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The Cytochemical Study of Oxygen-Dependent and Oxygen-Independent Components of Bactericidal Activity of Dog' Peripheral Blood Leukocytes

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ABSTRACT

The scientific literature does not discuss how the oxygen-dependent and oxygen-independent mechanisms of bactericidal activity of peripheral blood phagocytes relate to each other. For example, does oxygen-dependent and oxygen-independent bactericidal mechanisms act by themselves and independently? Or do they interact, potentiating each other's action? This work aimed to study the cooperation (clarification of the participation) of oxygen-dependent and oxygen-independent bactericidal systems of peripheral dog' blood neutrophils in the phagocytosis process. The mechanism of oxygen-dependent bactericidal activity of neutrophils was visualized in the NBT test. Study of the oxygen-independent bactericidal activity mechanism of neutrophils flowing through bactericidal proteins by Pigarevsky. Cytological preparations had been examining under immersion objective. Obtained results confirmed the well-known fact that a certain amount of peripheral blood granulocytes contains substances responsible for oxygen-dependent and bactericidal activity. These two mechanisms of bactericidal activity, responses different cells. In the process of phagocytosis, bactericidal substances gradually accumulated around the phagocytosed material and, after a while, completely impregnated it. In addition, particles phagocytosed by leukocytes containing cationic proteins were also gradually enveloped by the latter. However, in the research process, it was not established that these proteins penetrate deeply into phagocytosed particles. In the studied preparations, several such variants of the interposition of NBT-positive phagocytes and phagocytes containing cationic proteins are visualized, suggesting that these cells can interact in phagocytosis. It was found that different granulocytic cells have oxygen-dependent and oxygen-independent bactericidal activity. Also, bactericidal activity is exposed to bactericidal action by leukocytes, responsible for the oxygen-independent activity.

Key words: Phagocytosis, Superoxide anion, Cationic proteins, Granulocytes, Cytoplasm, NBT-Test.

INTRODUCTION

Despite the advances in veterinary surgery, surgical infection remains extremely relevant since its prevention and treatment in animals do not always provide a positive result. And in some cases, it leads to severe complications, including sepsis (Morrison et al. 1995). Regardless of the location, the first barrier to surgical infection is always the mechanism of resistance (Rechenberg et al. 2016). A priori, it can be argued that the more effective these mechanisms are, the less likely it is that the body's contact with microorganisms will lead to severe purulent complications (Lu et al. 2016). To increase the resistance of animals, doctors use various, both specific and nonspecific biological stimulants (Couturier et al. 2011). However, the effectiveness of such stimuli still leaves much to be desired. Therefore, it should be recognized that in most cases, no doctor will entrust the life of his patient with a purulent infection exclusively to stimulants of resistance or immunity and will resort to antibiotic therapy.

The very idea of preventing and/or treating infectious surgical diseases by stimulating resistance mechanisms is scientifically substantiated (Ulrich et al. 2020). However, at this stage, resistance mechanisms have not yet been studied to such an extent as to create effective drugs of this group that can completely replace antibiotics (Chammas and Hagiwara 1998). And this means that the scientific search in this direction should: be continuing.

Phagocytosis is one of the main components of the complex resistance mechanism (Uribe-Querol and Rosales 2020). If consider phagocytosis as a way to fight infection, then the following main stages can note 1) endocytosis, i.e.,

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the absorption of an infectious agent; 2) inactivation of the infectious agent; 3) digestion of the infectious agent and 4) exocytosis, i.e., removal of the products of inactivation and fermentation of the infectious agent from the cell. In this sequence of events, perhaps the most important is the inactivation of the carrier of the infection. As it is known, phagocytes, in particular neutrophils, have two: oxygen-dependent and oxygen-independent, bactericidal mechanisms (Forrest and Warrender 2004). The first is associated with the oxidation of glucose in the glucose-monophosphate shunt (GMPS) with the formation of various oxidants with bactericidal properties (Miceli et al. 1990). The second is associated with lysozyme, cationic proteins, lactoferrin, proteolytic enzymes, lipase (Li et al. 2020). In routine semi-quantitative screening tests, morphologically reflecting the patient's resistance level, either oxygen-dependent bactericidal activity is usually tested in the NBT test. In this test, live neutrophils are incubated in the presence of colorless nitro-blue tetrazolium absorb the latter and, by reaction with oxidants, convert it into dark blue formazan (Haghniaz et al. 2021). The number of formazan-positive cells and the amount of formazan formed in one cell are the evaluation criteria. Or cytochemical visualize cationic proteins, the activity of which is not associated with oxygen, but which, by binding to the surface membrane of bacteria and changing their permeability (Kolesnik et al. 2019).

