540 | 112 | 200 |
| Sup. | 3465 | 6930 | 2362 | 2310 |

| | Acid soluble N (mg) | Lipids N (mg) | Nucleic acid N (mg) | Protein N (mg) |
|---|---|---|---|---|
| Nucl. | 3.97 | 15.85 | 9.46 | 32.53 |
| I | 0.91 | 3.29 | 1.76 | 5.31 |
| II | 0.69 | 1.89 | 1.70 | 2.12 |
| III | 0.53 | 3.18 | 0.98 | 2.87 |
| IV | 0.82 | 3.58 | 1.68 | 4.88 |
| V | 0.48 | 2.13 | 1.44 | 2.75 |
| Sup. | 20.05 | 31.36 | 30.24 | 56.00 |

TABLE 3. Electrophoretic Analyses of Soluble High Molecular Substances of Each Fraction of the Horse Leukocyte

| | Numbers of the peak | Mobilities ( $\times 10^{-5}$ $cm^2$/Volt sec.) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| Nucl. | 3 | 3.4 | 7.5 | 9.5 | | | |
| I | 2 | 2.6 | 6.4 | | | | |
| II | 2 | 2.2 | 5.3 | | | | |
| III | 6 | 3.4 | 6.8 | 9.2 | 13.1 | 16.0 | 19.4 |
| IV | 4 | 2.5 | 5.8 | 8.8 | 11.2 | | |
| V | 4 | 4.4 | 7.7 | 9.4 | 16.3 | | |
| Sup. | 3 | 6.3 | 12.6 | 16.5 | | | |

Comparing with the mobility of the serum albumin ($5.9 \times 10^{-5}$ $cm^2$/volt sec.), mobilities of the components of the neutrophilic granular Fraction were greater. According to a study on the number of peaks and mobilities at peaks in the extract of Fraction III, the electrophoretic patterns of this fraction were supposed to consist of the components derived from mitochondria and neutrophilic granules.

3) *Glucosamine Content of Each Fraction:*

Glucosamine content of each fraction was 0.57 mg in the nuclear Fraction, 0.29 mg in the Fraction I, 0.34 mg in the Fraction II, 0.67 mg in the Fraction III, 0.67 mg in the Fraction IV, 0.69 mg in the Fraction V and 264.83 mg in the supernatant. Therefore, glucosamine of the leukocyte was localized mostly

