Application of a flow cytometry in differentiation of antibodies accompanying platelets in *Ehrlichia canis* and *Borrelia burgdorferi* infections in dogs

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Abstract

Sera of dogs (n = 30), with serologically confirmed monocytic ehrlichiosis (Ehrlichia canis, n = 21) or borreliosis (Borrelia burgdorferi, n = 9) were tested for presence of antibodies accompanying platelets (anti-platelet antibodies – APA). The analysis was carried out in the flow cytometer based on the principle of indirect anti-globulin test, using FITC-labeled antiglobulin conjugate against canine IgG. Out of the 30 examined dogs, antibodies directed against thrombocytes were indicated in total 10 cases (33.3%).

Key words: *flow cytometry, antiplatelet antibodies, Ehrlichia canis, Borrelia burgdorferi.*

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Introduction

Except for genetic, hormonal or environmental factors also infective factors belong to the commonly considered causes of auto-immunological disorders. In the group of causes of infective background able to induce or intensify auto-immunological disorders a number of pathogens is mentioned, among others *Rickettsia rickettsii*, the virus of equine infectious anaemia, *Babesia canis*, *B. felis* and *B. equi*, *Rochalimea henselae*, *Leptospira* spp. and *Ehrlichia* spp. or *Borrelia* spp.

One of the forms of the discussed auto-immunological disorders is immune mediated thrombocytopenia (IMT), which is accompanied by the presence of antiplatelet antibodies (APA). These antibodies represent a part of the pool IgG and may occur in 2 forms: as platelet-bound IgG antibodies and as antibodies washed out of the platelet surface – platelet-bindable immunoglobulins – Pa-Ig. The disorder of this type is a complication, which may accompany infections in dogs caused by *Ehrlichia canis* or *Borrelia burgdorferi*. Diagnosing it and differentiating from thrombocytopenias of different origin is essential as it may indicate – similarly as in human medicine – a necessity of combining the causal therapy oriented at one of the above mentioned infective factors with an additional therapy curbing autoagressive reaction of an animal's organism [1-5].

The purpose of the performed research based on the principle of indirect antiglobulin test was to indicate the presence of antibodies accompanying platelets (APA) in thrombocytopenic dogs, in which at the same time the presence of specific anti-*Ehrlichia canis* or anti-*Borrelia burgdorferi* antibodies were indicated.

Material and methods

The examined group (n = 30) included dogs, in which in the serological test (indirect immunofluorescence test) the presence of anti-*Ehrlichia canis* (n = 21) (3 P06 K 015 23) and anti-*Borrelia burgdorferi* (n = 9) antibodies was indicated. Haematological blood tests (thrombocytopenia, mean PLT 286.2 G/L) were performed in automatic haematological analyser MS 4 (Melet Schloesing). Description of examined group is presented in the Table 1.

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Table 1. Examined group (seropositive for *Ehrlichia canis* and *Borrelia burgdorferi*)

No.	Breed	Sex	Age	Antibody specific for *E. canis*	Antibody specific for *B. burgdorferi*	Titer
1.	rottweiler	♀	8 years	+	not examined	1 : 320
2.	mastino napoletano	♂	17 months	+	not examined	1 : 320
3.	boxer	♂	2.5 years	+	not examined	1 : 320
4.	mix-breed	♀	12 years	+	not examined	1 : 20
5.	boxer	♀	6 years	+	not examined	1 : 1280
6.	German shepherd	♂	1 year	+	not examined	1 : 20
7.	German shepherd	♀	1 year	+	not examined	1 : 20
8.	no data	♂	1 year	+	not examined	1 : 20
9.	no data	♂	5 years	+	not examined	1 : 20
10.	mix-breed	no data	no data	+	not examined	1 : 320
11.	mix-breed	no data	no data	+	not examined	1 : 320
12.	long-haired dachshund	♂	11 years	+	negative	1 : 1280
13.	German shepherd	♂	7 years	+	not examined	1 : 20
14.	German shepherd	♂	7 years	+	not examined	1 : 320
15.	boxer	♀	6 years	+	not examined	1 : 20
16.	Bobtail	♀	11 years	+	not examined	1 : 320
17.	no data	no data	no data	+	not examined	1 : 320
18.	Rhodesian Ridgeback	♂	4 years	+	not examined	1 : 320
19.	wire-haired miniature dachshund	♀	6 years	+	not examined	1 : 320
20.	mix-breed	♀	8.5 year	+	not examined	1 : 1280
21.	mix-breed	no data	9 years	+	not examined	1 : 320
22.	short-haired dachshund	♀	6 years	not examined	+	1 : 512
23.	German shepherd	♀	2 years	not examined	+	1 : 1024
24.	Hovawart	♀	5 years	not examined	+	1 : 64
25.	Golden Retriever	♂	6 years	not examined	+	1 : 512
26.	Weimaraner	♀	3 years	not examined	+	1 : 256
27.	wire-haired Fox Terrier	♂	7 years	not examined	+	1 : 64
28.	bernese mountain dog	♀	3.5 years	not examined	+	1 : 512
29.	mix-breed	♀	5 years	not examined	+	1 : 64
30.	American Staffordshire Terrier	♂	4 years	not examined	+	1 : 256