The available scientific literature does not discuss how these two different mechanisms of bactericidal activity of peripheral blood phagocytes relate to each other. For example, do oxygen-dependent, and oxygen-independent bactericidal mechanisms act independently and dependently? Or do they interact, potentiating each other's action? This work aimed to study the cooperation (clarification of the participation) of oxygen-dependent and oxygen-independent bactericidal systems of peripheral blood neutrophils in dogs in the process of phagocytosis.

MATERIALS AND METHODS

Ethical Statement

This research was conducted following ethical standards for using animals for scientific purposes, referred to Federal Law of 24.04.1995 N 52-FL (as amended on 08.12.2020) "On the animal world". The neutrophil source was peripheral dog' blood of healthy mongrel animals 3-7 years old. The blood had been taking with heparin from the saphenous vein of the forearm (Minutes No. 264 dated 04/05/2021).

The NBT Test

The oxygen-dependent bactericidal activity mechanism of neutrophils had been diagnosing by the NBT test (reaction with nitro blue tetrazolium to superoxide anion) (Gordon et al. 1975). The Spontaneous NBT test was performing next way: To heparinized blood (0.25mL) was adding 0.25mL of phosphate-buffered saline (pH 7.4) and 0.5mL of 0.2% nitroblue tetrazolium solution (Sigma) onto phosphate-buffered saline. The stimulated NBT test was doing so: To 0.25mL of heparinized blood was adding 0.15mL of phosphate-buffered saline (pH 7.4), and 0.1mL of pyrogenal ("Medgamal" Russia (100mg/mL)) and 0.5mL of 0.2% nitroblue tetrazolium solution (Sigma) onto phosphate-buffered saline.

The neutrophils' phagocytic reaction simultaneously with NBT test was doing such: To 0.25ml. of heparinized blood was adding 0.25mL of baker's yeast suspension onto phosphate-buffered saline (pH 7.4) and 0.5mL of 0.2% nitroblue tetrazolium solution (Sigma) in phosphate-buffered saline. Baker's yeast had been inactivating by boiling, then washed three times with phosphate-buffered saline. This baker's yeast suspension contained 50 thousand cells in 1.0μL, with a particle ratio of phagocyte/ phagocytirhemic approximately 1/4. All reactions were in Eppendorf tubes by 37ºC for 40min. The fixation of cytological preparations was by methanol for 10min.

The study of the mechanism of oxygen-independent bactericidal activity of neutrophils was carried out by staining for cationic proteins (Pigarevskiĭ et al. 1986) for 30min (painting with a 0.1% solution of strong green in an aqueous 12.5% solution of methyl alcohol). Nuclei staining was carried out in a 1% aqueous solution of sofranin for 15-20min. The prepared preparations composed of 1) native blood, 2) spontaneous NBT test, 3) induced NBT test and 4) phagocytic reaction + NBT-test (only coloring with sofranin). The following drugs/cells were obtained for the study:

1. Non-activated leukocytes stained for cationic proteins.
2. Non-activated leukocytes stained in the NBT test.
3. Non-activated leukocytes stained in the NBT-test and for cationic proteins.
4. Activated leukocytes stained for cationic proteins.
5. Activated leukocytes stained in the NBT test.
6. Activated leukocytes stained in the NBT test and for cationic proteins.
7. Leukocytes phagocytic yeast cells stained for cationic proteins.
8. Leukocytes phagocytic yeast cells stained in the NBT test.
9. Leukocytes phagocytic yeast cells stained in the NBT-test and for cationic proteins.

Cytological preparations were examined under a×100 immersion objective on a Lumam P-8 microscope equipped with a 5-megapixel digital camera. In addition, visualization and fixation of images from a digital camera were carried out on a personal computer using the ScopeTecScopt Photo program version 3.0.12.489.

