PAPERS

Secondary immune-mediated thrombocytopenia in dogs naturally infected by *Leishmania infantum*

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Forty-four dogs naturally infected by *Leishmania infantum* were divided into two groups: 20 thrombocytopenic dogs with fewer than 150 x 10^9 platelets/l, and 24 non-thrombocytopenic dogs with more than 200 x 10^9 platelets/l. Ten clinically healthy dogs were used as controls. A haematological profile was obtained and the dogs' serum was used to assess the presence of platelet-binding IgM and IgG antibodies using a flow cytometry technique. Nineteen of the 20 thrombocytopenic dogs, and 13 of the 24 non-thrombocytopenic dogs had detectable levels of platelet-binding immunoglobulins, but none of the control dogs did so. The differences were significantly different for both IgM and IgG platelet-binding antibodies.

LEISHMANIOSIS is a severe systemic infectious disease of dogs caused by protozoan parasites of the genus *Leishmania*. The disease is endemic in the Mediterranean basin where dogs are considered to be the main domestic reservoir of the parasite and transmit the disease to human beings. The clinical signs of leishmaniosis can vary widely in infected dogs as a result of their individual responses to the immunological changes mediated by the protozoan. Signs of bleeding, such as epistaxis, haematuria and haemorrhagic diarrhoea have been reported (Font and others 1994, Ciaramella and others 1997, Koutinas and others 1999, Ciaramella and Corona 2003). The pathogenesis of the bleeding is uncertain, but it may be caused by alterations in primary and/or secondary haemostasis (Slappendel and Ferrer 1998, Ciaramella and others 2005). Thrombocytopenia has been reported in 29·3 per cent of cases and a deficiency in platelet aggregation has been observed in the majority of infected dogs (Ciaramella and others 1997, 2005, Petanides and others 2008). Thrombocytopenia and thrombocytopathy may result from changes in the vessel wall due to vasculitis, from splenomegaly, changes in thrombocytopoiesis or an increase in platelet destruction, and/or renal or hepatic failure (Ferrer 1992, Slappendel and Ferrer 1998, Ciaramella and others 2005). Terrazzano and others (2006) observed a significant association between the presence of antiplatelet antibodies and moderate to severe clinical signs in infected dogs and, in particular, in animals with moderate to severe thrombocytopenia, and presumed that the data indicated a secondary immune-mediated thrombocytopenia.

The aim of the present study was to investigate, using immunofluorescence and flow cytometry, whether the presence of antiplatelet antibodies could be associated with the occurrence of an immune-mediated thrombocytopenia in a significant number of thrombocytopenic dogs naturally infected by *Leishmania infantum*.

Materials and methods

Forty-four mixed-breed dogs from the Campania region in southern Italy, naturally infected by *L infantum*, were used in the study. They were divided into two groups: group A consisted of 20 dogs with a platelet count fewer than 150 x 10^9 platelets/l and group B consisted of 24 dogs with a platelet count greater than 200 x 10^9 platelets/l. None of the dogs had a history of bleeding. In each case, the clinical diagnosis of leishmaniosis was confirmed by the detection of amastigotes in lymph nodes or bone marrow aspirate smears, and serologically by a positive immunofluorescent antibody test (IFAT), that is a titre of 1:160 or more. None of the dogs had received anti-*Leishmania* therapy, and other infective causes of secondary immune-mediated thrombocytopenia, such as ehrlichiosis, anaplasmosis, babesiosis and dirofilariosis, had been excluded. No *Ehrlichia canis* or *Anaplasma phagocytophilum* morulae, *Babesia canis* trophozoites or microfilariae were observed on peripheral blood smears. All these infections were also excluded using IFAT and/or PCR. Finally *Dirofilaria immitis* infection was also ruled out using the Snap Canine Combo Heartworm Antigen Antibody Test (IDEXX Laboratories). Ten clinically healthy dogs, negative for leishmaniosis and other infective diseases were used as controls. All the experiments were conducted according to national ethical guidelines.