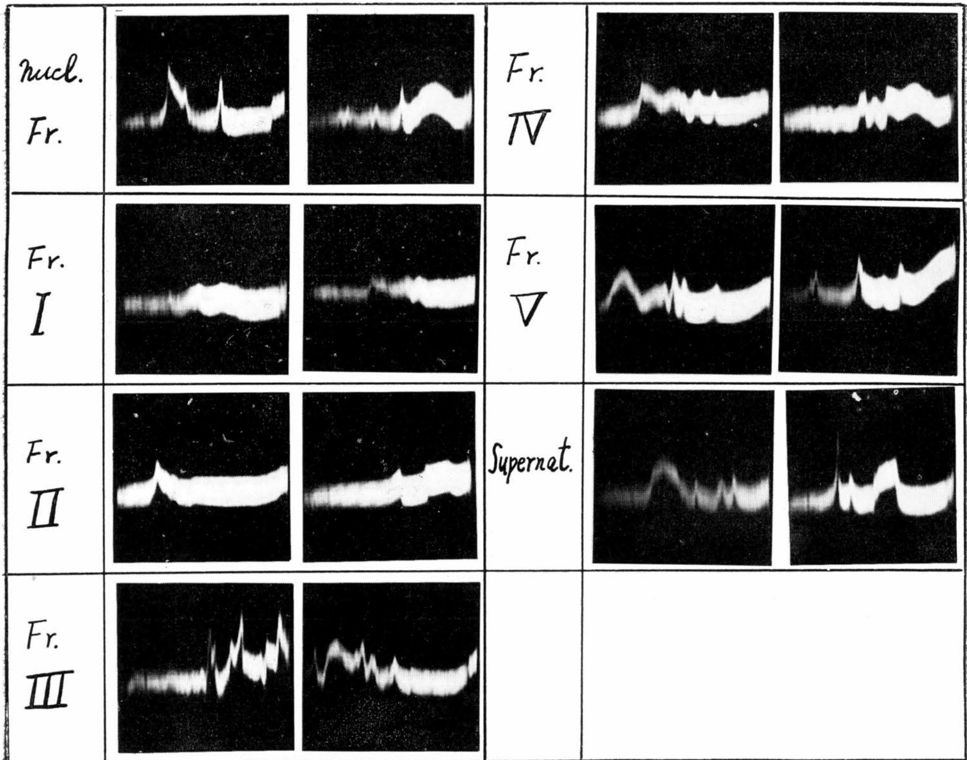


PHOTO. 1. Electrophoretic patterns of the soluble high-molecular substances of the horse leukocyte fraction.

The electrophoretic analyses were conducted in veronal buffer (pH 8.45, ionic strength-0.144) at 120 V., 15 mA. and 10° C, using a Tiselius electrophoresis apparatus (shlieren-scanning method).

left: ascending pattern right: descending pattern

TABLE 4. The Distribution of the Succinoxidase Activity in the Horse Leukocyte Fraction

| | Specific activity ($\mu l$ $O_2$/mg N) | Per cent activity |
|---|---|---|
| Nucl. | 30 | 26.8 |
| I | 150 | 9.0 |
| II | 255 | 14.6 |
| III | 270 | 24.6 |
| IV | 198 | 20.7 |
| V | 55 | 4.3 |
| Sup. | 0 | 0 |

in the supernatant.

4) *Succinoxidase Activity of Each Fraction* (Shown in the Table 4)

The specific activity and per cent activity of Fraction V (the pure neutrophilic granular Fraction) were distinctly low. The succinoxidase activity of the leukocyte localized in Fractions II, III and IV, was highest particularly in Fraction III (the mitochondria-rich Fraction).

## COMMENTS

The chemical composition of neutrophilic granules has been inferred by the cytochemical staining of neutrophils. In this report the biochemical property of neutrophilic granules was directly analysed by cytochemical staining and biochemical analyses of the neutrophilic granular fraction isolated in pure form horse neutrophils.

Mizuno's report on cytochemical staining of unbroken neutrophils revealed that the cytoplasmic portion of neutrophile leukocytes was negative to alloxan-Schiff staining, ninhydrin-Schiff staining and Millon's reaction.[14] In my study, the pure neutrophilic granular Fraction isolated was positive to alloxan-Schiff and ninhydrin-Schiff staining reactions, and slightly positive to Millon's staining reaction.

On the other hand, 40 per cent of the total nitrogen in the neutrophilic granular Fraction is protein-nitrogen according to Schneider's procedure. Consequently, the findings of alloxan-Schiff and ninhydrin-Schiff staining reactions agreed well with that of biochemical analysis. Millon's staining reaction was considered to be low in sensitivity by cytochemical staining because of the weak coloration by the stain.

The neutrophilic granular Fraction showed positive sulfhydril staining. However, this Fraction was supposed to contain other reducing substances in addition to the SH group containing substance because inhibition was hardly seen by treatment with bichloride of mercury before the staining.[5]

Neutrophilic granules have been suspected to contain lipids from the findings with Sudan black B staining and Baker's acid hematein staining of neutrophile leukocyte as a whole cell.[15][16] In my study, the neutrophilic granules isolated were stained clearly with Sudan black B and Baker's acid hematein and 50.12% of the total phosphorus of the neutrophilic granular Fraction was lipid-phosphorus according to biochemical analysis after Schneider. Consequently, phospholipin is an important constituent of the neutrophilic granules and the findings of these cytochemical stainings match well with the biochemical analysis.