The control group (n = 20) included clinically healthy dogs, not showing any deviations in blood morphological and biochemical analysis, in which in serological tests oriented at canine monocytic ehrlichiosis (*E. canis*) and borreliosis (*B. burgdorferi*) negative results were obtained. Mean PLT in this group was 386.2 G/L.

The material for test oriented at the presence of APA was serum. By the time the analysis was performed, the serum samples divided into small portions were stored in the temperature –65°C.

The presence of antibodies specific for *E. canis* and *B. burgdorferi* was indicated by use of an indirect fluorescent antibody test (IFAT). In both cases the test was performed in compliance with the instructions of the test manufacturers. In the case of *E. canis* a diagnostic set was used (VMRD, USA). The result was considered positive when

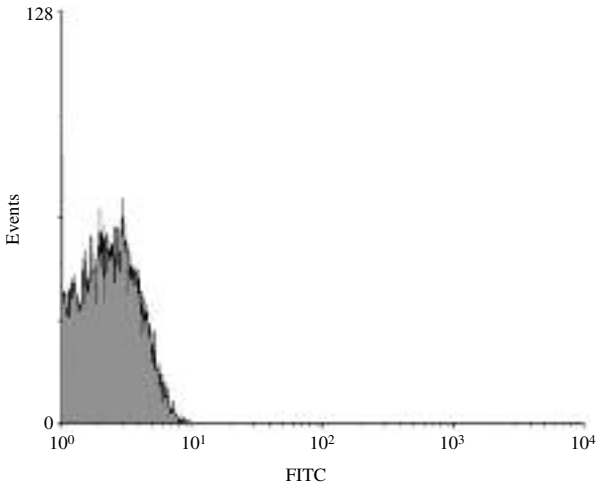

Fig. 1. Flow cytometry histograme demonstrate thrombocytes (control dog) gated on FSC versus FL1-H (FITC). The X-axis shows the fluorescence intensity (fluorescence channels, log scale) projected against the cell count (Y-axis)

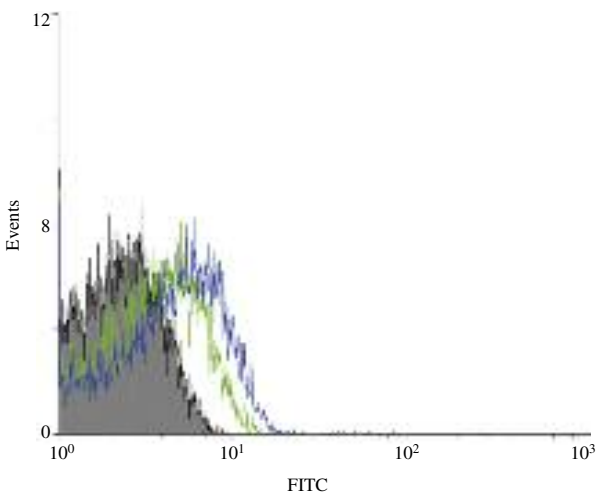

Fig. 2. Flow cytometry histograms (overlay) of the thrombocytes gated on FSC versus FL1-H (FITC). Gray linked: control sample; green and blue linked: results obtained for examined dog (positive for APA). The X-axis shows the fluorescence intensity (fluorescence channels, log scale) projected against the cell count (Y-axis)

stating lighter green-yellow fluorescence of cytoplasmatic inclusion bodies, grouped single elementary bodies (initial bodies) and free elementary bodies of the diameter close to 0.3 micron, at dilution of the serum amounting to at least 1 : 20. In the case of *B. burgdorferi* for serological tests a diagnostic set Mega Screen Fluoborrelia was used (Mega Cor Diagnostic GmbH, Germany). According to the producer's instruction, the result was qualified as positive when indicating specific fluorescence of *Borrelia* spp. for dilution of the serum amounting to at least 1 : 64. The preparations were assessed under a fluorescence microscope at the magnification 200 and 400-tuple.

