

Flow Cytometric Assessment of P-glycoprotein and Multidrug Resistance-associated Protein Activity and Expression in Canine Lymphoma

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Abstract. *Aim: To investigate the activity and expression of P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRPs) in biopsy samples from lymph nodes of 22 dogs at the time of lymphoma diagnosis. Materials and Methods: Protein activity and expression were investigated by flow cytometry. Expression was assessed with monoclonal antibodies (C494 for P-gp and MRPM6 for MRP1). P-gp activity was determined by rhodamine 123 (Rho 123) efflux inhibited by verapamil and MRP by 5(6) carboxyfluorescein diacetate (CFDA) efflux inhibited by probenecid. Cell lineage was determined based on the expression of CD79a, CD21, CD3, CD4 and CD8. Results: P-gp and MRP1 were expressed in all samples. Relative activity was low for both transporters and no correlation was found between transporter activity and expression. The majority of lymphomas were of B-cell origin. Conclusion: Low transporter activity and lack of correlation with expression suggest that spontaneous up-regulation of P-gp or MRP is not a common phenomenon in canine lymphoma.*

Lymphoma is one of the most common neoplasms in dogs. In contrast to solid tumors, which are treated by surgery or radiotherapy, most cases of canine lymphoma (CN) require systemic chemotherapy (1, 2). It is estimated that after chemotherapy the remission rate in CN may be as high as 80%-90% with an excellent improvement in the quality of life (3-5). However, most patients eventually die because of development of drug resistance and the average survival time is estimated to be 8-13 months (3, 4, 6). Immunophenotype

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and expression of membrane-bound transporters are considered to influence survival time (7-9). The transporters associated with drug resistance in cancer belong to the ATP-binding cassette superfamily (ABC) and are represented by P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs) and others (10, 11). Due to high expression on physiological barriers, their evolutionary role is associated with protection against xenobiotics (11, 12). However, ABC transporters may also be over-expressed by neoplastic cells and contribute to their drug resistance by enhancing the efflux of cytotoxic agents (10, 11). For P-gp and MRP, the spectrum of substrates is broad and includes anthracyclines, vinca alkaloids, epipodophyllotoxins and taxanes (13, 14). Earlier studies that applied immunohistochemistry reported positive staining for P-gp only in approximately one third of tumor tissue samples obtained from dogs with lymphoma (9, 15). Normal samples of canine lymph nodes were negative (15). Moore *et al.* (16), who applied western blotting, report P-gp expression in only 1 of 30 naïve CN samples and in 3 of 8 samples from dogs with chemotherapy-resistant disease. A more recent study that assessed the expression of P-gp and MRP1 in normal canine nodal lymphocytes by means of flow cytometry, reported the presence of transporter proteins in samples from all 21 investigated dogs (17). The authors concluded that flow cytometry is a more sensitive method for P-gp and MRP1 detection than immunohistochemistry or western blotting. The same study demonstrated the functional activity of both transporters in normal lymphocytes by means of flow cytometric assessment of dye efflux inhibition by transporter-specific inhibitors.

The aim of the present study was to assess, by means of flow cytometry, the P-gp and MRP1 expression, as well as P-gp and MRP function in tissue samples obtained from enlarged lymph nodes in CN patients. Since it is known that T- or B-cell origin of transformed lymphocytes is an important prognostic factor, the results of transporter expression and function were compared to the immunophenotype of the investigated cells.

Materials and Methods

Sample collection and diagnosis. Samples were obtained from 22 dogs diagnosed with CN between January and May 2012 at the Clinic of the Diseases of Horses, Dogs and Cats of the Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Poland. The study was approved by the II Local Ethics Committee for Animal Experiments in Wrocław (permit number 82/2011). All the dogs were diagnosed with CN for the first time and were not subjected to cytostatic therapy before. However, the vast majority of dogs were pre-treated with glucocorticoids before admission to the clinic. Due to the lack of reliable data on glucocorticoid pre-treatment, it was impossible to include this factor as a variable. Cells were obtained by fine needle aspiration biopsy of enlarged lymph nodes. Only microscopically confirmed lymphoma samples were included in the present study. Cytological smears were stained with hematoxylin and eosin (H&E) and the signs of neoplastic transformation were assessed based on pleomorphism, cell size, nuclear shape, chromatin density, mitotic index, the number, size and distribution of nucleoli and the extension and basophilia of the cytoplasm.

