Veterinary and Comparative Oncology

Short Communication

DOI: 10.1111/j.1476-5829.2011.00290.x

Use of CD9 and CD61 for the characterization of AML-M7 by flow cytometry in a dog*

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Abstract

Acute megakaryoblastic leukaemia (AML-M7) is a rare myeloproliferative disorder in domestic animals. Recently, thanks to the greater availability of immunophenotype techniques, precise diagnosis is more easily made. The morphological evaluation has its limitations, especially in the study of poorly differentiated cells. Few reports have described AML-M7 in dogs using flow cytometry. This clinical case points out the utility of flow cytometry in the characterization of AML-M7 in a 3-year-old German Shepherd dog. Flow cytometry investigation has established megakaryocytic lineage involvement by showing the presence of two megakaryocyte/platelet associated antigens (CD9 and CD61). In human medicine CD9 may be used as a platelet and megakaryocyte marker. There is an evidence of cross-reactivity of human anti-CD9 monoclonal antibody with canine samples. To our knowledge, the use of CD9 has never been described before, for this purpose in the dog.

Keywords
clinical pathology, comparative oncology, immunology, oncology, small animal internal medicine

Case report

A 3-year-old spayed female German Shepherd weighing 30 kg was presented for severe weakness, lethargy, anorexia and weight loss of several weeks duration. Physical examination revealed pale mucous membranes, generalized muscular atrophy, tachycardia and mild hypothermia. Splenomegaly was detected on abdominal palpation. Initial diagnostic plan included complete blood count (CBC), serum biochemical profile, urinalysis, thoracic radiography and abdominal ultrasound. Results from the diagnostic procedures listed were unremarkable except for the CBC (Table 1), which revealed severe anaemia [Hct 11.9%; reference interval (RI) = 37–55%; Hb = 4.4 g dL^{-1}; RI = 12–18 g dL^{-1}], severe thrombocytopenia (9.0 platelets $\times 10^9$ L^{-1}; RI = 150–500 $\times 10^9$ L^{-1}) and mild leukopenia (5.6 $\times 10^9$ L^{-1}, RI = 6.0–17 $\times 10^9$ L^{-1}). Circulating blast cells (30%) were also detected and morphologically evaluated (Fig. 1). Abdominal ultrasound examination confirmed splenomegaly. The additional diagnostic plan included bone marrow and spleen aspiration biopsy. The former revealed good cellularity and adequate cell morphology. All three haemopoietic cell lines (erythroid, myeloid and megakaryocytic) were almost completely replaced by large, variably sized round blasts that had a markedly increased nuclear-cytoplasmic ratio (N:C) and were often bi- or multinucleated. The nuclear chromatin was dense and fine and one or more distinct nucleoli were seen. Cytoplasmic microvacuolizations, basophilia and blebbing were noted. Cytological examination of the splenic aspirate revealed the same blast cells as those in the bone marrow. On the basis of these findings, the sample was suggestive of acute myeloid leukaemia probably of megakaryoblastic origin. To characterize

*This case report was submitted to the ESVONC-VCS Joint Meeting Copenhagen – February 28 to March 1, 2008, and was accepted as a poster.

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Table 1. CBC results with differential count

	Unit	Values	Reference interval
Erythrocytes	$\times 10^{12}$ L^{-1}	1.53	5.5–7.9
Haemoglobin	g dL^{-1}	4.4	12.0–18.0
Haematocrit	%	11.9	37–55
MCV	fL	78	60–77
MCH	pg	28.8	20–25
MCHC	%	37.1	30–35.3
RDW	%	18.3	12–16
Reticulocyte index		0.26	0–1
Platelets	$\times 10^{9}$ L^{-1}	9.0	150–500
Leucocytes	$\times 10^{9}$ L^{-1}	5.6	6.0–17.0
Band neutrophils	$\times 10^{9}$ L^{-1}	0.2	0–0.3
Neutrophils	$\times 10^{9}$ L^{-1}	2.4	3.0–10.5
Eosinophils	$\times 10^{9}$ L^{-1}	0.0	0.1 – 1.2
Basophils	$\times 10^{9}$ L^{-1}	0.0	Rare
Monocytes	$\times 10^{9}$ L^{-1}	0.5	0.1–1.4
Lymphocytes	$\times 10^{9}$ L^{-1}	0.8	1.0–4.8
Blast cells	$\times 10^{9}$ L^{-1}	1.7	0

