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PROPIONIBACTERIUM GRANULOSUM ACTIVITIES IN RATS DURING THE MUSCLE PHASE OF *TRICHINELLA SPIRALIS* INVASION

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ODDZIAŁYWANIE *PROPIONIBACTERIUM GRANULOSUM* NA FAZĘ MIĘŚNIOWĄ *TRICHINELLA SPIRALIS* U SZCZURÓW

Abstract. Immune response of Wistar rats, infected with 4000 L_1 *T. spiralis* and treated with *P. granulosum* during the muscle phase of nematode invasion were measured. The increase of spleen mass was observed in all groups infected and exposed to *P. granulosum*. Intraperitoneal injection of bacteriae results in higher level of T lymphocytes and activated neutrophils. The level of inhibition of macrophages migration was depended on relation to the time and doses of injection. In non-specific stimulated animals there were not statistically significant changes in the level of specific IgG_1 antibodies determined by ELISA, against the crude extract of infective larvae of *T. spiralis*. The reduction of intensity of nematode invasion during the muscle phase was not observed in rats after *P. granulosum* treatment.

INTRODUCTION

Trichinella spiralis is a unique in that it has a wide mammalian host range and it spends its larval and adult life in the same host. Several studies have demonstrated that every stage of *T. spiralis* can induce a phase-specific immunity in the host (MURRELL 1985, WANG et al. 1990). But more recently it has been shown that many parasites including *T. spiralis* can depress the immune response of the host (BROJEVIC et al. 1983). Several authors have reported that both specific and non-specific activation of host immune system might be expected to enhanced resistance to parasitic infections and hance to lead to reduction in the number of parasitic harboured (RUITENBERG and STEERENBERG 1973, KARMAŃSKA et al. 1985, KOCIĘCKA et al. 1989). Such immunostimulatory properties of *Propionibacterium* species were initially reported by HALPERN et al. (1964). *Propionibacterium granulosum* operates as non-specific activator of antibacterial, antiviral and antitumoural responses. Results of studies on experimental animals have found stimulation of reticulo--endothelial system and both humoral and cell-mediated immunity after the introduction of propionibacteria. The cytolytic activity of NK cells and non-

-specific stimulation of macrophages have been also demonstrated (ROSZKOWSKI et al. 1982, JANIAK et al. 1990).

Studies on *P. granulosum* activities in parasitized animals are limited. The influence on *Toxoplasma gondii* and *Moniezia* sp. infection has been noticed (ZEMBUROWA and ZIĘBA 1984, POŁEĆ 1992). The limitation on the intensity of *T. spiralis* intestinal phase of infection has been reported by RUITENBERG and STEERENBERG (1973), BANY et al. (1984), MOSKWA and POŁEĆ (1992b).

The purpose of the present experiment was to monitor parasitological and immunological parameters of rats exposed to *P. granulosum* intraperitoneal injection during the muscle phase of *T. spiralis* infection.

Material and methods

Experimental animals. Fourty male Wistar rats, aged 1 month were used in the study. The animals were divided into eight groups of five animals each. The experimental design is shown in Table 1. Animals receiving intraperitoneal

TABLE 1
Experimental design

Day of peritoneal injection	Non-infected control groups				Infected groups			
	I	II	II	IV	V	VI	VII	VIII
13 DAI	−	+	−	+	−	+	−	+
20 DAI	−	+	+	+	−	+	+	+
27 DAI	−	−	+	+	−	−	+	+
Dissection 42 DAI	All animals were killed on 42 DAI							

Symbols: + - day of *P. granulosum* treatment

injections were given 1 mg of lyophylized cell walls of *P. granulosum* in 0.5 ml PBS and 10,000 IU of penicillin. *P. granulosum* were given on 13 and 20 DAI (groups II and VI), on 20 and 27 DAI (groups III and VII) and on 13, 20 and 27 DAI (groups IV and VIII).

Rats of groups V–VIII were inoculated orally with 4,000 *T. spiralis* larvae obtained from infected hosts, using a standard pepsin-HCl design.

Evaluation of the muscle phase. Muscle larvae were recovered on 42 DAI. Killed rats were skinned and weighed. The carcasses were minced individually and incubated in the standard pepsin-HCl digestion fluid. The number of larvae per gram of tissue was calculated.

Immunological tests. Blood was sampled on 42 DAI. The changes of spleen mass were observed on the same day.

