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LEUKOCYTES PARAMETERS IN THE COURSE OF PIG INFECTION WITH III LARVAL STAGE OF *ANISAKIS SIMPLEX**

MAGDALENA PODOLSKA[1], JULIUSZ TOKARSKI[2] I JERZY ROKICKI[1]

[1] Katedra Zoologii Bezkręgowców UG
81-378 Gdynia, al. Piłsudskiego 46
[2] Katedra Fizjologii Zwierząt UG
80-822 Gdańsk, ul. Kładki 24

OBRAZ BIAŁOKRWINKOWY W PRZEBIEGU ZARAŻENIA ŚWIŃ LARWAMI III STADIUM *ANISAKIS SIMPLEX*

Abstract. Experimental anisakiosis covered 6 pigs infected with III larval stage of *A. simplex*. Changes in leukocytes were analysed. The blood showed an increased number of eosinophiles after 5th day. The number of High-affinity and EAC rosettes decreased after 8th day.

INTRODUCTION

The *Anisakis* larvae occurring widely on fish might prove an invasion form for terrestrial mammals and also for man. The final hosts for these nematodes are marine mammals, mainly cetaceans (KAGEI et al. 1967, OSHIMA 1972, SMITH & WOOTTEN 1978). Up to now, *Anisakis simplex* has not been found in porpoise *Phocoena phocoena* from the South Baltic (ROKICKI & BERLAND 1995). The infection occurs after eating raw fish containing alive larvae. In an untypical host's alimentary canal the *Anisakis* larvae penetrate the mucous causing damage (OSHIMA 1972). When confronted with the parasite's antigens an immunological response is directed against those antigens.

The aim of the present research basing on experimental pig infection with *A. simplex* larvae is to reveal the extent to which the basic populations' behaviour as well as lymphocyte subpopulations are coherent with their behaviour in their total number in the course of the experimental anisakiosis.

The joining ability of lymphocytes and erythrocytes of different animal species and thus formed so called rosettes have become the basis of the method of differentiating the lymphocyte into populations and subpopulations. T lymphocytes spontaneously form rosettes with sheep erythrocytes (SRBC) through

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a so called E receptor (JONDAL et al. 1972, BACH 1973, GILBERSTEN & METZGA 1973). The T lymphocyte number can be determined by a T-RFC test (Tot Rosette Forming Cells). The rosettes that are formed at the temp. 29°C wit a limited concentration of sheep erythrocytes and are described as High-affinit ERFC. The rosettes that are formed at the temp. 4°C in a highly enriche medium and an excess of erythrocytes are described as the Low-affinity ERF((WEST et al. 1976a, 1976b, 1977, 1978; HOCKLAND & HERON 1980). Rosettes ca also form B lymphocytes with sheep erythrocytes this, however, taking plac after coating by antibody and complement. The antibody-complement komple joins a receptor for C3 component of the complement (EAC rosettes) (KAT 1977). EAC rosettes tests are a method of differentiating B lymphocytes (JONDA et al. 1972). The method of differentiating the T and B lymphocyte population based on rosette tests which is widely used in diagnosis people clically can b used with pigs after some necessary modifications (PISKORZYŃSKA et al. 1993)

Material and method

Investigations in leukocyte parameter changes in the course of experimenta anisakiosis were conducted on 6 pigs from a pig farm. The animals were botl sexes, 8–10 weeks old with average body weight about 20 kg. The sex wa irrelevant as piglets were used for experimenting.

Every pig was infected *per os* with 50 alive *Anisakis simplex* at their III stage which had been collected from the Baltic herring *Clupea harengus membras*. Before the experiment the parasites were being stored in a physiological solution of NaCl at the temp. of 4°C for about 24 hours.

5 cm^3 of blood was collected into heparinised test tubes with "0" test being the collection before infection, then followed by collections on 2nd, 5th, 8th, 10th and 14th day after infection.