Determination of the immunophenotype. Classification of the immunophenotype of CN was based on the detection of T (CD3, CD4, CD8) or B (CD79 α , CD21) cell-specific markers. For this, the following set of monoclonal antibodies was applied: anti-CD3-FITC, anti-CD4-RPE, anti-CD8-Alexa Fluor 647[®] (clones CA17.2A12, YKIX302.9 and YCATE55.9, respectively; Serotec, Oxford, UK), anti-CD21-Alexa Fluor 647[®] (clone CA2.1D6; Serotec) and anti-CD79 α -RPE (Clone HM57; Dako, Glostrup, Denmark). Briefly, 5×10^5 cells/tube were incubated for 20 min at 4°C with monoclonal antibodies. Staining of CD79 α (cytosolic antigen) included a pre-incubation with permeabilization buffer (Dako) for 15 min. Cells were washed twice in phosphate buffered saline (PBS; Institute of Immunology and Experimental Therapy, Wrocław, Poland) supplemented with 1% bovine serum albumin (BSA; Sigma, Deisenhofen, Germany). Isotype-matched controls were included for each labeling. When acquisition could not be performed immediately, samples were fixed with 2% paraformaldehyde and analyzed within 2 days. Labeled cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) and the Cell Quest 3.1f software (Becton Dickinson). Expression of specific markers defined the lineage of CN: CD79 α and CD21 for B-cell and CD3 together with CD4 or CD8 for T-cell lymphomas. A positive staining referred to the antigen expression in at least 60% of cells. Classification to mixed cell type was made when the predominant population of cells could not be determined or when the level of expression did not exceed 60%.

Determination of transporter activity. P-gp and MRP activity were assessed as previously described (17) with modifications. The principle of this method is to measure the efficacy of selective transporter inhibitors in preventing the cellular efflux of fluorescent substrates of these transporters (18-21). The fluorescent dyes used in this study were rhodamine 123 (Rho123; Sigma, Deisenhofen, Germany), which is considered to be a selective substrate for P-gp, and 5 (6) carboxyfluorescein diacetate (CFDA; Sigma), which is considered to be a selective substrate for MRP. The transporter inhibitors used were verapamil for P-gp and probenecid for MRP (both from Sigma). Initially, cells were treated with 0.84% NH₄Cl for

erythrocyte lysis. After 2 washing steps in PBS with 1% BSA, cells were incubated at 37°C and 5% CO₂ for 30 min in dye-containing media (RPMI 1640; Institute of Immunology and Experimental Therapy, Wrocław, Poland) supplemented with 20% fetal bovine serum (FBS; Sigma) and the dye: 0.2 μ g/ml Rho123 or 1 μ M CFDA. Next, cells were incubated for 2 h (37°C, 5% CO₂) in either control medium (RPMI 1640, 20% FBS) or in medium containing the specific inhibitor (0.01 mM verapamil or 2.5 mM probenecid). Subsequently, the cells were washed in PBS with 1% BSA, transferred into cryoprotective medium (50% RPMI 1640, 40% FBS, 10% DMSO) and frozen at -70°C for up to few days. After rapid thawing, cells were washed and suspended in PBS with 1% BSA and their fluorescence was determined with a FACSCalibur flow cytometer (excitation 488 nm, fluorescence measured in channel 1, Cell Quest 3.1f software). Ten thousand events were measured per sample and the population of dye retaining cells was gated. Fluorescence was reported as a histogram and the mean value was calculated. The relative activity of a transporter was expressed as a ratio of mean fluorescence of cells treated with the inhibitor and of cells that were allowed to efflux the dye freely.