this type of myeloproliferative disorder accurately, samples from bone marrow were submitted for flow cytometry analysis.[1–5] The immunological panel (Table 2) revealed immunoreactivity for 7.2% CD3 (T lymphocytes), 5.8% CD79 (panB-lymphocytes), 2.3% CD34 (blast cells), 4.0% CD14 (monocytes), 64.5% CD9 (platelets), 59.5% CD61 (platelets) and 17.7% CD18 (beta2-integrins). CD34 immunoreactivity is usually considered supportive of acute leukaemia. However, this is not definitive, as it is known that a percentage of neoplastic blastic cells may lack CD34 expression in both canine and human patients.[6] Cells morphologically consistent with 'blast cells' with or without CD34 expression were then categorized as myeloid based upon further immunophenotyping results.[7] On the basis of morphological evaluation and the immunological profile, a diagnosis of acute megakaryoblastic leukaemia (AML-M7) was made.[5]

A transfusion of 250 mL of fresh canine blood was given. Ampicillin (20 mg kg^{-1} intravenous TID) and metronidazol (7 mg kg^{-1} intravenous TID) were administered. The blood transfusion increased the PCV to 17%. Following the patient's stabilization, chemotherapy was started with 100 mg m^{-2} of cytosine arabinoside administered subcutaneously divided into four daily doses. Cytosine arabinoside is a synthetic antimetabolite[8] that incorporates into DNA and acts as a relative chain terminator that blocks DNA synthesis. This drug is used to treat acute leukaemia in human beings and other animals.[9] The dog did not respond to chemotherapy and after 4 days the PCV dropped again to 12%. At this time the owner elected to euthanize the dog. Unfortunately the dog body was not available for necropsy.

Materials and methods

Samples

Both canine fresh whole blood in potassium ethylenediaminetetraacetic acid and bone marrow slides were submitted for haematological and cytological evaluation to the 'San Marco' Private

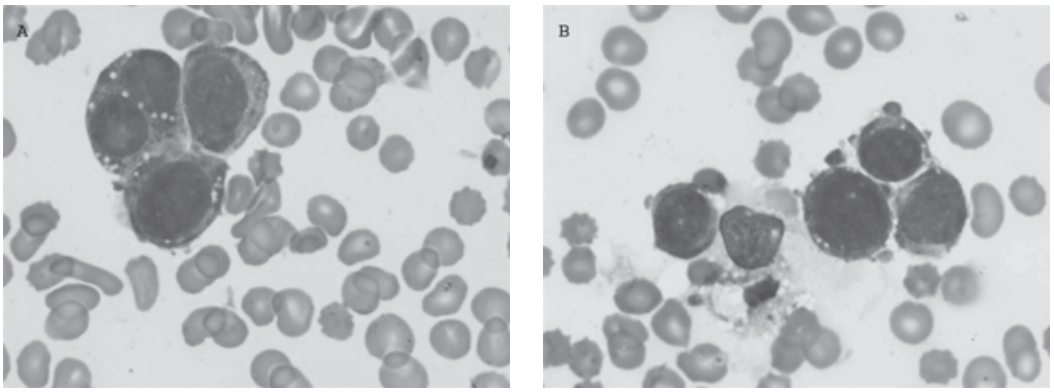

Figure 1. Peripheral blood from a dog with AML-M7: (A) large, variably sized and round-shaped blast cells, often bi- or multinucleated with marked increased N:C ratio. The nuclear chromatin is dense and fine and one or more distinct nucleoli are visible; (B) the cytoplasm is abundant, basophilic and shows microvacuolizations and projections, (blebbing). Modified Wright, ×50 objective.

Table 2. Antibodies used in the flow cytometric immunophenotyping

Specificity	Source	Designation	Isotype	Conjugation	Target
Canine CD3	Serotec, Oxford, UK	MCA1774F	Mouse IgG1	FITC	T lymphocytes
Human CD79[a]	Dako, Glostrup, Denmark	HM57	Mouse IgG1	PE	Pan-B lymphocytes
Human CD14	Serotec, Oxford, UK	MCA1568C	Mouse IgG2a	PE-Cy5	Monocytes
Canine CD34	Becton Dickinson, Erembodegem, Belgium	1H6	Mouse IgG2a	PE	Stem cells
Human CD9	Becton Dickinson, Erembodegem, Belgium	MCA 469	Mouse IgG1	FITC	Platelets, monocytes
Human CD61	Becton Disckinson, Erembodegem, Belgium	VI-PL2	Mouse IgG1	FICT	Platelets
Human CD18	Serotec, Oxford, UK	MCA503F	Rat IgG2b	FICT	Beta-2 integrins

FITC, fluorescein isothiocyanate.
[a]Cytoplasmic antibody.