Statistical Analysis

The variational-statistical processing of the research results was carried out on IBMPC/AT and "Pentium IV" in Windows 2000, using the data analysis package in the program "Excel Windows Office XP" and "Statistika 6.0" (Statsoft, USA) with the calculation of the arithmetic Mean±SE. In the statistical analysis of the data obtained, the student's t-test was used for independent samples, while the differences were considered significant at $P<0.05$. All anatomical and histological terms according to Constantinescu and Constantinescu (2013) and International histological nomenclature.

RESULTS

After a spontaneous NBT test (incubation with nitro blue tetrazolium in the absence of activators), a slightly dark blue granularity was visualized in the cytoplasm of some leukocytes (Fig. 1-1-a). According to Pigarevsky, green granularity was visualized in the cytoplasm of some

leukocytes (Fig. 1-3-b). In the case when, after the spontaneous NBT test, cytological preparations were additionally stained by (Pigarevskiĭ et al. 1986), cells containing only dark blue cytoplasmic granularity and cells containing only green granules in the cytoplasm were visualized separately.

Cells that simultaneously contain both dark blue and green granularity were not identified. If pyrogen was added to the incubation medium during the NBT test, the granularity in the corresponding cells' cytoplasm increased (Fig. 1-2-a). At the same time, both the quantitative and qualitative characteristics of granularity in cells stained for cationic proteins remained unchanged (Fig. 1-4-b). The color pattern of granulocytes in the NBT test, in the process of phagocytosis of baker's yeast cells, changed in such a way that at first, the formazan granules were localized in the cytoplasm of cells intact from phagocytosed yeast (Fig. 2-1-a, e). Then the number of formazan granules increased significantly, and they surrounded the phagocytosed yeast cells (Fig. 2-2-a, e). In the final version, individual formazan granules were not visualized in the cytoplasm of leukocytes, and phagocytosed yeast cells themselves were diffusely stained dark blue (Fig. 2-3-e-1).

A similar, but not analogous, the picture was when leukocytes were stained in the process of phagocytosis of yeast cells for cationic proteins by Pigarevsky. Initially, granules containing cationic proteins were localized in the cytoplasm of cells, intact from phagocytosed yeast (Fig. 2-4-b, e). Then they began to surround the phagocytosed yeast cells (Fig. 2-5-b, e). Finally, and in the final version, the granules completely enveloped the phagocytosed material, at the same time, for the most part, remaining on its periphery (Fig. 2-6-e).

With light-optical microscopy of preparations prepared from a suspension of peripheral blood leukocytes, which were in the process of phagocytosis of yeast cells, variants of close contact of two phagocytes were visualized, one of which was stained in the NBT test, and the second - for cationic proteins by Pigarevsky (Fig. 3).

In the studied preparations, three morphological variants of granulocyte contact were identified. The first option – granulocytes were located side by side. One of them had phagocytosed material and granules stained for cationic proteins. Another granulocyte had phagocytosed material stained with nitro blue tetrazolium (Fig. 3-1). The second option – the granules stained for cationic proteins not only surrounded the phagocytosed yeast in the corresponding granulocyte but also closely contacted the yeast cells that were phagocytosed by the granulocyte stained in the NBT test (Fig. 3-2). The third variant – yeast cells diffusely stained with nitroblue tetrazolium- were visualized, not in NBT positive phagocytes, but in phagocytes with cationic proteins in the cytoplasm (Fig. 3-3, 4). Moreover, granules of cationic proteins accumulated around yeast cells stained with nitro blue tetrazolium.

Additionally, it should be noted that in the investigated cytological preparations, two variants of yeast cells outside of phagocytes were identified. Some of them were stained red with safranin (Fig. 4-2). At the same time, others had a dark blue coloration by analogy with yeast cells found inside NBT-positive phagocytes (Fig. 4-1). Probably, the former was not yet phagocytosed yeast cells. In contrast, the latter were yeast cells phagocytosed with NBT-positive leukocytes and released outside the phagocyte through exocytosis.

