FLOW CYTOMETRY EVALUATION OF PHAGOCYTIC ACTIVITY AND OXYGEN METABOLISM OF GRANULOCYTES IN PERIPHERAL BLOOD OF RABBITS WITH NATURAL TRICHOPHYTOSIS

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Abstract

Cytometric evaluation of blood of rabbits naturally affected with trichophytosis showed a successive and significant increase in the percentage of phagocytic cells and the highest level of phagocytes was found on the 42nd day of the investigation. However, the analysis of phagocytic activity, oxygen metabolism (% of activated cells), and mean channel of fluorescence of granulocytes demonstrated a significant decrease on the 14th day and the highest mean values were obtained on the 42nd day of the investigation. An increase in phagocytic activity and oxygen metabolism of granulocytes on the 42nd day were observed during spontaneous recovery. The results of the presented study indicated that the non-specific cell-mediated immunity contributed to the elimination of the infection.

Key words: rabbits, Trichophyton mentagrophytes, non-specific immunity, phagocytosis, oxidative burst, flow cytometry.

In recent years, intensive mass-production as well as private rabbit breeding has started in Poland. Rabbits are raised for a variety of reasons; however, they are the most important value as a source of food. Rabbits produce white meat that is high in protein, low in fat and cholesterol, and can be substituted for poultry. Simultaneously, the incidence of dermatophytosis in rabbits has significantly increased. Dermatophytosis in large commercial rabbitries represents a costly and serious health problem. It is also considered as an important epidemiological and clinical problem. Trichophyton mentagrophytes is a dominant agent of rabbit dermatophytosis. Trichophytosis is also an important zoonosis (4, 16).

The mechanisms of innate and acquired immunity are involved in the protection against dermatophytosis. The importance of these mechanisms is supported by clinical observations demonstrating that the dermatophyte infections occur in individuals with defective both innate and acquired immunity (6).

The pathogenesis of dermatophytosis may be related to a number of factors such as immunogenicity of the infecting microorganism, the site of infection, and the animal’s immune response. Non-specific cell-mediated immunity and acute inflammation are the essential elements of the host defence mechanism against dermatophytes. Similarly, to other animal species, the development of clinical signs of trichophytosis in rabbits greatly depends on the efficiency of non-specific immunity. Phagocytosis and oxidative burst by polymorphonuclear leukocytes (PMNs) are a part of the innate defence mechanism of the phagocytic cells (1).

In dermatophytosis, the fungal focus is infiltrated mainly by neutrophils activated by the chemotactic factors (complement fragment C3, IL-8) released by keratinocytes. The neutrophils have oxygen dependent and oxygen independent mechanisms (defensins, cathepsin, lysozyme, elastase), which strongly inhibit the growth of fungi. Moreover, they contain a large amount of proteins binding calcium, and zinc (calproteins) and thus have fungistatic effects. Therefore, in the early period of fungal infection, the neutrophils play an essential role in phagocytosis. Small particles of the fungus (e.g. microconidia) are easily absorbed and destroyed in the phagolysosome by phagocytosis. The myeloperoxidase system of neutrophils exerts destructive effects on dermatophyte spores, even in the presence of catalase released by fungi, whose role is to prevent this phenomenon. The mycelium hyphae, on the other hand, are surrounded by the neutrophils, which join together by means of lectin receptors and release the fungicidal substances (mainly oxidising metabolites) to the direct surrounding.
Furthermore, the cells involved in phagocytosis of dermatophytes are the source of the production of lactoferrin, free oxygen radicals, and lysozomal peroxidise, and cooperate in the initiation of the process of repair for the damaged skin elements (3, 7, 10).

Better understanding of innate immunity may widen our knowledge about the mechanisms involved in host immunity to fungal infections. Non-specific cell-mediated immunity involved in the prevention and control of dermatophytosis has not been investigated in detail by using flow cytometric method. Although some flow cytometric methods of quantifying phagocytosis have been described for human PMN leukocytes, there are no reports dealing with the quantification of phagocytosis in rabbit leukocytes during natural trichophytosis (5).

The aim of the present study was to evaluate the phagocytic activity and oxygen metabolism of granulocytes in peripheral blood of rabbits with natural trichophytosis by using the flow cytometry method. The profile and intensity of non-specific immune responses in rabbits with natural trichophytosis are not fully known in rabbits. Therefore, better understanding of the immunological phenomena of natural trichophytosis can make possible developing efficient immunoprophylaxis and immunotherapy of the disease in rabbits.