Veterinary Laboratory, Padua, Italy. The iliac crest was used as the site for bone marrow aspiration. The blood cell count (CBC) was performed with ADVIA 120 (Siemens® Healthcare Diagnostics, Deerfield, IL, USA). The blood- and bone marrow-dried smears were stained with modified Wright and May-Grünwald-Giemsa techniques, respectively (Aerospray slide stainer 7120, Wescor®, Delcon, Italy). The morphological classification criteria were based on the cell size, the shape of the nuclei, the density of the chromatin, the number, size and distribution of the nucleoli and the volume and basophilia of the cytoplasm, according to the FAB classification.[5]

Labelling of surface antigens

One hundred microlitres of blood was mixed with 5–10 μL of each antibody in a plastic tube and incubated in the dark at room temperature for 30 min, according to manufacturer recommendations. The specificity designation and the reactivity of the antibodies employed are listed in Table 2. Two millilitres of lysing solution was added to the samples which were left for 15 min at room temperature. Afterwards, the samples were centrifuged for 10 min at 1200 × *g*, and the supernatant was discarded. The leucocytes were resuspended in 4 mL of phosphate-buffered saline (PBS) and centrifuged for 10 min at 1200 × *g* and then they were resuspended in 1 mL of PBS prior to flow cytometry analysis.[5]

Labelling of cytoplasmic antigens

The Leucoperm® reagent (Serotec®, Kidlington, UK) system for fixing and permeabilizing cells in suspension was used. This procedure gives antibodies access to intracellular structures and leaves the morphological characteristics of the cells intact. For each sample, 100 μL of reagent A (fixation medium) was added to 50 μL of whole blood. Four millilitres of PBS was added after 15 min of incubation at room temperature, and the mixture was centrifuged for 5 min at 1200 × *g*. Supernatant was removed and 100 μL of reagent B (permeabilization medium) was added to the cell pellet along with 10 μL of the appropriate antibody directly conjugated with phycoerythrin (PE). The sample was vortexed at low speed for 1–2 s and incubated for 30 min at room temperature in the dark. After incubation, cells were resuspended in 4 mL of PBS and centrifuged for 10 min at 1200 × *g*. Supernatant was then discarded. The sample was resuspended in 4 mL of PBS to wash the cells and centrifuged for 10 min at 1200 × *g*. Supernatant was discarded and the sample was resuspended in 1 mL of PBS prior to flow cytometry analysis.[5]

Flow cytometric analysis and evaluation of measured data

Flow cytometry was performed with the Epics XL-MCL flow cytometer (Beckman Coulter®, Milan, Italy). Measured data were evaluated with the computer program System II™ (DOS 6.22; Beckman Coulter). The gate was established using forward scatter (FS) and side scatter (SS); these two parameters take into account cell size and cytoplasm complexity, respectively (Fig. 2). The choice of gate region was made, according to cytological evaluation, around the monocyte/neutrophil region because of the large size of the cell population. Platelets were not considered in the gate for two reasons: they are not usually expected to be found