The immunological reactions were determined by mean of the percentage of T lymphocytes (MUELLER et al. 1975), circulating neutrophilic granulocytes (PARK et al. 1968), peritoneal macrophage inhibition test (CYPESS et al. 1971) and ELISA test. The level of T lymphocytes was measured basing on an acid Fα-naphthyl acetate esterase activity (ANAE). Procedure found that cytoplasm of cells reacting positively contained a single or a few distinct spoots of the reedish-brown reaction product. In the test in which neutrophilic granulocytes were determined, nitroblue tetrazolium penetrated to the cell stimulated by the infective factor and was reduced to isoluble diformozan under the influence of pirymidine nucleotides NADP and NADPH released as the result of cellular metabolism.

Peritoneal excudate of macrophages were obtained from rats after intraperitoneal injection of 0.1% bovine serum albumin in 10 ml sterile PBS and 50 IU heparinum. The viability of the cells was assessed by trypan blue and was greater than 95%. Concentration of 1×10^6/ml cells was used in the migration inhibition assy, all chambers were incubated at 37°C for 24 h. The migration assay was determined in triplicates. The antigen used contained 135 μg protein per 1 ml solution.

The migration index (MI) was calculated as follows:

$$MI = \frac{\text{mean area of migration of cells with Ag}}{\text{mean area of migration of control cells}} \times 100\%$$

Dynamic of occurrence of specific antibodies was determined by ELISA test. ELISA plates (Dynatech Lab.) were coated with crude extract of infective larvae of *T. spiralis* at concentration 10 μg/ml. The plates were washed five times with PBS-Tween 20. Nonspecific reaction were blocked by adding PBS-SM and incubating for 2 h at room temperature. The plates were washed as above and rat serum diluted 1 : 2000 were added to the wells. After incubating the plates were washed as above. Monospecific rabbit anti-rat IgG_1 (Serotec) diluted 1 : 5000 were added and incubated for 30 min at 37°C. Than the plates were washed as above. Horseradish peroxidase labelled sheep anti rabbit IgG (Serotec) at a dilution 1 : 5000 were added and plates were incubated for a further 30 min at 37°C. Plates were washed before the addition of 100 μl of substrate solution (TMB). Development of colour was allowed for 30 min, then 100 μl of 2 M H_2SO_4 was added to stop the reaction. Absorbance was read at 450 nm in a Dynatech reader. All samples were run in triplicate. Results are expressed as optical density at 450 nm (O.D.). Optical density (D.O.) values over the mean value of negative sera plus three times the standard deviation were considered as positive (CHAPA-RUIZ et al. 1992). STUDENT's *t*-test and DUNCAN's test were used to assess the significance of differences between groups, and values smaller than 0.05 considered to be statistically significant.

Results

Parasitological observations. The range of weights of carcasses was from 81.1 g ± 10.3 g to 105.2 g ± 9.7 g. Muscle larvae of *T. spiralis* were recovered from all infected groups (Tab. 2). A significantly higher number of larvae was

TABLE 2
Muscle larvae recovered from rats on 42 DAI
(*n* of rats in particular groups – 5)

Group	Mean body weight of carcasses ± SE	Mean of larvae recovered ± SE	Mean no. of larvae/g of carcasses ± SE
V	87.96 ± 4.70	276,700 ± 19,300	3200 ± 150
VI	81.10 ± 5.50	324,066 ± 47,900	3990 ± 410
VII	94.07 ± 12.80	283,641 ± 14,400	3020 ± 520
VIII	105.20 ± 19.35	251,191 ± 75,900	2380 ± 450

recovered from carcasses in group V (324,066 ± 47,900). The results in groups VII and VIII were not statistically significant.

Spleen mass. In the control groups the spleen weights were from 378.7 mg ± 19.1 mg (gr. I) to 496.3 mg ± 89.4 mg (gr. III). In infected control group (V) the spleen weight was 384.6 mg ± 46.9 mg (Fig. 1). The increase of spleen mass

