

## Diagnosis of Mediastinal Masses in Dogs by Flow Cytometry

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**Background:** Biopsy of mediastinal masses can be invasive, but the procedure may be necessary if cytology of a mass aspirate is inconclusive. The 2 most common mediastinal masses, lymphoma and thymoma, may both be comprised of small lymphocytes. We investigated the ability of flow cytometry to distinguish between these 2 neoplasms.

**Hypothesis:** Flow cytometry of mediastinal mass aspirates may provide a definitive diagnosis of thymoma or lymphoma, reducing the need for biopsy.

**Animals:** Dogs with mediastinal masses presenting to the Veterinary Teaching Hospital/Animal Cancer Center were included in the study.

**Methods:** Aspirates obtained over 2 years that met the inclusion criteria (i.e. sufficient viable cells and a definitive diagnosis by means other than flow cytometry) were analyzed by flow cytometry to determine the percentage of cells expressing B- and T-cell markers, and co-expressing CD4 and CD8.

**Results:** All cases of thymoma (n = 6) consisted of  $\geq 10\%$  lymphocytes coexpressing CD4 and CD8, a phenotype that is characteristic of thymocytes, whereas 6 of 7 lymphomas contained  $< 2\%$  CD4+CD8+ lymphocytes. The CD4+CD8+ lymphoma could be readily distinguished flow cytometrically from thymoma by light scatter properties. The phenotypes of the remaining lymphomas were CD4+ T cell (4), CD34+ (1) and B cell (1).

**Conclusions:** Our studies demonstrate that flow cytometry is a useful tool for discriminating mediastinal masses. Lymphocyte-rich mediastinal masses could be unambiguously identified by flow cytometry in 13/13 cases.

**Key words:** Carcinoma; Flow cytometry; Lymphoma; Mediastinal masses; PARR; Thymoma.

Obtaining a definitive histopathologic diagnosis from a mediastinal mass can be an invasive procedure, whereas distinguishing the 2 most common causes of mediastinal masses, lymphoma and thymoma, by cytology can be difficult.<sup>1,2</sup> Cytology may be inconclusive because both types of tumors can be comprised of small, benign-appearing lymphocytes. In one retrospective study, only 7 of 17 mediastinal masses could be definitively diagnosed by cytology.<sup>3</sup> Therefore, less invasive diagnostic testing would be useful for clinicians in making this diagnosis.

Thymomas are neoplasms of thymic epithelial cells, and they are colonized to a variable extent by T cells undergoing normal development. When T-cell precursors enter the thymus, they are negative for surface markers CD3, CD4, CD5, and CD8. As they proliferate, they rearrange T-cell receptor genes and express T-cell markers. The majority of thymocytes ( $> 80\%$ ) simultaneously express CD4 and CD8.<sup>4</sup> This unique phenotype allows thymocytes to be distinguished from peripheral lymphocytes in all other organs. This feature and other flow cytometric characteristics have been used in numerous studies of human patients to distinguish the

2 main causes of mediastinal masses, lymphoma and thymoma.<sup>5-8</sup> Here, we analyze the immunophenotype of a series of thymomas that were definitively diagnosed by histology, and compare these findings with the phenotypes of mediastinal lymphomas, as well as those of other mediastinal masses for which a definitive diagnosis was available. Our primary objective was to determine if flow cytometry could be used as a diagnostic tool to accurately distinguish thymoma from lymphoma and other types of mediastinal masses.

### Materials and Methods

#### Selection of Cases

Clinicians were asked to submit mediastinal mass aspirates from all patients presenting with mediastinal masses between February 2003 and February 2005, regardless of the working diagnosis. 25 samples were studied during this interval. Samples which, when analyzed, had  $< 300$  cells per antibody combination (n = 3) or  $> 50\%$  propidium iodide positive cells (dead cells, n = 2) were considered unsuitable for analysis and were excluded. One of these was definitively diagnosed as thymoma, 1 as mammary tumor metastasis, 1 as a sarcoma, and 1 as malignant histiocytosis. One of these cases never had a final histologic or cytologic diagnosis. Of the remaining 20 samples, a definitive histologic or cytologic diagnosis was available for 15, and a combination of clinical findings and the results of clonality assays were used for a definitive diagnosis of lymphoma in 1 dog. Therefore, there were 16 cases available for analysis. All histologic and cytologic assessments were made without knowledge on the part of the pathologist of the flow cytometry or clonality assay results.

Diagnosis was based on the following criteria: for lymphoma, definitive cytology or histology findings (6 of 7 cases) or strongly suggestive clinical findings together with a positive clonality result by polymerase chain reaction (PCR) for antigen receptor rearrangement (PARR; 1 of 7 cases). For thymomas, definitive histologic diagnoses were made for all 6 cases. The 3 remaining masses were diagnosed as carcinomas. Two of the 3 were diagnosed by histology (neuroendocrine and thyroid carcinoma) and 1 by cytology without further classification.