Determination of transporter protein expression. For transporter expression, 5×10^5 cells/tube were incubated with permeabilization buffer (Dako) for 15 min, washed twice in PBS and stained with monoclonal anti-P-gp (clone C494; Covance, Dedham, MA, USA) or anti-MRP1 (clone MRPm6; Enzo, Farmingdale, NY, USA) antibodies. After 1 h incubation at 4°C and 2 washing steps, secondary antibodies, rat anti-mouse IgG1-RPE for MRP1 (clone SB77e; Southern Biotech, Birmingham, AL, USA) and rat anti-mouse IgG2a-FITC for P-gp (clone SB84a; Southern Biotech) were used. After 20 min of incubation and two washing steps, cells were re-suspended in PBS. For each sample, isotype-matched control was performed based on the manufacturer's instructions. The relative fluorescence was calculated as a ratio of the fluorescence of the sample divided by the fluorescence of the isotype control (17, 22).

Statistical analysis. To determine the relationship between the transporter function and expression, linear regression analysis was performed and the Pearson's correlation coefficient (r) was determined. Statistical significance of the correlation coefficient was assessed by the Student's *t*-test ($p \leq 0.05$ was considered significant).

Results

Both, P-gp and MRP1 expression was detected in all investigated samples. The mean relative transporter activity and the mean relative transporter expression are shown in Figure 1. The mean activity of P-gp was 1.26 ± 0.54 (range=0.99-2.79) and the mean activity of MRP was 3.26 ± 2.32 (range=1.05-10.65). For P-gp, the relative expression was 26.67 ± 9.12 (range=13.21-52.19) and for MRP1 the relative expression was 11.27 ± 6.52 (range=4.29-29.94). Linear regression analysis did not reveal any relation between P-gp or MRP function and expression (only MRP1 expression was investigated). Figure 2 shows representative semi-logarithmic histograms of P-gp (A) and MRP (B) activity. The white area represents the autofluorescence of control cells, whereas the dark-grey area depicts the fluorescence of the cells that were

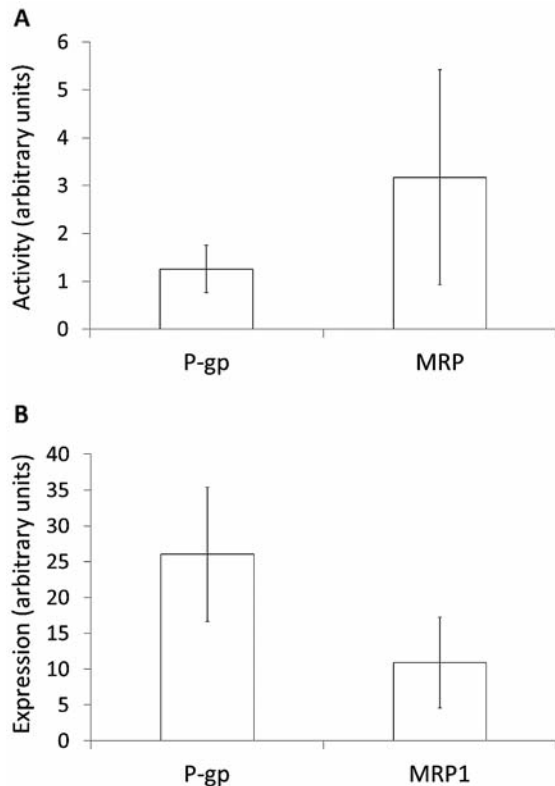


Figure 1. Mean (\pm SD) relative transporter activity of P-gp and MRPs (A) and mean (\pm SD) relative transporter protein expression of P-gp and MRP1 (B) in the biopsy samples from enlarged lymph nodes of canine lymphoma patients (n=22).

incubated with a dye and an inhibitor. The light-grey area depicts the fluorescence of cells that were allowed for free dye efflux. The prominent shift of the light-grey area towards the control in the lower diagram (B) indicates higher activity of MRP compared to P-gp (upper diagram, A). Mean relative activity and expression of transporters in relation to the immunophenotype are shown in Table I. Small number of T- cell and mixed phenotype lymphoma cases prevent statistically reliable conclusions.