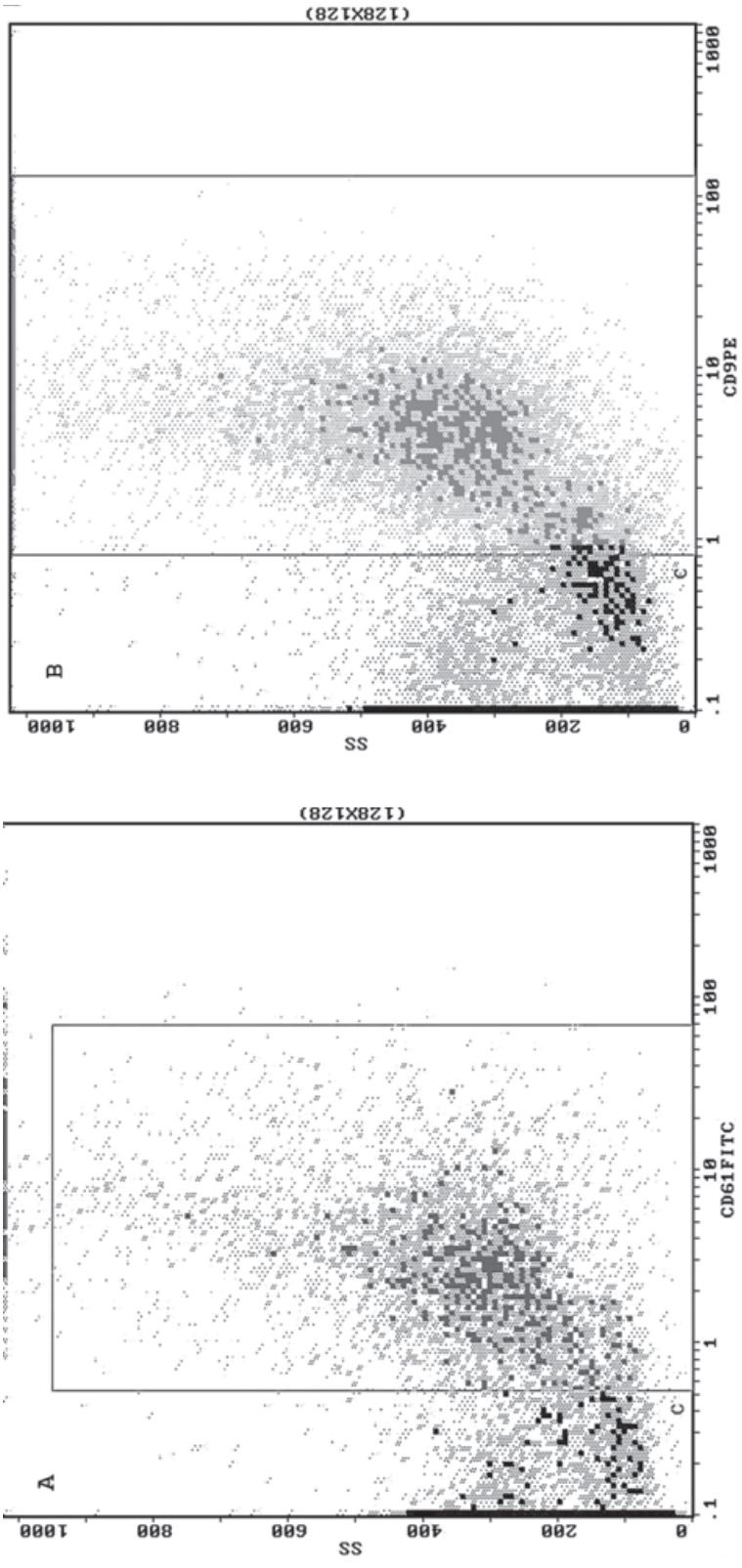

Figure 2. Flow cytometry of peripheral blood from a dog with AML-M7; according to the morphological evaluation, this neoplastic population shows high levels of both FS (dimension) and SS (complexity). (A) neoplastic cells expressing CD61 detected by anti-CD61-FITC antibodies; (B) same population of cells expressing CD9 detected by anti-CD9-PE antibodies.

in the bone marrow and even if they are present in aspirates as a result of almost inevitable, although variable, degree of haemodilution, the scatter of platelets is very different indeed they have no nuclei and are very small.

The discriminator was fixed to 100 V on FS channel to eliminate the interference resulting from the debris: it works as a threshold in which a signal must exceed to be saved in order to minimize the recording of background noise. Regarding the gate, neoplastic cells had replaced most of the normal haematopoietic cells, so the gate was drawn around a single population. Within the gate, percentages of subpopulations were calculated by establishing parameters for SS and fluorescence (fluorescein isothyocyanate, PE or PE-CY5).[1] Negative and single control are not usually employed for each antibody, but every antibody previously to be introduced in routine panel, was tested to confirm its reactivity and to create a specific protocol for that antibody. Before each analysis, the flow cytometer is controlled for the alignment of fluorescence channels.