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**Table 1.** Characteristics of patients with mediastinal masses.

|           | N              | Median age at diagnosis (range) | Sex             | Breed                         |
|-----------|----------------|---------------------------------|-----------------|-------------------------------|
| Lymphoma  | 7              | 7.6 (3.5–10.3)                  | 4 MC, 1 M, 2 FS | 7 different breeds            |
| Thymoma   | 6 <sup>a</sup> | 9.5 (6–11.3)                    | 2 MC, 4 FS      | 4 Labrador Retriever, 2 other |
| Carcinoma | 3              | 6 (5.6–13)                      | 1 MC, 1 M, 1 FS | 3 other                       |

MC=male castrated, M=male intact, FS=female spayed.

<sup>a</sup> One of these cases was diagnosed cytologically as both a thymoma and a neuroendocrine tumor, and this finding was confirmed by histology. Nonetheless, CD4+CD8+ lymphocytes were readily detectable (comprising 45% of the lymphocytes in this sample). This case will be reported separately (Prandhar, K. manuscript in preparation).

### Immunophenotyping

Ultrasound-guided aspirates from mediastinal masses were transferred into media consisting of RPMI 1640 with 5% fetal bovine serum (FBS). Samples were stored at 4° until analysis, which was carried out within 48 hours. For staining, samples were pelleted and resuspended in 1 mL of erythrocyte lysis buffer (0.15 M NH<sub>4</sub>Cl, 1 M KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA, 1 N HCl to a pH of 7.2–7.4) for 5 minutes at room temperature. The cells were pelleted, erythrocytes lysed a second time, and then resuspended in 300 µL of phosphate buffered saline (PBS) with 2% FBS with 0.1% sodium azide. 25 µL of antibody mixture was added to 25 µL of the cell suspension and incubated for 20 minutes at room temperature in the dark. Samples were stained with a panel of antibodies including anti-CD3 (clone CA17.2A12)<sup>a</sup>, CD4 (clone YKIX302.9)<sup>a</sup>, CD5 (YKIX322.3)<sup>a</sup>, CD8 (YCATE55.9)<sup>a</sup>, CD14 (clone TUK4)<sup>a</sup>, CD18 (clone YFC118.3)<sup>a</sup>, CD21 (clone CA2.1D6)<sup>a</sup>, CD34 (clone 1H6)<sup>b</sup>, CD45 (YKIX716.13)<sup>a</sup> and class II MHC (YKIX334.2)<sup>a</sup>. All antibodies were directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and used at a 1:10 dilution except anti-CD45, which was used at 1:50, and anti-CD34, which was used at a final concentration of 2 µg/mL. After incubation, cells were washed twice in 200 µL of PBS-FBS, and each sample was resuspended in 300 µL of PBS-2% FBS with 0.1% sodium azide and 0.01% propidium iodide to exclude dead cells. Samples were then immediately analyzed on a Coulter XL flow cytometer. When possible, 5000 cells in the lymphocyte gate were collected.

As a guideline to determine the percentage of cells stained with each antibody, gates were set based on the isotype controls (mouse IgG1 conjugated with FITC or PE and supplied by the same manufacturer) such that 2% or fewer cells were positive.

### PARR Assessment of Clonality

The presence of clonally expanded lymphocyte populations was detected by PARR (PCR for clonal antigen receptor rearrangements) as described in Burnett et al.<sup>9</sup> The only change from the described methods was the replacement of the TCR $\gamma$  primers with the following sequences, which were derived by examining cDNA and the published canine genome.

DPA ctgttgktgcagaarctggagaag (forward primer)

DPB aacctgagaattgtgccaggac (reverse primer)

DPC gagttactataatcctgttamctctctg (reverse primer)

All 3 primers were included in the same reaction. Briefly, each sample was amplified by 2 sets of primers for immunoglobulin gene segments in 2 separate reactions, and 3 primers for TCR $\gamma$  gene segments in a single reaction. A DNA positive control reaction was also run with each sample to ensure adequate DNA in the sample. A reaction was considered positive (clonal) if 1 or more dominant and discrete PCR products were present on the gel after electrophoresis, and the intensity of the band(s) was similar to the positive control. A reaction was considered negative if no bands, a diffuse smear or a ladder of faint bands was observed.