Discussion

Investigation of P-gp and MRP activity or expression in malignant tissues has a long history in human oncology. The important role of these transporters is also commonly acknowledged in veterinary oncology. This is, however, based more on implications from human medicine than actual scientific data, which is much less robust for veterinary cancer patients compared to their human counterparts. Since spontaneous CN is often considered a valuable animal model

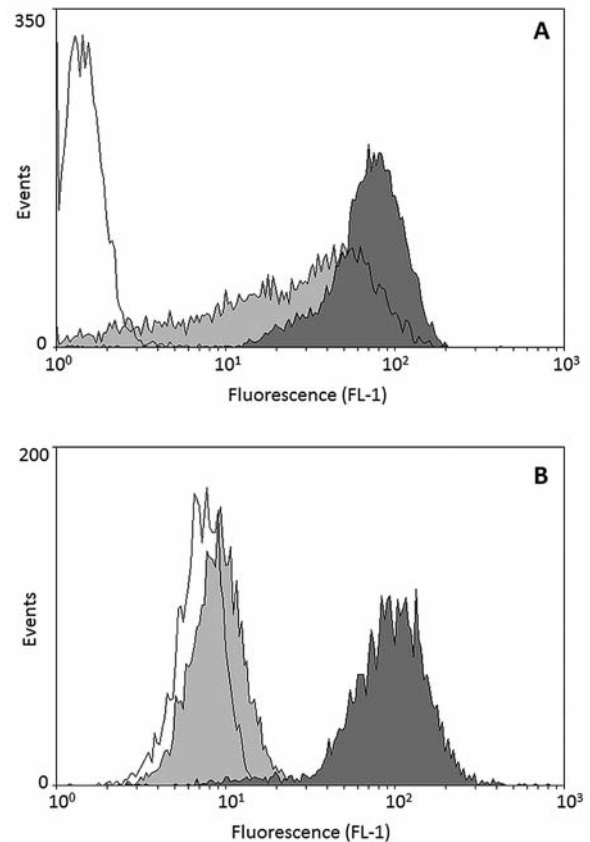


Figure 2. Representative semi-logarithmic histograms of P-gp (A) and MRPs (B) activity in canine lymphoma cells determined by fluorescent dye efflux assay. White area, autofluorescence of control cells; dark-grey area, fluorescence of cells incubated with a dye and appropriate transporter inhibitor; light-grey area, fluorescence of cells that were incubated in dye-containing medium and allowed for subsequent free dye efflux. The extent of the leftward shift of the light-grey area indicates the intensity of transporter-mediated dye efflux from a cell.

for non-Hodgkin lymphoma in humans, the assessment of the actual role of ABC transporters in the development of multidrug-resistant (MDR) phenotype in canine patients seems very important.

Up to date, most research on transporter expression in CN was carried out by means of western blotting (16), immunohistochemistry (9, 15) and, to a lesser extent, flow cytometry (17) and reverse transcription polymerase chain reaction (23). Flow cytometry was found to be more sensitive compared to the first two methods (17), which allowed to observe transporter expression only in relatively few cases (described as over-expression) (9, 15, 16). Moreover, flow cytometry enables for assessment of transporter function in fluorescent dye efflux assays. Although some controversies exist regarding the selectivity of different dyes as transporter substrates and the selectivity of transporter inhibitors (21), it

Table I. Mean (\pm standard deviation) relative activity and expression of respective transporters in relation to the immunophenotype of the lymphoma (n=22).

Immunophenotype	T cell (n=3)	B cell (n=16)	Mixed phenotype (n=3)
P-gp activity	1.15 (\pm 0.19)	1.15 (\pm 0.44)	1.95 (\pm 0.84)
MRP activity	4.57 (\pm 2.81)	2.72 (\pm 2.31)	4.85 (\pm 0.44)
P-gp expression	31.88 (\pm 17.87)	24.93 (\pm 7.30)	30.75 (\pm 7.99)
MRP1 expression	4.84 (\pm 0.63)	11.58 (\pm 5.61)	16.00 (\pm 10.64)

P-gp: P glycoprotein; MRP: multidrug resistance-associated protein; MRP1: multidrug resistance-associated protein 1.