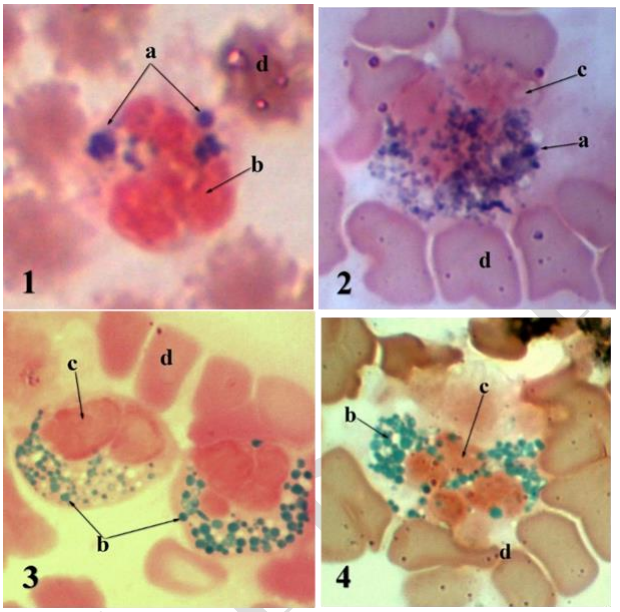

Fig. 1: Cytochemical visualization of bactericidal activity mechanisms in dogs' peripheral blood neutrophils (optical zoom×1600). 1 and 2 are oxygen-dependent mechanism visualization in the test with reduce nitroblue tetrazolium (NBT test). 3 and 4 are oxygen-independent mechanism visualization when staining for cationic proteins. 1 and 3 are spontaneous activity. 2 and 4 are stimulated activity. a: formazan granules in NBT positive phagocytes; b: lysosomes containing cationic proteins; c: cell nuclei stained with sofranin and d: erythrocytes.

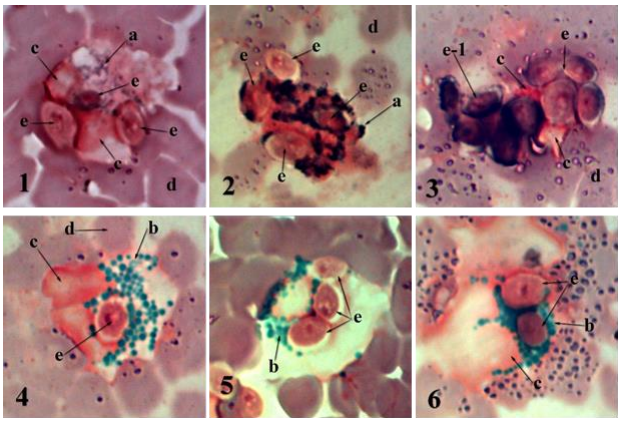

Fig. 2: Cytochemical visualization of the mechanisms of bactericidal activity in peripheral blood neutrophils of dogs in the process of phagocytosis of baker's shiver bodies (optical zoom×1600). 1, 2, and 3: Successive changes in the visualization of the oxygen-dependent mechanism in the test with the reduction of nitroblue tetrazolium (NBT test). 4, 5, and 6: Successive changes in the visualization of the oxygen-independent mechanism when staining for cationic proteins. a: formazan granules in NBT positive phagocytes; b: lysosomes containing cationic proteins; c: cell nuclei stained with sofranin; d: erythrocytes; e: unchanged or slightly altered phagocytosed yeast fungi; and e-1: phagocytosed yeast fungi intensely stained with formazan.