Sample collection and haematological evaluations

After the dogs had fasted overnight, blood samples were collected into plastic tubes containing 3·8 per cent sodium citrate. A complete blood count was performed on all the samples within 30 minutes of their collection, using a semiautomatic cell counter (Genius S; SEAC Radom

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TABLE 1: Haematological data and serum levels of platelet-bound (PB) IgM and IgG in the 20 dogs with fewer than 150 x 10^9 platelets/l (group A) and the 24 dogs with more than 200 x 10^9 platelets/l (group B)

Dogs	WBC (x 10^9/l)	RBC (x 10^{12}/l)	Hb (g/l)	HCT (%)	MCV (%)	MCH (%)	MCHC (g/l)	PLT (x 10^9/l)	PB IgM*	PB IgG*
Group A										
1	6·4	3·3	8·0	22·7	68	24·2	35·2	88	–	–
2	10·5	6·0	14·4	42·6	70	23·6	33·7	128	+	+
3	7·2	5·7	12·7	37·2	65	22·2	34·2	132	++	+
4	8·8	5·7	14·1	40·1	70	24·8	35·2	79	+	–
5	11·3	6·2	15·4	43·5	70	24·7	35·3	114	+	+
6	6·2	3·8	8·0	22·3	58	21·0	35·8	134	++	++
7	11·5	4·1	7·9	23·0	56	19·3	34·5	115	++	++
8	7·9	4·5	10·2	29·2	65	22·9	35·1	101	+	–
9	10·0	4·4	10·3	28·9	65	23·1	35·5	95	++	+
10	12·4	6·5	14·3	38·0	59	22·1	37·6	60	++	++
11	10·3	5·8	11·8	33·5	59	20·4	35·2	124	++	+
12	10·1	6·2	12·7	35·4	57	20·6	35·9	68	++	+
13	7·9	4·7	9·9	27·3	58	21·0	36·2	100	++	++
14	7·7	6·9	15·1	42·2	61	21·7	35·8	95	++	++
15	9·8	7·6	16·9	46·0	61	22·3	36·7	65	++	+
16	8·2	8·0	18·8	49·2	62	23·5	38·2	62	++	++
17	9·6	8·2	19·8	48·1	59	24·1	41·1	136	++	++
18	9·7	7·0	16·2	42·0	60	23·1	38·5	89	++	++
19	9·9	6·0	12·4	36·7	61	20·5	33·7	61	++	+
20	4·9	4·8	10·3	28·9	60	21·6	35·7	130	++	++
Group B										
21	13·0	6·1	12·0	39·6	65	19·6	30·3	395	+	–
22	9·1	5·4	10·0	36·4	67	18·5	27·5	210	–	–
23	5·8	5·3	13·2	35·9	67	24·8	36·8	212	++	–
24	8·9	6·8	18·1	47·9	69	26·3	37·9	221	–	–
25	5·9	7·0	17·4	46·0	65	24·7	37·8	238	–	–
26	11·5	5·3	12·9	35·2	67	24·5	36·6	220	–	–
27	11·1	6·9	17·8	44·8	64	25·0	39·7	211	+	+
28	7·0	6·3	14·8	41·0	64	23·2	36·0	206	+	–
29	9·2	6·7	16·1	44·2	65	23·8	36·5	220	–	–
30	8·2	5·7	12·0	38·1	66	21·0	31·4	296	+	+
31	11·8	5·3	13·2	35·9	67	24·8	36·8	240	+	–
32	11·2	7·0	17·1	47·6	67	24·3	36·0	235	+	–
33	8·9	6·6	17·2	43·8	66	26·1	39·4	320	–	–
34	8·7	6·6	15·5	42·4	63	23·1	36·4	220	+	+
35	11·6	6·7	16·8	46·0	68	24·9	36·6	352	+	–
36	5·8	7·7	18·5	53·1	69	24·0	34·9	273	+	–
37	16·8	5·3	13·2	35·9	67	24·8	36·8	256	+	–
38	11·0	5·7	11·8	39·3	68	20·7	30·0	368	–	–
39	9·3	6·9	16·4	44·1	64	23·6	37·1	263	+	–
40	10·7	6·5	16·7	43·7	67	25·6	38·1	339	–	–
41	8·3	6·9	16·7	46·1	67	24·4	36·2	256	–	–
42	10·6	5·6	13·8	36·7	65	24·5	37·0	248	–	–
43	10·5	5·9	13·8	39·9	68	23·4	34·6	286	++	–
44	11·0	7·2	12·9	46·6	65	28·5	43·9	293	–	–

