Specific cell-mediated immune response in rabbits immunised and infected with *Trichophyton mentagrophytes*

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Abstract

Deep crusty dermatophytosis was demonstrated in 10-week-old rabbits naturally and experimentally infected with *T. mentagrophytes*. Moreover, specific cell-mediated immune response in *T. mentagrophytes*-infected rabbits occurred in two phases. During the presence of clinical symptoms of trichophytosis, slightly positive results of leukocyte migration inhibition factor (LMIF) assay were noted, whereas the highest values were observed during spontaneous regression of fungal lesions and complete recovery. The slightly positive LMIF values in rabbits with fungal lesions reflect the mechanism of cell-mediated immune response during *T. mentagrophytes* infection. On the other hand, the highest positive LMIF values during spontaneous recovery indicate that regression of fungal lesions is attributed to acquired specific cellular immunity. The findings also confirm that the used vaccine against trichophytosis quite strongly stimulates the cell-mediated immune mechanisms, which is confirmed by positive LMIF values in immunised rabbits.

Key words: rabbits, dermatophytosis, *Trichophyton mentagrophytes*, cell-mediated immunity.

Introduction

The protection of rabbit health and welfare under the conditions of mass-scale breeding and commercial production is associated with numerous new health-related problems. In Poland, the current and relevant economic and sanitary issues in large rabbit farms regard dermatophytosis caused mainly by *Trichophyton mentagrophytes* (10, 12). Therefore, new challenges concerning prevention, control, and eradication of this disease have to be faced. The treatment and elimination of trichophytosis in rabbits in indoor breeding system, in which the disease occurs stationary, is difficult and not always effective. The difficulties are associated with a high resistance of *T. mentagrophytes* spores to the environmental factors and disinfectants, as well as hindered penetration of antifungal drugs into the site of active disease. Moreover, local application of antifungal chemotherapeutics is time-consuming and it is not efficient. Additionally, some systemic drugs are highly toxic and have immunosuppressive and teratogenic effects (1, 6). The available studies indicate that specific prophylaxis and therapy are the most effective methods of rabbit dermatophytosis control in farms, where the disease occurs stationary (10, 12, 16, 20).

The knowledge about specific immune response in rabbits with natural and experimental trichophytosis, as well as in animals vaccinated against this disease will enable controlled stimulation of antifungal immunity for prevention and treatment of rabbit trichophytosis. The aim of the study was to analyse specific cell-mediated immune response in rabbits naturally and experimentally infected with *T. mentagrophytes* and those immunised with an inactivated vaccine against trichophytosis.

Material and Methods

Experimental animals. The first part of examinations was carried out in eight New Zealand rabbits, aged 10 weeks, with early trichophytosis, *i.e.* 2 weeks after the symptoms were observed (Group I). In this group, *T. mentagrophytes var. granulosum* was isolated. The severity of symptoms in individual animals was assessed according to the following scale (10): (+) single fungal foci on the head or limbs, (+++) single fungal foci on the head and ear pinnae, (++++ fungal foci on the head, pinnae, and limbs, and (++++) the generalised form - fungal foci scattered all over the skin surface.
In the second part, examinations were performed in eight New Zealand rabbits, aged 10 weeks, from the dermatophytosis-free farm, which were experimentally infected (Group II). Before the infection, hair samples were collected from various body areas for microbiological testing. No increased numbers of dermatophytes were observed in any sample. The rabbits were experimentally infected with the virulent species of *T. mentagrophytes var. granulosum* (Tm-K) isolated from rabbits with natural trichophytosis (10). The initial examinations demonstrated that the Tm-K species induced typical lesions in the form of fungal foci in animals infected with a dose of 10^5 cfu/mL, which was considered the lowest infective dose for rabbits. The rabbits were infected by rubbing the suspension (10^5 cfu/mL) into the shaved and slightly scarified skin for successive 2 d around the scapula, thighs on both sides, and back (in total, five areas). The daily dose, *i.e.* 5 mL of suspension, was divided into five portions and rubbed into the skin of each rabbit. Animals with natural and experimental trichophytosis were under clinical observation to assess the dynamics of the disease process, time of regression of fungal lesions, and survival of fungal spores. The survival of *T. mentagrophytes* spores was evaluated by culturing the scrapings collected from the lesions onto the Sabouraud medium with the addition of actidione (27°C, 14 d) at the same time intervals as leukocyte migration inhibition factor (LMIF) assay.