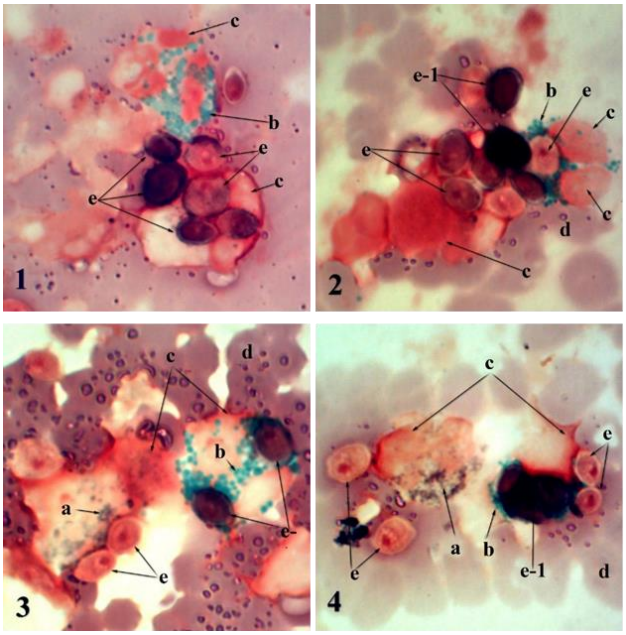

Fig. 3: Variants of cooperation of NBT+and CTB+leukocytes of peripheral blood of dogs in the process of phagocytosis of yeast cells. (Optical zoom×1600). 1) CTB+phagocyte (above) and NBT+phagocyte (below) is located side by side; 2) CTB+phagocyte (right) and NBT+phagocyte (left) are in close contact, and granules containing cationic proteins begin to cover yeast cells stained with nitro blue tetrazolium, and 3 and 4) in CTB+phagocyte (right), granules of cationic proteins surround yeast cells strongly stained with nitro blue tetrazolium. NBT+phagocyte (left) has a small number of reduced tetrazolium granules and phagocytosed yeast cells not stained with tetrazolium. a: formazan granules in NBT positive phagocytes, b: lysosomes containing cationic proteins, c: cell nuclei stained with sofranin, d: erythrocytes, e: unchanged or slightly altered phagocytosed yeast fungi, and e-1: phagocytosed yeast fungi intensely stained with formazan.

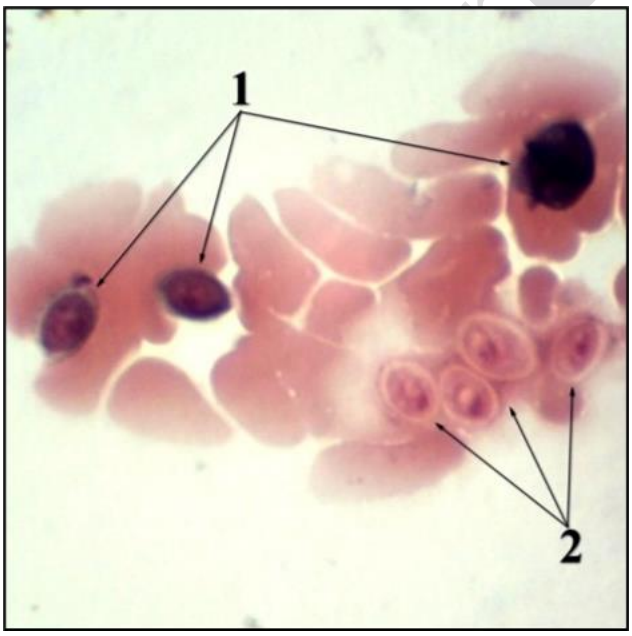

Fig. 4: Fungal cells are located extracellularly. (Optical zoom×1600). 1: fungal cells stained with reduced nitro blue tetrazolium and 2: fungal cells not stained with reduced nitro blue tetrazolium.

DISCUSSION

The study results correspond to the well-known fact that a certain number of peripheral blood granulocytes contains substances responsible for oxygen-dependent (Smirnova et al. 2018) (spontaneous NBT test (Fig. 1-1) and oxygen-independent (Tovmasyan et al. 2020) (cationic proteins (Fig. 1-3) bactericidal activity (Tohyama et al. 2019). Studies have shown that different cells are responsible for these two mechanisms of bactericidal activity. If soluble activators are present in the culture medium simulating the in vitro process of phagocytosis, then the number of leukocytes that react in the NBT test increases (Nowicka et al. 2018). In addition, the individual oxygen-dependent bactericidal activity of individual cells also increases (Fig. 1-2). At the same time, the number of cells containing cationic proteins does not increase, just as the number of cationic proteins in their cytoplasm does not increase (Fig. 1-4).