For detecting antiplatelet antibodies the method of flow cytometry based on the principle of an indirect anti-globulin test was applied. The principle of the test consists in incubation of allogenic blood platelets with the tested serum, and then with anti-globulin serum, combined with fluorochromium. Allogenic blood platelets suspended in platelet rich plasma (PRP) were obtained by centrifugation of blood of the dogs from the control group in the temperature of 20°C for 10 minutes at 250 × g.

The tested sera (30 µl) of the tested group were put into the cuppings of the micro-platelet, simultaneously with the control group, at the dilution 1 : 10. Then 30 µl of the isolated blood platelets with the plasma were added to the sera. The micro-platelet was incubated in the temperature of 37°C for 60 minutes. The samples were washed twice in the volume of 200 µl of Tyrode's buffer (0.14 mM NaCl, 0.003 M KCl, 0.005 M NaH_2PO_4, 0.001 M $NaHCO_3$, 0.006 M Na_2EDTA, pH 7.1), centrifuging them for 3 minutes at 800 × g. After washing the samples were suspended in 50 µl of Tyrode's buffer. Then, 30 µl of antiglobulin serum (goat anti-dog IgG) combined with FITC (Serotec, Austria) was added to the micro-platelet cuppings. The micro-platelet protected with aluminium foil against light was incubated for 60 min in the temperature of 37?C, and then it was washed twice according to the above procedure. After incubation the samples were fixed with paraformaldehyde (Becton & Dickinson, USA) for 5 min at the temperature of 20°C.

Cytometric analysis was performed transferring from each cupping of the micro-platelet 5 µl of the cell sediment suspended in 700 µl of Tyrode's buffer. Specimens were analysed on the FACScan (Becton & Dickinson, USA) and data were acquired with the log amplification for the forward scatter (FSC), side scatter (SSC) and fluorescence (FL1-FITC). The result was qualified as positive when the average value in the mean fluorescent channel was > 2 SD of the values obtained in the control group.

Results

The histogram of the thrombocytes (control dog) gated on FSC-H versus FL1-H (FITC) is presented on the Figure 1. The representative histogram of plasma (examined dog 1 and 2) containing an antiplatelet antibody that binds to normal platelets is shown on the Figure 2. The total mean value of the fluorescence (X mean) in antiplatelet antibodies negative-control serum samples from control dogs amounted to 2.69 (SD 0.13). Out of 30 dogs from the examined group, the value of fluorescence proving the presence of anti-platelet antibodies was obtained for 10 dogs (33.3%). The mean value of the fluorescence for these samples amounted to 3.49 and ranged from 3.09 to 4.98 (> 3-17 SD of the control group). The distribution of this parameter in

the control group and in dogs with positive results for APA is summarised in Table 2.

Discussion

Thrombocytopenia is well-know complication of many bacterial infections. It may be a result of the decreased production of platelets in the bone marrow, their increased usage or sequestration or increased destruction combined with the presence of antibodies [5-7]. Another proposed model of thrombocytopenia accompanying infections is a formation of platelet-microbe complexes [8]. Immune Mediated Thrombocytopenia (IMT) is a relatively frequent disorder occurring in dogs. Immune mediated thrombocytopenia may assume primary nature (autoimmunological or idiopathic) or secondary caused by infections, drugs or cancer process. However it is always accompanied by the increased destruction of platelets, which leads to decrease of a number of peripheral thrombocytes, and the presence of antibodies related to platelets [3].

In primary thrombocytopenias antibodies related to thrombocyte surface are usually oriented against glycoproteins such as the receptor for fibrinogen gpIIb/IIIa [9, 10]. At thrombocytopenias of secondary origin, damage of platelets is caused by the presence of immunological complexes connecting with the receptors for the fragment of immunoglobulins FCR, non-specific antigens absorbed on the surface of thrombocytes or newly-occurring antigens revealing themselves, among others, in course of infectious diseases. The basic class of antibodies participating in these reactions are immunoglobulins of the class G. Antiplatelet antibodies APA cause premature destruction of the platelets [3, 11].