Wislocki, Rheingold and Dempsy,[17] and, Gibb and Stowell[18] have noted a PAS positive substance which was inhibited by treatment with saliva before staining, in the cytoplasmic portion of the cell body of neutrophilic leukocyte, and identified it as glycogen. However, Pearse has stained a PAS-positive but salivary digestion-fast substance in the cytoplasm of the neutrophile leukocyte, and supposed it to be a substance other than glycogen.[4] In my experiment,

neutrophilic granules contained a PAS-positive material that resisted salivary digestion. PAS, peroxidase and Giemsa stainings of neutrophilic granules were not altered by treatment with testicular-hyaluronidase before the staining. Metachromasia of neutrophilic granules was not observed. Therefore, according to Pearse's table concerning the identification of PAS-positive tissue component,[4] it was assumed that the neutrophilic granules contain some of the following: 1) Mucoprotein or neutral muco-polysaccharides, 2) glycolipids, 3) unsaturated lipids, phospholipids or lipoprotein.

Determination of reducing sugar content in the hydrolized products of the neutrophilic granular fraction is considered to be unsatisfactory, because the sucrose solution is used as a medium for homogenization and differential centrifugation. Therefore, the glucosamine content was determined in the hydrolyte of the neutrophilic granular fraction. Glucosamine content was only 0.60 mg per 100 mg (dry weight) of neutrophilic granules. However, the supernatant contained the highest content of glucosamine.

In my study, the saliva-labile P.A.S. positive substance *i.e.* glycogen was not found in any of the granular Fractions. Therefore, it was considered to be dissolved in the supernatant during homogenization and differential centrifugation.

Four kinds of substances which had larger mobilities than serum albumin were found in the electrophoretic pattern of soluble high-molecular constituents extracted from the neutrophilic granular fraction. Therefore, the chemical composition of neutrophilic granules may be considerably complex.

Succinoxidase staining method with neotetrazolium revealed a positive reaction in mitochondria, according to the reports Siligman *el al.*[7] and Oda.[8] In my study, diformazan formed by the activity of succinoxidase was found in the mitochondria-rich Fractions and stained the mitochondria. In the neutrophilic granular Fraction, very few granules demonstrated positive staining, but the neutrophilic granules did not show any staining. According to Hogeboom *et al.*,[19] succinoxidase activity is localized in the mitochondria of liver cells. Therefore, in this study, the relationship between the enzyme and mitochondria was studied in the granular fractions of neutrophilic leukocytes. The activity of succinoxidase analysed manometrically was found to be apparently highest in the mitochondria-rich Fractions both in specific activity and in per cent activity. Accordingly, succinoxidase of the neutrophile leukocyte is localized in the mitochondria. The neotetrazolium method of succinoxidase staining ran parallet, with the biochemical analysis.

Wachstein[20] has reported that nucleated blood cells in smear preparations of blood and bone marrow were not stained by Seligman's method for $\beta$-glucuronidase. Takeuchi's report[21] revealed that pseudoeosinophile leukocytes in bone marrow specimens from rabbits were positive to $\beta$-glucuronidase staining. In my examination, the neutrophile leukocyte from horse blood showed negative or weak positive staining of $\beta$-glucuronidase in smear preparations. Further, $\beta$-glucuronidase staining revealed negative finding in the nuclear Fraction, strong positive reaction in the mitochondria rich Fraction, and weak positive staining in the neutrophilic granular Fraction. Diffuse staining seen in the

positive specimens of the β-glucuronidase staining indicates a tendency for diffusion of the product formed by decomposition of the substrate by β-glucuronidase activity. Ishiguro[22] stated that β-glucuronidase activity was demonstrated most actively in the mitochondrial fraction of horse leukocyte according to biochemical analysis. Consequently, it was shown by the β-glucuronidase staining method after Seligman and by biochemical analysis that the mitochondria of the neutrophile leukocyte contained β-glucuronidase. Results of Seligman's staining for β-glucuronidase correspond to those of biochemical analysis of the enzyme.

## CONCLUSION

1) The neutrophilic granular fraction isolated in pure form by differential centrifugation of the homogenate of horse neutrophile leukocytes were studied by cytochemical staining and biochemical analyses. Further, the reliability in localization and specificity of cytochemical stainings was discussed.

2) The neutrophilic granular fraction purely isolated was demonstrated to contain protein and lipid components and was suspected to contain some polysaccharides.

3) Four kinds of substances which had larger mobilities than serum albumin, by electrophoretic analysis, were shown to be soluble high-molecular substances.

4) Succinoxidase and β-glucuronidase of neutrophilic leukocyte were found in the mitochondria but not found in the neutrophilic granules.

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