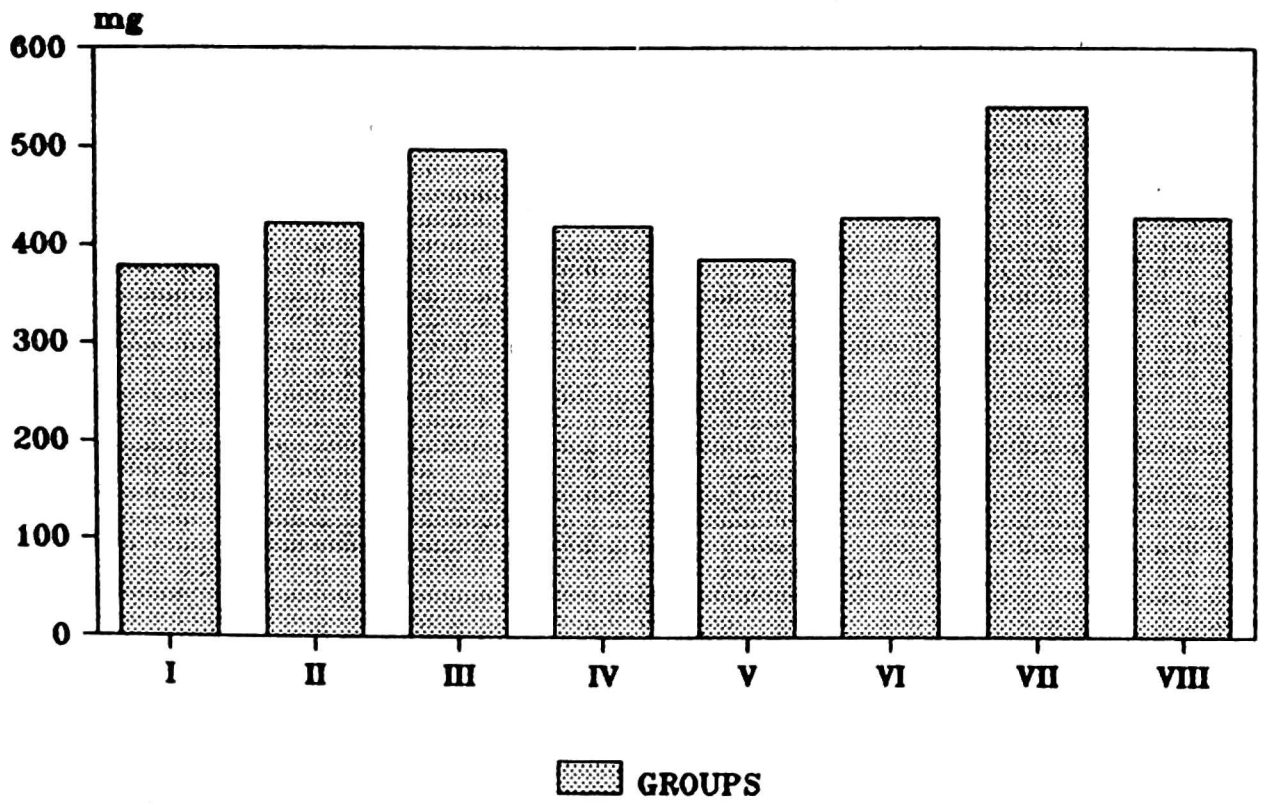

Fig. 1. The changes in the spleen mass: (I–III) non-infected control groups, (IV) infected with *T. spiralis* control group, (V–VIII) groups infected with *T. spiralis* and treated with *P. granulosum*. The same symbols for Figs 2–5

was observed in group VI (428.6 mg ± 30.9 mg), VIII (428.5 mg ± 90.0 mg). Only the result observed i group VII (540.0 mg ± 23.5 mg) was statistically significant as compared with non-infected, non-treated group I and infected group V ($p<0.05$).

T lymphocytes. In non-infected control groups (I–IV) percentage of T lymphocytes varied from 35.7% (IV) to 54.3% (II). In infected control group V the percentage of T lymphocytes was 32.8%. In all infected and exposed to intraperitoneal injection groups (VI–VIII), the higher T lymphocytes level was observed (VI–43.5%, VII–39.8%, VIII–42.6%). The results were statistically significant only was compared with infected control group (V) (Fig. 2).