was shown that, for normal canine lymphocytes, the use of fluorescent dyes, Rho123 for P-gp and CFDA for MRP with verapamil and probenecid as respective inhibitors, is a valuable approach (17). Since non-functional P-gp has been frequently found in *e.g.* human acute myeloid leukemia patients and cell line models (24, 25), it seems reasonable to investigate both transporter expression and function in order to assess the actual role of ABC transporters in the development of the MDR phenotype. Such an approach, however, does not always clarify the situation. In the present study, no correlation between protein expression and function was observed. A possible reason for the lack of correlation between these two parameters could be the generally low transporter activity. Other explanations include loss of protein functionality or change in the epitope recognized by the antibody. Such a lack of correlation is sometimes described in samples obtained from human patients (21). It is much easier to observe such a relation in *in vitro* models that have been successfully developed based on CN cell lines (26).

The material used in the present study was obtained from biopsies performed during the initial diagnosis of CN at the local reference clinic. The majority of patients were treated with glucocorticoids (GC) during two weeks before their admission to the clinic. We decided to include these patients since GC pre-treatment is a common procedure and reflects the “real-life” situation. The aim of this study was to investigate transporter expression and function in typical patients as they appear on a daily basis rather than in a desired, but otherwise abstract, condition of no earlier drug administration. It was expected that GC pre-treatment could increase transporter activity or expression, as hypothesized by different researchers (27, 28). In fact, both parameters were low suggesting that the pre-treatment had no significant stimulatory impact on the investigated parameters, at least during the time frame preceding diagnosis and application of the chemotherapeutic protocol. This is in accordance with an *in vitro* study where prednisolone pre-treatment did not induce P-gp expression in either GL-1 or 17-71 canine lymphoid cell lines (28). There is evidence that GC

pre-treatment may cause other, P-gp- and MRP-independent, mechanisms of chemoresistance in canine cells (27).

Surprisingly, the authors did not find any data published in peer-reviewed Journals on P-gp or MRP activity in lymphoma cells obtained from clinical canine patients. However, there is data available for transporter activity in normal lymphocytes. In a previous study (17) that used a similar protocol to investigate P-gp and MRP function and expression in normal canine nodal lymphocytes, the parameters of P-gp and MRP activity were 1.22 and 20.4, respectively (arbitrary units). Although it is impossible to compare these values directly with the results of the present study, a common tendency may be observed; the mean activity of P-gp was close to 1 and the mean activity of MRP was apparently higher (3.26). A similar tendency was observed for relative transporter expression, although the differences (low expression of MRP1 and high expression of P-gp) were even more pronounced in normal canine nodal lymphocytes (17) compared to the lymphoma cells in our study. Similarly, no correlation between transporter function and expression was observed in normal canine nodal lymphocytes. Taken together, it seems that neoplastic transformation in the canine patients included in this study was not associated with either P-gp or MRP over-expression. Although the study group was relatively small, the results suggest that initial over-expression of these transporters is not common in CN. Unfortunately, poor client compliance, premature euthanasia or other conditions did not permit for re-evaluation in a representative group of patients. Thus, it is impossible to assess whether chemotherapy-induced transporter over-expression developed thereafter.

Comparison of the immunophenotype and transporter activity or expression did not provide firm conclusions. This is due to the small number of T-cell and mixed-phenotype CN cases that prevented reliable statistical analysis. It may be, however, interesting to note that highest values for both function and expression of both transporters were observed in mixed-phenotype lymphomas (Table I). These cells may represent less differentiated lymphoid cells. It is known that early hematopoietic cells show higher function of P-gp (29).

It is concluded that flow cytometry is a very useful tool in the assessment of P-gp and MRP expression and activity in CN. Initial over-expression of P-gp and MRP1 was not observed in the investigated malignant cells. The activity of both transporters was low and probably unchanged compared to normal lymphocytes. Further studies, including re-evaluation of transporter function and expression in the course of treatment, should provide more insight into the role of P-gp and MRP in the development of the MDR phenotype in CN. The addition of other techniques that determine transporter activation at the level of gene transcription (RT-PCR) (23) or epigenetic regulation (DNA methylation profile) (30) would, perhaps, help improve the development of novel biomarkers of chemoresistant phenotype related to P-gp and MRP.

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