In the process of phagocytosis, bactericidal substances, which were visualized in the NBT test as substances converting colorless nitro blue tetrazolium into dark blue formazan, gradually accumulated around the phagocytosed material (Fig. 2-2) and, after a while, completely impregnated it (Fig. 2-3). Furthermore, the particles phagocytosed by leukocytes containing cationic proteins were also gradually enveloped by the latter (Fig. 2-4, 5, 6). However, it was not established during the study that these proteins penetrate deeply into phagocytosed particles (Table 1).

In the studied preparations, the most exciting fact is that many such variants of the interposition of NBT-positive phagocytes and phagocytes containing cationic proteins are visualized, suggesting that these cells can interact during phagocytosis (Barillet et al. 2019). If assume that such cooperation has a certain vector of development, then it most likely looks like this (Fattori et al. 2020). Initially, the NBT-positive phagocyte and the phagocyte containing cationic proteins converge on their cell membranes (Fig. 3-1). Then, the phagocytosed material, treated with substances of the oxygen-dependent bactericidal mechanism, comes into contact with the cationic proteins of another phagocyte (Fig. 3-2) (Abakumova et al. 2016). And at the final stage, the cationic proteins of the second phagocyte surround the particles phagocytosed and treated with oxidants by the first phagocyte (Fig. 3-3, 4) (Mandala et al. 2020).

However, the question arises, how can phagocytosed particles of NBT-positive leukocyte contact with cationic proteins of another leukocyte? Theoretically, these two phagocytes should be separated by two plasma membranes (Yousef et al. 2021). Then, they either merge into one binuclear cell by analogy with macrophages (Iqbal et al. 2015). Or particles phagocytosed by NBT-positive leukocytes and treated with bactericidal oxidants are removed from it by exocytosis. And after exocytosis, the particles are re-phagocytosed by leukocytes containing cationic proteins (Vandendriessche et al. 2021). The latter option is indirectly confirmed because the studied preparations contained many yeast cells located extracellularly and stained with formazan (Fig. 4-1). This indicates that they have been inside NBT-positive phagocytes and reacted with substances of the oxygen-dependent bactericidal mechanism.

Table 1: Quantitative, qualitative, and distribution characteristics of oxygen-dependent and oxygen-independent mechanisms of bactericidal activity of canine peripheral blood phagocytes

Phagocytosis stages	Cytochemical visualization of the					
	Percentage of phagocytes containing bactericidal substances		Quantitative changes in bactericidal substances in the cytoplasm of phagocytes		Localization of bactericidal substances in phagocytes relative to phagocytosed particles	
	A*	B**	A*	B**	A*	B**
1 Before phagocytosis	5 - 12	12 - 23	= ***	=***	In the cytoplasm	In the cytoplasm
2 At the beginning of phagocytosis	35 - 45	12 - 23	> ****	=	At the periphery of phagocytosed particles	Migrate side of phagocytosed particles
3 In the active phase of phagocytosis	60 - 85	12 - 23	>>*****	=	On the periphery and inside phagocytosed particles	Densely around the periphery of phagocytosed particles

A=Oxygen Dependent*, B= Oxygen independent**. *=Detected visually in the NBT test. **=Detected visually when staining for cationic proteins. ***=Content in non-stimulated cells. ****=The number of cells in the cytoplasm increases. *****=The number of cells in the cytoplasm increases significantly.

Conclusion

In an inactivated state, the granulocytes of the peripheral blood of dogs have either oxygen-dependent bactericidal activity (oxidants) or oxygen-independent bactericidal activity (cationic proteins). In activated neutrophils with oxygen-dependent bactericidal activity (oxidants), the quantitative parameters of the latter increase. At the same time, the quantitative parameters of the components of oxygen-independent bactericidal activity (cationic proteins) in the corresponding granulocytes remain unchanged. Phagocytes responsible for the oxygen-dependent mechanism of bactericidal activity interact with phagocytes responsible for the oxygen-independent mechanism of bactericidal activity. In this case, phagocytosed particles treated with bacteriotoxic oxidants in one phagocyte are additionally surrounded and treated with cationic proteins in another.

Author's Contribution

AS conceived of the presented idea and supervised the project. AB developed the theory and performed the computations. AK verified the analytical methods. AM carried out the experiment. MN wrote the manuscript with support from AM, AK and AB.

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