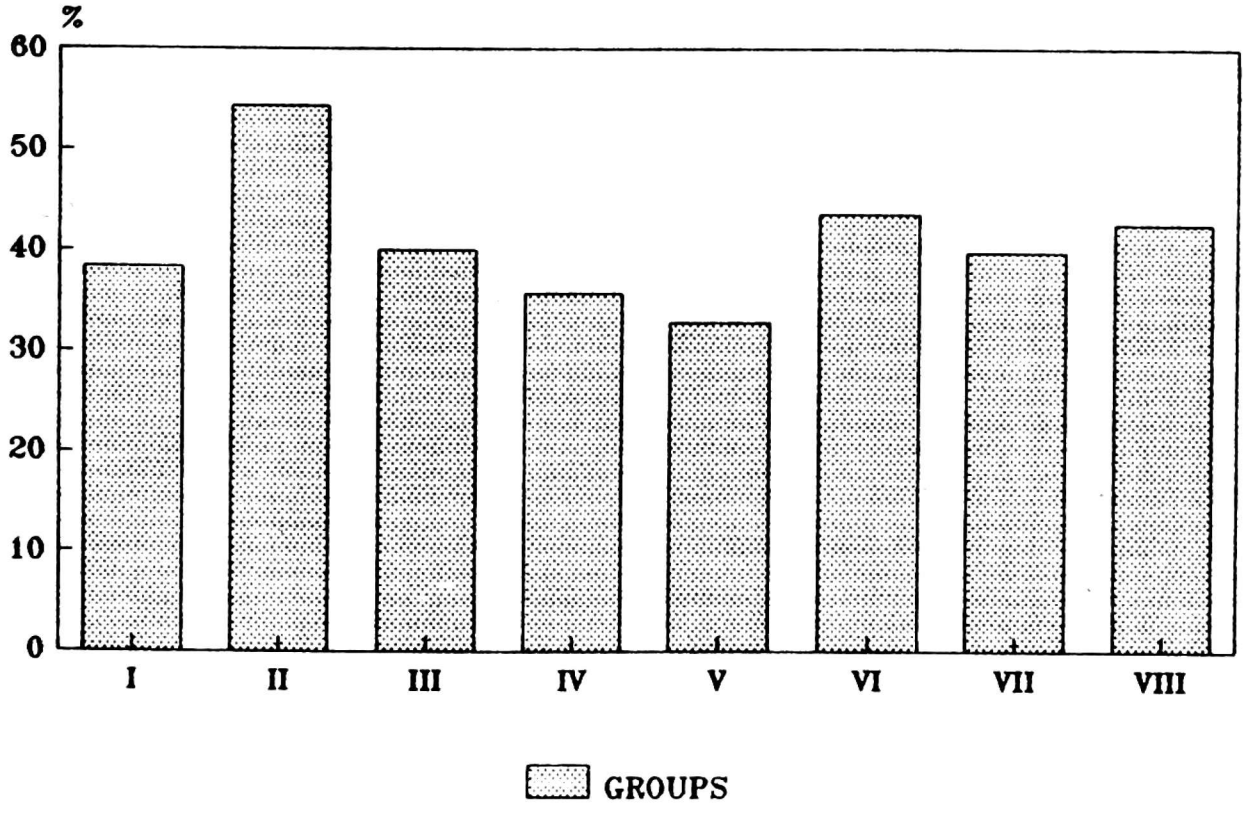

Fig. 2. The level of T lymphocytes in peripheral blood groups

NBT-positive cells. In non-infected control group I the percentage of the circulating neutrophilic granulocytes level was 7.3%. In infected control group V the percentage of NBT-positive cells was 11.5%. Following injection in non-infected control groups (II–IV), the higher NBT-positive cells level was observed, but the highest results were noticed after injections on 20 and 27 DAI (14.0%).

In rats infected and exposed to *P. granulosum* injections (VII–VIII) granulocytes level varied from 12.2%–14.4%. The changes were statistically significant as compared with non-infected group I ($p<0.01$). Results in groups VII and VIII were significant as compared also with infected control group V ($p<0.05$) (Fig. 3).