* Samples with more than 30 or 50 per cent of platelets with a mean fluorescence intensity of 100 intensity channels on a log scale higher than the negative control, are indicated by + and ++, respectively
Hb Haemoglobin, HCT Haematocrit, MCH Mean corpuscular hb, MCHC MCH concentration, MCV Mean cell volume, PLT Platelet count, RBC Red blood cell, WBC White blood cell

Group). May-Grünwald-Giemsa-stained blood smears were evaluated for additional confirmation of thrombocytopenia or evidence of platelet clumping. Samples of serum were obtained by centrifugation within an hour of collection and stored at –20°C until their platelet-binding ability was assessed by flow cytometry.

Platelet isolation and immunofluorescence assay

Blood samples were collected into EDTA from healthy dog donors. The platelet-rich plasma fraction was purified by centrifugation at 300 *g* for 10 minutes at 20°C, as described by Kristensen and others (1994b). The platelet-rich plasma was transferred into a plastic tube and washed three times with PBS containing 3 mM EDTA and 1 per cent bovine serum albumin (PBS-EDTA-BSA). After the washes, the platelets were adjusted to a concentration of 40 x 10^8 cells/ml and used for the immunofluorescence assay. Freshly isolated platelets were used each time.

Twenty microlitres of the platelet suspension were incubated in plastic tubes (Falcon; Becton Dickinson) for 30 minutes at room temperature with 20 µl of a 1:10 dilution of samples of serum from the two groups of dogs and from the control dogs. After two washes with PBS-EDTA-BSA, the platelets were incubated for 30 minutes with 1:20 diluted fluorescein isothiocyanate (FITC)-labelled goat antidog IgM or IgG (Bethyl Laboratories).

The platelet population was gated using forward scatter and side scatter parameters, as described by Kristensen and others (1994b). Flow cytometry and data analysis were performed using a two-laser FACScalibur apparatus and CellQuest software (Becton Dickinson). To establish a reference negative value, staining profiles with platelets obtained from the 10 healthy dogs were assessed. Only samples with more than 30 per cent of platelets showing a mean fluorescence intensity on a log scale 100 channels higher than the negative control value, represented by the binding level obtained by incubating platelets with the healthy dog sera, were considered positive for the presence of platelet-binding IgM and IgG antibodies.

Statistical analysis

The data were analysed using Fisher's exact test with a two-sided P value. Results were considered significant when $P<0·05$.