Material and Methods

Animals. The investigations were carried out on sixteen 10-week-old rabbits (New Zealand White). The rabbits were housed singly and supplied with feed and water ad libitum. The rabbits of experimental group I (eight) came from a commercial rabbit production farm where clinical signs of dermatophytosis were observed for several years. The infection was confirmed by microscopic examination and fungal culture. The skin and hair samples were inoculated into Sabouraud Agar and incubated at 28ºC for up to four weeks. Dermatophytes were identified according to colony morphology and microscopic appearance using standard criteria. The group I was observed for 56 d and clinical signs of mycosis were noted (loss of hair, indurated well-demarcated plaque).

The infection intensity was recorded as (-) none, (+) mild, (+++) intermediate, (++++) intensive, and (+++++) disseminative trichophytosis, according to own scale.

The control group II consisted of eight healthy rabbits from uninfected rabbitries with no history of dermatophytosis.

Collection of blood samples. Peripheral venous blood (2 ml) was collected by venipuncture into tubes containing heparin as an anticoagulant and stored at room temperature for no longer than 4 h prior to use. In all the groups, the blood samples were collected at 0, 14, 28, 42, and 56 d of the experiment.

Skin test. The control group was tested with 0.2 ml of purified trichophytin TM-GP (Trichophyton mentagrophytes glycoprotein fraction) intracutaneously. The protein content (0.2 ml protein/mL) of the antigen preparation was measured according to Woloszyn and Umiński (19). Prior to the skin test, dermatophyte infestation was excluded. Skin tests were read after 24, 48, and 72 h, perpendicular diameters of the indurations were measured and mean values calculated. The reactions with a mean diameter of at least 5 mm were considered to be positive. None of the controls showed reactivity.

Phagocytosis assay. The phagocytic activity was measured in whole blood by flow cytometry using the Phagotest kit (Orpegon Pharma, Germany) according to the instructions of the manufacturer. Heparinised peripheral blood (100 µl) was incubated with 20 µl of FITC-labelled heat-killed E.coli (10 cfu) for 20 min at 37ºC, a negative control remained in the ice bath for 20 min at 0ºC. At the end of the incubation, all the samples were placed in the ice bath in order to stop phagocytosis. A volume of 100 µl of ice-cold Quenching Solution was added to each sample. The samples were mixed (vortex mixer) for 2 min at 25ºC. Then, 3 ml of cold washing solution was added to each sample and centrifuged at 1,250 rpm for 5 min at 4ºC. After two washing steps, the erythrocytes were removed with 2 ml of lysing solution added for 20 min at room temperature. The supernatant was discarded; 3 ml of cold washing solution was added and then centrifuged at 1,250 rpm for 5 min at 4ºC. DNA staining solution (100 µl) was added and incubated in the ice bath for 10 min. The samples were analysed by flow cytometry (EPICS XL, Beckman Coulter) within 30 min. The percentage of FITC-positive cells and mean channel of fluorescence were measured and the phagocytic index (% of positive cells x mean channel/100) calculated after gating the granulocytes by size and granularity. Phagocytic activity was expressed as the mean channel of fluorescence, emitted by the cells, which was proportional to the number of phagocytosed bacteria.

Oxidative burst assay. Oxidative burst was measured in whole blood with flow cytometry by using the Bursttest kit (Orpegon Pharma, Germany), according to the instructions of the manufacturer. Heparinised whole blood (100 µl) was incubated for 10 min at 37ºC in the water bath with 20 µl of E.coli suspension (E.coli opsonised), 20 µl of PMA working solution (high control), 20 µl of FMLP working solution (low control), and 20 µl of washing solution (a negative control). After incubation, 20 µl of substrate solution was added to each sample and incubated for 10 min at 37ºC in the water bath. The erythrocytes were removed by the addition of 2 ml of lysing solution to each tube for 20 min at room temperature. After incubation, the samples were washed in 3 ml of washing solution to each tube for 20 min at room temperature. After incubation, the samples were washed in 3 ml of washing solution and centrifuged at 1,250 rpm for 5 min at 4ºC. Then DNA staining solution (200 µl) was added and incubated for 10 min in the ice bath. The samples were analysed by flow cytometry (EPICS XL, Beckman Coulter) within 30 min. Oxygen metabolism of granulocytes (% of activated cells) and phagocytic activity of granulocytes (mean channel of fluorescence) in peripheral blood were measured.

Statistical analysis. Data were compared by calculating means, standard deviations and the
significance of differences between groups and successive determinations using the Student’s t-test. The level of significance was set at P≤0.05 and P≤0.01.