## Results

### Distribution of Cases

Seven of the 16 cases were diagnosed as lymphoma, and 6 were diagnosed as thymoma. The remaining 3 were carcinomas. Patient characteristics are reported in Table 1. Consistent with previous reports, Labrador Retrievers were highly represented among the thymoma cases.<sup>2</sup>

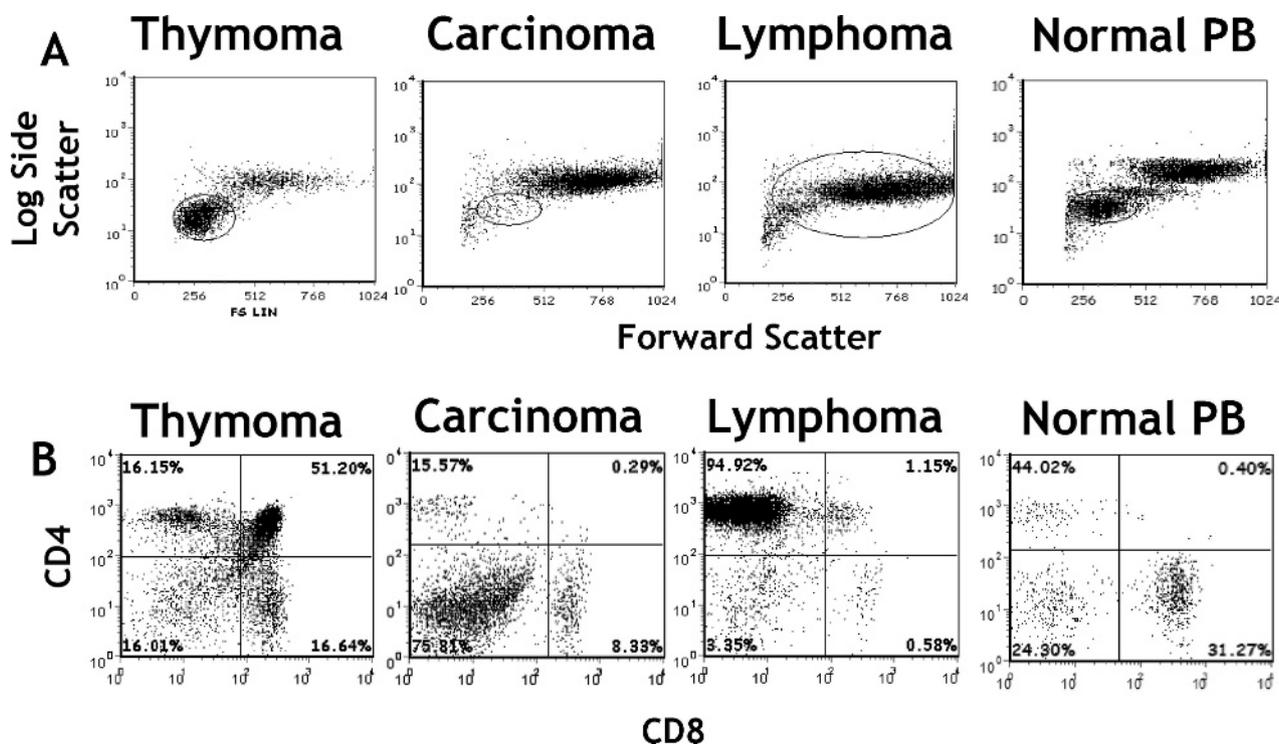
### Flow Cytometric Assessment of Lymphocyte Content of Mediastinal Masses

The first step in analysis of mediastinal mass aspirates by flow cytometry was to determine where the lymphocyte gate should be placed. In peripheral blood, lymphocytes are easily distinguished from neutrophils and monocytes by their low forward and side scatter (which are roughly correlated to small size and low granularity). Neoplastic lymphocytes may be large, however, and nonlymphoid neoplastic cells (such as those in carcinoma samples) may also be similar to neutrophils with respect to light scatter. Neoplastic lymphocytes usually exhibit lower side scatter than neutrophils. To make a more objective assessment of these populations, we used a combination of antibodies to ensure that the lymphocyte gate was properly drawn. Thus 1 pair of antibodies used for analysis was anti-CD4, which binds to neutrophils and a subpopulation of T lymphocytes, and anti-CD14, which binds to neutrophils and monocytes. Expression of these 2 markers on the same cell allowed us to determine if the population of large cells consisted primarily of neutrophils or were indeed lymphocytes. Staining for CD4 and CD14 was performed on each of the samples, and was the basis for drawing the lymphocyte gates shown in Figure 1.

The above staining combinations allowed us to conclude that, as expected, lymphocytes tended to predominate in aspirates from thymomas and lymphomas, but tended to be less frequent in aspirates from carcinomas (Table 2). The presence of a substantial number of neutrophils in some samples, however, indicates that peripheral blood contamination should be expected and taken into account.