Results

Analysis of haematological parameters in the infected dogs

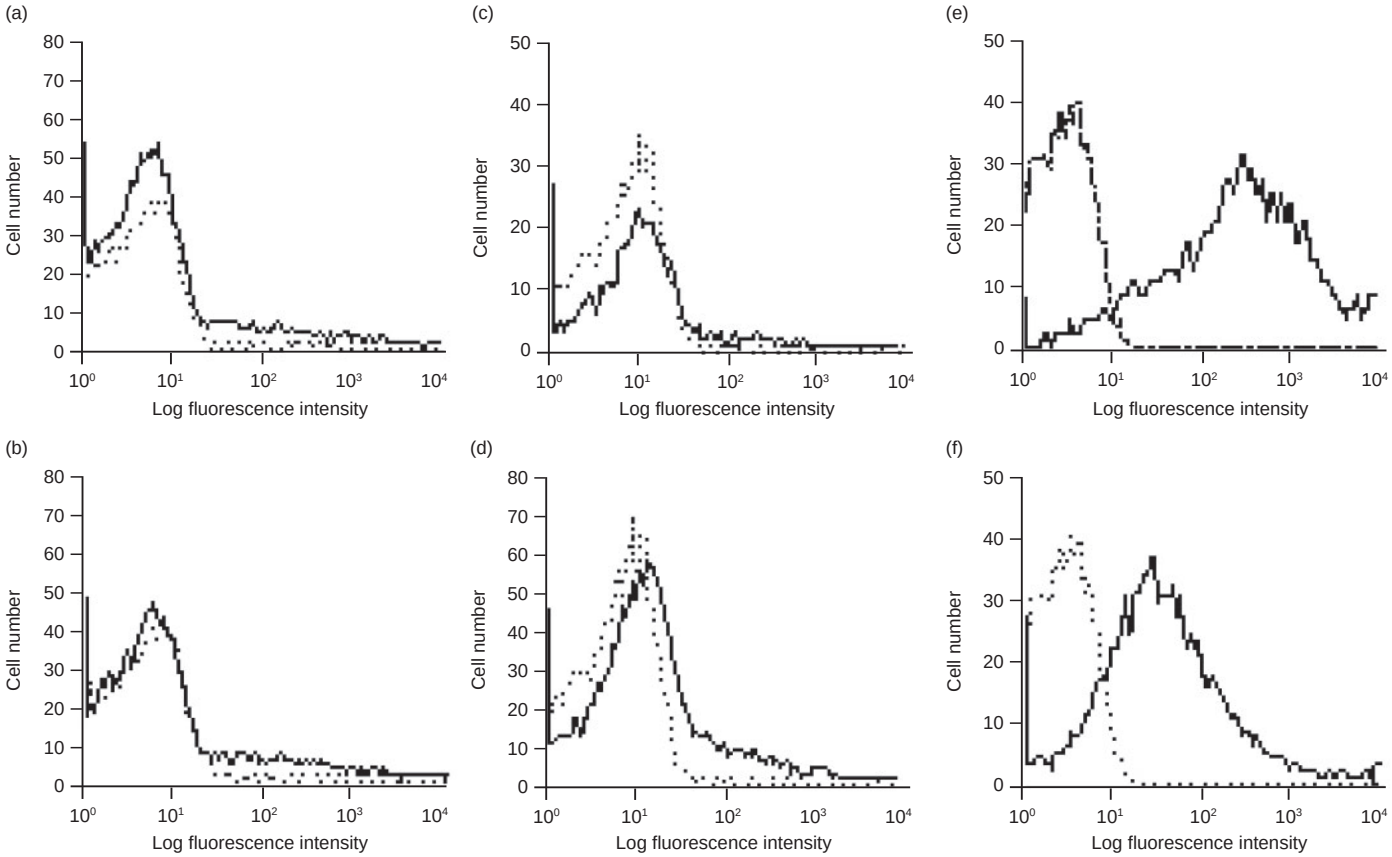
The haematological data for the infected dogs are shown in Table 1. Their platelet counts ranged from a minimum of 60 x 10^9/l to a maximum of 395 x 10^9/l. Among the dogs with fewer than 150 x 10^9 platelets/l, a mild or moderate normochromic and normocytic anaemia was observed in five (dogs 1, 8, 9, 19 and 20) and a mild or moderate normochromic and microcytic anaemia was observed in five (dogs 6, 7, 11, 12 and 13). Among the dogs with more than 150 x 10^9 platelets/l a mild normochromic and normocytic anaemia was observed in five (dogs 23, 26, 31, 37 and 42) and a mild hypochromic and normocytic anaemia was observed in dog 22.

The 10 healthy dogs had normal haematological profiles.

Analysis of platelet-binding antibodies in the serum of the infected dogs

Fig 1 shows examples of the staining profiles of healthy donor platelets treated with FITC-labelled antidog IgM or IgG after they had been incubated with samples of serum from the healthy dogs or the dogs infected with *L infantum*. Three typical binding profiles were obtained in the infected animals: a single binding peak for both IgM and IgG antiplatelet antibodies (panels E and F); a double peak for both IgM and IgG antiplatelet antibodies (panels G and H); and a double peak for IgM and a single peak for IgG antiplatelet antibodies (panels I and L).