Numerous reports exist on the thrombocytopenia accompanying monocytic ehrlichiosis and only a few studies have investigated the possible association of *B. burgdorferi* serologic status with immune mediated thrombocytopenia [12]. In ehrlichiosis, it is mentioned as one of the permanent symptoms of manifestation of the discussed infections. In the event of ehrlichiosis thrombocytopenia

Table 2. The value of the fluorescence in the control group (n = 20) and in dogs positive for APA (n = 10) [movement in the fluorescence channel > 2.95]

No.	Control group the value of Fluorescence (X mean)		Examined group the value of Fluorescence (X mean) in dogs positive for APA	
1.	2.77	1.	3.25	*E. canis* (+) No. 3*
2.	2.66	2.	3.09	*B. burgdorferi* (+) No. 25*
3.	2.68	3.	3.39	*E. canis* (+) No. 14*
4.	2.74	4.	3.70	*E. canis* (+) No. 5*
5.	2.66	5.	4.98	*E. canis* (+) No. 20*
6.	2.84	6.	3.75	*E. canis* (+) No. 12*
7.	3.0	7.	3.27	*E. canis* (+) No. 14*
8.	2.88	8.	3.12	*E. canis* (+) No. 21*
9.	2.69	9.	3.19	*E. canis* (+) No. 19*
10.	2.61	10.	3.20	*E. canis* (+) No. 17*
11.	2.60			
12.	2.78			
13.	2.61			
14.	2.60			
15.	2.60			
16.	2.34			
17.	2.78			
18.	2.64			
19.	2.70			
20.	2.73			

**No – number of the dog described in the Table 1*

accompanies acute and subclinical phase of the disease (aboute 89% of cases), in which in comparison to the pre-infection level the reduction of blood platelets amounts to 42%. In natural infections, only 50% of the infected dogs indicated thrombocytopenia [13, 14]. Pathogenesis of the monocytic ehrlichiosis in dogs in reference to effector mechanisms related to thrombocytopenia remains still unexplained. The participation of immunological mechanisms was confirmed. The role in development of thrombocytopenia is also attributed to the platelet migration inhibition factor (PMIF), produced by the activated lymphocytes, which inhibits migration of platelets. Stimulation of lymphocytes secreting not yet recognised mediators seems to be caused by the infection of monocytes. In pathogenesis of thombocytopenia accompanying ehrlichiosis participation of specific antibodies is also suggested [5, 13, 14]. Therefore in subsequent stages of the disease thrombocytopenia may develop as a result of operation of various mechanisms. Antiplatelet antibodies may occur already after 17 days of the infection, so their presence may even precede occurrence of immunoglobulins specific for *E. canis.*

A highly sensitive method of detecting APA, is direct indication of antibodies related to thrombocytes. However, practical limitation of suitability of this method in dogs results from the necessity of using fresh blood and immediate isolation of thrombocytes at maximal inhibition (occurring already after sampling) of the processes of activation of platelets and coagulation. An alternative method developed in order to differentiate IMT is an indirect test. This test detects the total quota of IgG antibody, showing specific, unbound APA (washed out from the platelets' surface) in the serum and any other IgG antibody associated with platelets. These antibodies may be indicated with various techniques, among others with ELISA test, tests based on platelet factor 3 (PF3) and direct immunofluorescence test for megakaryocytes (MK-DIF) [3, 11]. At present the most common method of their detection is cytometric analysis applied among others authors [1, 3, 5, 7, 10, 15]. Detecting the discussed antibodies enables us to confirm participation of the immunological components in pathogenesis of thrombocytopenia however it does not give a possibility of differentiating between primary and secondary IMT.

Sensitivity of cytometric analysis based on the experiments of Lewis' *et al.* [3] is significantly higher in direct tests, commercial tests available for humans detecting IgG bound with the platelets are not however licensed for dogs. Differences in sensitivity of indirect and direct tests result probably from the fact that majority of antibodies may be bound with platelets (APA are in relative equilibrium with antigen) and only a small pool is present in the serum. There is not much data regarding time of maintaining of APA in serum either. The research of Harrus *et al.* [16], regarding kinetics of APA indicated their presence in the serum at the earliest on the 7th day after *Ehrlichia canis* infection, however only in one animal they were indicated on the 50th day after infection [10, 13].

In conclusion, our data suggest that quite large number of diagnosed as seropositive for *E. canis* dogs in Poland, should be considered as a risk group for immunemediated thrombocythopenia. Our research in the discussed subject is the supplement of the laboratory diagnostics of canine monocytic ehrlichiosis and borreliosis. The indication for its performance was thrombocytopenia in dogs with positive result of the serological tests. However it should be remembered that the limitation of the results of such tests shall always be a lack of possibility of precise determination of the moment of infection of an animal by ticks. If thrombocytopenia has no immunological origin, the result of the test oriented at the presence of APA should be negative. In the event of positive results one should consider a possibility of making a decision regarding additional therapy. Treatment in the discussed scope comes down to an attempt of blocking autoagressive reaction. In human medicine for over 20 years homologous polivalent sera have been applied in this respect. At present in treatment of humans application of high doses of monoclonal antibodies is implemented successfully [5]. It represents a wide and open field for research in veterinary medicine where still in the therapy of autoimmunological disorders most often corticosteroidal drugs are used.

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