Discussion

AML-M7 is a rare form of myeloid leukaemia in humans, first described in 1931.[10,11] It may occur as a spontaneous disease or as therapy-related acute leukaemia. It accounts for approximately 1–2% of all *de novo* AML in the adult population, but the incidence in children is higher, partly because of an association with Down's syndrome where cytogenetic abnormalities of chromosome 21 have been found.[12,13] The simultaneous use of several differentiation markers is required to diagnose this type of leukaemia in people.[12,13] Cytogenetics has provided a new approach to the diagnosis and classification of AML in humans.[14]

Megakaryoblastic leukaemia is a rare myeloproliferative disorder in domestic animals as well.[15] AML-M7 was first described in a dog by Rudolph and Hubner in 1972.[16] Megakaryoblastic leukaemia should not be diagnosed based on cell morphology alone. This may be possible if blasts show some criteria of differentiation otherwise it is difficult to identify blast cells as megakaryoblasts. The presence of cytoplasmic projections and binucleate and tetranucleate cells may be suggestive of cells of megakaryocytic lineage.[17,18] Poorly differentiated megakaryoblasts may resemble lymphoblasts or myeloblasts based on routinely stained blood or bone marrow smears. To make a specific diagnosis for AML-M7 immunophenotype technique is required.[19] Immunophenotyping is the most sensitive and specific diagnostic tool for determination of cell origin in poorly differentiated acute leukaemias, making the diagnosis more accurate.[20,21] Immunophenotype markers that have been used previously to confirm a megakaryoblastic lineage in dogs include factor XIII, CD61, vWF, CD79a, CD34 and CD41[19]; however, factor XIII can also be present in hepatocytes, monocytes and macrophages; CD61 may be expressed by monocytes/macrophages and endothelial cells; vWF expression in dogs is extremely variable and often weak; CD79a is not specific for megakaryocyte lineage but mostly for B-lymphocyte lineage; immunocytochemical detection of cytoplasmic CD41 by leukaemic blasts is highly specific for megakaryocytic origin.[19] Cytogenetic studies in canine leukaemias are rare. A case of canine AML showing a trisomy 1 and a t(X;8) has been reported.[27]

To classify myeloproliferative disorders in veterinary medicine, precise criteria have been proposed by the Animal Leukemia Study Group. On the basis of these criteria all acute myeloproliferative disorders are designated with AML followed by an acronym indicating the cell lineage involved in the neoplastic proliferation. AML-M1 refers to acute myeloblastic leukaemia without maturation, myeloblastic leukaemia with maturation (M2), promyelocytic leukaemia (M3), myelomonocytic leukaemia (M4), monocytic leukaemia (M5) and erythroleukaemia (M6). Megakaryoblastic leukaemia (M7) is the proposed designation for myeloproliferative neoplasms involving megakaryocytic lineage.[22–24]

The prognosis for dogs with megakaryocytic neoplasia is extremely poor. Survival time is reported to be only a few weeks once clinical signs have become apparent. The few attempts to treat dogs have been disappointing.[17]

To our current knowledge, only 14 cases of spontaneous AML-M7 have been previously described

and only two publications describe the use of flow cytometry for definitive diagnosis of AML-M7[19,25] in veterinary medicine. On the basis of this further report, the German Shepherd dog seems overrepresented[6] accounting for six cases in the literature including this case. In accordance with other previous works, clinical outcome and prognosis for both people and dogs affected by AML-M7 are poor.

This clinical case points out the utility of flow cytometry in the characterization of AML-M7 in the dog. Nowadays, the association between morphological and immunophenotypical evaluation can increase the accuracy of the diagnosis. Compared with immunohistochemistry, flow cytometry can be used with blood and/or bone marrow samples without the need for tissue or core biopsy. Furthermore, flow cytometry allows the evaluation of several parameters in a few minutes and provides the clinician with daily results. In this work, flow cytometry has established megakaryocytic lineage showing the co-expression of two megakaryocyte/platelet associated antigens (CD9 and CD61). In human medicine, CD9[1–3] is used as a platelet and megakaryocytes marker. There is an evidence of cross-reactivity of human anti-CD9 monoclonal antibody with canine samples.[4] The anti-CD9 monoclonal antibody MCA469 is a 24-kD cell surface glycoprotein expressed principally by platelets, monocytes, some lymphocytes and endothelial cells. It is a member of the tetraspan gene family.[26] The use of CD9 alone does not permit platelet identification, but in association with other platelet markers, such as CD61, it is possible to confirm a platelet/megakariocyte origin. In addition, in this case, the population showed a low expression of CD14 (monocytes) and of all lymphocyte antigens, ruling out a lymphocyte and monocyte origin. To our knowledge, the use of CD9 has never before been described, for this purpose in the dog.

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