Table 1 shows how many of the infected dogs had platelet-binding IgM and/or platelet-binding IgG. Thirty-two of the 44 infected dogs (72·7 per cent) had detectable platelet-binding immunoglobulins; 19 of the 20 dogs in group A (all except dog 1), and 13 of the 24 dogs in group B had platelet-binding IgM, or IgM and IgG. There was a significant association between the occurrence of thrombocytopenia and the presence of both IgM ($P<0·01$) and IgG ($P<0·01$) antiplatelet antibodies in the infected dogs. The isotype analysis of the platelet-bound immunoglobulins showed that all the dogs with detectable antiplatelet



immunoglobulins had both IgM and IgG, except for two dogs in group A (dogs 4 and 8) and 10 in group B (dogs, 21, 23, 28, 31, 32, 35, 36, 37, 39 and 43) that had only IgM.

No significant binding was observed after the incubation of platelets with the serum samples from the 10 healthy dogs (Fig 1).

Discussion

Immune-mediated thrombocytopenia is the most common cause of abnormal primary haemostatic disorders in dogs (Makin 1995a). It may occur alone and, in the absence of other identifiable diseases, it is referred to as a primary immune-mediated thrombocytopenia or idiopathic thrombocytopenic purpura. More frequently, a secondary immune-mediated thrombocytopenia occurs as a result of autoimmune disorders, treatment with drugs and blood products, cancer or many infectious agents. Antibodies that can bind to platelets have been identified in dogs with dirofilariosis, ehrlichiosis and babesiosis (Cockburn and Troy 1986, Breitschwerdt 1988, Taboada 1990, Troy and Forrester 1990). In particular, antiplatelet antibodies have been detected in the serum of dogs with naturally occurring and experimentally induced *Rickettsia rickettsi* infection (Grindem and others 1999). There is also evidence for the presence of antiplatelet antibodies in the serum of dogs with *E canis* infection (Lewis and others 1995b, Waner and others 1995, 2000, Harrus and others 1996). A secondary immune-mediated thrombocytopenia has also been presumed in canine leishmaniosis (Chabanne and others 2000, Terrazzano and others 2006). Infections may result in immune-mediated destruction of platelets by exposing antigenic sites on their surfaces or by immune complex injury to their membranes (Breitschwerdt 1988).

The diagnosis of antibody-mediated thrombocytopenia in dogs is complicated by the lack of a sensitive and specific assay for the detection of antiplatelet antibodies. Establishing a definitive diagnosis requires the detection of antibodies either directly on the surface of platelets or megakaryocytes (direct tests), or in serum (indirect tests). Flow cytometry has been applied extensively to improve the diagnosis of the condition (Lewis and others 1995a, Kohn and others 1999, Davis and others 2002, Kimberley and others 2004) in dogs and in horses, and it is considered to be one of the most specific and sensitive laboratory tests for platelet-binding antibodies in thrombocytopenic disorders in dogs (Chabanne and others 2000). The technique has several advantages over other platelet antibody assays. It can quantify antibody binding at the single cell level, it is highly sensitive owing to its ability to detect levels of fluorescence undetectable by visual observation, and it can be applied to small samples (Kristensen and others 1994b).

The results of this study show that the serum samples obtained from the dogs infected with *L infantum* contained IgM and IgG antibodies that were able to bind to the platelets. No binding activity was observed in the samples from the healthy dogs. The platelet binding activity was observed against different samples of normal platelets because in each experiment different dog donors were used. There was a significant association between the serum antiplatelet antibodies and thrombocytopenia in the infected dogs. Nineteen of the 20 dogs with fewer than 150 x 10^9 platelets/l had platelet-binding IgM, or IgM and IgG antibodies, whereas only 13 of the 24 dogs with more than 200 x 10^9 platelets/l had platelet-binding immunoglobulins ($P<0{\cdot}01$). These results suggest that the antiplatelet antibodies may play a role in the dogs' thrombocytopenia, and that it was an antibody-dependent secondary immune-mediated form of leishmaniosis. However, the absence of platelet-binding immunoglobulins from one of the thrombocytopenic dogs suggests a more complex pathogenesis, and decreased bone marrow production and/or splenic sequestration have been suggested (Makin 1995a). It is also possible that the sensitivity of the indirect method applied here was suboptimal and failed to detect antibodies already bound to platelets (Scott and others 2002).