Conventional methods were used to calculate total leukocyte number. Blood was diluted 20-fold with the use of TÜRK liquid. Total leukocyte number were calculated according to the following formula:

$$x = \frac{a \times 4000 \times c}{b}$$

where: x – cells number in 1 mm^3 of tested blood
a – cells number found
b – number of little squares for counting cells
c – applied blood dilution

The percentage of lymphocytes, eosinophiles, neutrophiles and monocytes was calculated against 100 leukocytes found in stained smear. Total number of lymphocytes, eosinophiles, neutrophiles and monocytes was calculated on the basis of general leukocytes number and SCHILLING parameters.

Lymphocyte isolation

The collected animal blood was diluted with buffered physiological salt solution (PBS) in the 1:1 ratio then layered on Ficoll 400 (Pharmacia) with a dash of Uropoline (Polfa), the density of 1.0779/cm^3. All was then being centrifuged for 30 minutes at 2700 rotations/min. After centrifugation, mononuclear cells recovered from the interphase, were washed three times in PBS by means of a 10 min. centrifugating at 1200 rot/min.

The washed lymphocytes were suspended in PBS so that were 10^7 cells/cm^3. Thus prepared suspension was used for rosette forming tests.

EAC preparation

In order to obtain EA cells, a 3% suspension of sheep erythrocytes (SRBC) were incubated for 30 min. at 37°C along an equal volume of rabbit hemolytic serum against sheep erythrocytes (Biomed, Kraków) marked 1024. After the incubation the EA cells, were washed three times in PBS, each time centrifuged for 10 min. at 1200 rot/min. In order to obtain the EAC cells, the EA were coated with guinea pig complement. The complement used for coating the EA was diluted by PBS in 1:1000 ratio and incubated with an equal number of EA for 30 min. at 37°C. After the incubation, the EAC cells were washed three-fold being centrifuged for 10 min. at 1200 rot/min. A 2% EAC suspension was used for tests.

Rosette tests procedure

0.5 cm^3 of PBS, 0.2 cm^3 of lymphocytes suspension and 0.2 cm^3 sheep erythrocyte suspension were placed in a test-tube. The cell mixture thus formed was centrifuged for 5 min. at 500 rot/min.

For the High-affinity E-RFC test, the cells were incubated for 1 hour at 29°C. A parallel T-RFC test was on by incubating the cells for 2 hours at 4°C. Thus formed rosettes were fixed by adding 0.2 cm^3 of 4% formalin. Low-affinity rosettes percentage was calculated on the basis of differences between the levels of T-RFC and High-affinity.

EAC test

0.5 cm^3 of PBS, 0.2 cm^3 of lymphocyte suspension and 0.25 cm^3 of EAC suspension were placed in a test-tube. The cell mixture was incubated for 30 min. at 37°C and then centrifuged for 5 min. at 500 rot/min. The rosettes were fixed by adding 0.2 cm^3 of 4% formalin.

In the all tests the percentage of formed rosettes was calculated under a light microscope recognising a lymphocyte with minimum 3 erythrocytes adhesing to it to be a rosette. In each test at least 200 lymphocytes were counted. The total lymphocyte number in particular fractions was calculated on the basis of the overall leukocyte number and the SCHILLING parameters.

The results are pesented in tables in mean absolute values. A statistical analyse of the results covered the following:

1. Basic mean values characteristics method by means of standard error (SE) of the average ($\bar{x}$). Evaluation of statistical difference significants was done by means of *t*-STUDENT test between the onset level and the subsequent measurements and particular measurements carried out in relation to each other.

2. Analysis of variance (ANOVA) for determining the infection influence on changes in leukocyte parameters and the factor of time in course of the experiment.