During the final stage, eight New Zealand dermatophytosis-free rabbits, aged 10 weeks, from the same farm as group II animals, were examined (Group III). Specific immunity against trichophytosis was provided using the commercial inactivated vaccine Alopevac containing highly immunogenic species of *T. mentagrophytes* and *T. verrucosum* (Biowet, Pulawy, Poland) used for the prevention and treatment of trichophytosis in foxes. The field examinations demonstrated that the vaccine had good therapeutic properties and effectively prevented the clinical symptoms of trichophytosis in rabbits in the infected environment (10). Moreover, the initial findings showed that the vaccine induced specific antifungal immunity even in the 10-18-day-old suckling rabbits (12). Alopevac, at the dose of 0.5 mL, was administered intramuscularly twice at the 14-day interval. The control group consisted of 16 healthy rabbits, uninfected and unimmunised from the same farm as animals of groups II and III (group IV). Before the experiment, hair samples were collected from various body areas of control animals for microbiological testing. No increased numbers of dermatophytes were observed in any sample. During the study, experimental and control animals received full-portion granulated fodder with the addition of coccidiostatics and water *ad libitum*.

The rabbits were under observation to determine the extent of specific cell-mediated immune response during natural and experimental *T. mentagrophytes* infections as well as in immunised animals using the LMIF assay. In group I, testing was performed on day 0 (2 weeks after the development of clinical symptoms of trichophytosis) and repeated at 14-d intervals throughout the 8-week observation. In group II, the tests were carried out on day 0 and days 8, 14, 22, 30, and 40 after experimental infection, whereas in group III - on days 0, 7, 14, 21, 28, 42, and 56 after immunisation.

The results were statistically analysed using the Student’s t-test.

**Leukocyte migration inhibition factor assay.** The test was performed using the capillary tube method according to Bendixen and Soborg (2). The blood (10 mL) was sampled from the heart to the sterile syringes with the addition of 20 U/mL preservative-free heparin (Heparin, Sigma, USA), and 10 μg/mL gentamicin (Gentamicin, Gibco, USA). The mononuclear cells were isolated by centrifugation of twice-diluted blood deposited over the one-step gradient, density 1.119 g/cm³, (Gradisol G, Aqua-Medica, Poland), according to Ferrante and Thong (5). The viability of cells, assessed using the trypan blue staining, exceeded 95%. The leukocyte suspension of 5x10⁶/mL density was placed in sterile, silicone capillary tubes, which were centrifuged at 600 rpm for 5 min. The fragments with the deposited leukocyte layer of about 2 mm were stuck horizontally to the bottom of the Davis chamber using a silicone paste (Bayer). The chambers were closed with cover glasses glued with melted paraffin. One chamber was filled with the Eagle medium with the addition of 15% horse serum (Biomed, Poland) and the plasmatic antigen obtained from the *T. mentagrophytes* (Tm-7) species according to the method described by Kostro (9). The antigen was standardised in relation to the amount of protein, which concentration was determined using the Lowry et al. (13) method. The final antigen concentration in the test was 40 μg/mL. The second chamber, filled with the medium with horse serum without antigen, was the control. Chambers were incubated at 37°C for 24 h. The migration areas were outlined on the filter paper using a microfilm reader; the discs were cut out and weighed to calculate percentage inhibition according to the formula: average weight of migration areas in the environment with antigen/average weight of migration areas in the antigen-free environment x 100.

The inhibition values above 20% were considered as specific.

**Results**

The rabbits under observation developed deep crusty dermatophytosis. The fungal foci in the form of oval alopecia covered with the asbestos crusts strongly adhered to the skin were most commonly located on the head, pinnas, and limbs. Among eight rabbits selected on the day of initial examinations, four had fungal lesions classified into group (+++) of severity, other two animals showed lesions classified as (+++), and the remaining two developed the generalised form of dermatophytosis (++__).
**Fig. 1.** Peripheral blood leukocytes migration inhibition in rabbits with natural trichophytosis (x ± SD)  
** - P<0.01; comparison with initial value in the experimental group;  
20% migration inhibition was considered as positive

**Fig. 2.** Peripheral blood leukocyte migration inhibition in rabbits experimentally infected with *T. mentagrophytes* (x ± SD)  
** - P<0.01; comparison with initial value in the experimental group;  
20% migration inhibition was considered as positive

**Fig. 3.** Peripheral blood leukocyte migration inhibition in rabbits immunised against trichophytosis (x ± SD)  
** - P<0.01; comparison with initial value in the experimental group;  
20% migration inhibition was considered as positive
During the first period of observation, *i.e.* until day 28, the fungal foci persisted in all rabbits. After this period, the fungal lesions on the head, pinnae, and front extremities started to subside gradually, *i.e.* the crusts were found to exfoliate and separate. On day 42, the fungal lesions (single foci on the limbs) were present only in two rabbits with generalised trichophytosis diagnosed at the beginning of experiments. On day 56, the fungal lesions spontaneously regressed in all rabbits. The pure *T. mentagrophytes* culture was isolated from the scrapings of infected rabbits before and during the examinations, *i.e.* until day 42 of observation. On day 56, the cultures from all rabbits were negative. In the control group no symptoms of the disease were observed and the culture results were negative.