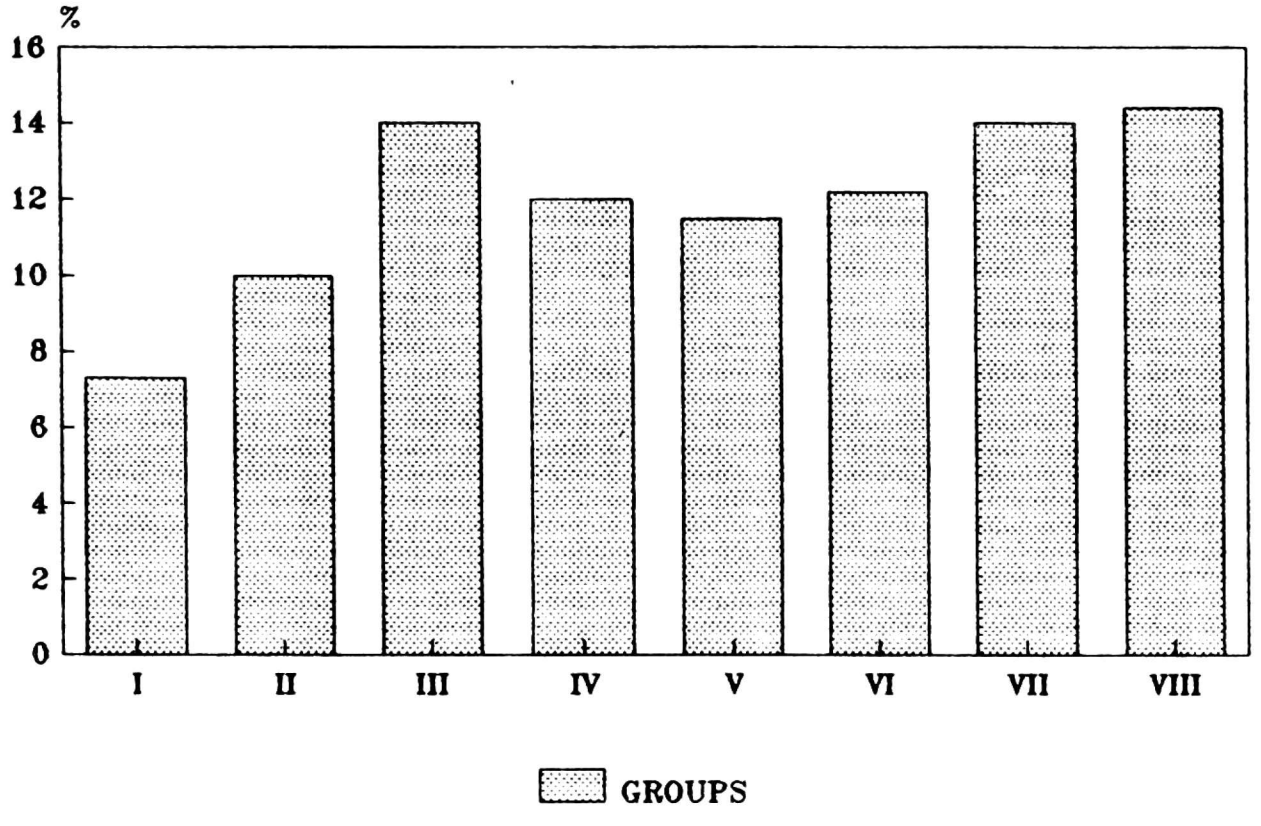

Fig. 3. The level of NBT-positive cells in the peripheral blood

Peritoneal macrophages inhibition test. Only in control group I inhibition of macrophages migration was not observed (MI = 149%). In all non-infected and exposed to propionibacteria control groups (II – IV) the inhibition of macrophage migration was observed (MI > 50%) ($p < 0.01$). The level on inhibition of macrophages migration was depended on relation to the time and doses of