In human beings with immune-mediated thrombocytopenia, the platelet-bound antibodies are predominantly IgG (in approximately 65 per cent of cases) or IgG and IgM (in approximately 30 per cent of cases) (Aster 1989, Tijhuis and others 1991). The antiplatelet antibodies in affected dogs are frequently IgG, having been detected in the serum of 55 to 80 per cent of cases (Wilkins and others 1973, Campbell and others 1984, Kristensen and others 1994c, Lewis and Meyers 1996). In this study, the isotype analysis of the platelet-bound immunoglobulins revealed that 17 of the 20 thrombocytopenic dogs had both IgM and IgG platelet-binding antibodies. Such a result may be due to the frequent recurrent

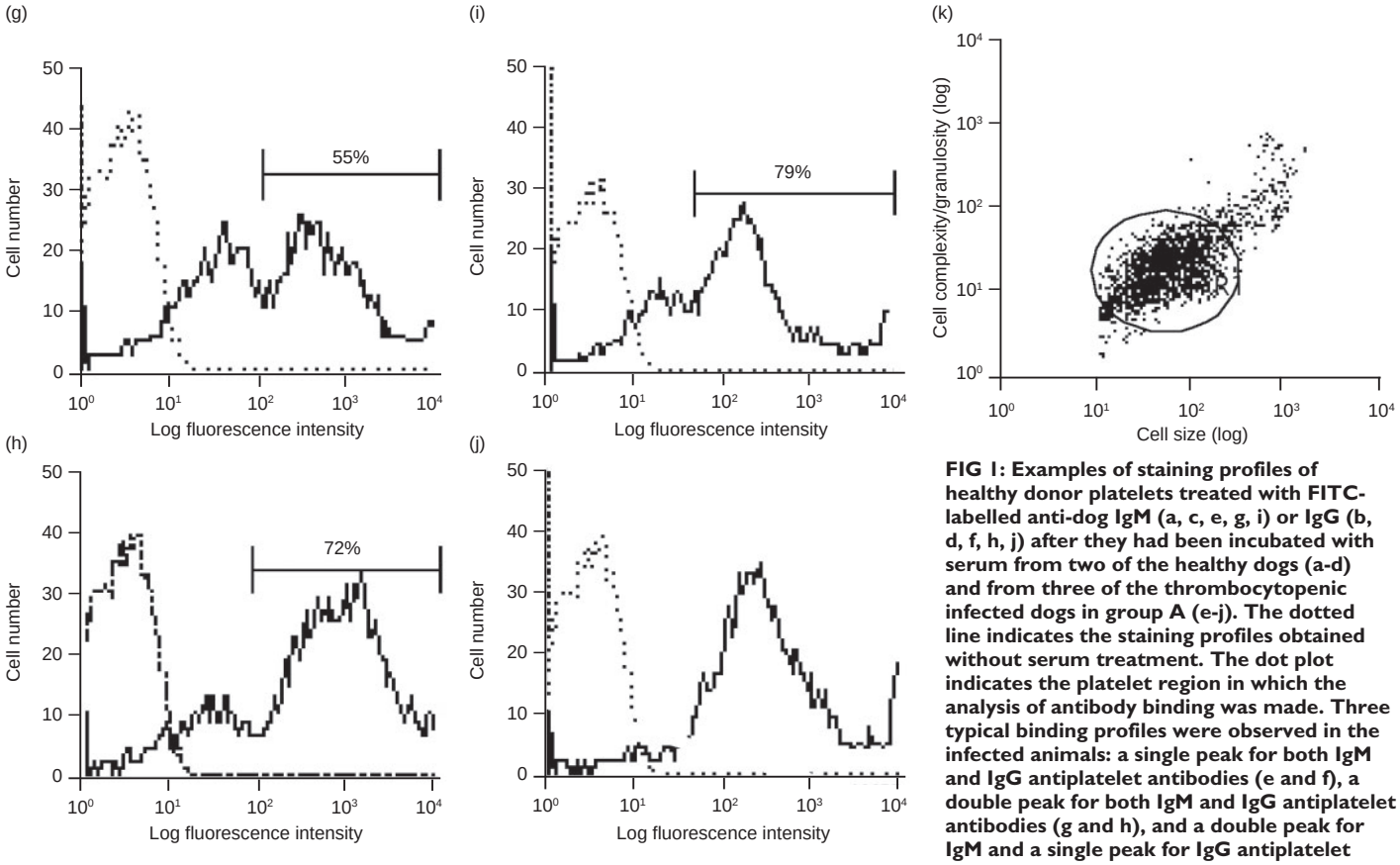


FIG 1: Examples of staining profiles of healthy donor platelets treated with FITC-labelled anti-dog IgM (a, c, e, g, i) or IgG (b, d, f, h, j) after they had been incubated with serum from two of the healthy dogs (a-d) and from three of the thrombocytopenic infected dogs in group A (e-j). The dotted line indicates the staining profiles obtained without serum treatment. The dot plot indicates the platelet region in which the analysis of antibody binding was made. Three typical binding profiles were observed in the infected animals: a single peak for both IgM and IgG antiplatelet antibodies (e and f), a double peak for both IgM and IgG antiplatelet antibodies (g and h), and a double peak for IgM and a single peak for IgG antiplatelet antibodies (i and j). The numbers indicate the percentage of binding when a double peak was identified. (k) Scatter graph showing cell size against cell complexity/granulosity

Leishmania species infections that probably occur in an endemic area such as Campania. In addition, the occurrence of thymus-independent B cell responses, driven by *Leishmania* species antigens in the naturally infected dogs, could explain the greater prevalence of IgM antiplatelet antibodies in the two groups (Schofield 1991, Wabl and Steinberg 1996).