Results

The generalised form of trichophytosis was found in all experimental rabbits. The clinical signs of infection included: patchy, crusty, and erythematous areas of alopecia of variable severity, most commonly located on the head, auricles, and limbs. The infection was found to be intermediate (++) in four rabbits, intensive (+++) in two, and disseminate (++++) in another two. The control rabbits remained uninfected. In all infected rabbits, the clinical signs of trichophytosis were observed until the 28 d of observation. After that period, the gradual regression of clinical signs was noted, which manifested itself in exfoliation and come off with the crust. On the 42nd d, the clinical signs, as single foci on the limbs, were found only in two rabbits (with previous disseminated trichophytosis). On the 56th d, the clinical signs of trichophytosis disappeared spontaneously in all rabbits. *Trichophyton mentagrophytes* was isolated up to the 42nd d. The results of mycological examination on the 56th d were negative. There were no signs of trichophytosis in the control group and the mycol ogical examination findings were negative.

The results of phagocytosis of FITC-labelled bacteria are presented in Fig. 1. The mean baseline values of the percentage of phagocytes with phagocytosed bacteria in the experimental group were significantly higher (P≤0.01) compared to the control group until the 14th d of the experiment. In the successive measurements, a significant increase in the percentage of phagocytes was observed in the infected rabbits. Compared to baseline values and control group, the highest level of phagocytes was found on the 42nd d. On the day 56, the percentages of phagocytes were significantly lower (P≤0.05) compared to baseline values but higher (P≤0.01) than controls. The percentages of phagocytes in control rabbits were at the same level throughout the study.

The values of mean channel of fluorescence are shown in Fig. 1a. The initial mean channel of fluorescence in the experimental group was significantly higher compared to controls. On the 14th d, a significant and rapid decrease was observed in comparison to baseline (P≤0.01) and control (P≤0.01) values followed by a gradual and significant increase. The highest mean values were obtained on the 42nd d. At the final measurement (day 56), the mean channel of fluorescence was similar to baseline values yet significantly higher compared to the control group. The values in controls were significantly lower compared to the experimental group, except for the day 14.

In rabbits with natural trichophytosis, the percentage of *E. coli* activated cells at day 0 was significantly (P≤0.01) higher than that in controls (Fig. 2). During the next measurement, its mean values significantly decreased compared to baseline (P≤0.01) and control (P≤0.05) values. Then, a gradual significant increase (P≤0.01) was observed compared to baseline and control values; the highest mean values were demonstrated on the 42nd d. During the final measurement, the mean values of percentage of activated cells were similar to the baseline values but significantly (P≤0.01) higher than in controls. In the control group, the percentage of activated cells was significantly (P≤0.01) lower than that in the experimental group, except for the day 14.

![Fig. 1. Cytometric analysis of phagocytic activity of granulocytes (% of phagocytic cells) in peripheral blood in rabbits with natural trichophytosis (x ±SD).](image-url)

A – statistically significant differences between the experimental and control groups (P≤0.01)
b - statistically significant differences between successive collections and day “0” (P≤0.05)
B – statistically significant differences between successive collections and day “0” (P≤0.01)
Fig. 1a. Cytometric analysis of phagocytic activity of granulocytes (mean channel of fluorescence) in peripheral blood in rabbits with natural trichophytosis (x ±SD).

A – statistically significant differences between the experimental and control groups (P ≤ 0.01)

b - statistically significant differences between successive collections and day “0” (P ≤ 0.05)

B – statistically significant differences between successive collections and day “0” (P ≤ 0.01)

Fig. 2. Evaluation of oxygen metabolism of granulocytes (% of activated cells) in peripheral blood following E. coli activation in rabbits with natural trichophytosis (x ±SD).

A – statistically significant differences between the experimental and control groups (P ≤ 0.01)

a – statistically significant differences between the experimental and control groups (P ≤ 0.05)

B – statistically significant differences between successive collections and day “0” (P ≤ 0.01)
Fig. 2a. Evaluation of oxygen metabolism of granulocytes (mean channel of fluorescence) in peripheral blood following E.coli activation in rabbits with natural trichophytosis (x ±SD).

A – statistically significant differences between the experimental and control groups (P≤0.01)
a – statistically significant differences between the experimental and control groups (P≤0.05)
B – statistically significant differences between successive collections and day “0” (P≤0.01)

The initial mean channel of fluorescence in the experimental group was significantly lower than that in the controls (Fig. 2a). During the next measurement, a further significant decrease was observed compared to baseline and control values. Then, the mean channel of fluorescence in the infected rabbits significantly increased reaching its highest values on the 42nd d. During the final measurement the mean values were found to be significantly (P≤0.01) higher than the baseline values yet lower compared to the controls. In the control group, the values of fluorescence channel remained at the level similar to the initial one throughout the observation period, however at the first, second, and final measurements they were significantly higher compared to experimental rabbits.