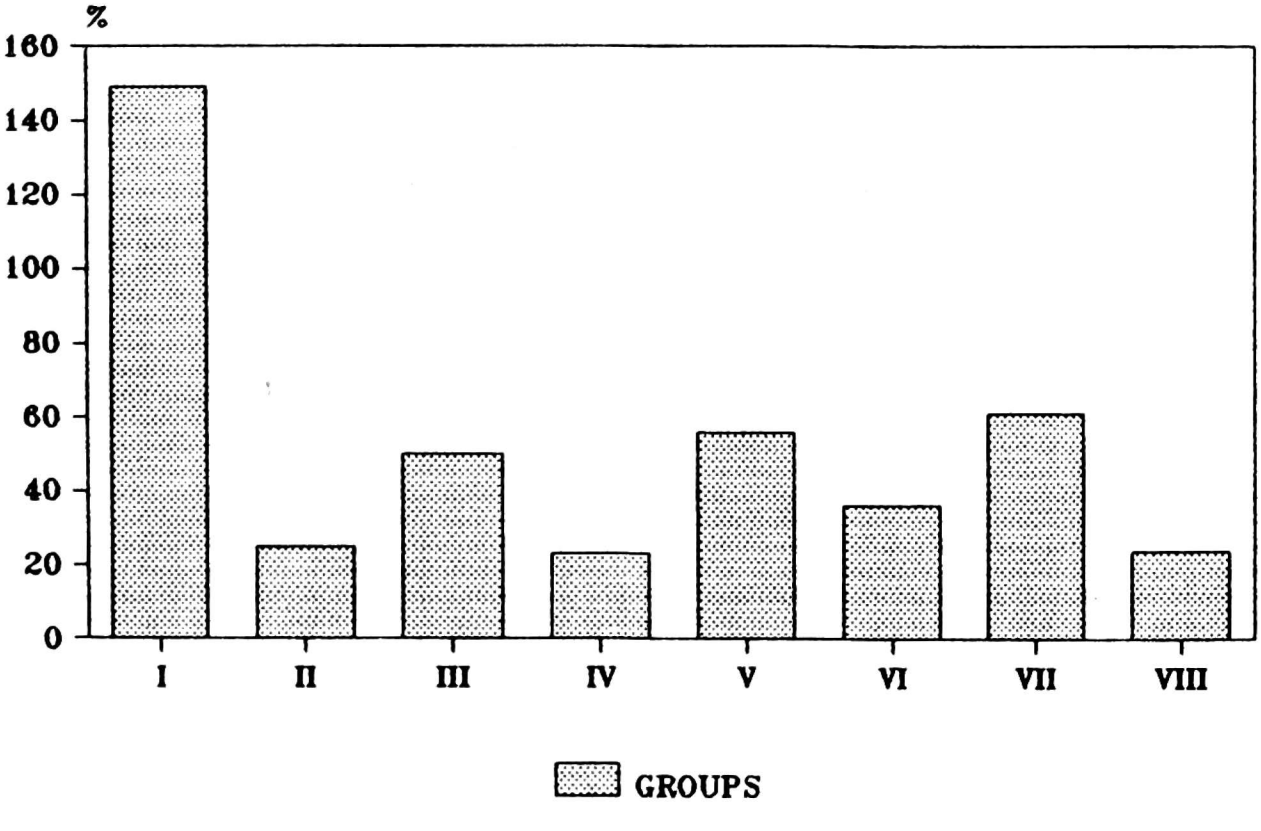

Fig. 4. The results of macrophage migration inhibition test. The results are expressed as the migration index (MI)

injection. In non-infected control groups the highest inhibition was observed in group IV (MI=23%) (Fig. 4). In all animals infected and exposed to peritoneal injection the inhibition of reaction was observed. The highest inhibition of macrophage migration was observed in group VIII (MI=24%) ($p<0.01$).

Antibodies. The antibody response to the crude extract of infective larvae of *T. spiralis* determined by ELISA is shown in Fig. 5. In non-infected control groups (I–IV) we can appreciate very low IgG_1 antibody level. All serum