Results

In the course of the infection a significant difference in eosinophil number was noted (Tab. 1). The value of the F variable for the influence of infection on eosinophil number change was 7.28 ($p<0.001$). A maximum increase in eosinophil value was noted on the 5th day. The F variable value in relation to preinfection level was 8.95 ($p<0.01$). On the 8th day of the experiment the eosinophil level decreased ($F=5.70$; $p<0.05$). On the 10th day the eosinophil number decreased further ($F=7.86$; $p<0.05$ in relation to the level on the 5th day of the experiment). On the 14th day the eosinophil number decreased to the minimal level ($F=40.42$; $p<0.001$ in relation to the 5th day of the experiment).

Slight fluctuations of overall leukocyte number was noted in the course of the experiment (an increase at the beginning and then a decrease in number). The changes did not differ significantly.

No significant differences to statistics were noted in the overall lymphocyte number, which oscillated within the normal during following days (Tab. 1).

TABLE 1

Changes in the total number of leukocytes and their fractions in the peripheral blood of pigs infected with larval *Anisakis simplex* (infection – day 0) (average number of cells/mm^3 ± SE; $n=6$)

Days	Leukocytes	Lymphocytes	Eosinophiles	Neutrophiles	Monocytes
0	20059±1363	12559±1066	653±94	6068±739	552±68
2	22425±2516	13493±1440	633±133	7748±1177	424±67
5	26369±3022	13268±1134	1068±102**	7720±1267	862±262
8	22517±1915	12673±725	918±59*	7030±1666	331±72
10	19622±606	13426±703	708±78#	4943±617	350±98
14	18816±1912	10941±499	376±38###	6286±1653	475±171

** $p<0.01$

* $p<0.05$ with respect to day 0

$p<0.001$

\# $p<0.05$ with respect to day 5

The initial neutrophil level was slightly oscillating over the following days of the experiment (Tab. 1). No case gave any significant differences.

Number change of monocytes never exceeded the level of statistical significance (Tab. 1).

The mean value of T-RFC rosettes (cell/mm^3) and of High-affinity and Low-affinity relationship as well as EAC rosettes on the days following the infection are presented in Tab. 2.

TABLE 2

Populations and subpopulations of lymphocytes in experimental infection of pigs with larval *Anisakis simplex* (infection – day 0) (average number of cells/mm^3 ± SE; $n=6$)

Days	E rosettes			H/L	EAC-rosettes
	T-RFC	High-affinity	Low-affinity		
0	4481±735	3254±635	1227±402	2.65	2945±271
2	3284±507	1929±695	1355±495	1.42	1912±839
5	4383±325	2258±305	2125±462	1.06	3621±191
8	3028±696	974±378**	2054±485	0.47	1464±188***
10	4990±310	2890±309##	2100±304	1.38	1761±298
14	4146±203	3104±64###	1042±262	2.98	3043±211###

*** $p<0.001$

** $p<0.01$ with respect to day 0

$p<0.001$

$p<0.01$ with respect to day 5

Over the next days of experiment there occurred statistically insignificant fluctuations of total number of the forming T-RFC rosettes (Tab. 2).

The F variable value calculated for the variable of High-affinity forming rosettes throughout the experiment was equal 3.67 ($p<0.01$). On the 8th experiment day there was noted a maximum decrease in forming rosettes number ($F=9.50$ as compared to initial level; $p<0.01$). Over the following experiment days the number of forming rosettes was increasing on the 10th day ($F=15.40$ as compared to the level on the 8th day; $p<0.01$) and on the 14h day ($F=30.87$ as compared to the level on the 8th day; $p<0.001$) (Tab. 2).

The number of Low-affinity rosettes before the infection fluctuated over the following experiment days. The fluctuations, however, never exceeded the level of significance (Tab. 2).

The H/L coefficient underwent changes over consecutive days after the infection, that is the relation of High-affinity to Low-affinity rosettes, calculated on the basis of mean numbers of rosettes (cells/mm^3) (Tab. 2).