**Discussion**

The clinical picture of trichophytosis in rabbits mainly depends on age, immunological status, and living conditions. In our study, the 10-week-old naturally infected rabbits showed deep crusty trichophytosis characterised by typical fungal foci in the form of oval alopecia covered with thick crusts tightly united with the skin. Our findings confirm the previous results published by Kostro et al. (8). Pure *T. mentagrophytes var. granulosum* were cultured from the hair and epithelium scrapings collected from all experimental animals, which confirms the opinion of many authors that this species plays a dominant role in the aetiology of dermatophytosis in rabbits (11, 17).

On the 1st d, the clinical picture in the individual rabbits varied. The results are likely to indicate that the susceptibility of rabbits to *T. mentagrophytes var. granulosum* infection under natural conditions depends on the individual susceptibility, which is mostly related to the status of their immune system, *i.e.* reactivity of the mechanisms determining the initiation of the immune response due to infection. In organisms with normal defensive mechanisms, the contact with the fungus results only in the long-term carrier state. When the defensive mechanisms are impaired, the animals in the infected environment develop overt trichophytosis with the tendency to its chronic form (12). The clinical signs of trichophytosis in naturally infected rabbits persisted up to the 42nd d and then spontaneously subsided. Our findings confirm the observations reported by Wołoszyn *et al.* (18) that dermatophytosis in rabbits tends to be chronic, without clinical signs.

The flow cytometry method with the adapted Phagotest and Bursttest kits was used in our study to assess the phagocytic activity and oxygen metabolism in rabbits with natural trichophytosis. Our study revealed the two-stage immune response in rabbits naturally infected with *T. mentagrophytes*. In the first period of trichophytosis, the indices of non-specific cellular immunity significantly decreased, while in the second one the immune responses increased and clinical signs of the disease subsided. The available literature reports contain no data about immune phenomena in natural trichophytosis in rabbits. The analysis of indices of the phagocytic activity and oxygen metabolism in granulocytes of peripheral blood in rabbits infected with
T. mentagrophytes shows that their changes were related to the course and regression of the disease. A significant decrease in phagocytic activity and oxygen metabolism of granulocytes determined with flow cytometry was observed during the development of fungal lesions and was similar throughout the disease process. This concerned a significant mean decrease in the channel of fluorescence, in particular, which is a feature of the granulocyte phagocytosis intensification. The highest mean values of the percentage of phagocytic and activated cells and mean channel of fluorescence in rabbits naturally infected with T. mentagrophytes were observed during spontaneous recovery.

The changes observed – a drop or increase in indices of phagocytic activity and oxygen metabolism of granulocytes - are likely to be related to the infection and suppressive effects of the fungus, which removing during the recovery resulted in significantly increased indices of non-specific antimycotic cellular immunity. The suppressive effects on the cellular mechanisms of non-specific antimycotic immunity are exerted by mammans contained in the cell wall of T. mentagrophytes, whose target cells are mainly monocytes and macrophages. The inhibitory effects of these substances are likely to consist in blocking of the surface receptors of phagocytic cells (9, 14, 15).

Therefore, it may be assumed that the impaired biological function of granulocytes in rabbits with natural trichophytosis resulted in the lack of ability to destroy the fungus in the process of exo- and endophagocytosis and to eliminate the clinical signs in the first period of dermatophytosis. A significant decrease in phagocytic activity and oxygen metabolism of granulocytes manifested in the lowest channel of fluorescence in rabbits during the disease process was also likely to affect the development of effective specific cellular antimycotic immunity. It is generally accepted that the efficient system of phagocytic cells is one of the basic mechanisms of non-specific cellular antimycotic immunity involved in the elimination of dermatophytes (13). In the early period of fungal infection, the key role in phagocytosis is played by the granulocytes, mainly neutrophils, which are the dominant population in peripheral blood and react quickly when the dermatophyte infection develops. The fungus spores are destroyed by two different mechanisms: the first one is oxygen dependent while the second is connected with digestion by lysosomal enzymes. It is thought that the first mechanism is more effective against T. mentagrophytes as the neutrophils are capable of destroying spores as well as hyphae by releasing the fungicidal substances (mainly oxidising metabolites) into the direct surrounding (2). This fact is confirmed by our results, which demonstrate that oxygen dependent mechanism of granulocytes is an important element of antimycotic immunity.

This is the first report that contains the data about immune phenomena in natural trichophytosis in rabbits. Better understanding of these interactions will significantly widen our knowledge about the host defence in dermatophytosis.

References