The clinical symptoms in experimentally infected rabbits, such as congestion, skin oedema, and desquamation of the epidermis at the site of inoculation with *T. mentagrophytes* developed between day 6 and 8 after infection. After 12-14 d, blisters and crusts of various thicknesses, grey-yellow, and then asbestos in colour occurred; they covered the entire foci and were always delimited from the infection area. The crusts separated and came off spontaneously after several days, exposing the healthy epidermis. In rabbits infected with *T. mentagrophytes*, the lesions regressed between day 30-40 after infection, which was manifested in crust exfoliation and coming off. In all animals, the culture examinations demonstrated the presence of *T. mentagrophytes* in the diseased foci between day 6 and 22 after infection. After 30 and 40 d, the culture results were negative in all rabbits. In the control group, the disease symptoms were not observed and the culture results were negative.

In the LIMF test, in the case of rabbits with natural trichophytosis, the positive values within the limits of 28.3%-29.4% were noted until the observation day 28. (Fig. 1). Starting on day 42, the values of specific migration inhibition further significantly increased to reach the highest values on day 56 (37.4%). At that time, clinical symptoms of mycosis disappeared and microbiological results were negative. In the control group, the test results were negative at all examination points.

The data presented in Fig. 2 demonstrates that in rabbits experimentally infected with *T. mentagrophytes*, specific leukocyte migration inhibition was noted during the period of maximum development of clinical symptoms of trichophytosis (day 14) and maintained at the similar level until day 22. During the regression of lesions, the migration inhibition rate further increased and at the final determination point (day 40), the maximum test values were observed (34.9%).

The positive LIMF values were noted in rabbits immunised against trichophytosis on day 21 after immunisation (7 d after the 2nd vaccine dose). The highest mean LIMF values in the experimental animals were observed on day 28 (Fig. 3). At further determination points, the extent of migration inhibition significantly decreased and on day 56 the test values were negative (below 20%) in immunised rabbits. In control rabbits, the mean LIMF values were significantly below the borderline value at all determination points.

**Discussion**

The examinations of 10-week-old rabbits naturally and experimentally infected with *T. mentagrophytes* demonstrated deep crusty trichophytosis characterised by typical fungal foci in the form of oval alopecia covered with thick crusts strongly adhered to the skin. The observations confirmed the results published by Wołoszyn *et al.* (20) and Kostro *et al.* (10, 12) indicating that young rabbits were most susceptible to dermatophytosis, revealing the highest severity of clinical symptoms and developing the deep crusty form of the disease.

The findings confirm the induction of specific cell immune response in rabbits during natural and experimental *T. mentagrophytes* infection, which was evidenced by positive LIMF test results. Specific leukocyte migration inhibition was observed in all rabbits infected naturally or experimentally with *T. mentagrophytes*, and the extent of inhibition was correlated with the severity of clinical lesions. On the other hand, there were marked differences in positive LIMF values in rabbits between the period of occurrence of clinical symptoms of dermatophytosis and the period of regression of fungal lesions and complete recovery. Slightly positive values of LIMF testing in rabbits during the development of fungal lesions reflect the mechanism of cell-mediated immune response in the course of *T. mentagrophytes* infection. It can be suggested that during the presence of clinical symptoms, the specific cell immunity is suppressed by the presence of the fungi in the diseased skin and their direct inhibitory effect on lymphocytes Th1 producing cytokines involved in the support of non-specific and specific cell immunity (4, 19). During spontaneous recovery, the LIMF values reached the highest values throughout the entire observation period. This can be explained by the elimination of the fungi from lesions inhibiting the development of specific Th1 cell immunity (3, 4). Thus, spontaneous recovery from trichophytosis, *i.e.* complete regression of inflammatory and crusty lesions, as well as covering of the entire focus with healthy epidermis, and visible hair re-growth was attributed to the development of specific cell-mediated immunity, which is essential for elimination of infections caused by dermatophytes (7, 8, 14, 15, 17). The above data is consistent with the findings of other authors studying experimentally infected guinea pigs, mice, rats, foxes, and calves, which confirms that the extinction of the disease is associated with the development of specific antifungal cell immunity (9, 11, 16-18). The vaccine Alopevac used in the study quite strongly stimulated cell-mediated immune mechanisms, which was evidenced by positive LIMF values in immunised rabbits. The highest values of specific leukocyte migration inhibition were noted between day 21
and 28 after the second dose of dose. These findings confirm the observations of Wawrzkiewicz and Wawrzkiewicz (18), demonstrating that the highest immune response after administration of inactivated antifungal vaccines is observed between day 7 and 14 after the second vaccine dose.

References