Number change of forming EAC rosettes over the consecutive days of the experiment is presented in Tab. 2. The F variable in the course of the experiment was 4.52 ($p<0.01$). The 8th day of the experiment witnessed

a maximal drop of forming rosettes down ($F=20.16$; $p<0.001$ in relation to preinfection level). In the course of further experiments days an increasing number of forming rosettes was noted which culminated on the 14th day ($F=31.22$; $p<0.001$ in relation to 8th day).

Discussion

In the course of experimental anisakiosis in pigs there occurred changes in leukocyte parameters. Occurrence of eosinophilia was noted in the peripheral blood. There was no changes in the lymphocyte number, yet their populations and subpopulations showed significant differences. Also relation of T-helper to T-suppressor lymphocytes changed.

In most cases of helminthosis there occurs eosinophilia in peripheral blood which was confirmed by the results of our experiments presented in this paper. RUITENBERG (1970) described changes in eosinophil number in the course of anisakiosis in rabbits. According to OSHIMA (1972) eosinophilia accompanies most anisakiosis cases in man whereas leukocytosis is irrelevant or none. BIER et al. (1976) observed a slight eosinophilia in infected pigs' blood as early as next day after infection, which dissappeared during next 7 days. VALDISERRI (1981) found an occurrence of temporal eosinophilia on the 9th day of patient's hospitalisation who suffered from anisakiosis. SOBECKA (1983) revealed leukocyte and eosinophil number increase in peripheral blood in Syrian hamsters infected with *A. simplex* larvae as well as presence of juvenile forms of leukocytes in the blood parameters and some forms with pathological changes. SHIRAHAMA et al. (1990) found peripheral eosinophilia in a patient suffering from colon anisakiosis. ROKICKI et al. (1993) revealed the occurrence of eosinophilia in experimental anisakiosis in pigs on the 5th day after the infection. Eosinophilia is also to be found in other parasite infections such as schistosomosis (GOUNNI et al. 1994).

On the basis of the rosette tests carried out in this experiment one may hypothesize that in the course of anisakiosis the T-lymphocyte ability to form High-affinity E-RFC rosettes decreases as in the case with B-lymphocyte ability to form EAC rosettes. It is generally assumed that High-affinity E-RFC rosette fraction supports activity of B lymphocyte whereas Low-affinity E-RFC rosette supports the suppressor one (MORETTA et al. 1975, 1976; GUPTA & GOOD 1977a, 1977b, 1978). In the course of experiment changes were noted of the T-helper lymphocyte relation to T-suppressor lymphocytes in peripheral blood expressed as H/L coefficient (High-affinity/Low-affinity). The coefficient's minimal value occurs on the 8th day after infection which is followed by a gradual increase up to the value slightly overgrowing the initial level.

It must be emphasized, that all the blood parameters changes described here, were observed with stable values of the total lymphocyte number in

peripheral blood. It is, however, unknown if the changes reflect an appearance of greater percentage of lymphocytes devoid of T & B surface markers, being part of so called „third fraction" – Null lymphocytes, or they are changes, possibly deformating of lymphocyte membranes thus having no ability of rosette forming.

Changes in function mechanisms of cell mediated immunity in parasite diseases were described at *Trichinella spiralis* infections (BANY 1988). The results clearly point to a modulation of T-lymphocyte activity within experimental trichinellosis in mice which most probably plays an important part in the immunological response to parasite infection. The modulation mechanisms (stimulating or inhibiting) of host's immunity is not clear. The parasites antigens (cell membrane components or metabolites) may activate T-suppressor lymphocytes which, in turn, delay the activity of T-helper lymphocyte subpopulation (WASSOM et al. 1987). In the experimental anisakiosis in pigs, being the objective the present paper, there happened significant shifts within the whole T-lymphocyte population, which was revealed by an increase of the relation of T-suppressor lymphocytes to T-helper ones.

Conclusions

1. Infection with *Anisakis simplex* 3rd stage larvae cause eosinophilia in the peripheral blood of pigs.
2. Larval *Anisakis simplex* inhibit host's immunological response.

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