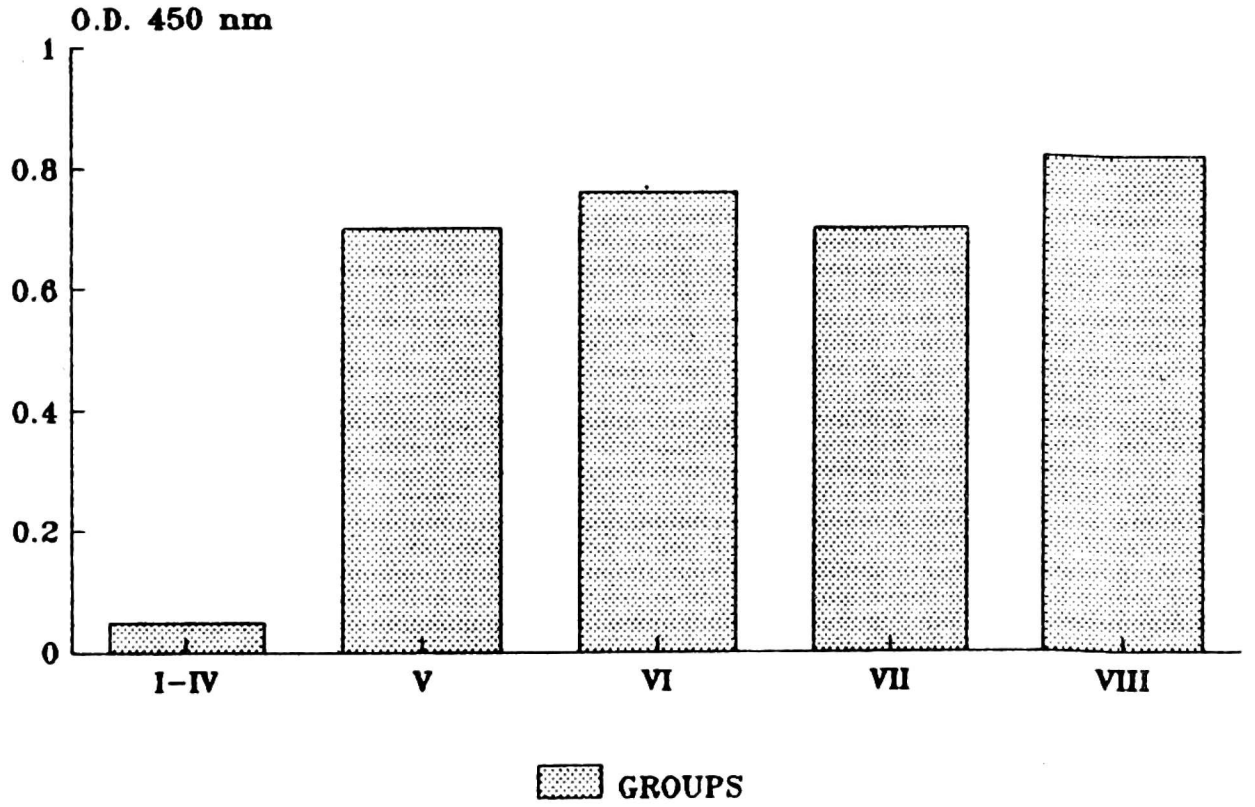

Fig. 5. ELISA values for specific IgG_1 antibodies against the crude extract of infective larvae of *T. spiralis*. Results are expressed as optical density at 450 nm (O.D. 450 nm)

samples from control groups gave similar O.D. values with nematode antigen, the mean values were lower than 0.05. All serum samples from infected groups (V–VIII) gave higher level of specific antibodies. In group V (infected with nematode but not treated with bacteriae) optical density was 0.7. The same level of antibody was found in group VII (infected and injected with bacteriae on 20 and 27 DAI). Very similar results was detected in group VI (O.D. was 0.76). The highest IgG_1 level were determined in group VIII (O.D. was 0.82). Results obtained in groups infected with nematode and treated with *P. granulosum* (VI–VIII) were not statistically significant as compared with infected group V not treated with bacteriae. But the results obtained in all infected groups (V–VIII) were statistically significant as compared with non-infected groups (I–IV) ($p<0.001$).

Discussion

Infection with parasites causes significant structural and biochemical changes in tissue of host's organs. It has been demonstrated that the higher level of mutual host parasite adaptation, the lower deformation of the host system (GUTTOWA 1990). Results of several studies have shown the effects on parasitic infection could be changed following stimulation of the host's immune system by non-specific immunomodulators (GOLIŃSKA et al. 1989, KOCIĘCKA et al. 1989). The consequence of this kind of immunization was limitation of the intensity of infection (BANY et al. 1984, MOSKWA and POŁEĆ 1993).

In the study presented here *P. granulosum* operated as non-specific activator. Their immunostimulatory activities were connected with glycoproteins and the esters of 6,6L-trehalose occurring in the cell wall (JANIAK et al. 1990). It has been known that administration of propionibacteria results in enlargement of spleen, thymus and liver mass. Histological examinations have shown stimulation of proliferation of lymphocytes and macrophages in these organs (ROSZKOWSKI et al. 1982). Results of studies with experimental animals have found the stimulation of reticuloendothelial system, the highest level of humoral and cell-mediated immunity following propionibacteria injection. Inactivated cell walls of this bacteria could stimulate antibacterial, antiviral and antitumoural responses (FUROWICZ et al. 1989, JANIAK et al. 1990). Lymphatic cells isolated from thymus, spleen and PEYER'S pathes of rats exposed to *P. granulosum* injections have shown the higher ATP level (MOSKWA and POŁEĆ 1992a).

Our earlier studies have shown that the administration of propionibacteria during the early phase of *T. spiralis* infection have had the effect on the intensity of intestinal phase of infection, the increasing of leucocytes counts, haematocrit and haemoglobin levels (MOSKWA and POŁEĆ 1993). The influence of *P. granulosum* on immunological reactions of rats infected with *T. spiralis* was observed. The results have shown the higher percentage of T lymphocytes, the increase of circulating neutrophilic granulocytes and stimulation of peritoneal macrophages (MOSKWA and POŁEĆ 1992b). The limitation of the intensity of the muscle phase of infection depended on the time of injection (MOSKWA and POŁEĆ 1993). In purpose of the present study was to monitor the effect of propionibacteria administered during the muscle phase of *T. spiralis* infection.

The first effect occurred following propionibacteria injection used to be the enlargement of spleen mass as a result of stimulation of the cells precursor (HALPERN et al. 1964). In the experiment presented here, the enlargement of the organ was observed after intraperitoneal injection in both non-infected and infected groups.