Antiplatelet antibodies may also affect platelet function and further compromise primary haemostatic function (Makin 1995a, Boudreaux 1996). Autoantibodies recognising functionally relevant platelet-receptor glycoproteins and able to interfere with platelet function have been detected in human patients and in dogs with immune-mediated thrombocytopenia. A severe decrease in platelet aggregation has been observed in dogs infected with *Leishmania* species (Balduini and others 1987, George and Shattil 1991, Kristensen and others 1994a, Ciaramella and others 2005).

Immunosuppressive therapy to restore normal haemostasis by increasing the platelet count to more than 50,000/µl (usually considered the threshold value for bleeding control) has been reported to be effective for treating immune-mediated thrombocytopenia (Makin 1995b). Glucocorticoids are the mainstay of immunosuppressive therapy and have the most immediate effect. For patients with the primary form of the disease that fail to respond to glucocorticoids, additional immunosuppressive medications such as azathioprine or cyclophosphamide may be used (Makin 1995b, Grindem 2000). In cases associated with infectious diseases, the treatment or removal of the underlying infection in addition to therapeutic approaches can restore the platelet count and haemostatic function. In dogs with leishmaniosis, prednisone is commonly used at subimmunosuppressive doses to control immunopathological effects, such as glomerulonephritis, keratitis, uveitis or polyarthritis (Slappendel and Ferrer 1998), but immunosuppressive doses are considered dangerous because of their potentially adverse effects. Because of their catabolic, hypoalbuminaemic and immunosuppressive activities, high doses of corticosteroids should be used with care. Recently, the addition of an anti-inflammatory dose of prednisone to the classical anti-*Leishmania* chemotherapy of meglumine antimoniate and allopurinol has been observed to increase the rate of improvement of platelet aggregation in leishmaniotic dogs (Cortese and others 2008).

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