Granulocytes, the cellular factors of non-specific defence of an organism are known as professional cells of phagocytosis. They are considered as cells

controlling the immunological response. Neutrophils present the first line of defence and the first to appear on the site of demaged tissue (BANY et al. 1990). Results of studies with human and experimental animals have found that propionibacteria injection could stimulate the higher percentage of neutrophilic granulocytes. That activated granulocytes could able to reduce tetrazolium stain in correlation with the level of oxygen metabolism (BAEHNER and NATHAN 1968). BANY et al. (1990) have demonstrated the increased stimulation of the metabolic of circulating neutrophilic granulocytes depending of the phase of *T. spiralis* infection in mice. Moreover, slight activity of NBT-positive cells in lymphatic organs were observed in mice during the muscle phase of *T. spiralis* infection (KARMAŃSKA et al. 1989). Our results obtained on 42 day after *T. spiralis* infection have also shown the higher NBT-positive cells level in animals exposed to bacteria injection as compared with the control groups (I or V). The results have confirmed earlier conclusion that cicrulating neutrophilic granulocytes could participate as one of the mechanisms of non-specific defence of *T. spiralis* during the phase of migrating larvae and the muscle phase (BANY et al. 1990).

The studies made by CHAPES and HASKILLS (1983) shoved that neutrophils could also play a part in activation of macrophages. Much attention has been paid to macrophage migration inhibition phenomenon with have occurred only in delayed hypersensitivity and not in the presence of humoral antibodies. The capillary method of macrophage migration inhibition test was adapted for studies on trichinellosis (KOZAR and PIOTROWSKI 1971, VARNES et al. 1975). HALPERN et al. (1964) have found that following activation by propionibacteria, macrophages exhibit increased. In stimulated cells some changes in the structure of membrane, vacuolization and distribution of lysosomes were observed. Propionibacteria-stimulated macrophages could release special substances distinct from interferon wich play an important role in their antiviral, antitumoural and antibacterial activity (ROSZKOWSKI et al. 1982).

Results presented here confirm earlier findings. Positive reaction of the test could be seen following propionibacteria intraperitoneal injection in all non-infected control groups and in infected groups of rats. The level of inhibition of migration was depended on the time of injection. The highest positive reactions were observed in rats injected twice in the early muscle phase and in rats injected three times.

The influence of propionibacteria on T lymphocytes has not been completely determined. It was found that stimulated macrophages may caused increased trapping of lymphocytes in spleen. Several results have suggested immunosuppresive effect of propionibacteria on T lymphocytes (MARKOWSKA-DANIEL 1993). In JANIAK et al. (1990) opinion the inhibition could be connected with limitation of T cell precursors. But in the contrast increase of T lymphocytes in the blood was observed by FUROWICZ et al. (1979), SZMIGIELSKI et al. (1983), MOSKWA and POŁEĆ (1992). Results presented here have shown

statistically significant higher T cells level only in groups infected and exposed to *P. granulosum* (VI–VII). But the percentage of T lymphocytes noticed on 42 DAI decreased as compared with non-infected groups (II). The lower activity of the cells in the blood could be the results of T cells infiltrating and penetrating into the capsules of parasites in larger number (KARMAŃSKA and MICHALSKA 1978).

The second part of presented work concerned humoral response against *T. spiralis* in stimulated rats. SZMIGIELSKI et al. (1983) concluded that propionibacteria administration resulted in B lymphocytes mobilization and the higher level of antibodies, especially in IgM and IgG. In this study attention has been drawn to specific IgG_1 antibodies level in sera of infected groups. Higher level of specific antibodies against *T. spiralis* antigen was found in infected groups than in non-infected animals. In the contrast, there were not statistically significant changes in the level of IgG_1 antibodies in infected animals after *P. granulosum* injections.

The higher activity of some special immunocompetent elements: T lymphocytes, NBT-positive neutrophils and peritoneal macrophages could suggest the slight decreased of the intensity of nematode infection. But the mean of larvae recovered from carcasses of infected rats have no confirm it. In two of tree infected groups the changes of the intensity of the muscle phase of infection were no statistically significant. In one of inoculated groups the higher number of *T. spiralis* larvae was recovered as compared with the control. It seems that effect of propionibacteria on the dynamic of *T. spiralis* infection was no depended on the level of adaptation of the parasite, but on the time of intraperitoneal injection.

Infection with *T. spiralis* made a deep structural, biochemical and functional changes in tissue, specially in host myofibers that propionibacteria intraperitoneal injection administered during the muscle phase of infection couldn't dance as an real stimulator. The injection have activated some immunoeffectors but it seemed to be too late to reduce the mean of larvae in tissue.

Conclusions

1. *Propionibacterium granulosum* administered during the muscle phase of *T. spiralis* invasion effected on the higher activity of T lymphocytes, NBT-positive neutrophils and peritoneal macrophages.
2. There were not statistically significant changes in the level of specific IgG_1 antibodies against the crude extract of infective larvae of *T. spiralis* after propionibacteria injection.
3. The limitation of the intensity of *T. spiralis* invasion was not observed in rats after *P. granulosum* non-specific stimulation.

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