### Immunophenotype of Thymomas

Because thymomas contain normally developing T cells, such tumors are expected to contain a high number of lymphocytes that co-express CD4 and CD8. When we



**Fig 1.** Cell distribution and expression of CD4 and CD8 on mediastinal masses and normal peripheral blood. (A) Forward versus side scatter plots showing placement of the lymphocyte gate in 3 types of tumors and in normal peripheral blood (normal PB). (B) Staining of 3 tumor types with anti-CD4 and anti-CD8. Quadrants were set based on negative isotype controls such that no negative control was > 2% positive. The cells shown in B were gated on the lymphocyte populations shown in A.

examined coexpression of CD4 and CD8 on histologically confirmed thymomas we found that all but 1 case of thymoma had >40% CD4+CD8+ cells (Table 2). The remaining case had 10% CD4+CD8+ T cells. By contrast, all but 1 of the nonthymoma cases had <2% CD4+CD8+ lymphocytes. The remaining case (lymphoma) is discussed in detail in the next section.

In the single case of thymoma with 10% CD4+CD8+ T cells, 14% of the remaining cells were CD8+ only and 23% were CD4+ only. The other cells in the lymphocyte gate were not B cells (none of the cells expressed CD21), but appeared to be leukocytes (CD45+). This particular thymoma may have contained a high percentage of early thymocytes, cells that have not yet begun to express markers of mature T cells.

Our findings demonstrate that the presence of CD4+CD8+ lymphocytes is strongly suggestive of thymoma, and in this series of cases, the absence of such cells ruled out thymoma.

**Table 2.** Flow cytometric characteristics of mediastinal mass aspirates.

|           | % Lymphocytes<br>median (range) | % CD4+CD8+ lymphocytes<br>median (range) |
|-----------|---------------------------------|--|
| Carcinoma | 29.7 (5.7–36.4)                 | 0.7 (0.1–0.9)                            |
| Lymphoma  | 96 (62–99)                      | 0.6 (0–59)                               |
| Thymoma   | 79.7 (43–95)                    | 47.4 (10–65)                             |

### *Immunophenotype of Mediastinal Lymphomas*

Flow cytometry of the mediastinal lymphomas revealed a phenotypically heterogeneous group of tumors. One tumor expressed CD21 and CD3, but was negative for all other T-cell markers (CD4, 5, and 8). The finding of a clonal immunoglobulin gene rearrangement and failure to express any other T-cell markers suggests that this tumor was B cell in origin. Four of the lymphomas were CD4+ and had clonally rearranged T-cell receptor genes (Table 3 and Fig. 2). One of the lymphomas (diagnosed by cytology) expressed CD34, CD45, and CD14 but was negative for all other markers (CD3, CD4, CD5, CD8, CD18, CD21 and class II MHC). This lymphoma carried a clonally rearranged T-cell receptor gene.

One of the lymphomas coexpressed CD4 and CD8. The forward scatter of these cells, however, was substantially higher than that seen with the thymomas, and this feature can be used to distinguish the 2 types of tumors.<sup>5</sup> In addition to the forward scatter properties, the CD4+CD8+ lymphoma contained a clonal population of T cells as assessed by PARR (Fig. 2), whereas none of the 6 thymomas had clonal T-cell receptor or immunoglobulin gene rearrangements.

### *Immunophenotype of Carcinomas*

Three of the mediastinal masses examined were carcinomas. None of these samples contained >1%

**Table 3.** Phenotype of mediastinal lymphomas.

|   | Immunophenotype | PARR result | Age  |
|---|-----------------|-------------|------|
| 1 | CD21/CD3        | Clonal Ig   | 9.4  |
| 2 | CD34            | Clonal TCR  | 9.5  |
| 3 | CD4             | Clonal TCR  | 4.0  |
| 4 | CD4             | Clonal TCR  | 7.6  |
| 5 | CD4             | Clonal TCR  | 7.3  |
| 6 | CD4             | Clonal TCR  | 10.3 |
| 7 | CD4+CD8+        | Clonal TCR  | 8.0  |

CD4+CD8+ T cells, and the remainder of the lymphocytes had a distribution similar to what is found in normal peripheral blood. The normal distribution of lymphocytes together with the low proportion of lymphocytes in these samples (Table 2) could be used together to rule out lymphoma and thymoma.

### Discussion

In this study, we have demonstrated the use of flow cytometry in distinguishing 2 common causes of mediastinal masses, lymphoma and thymoma. The finding of  $\geq 10\%$  CD4+CD8+ small lymphocytes was diagnostic for thymoma in all cases evaluated (6/6). Flow cytometry could also accurately diagnose lymphoma in 7/7 cases based on the homogeneous expression of CD4 (4 cases), CD21 (1 case), CD34 (1 case) and the co-expression of CD4 and CD8 together with light scatter properties (1 case). Clonality assessment can contribute to the diagnosis as well, because clonal lymphocyte populations are found in lymphoma but not thymoma. Because this assay can also be performed on aspirates and uses a small number of cells, it provides an additional method for obtaining a diagnosis without biopsy.

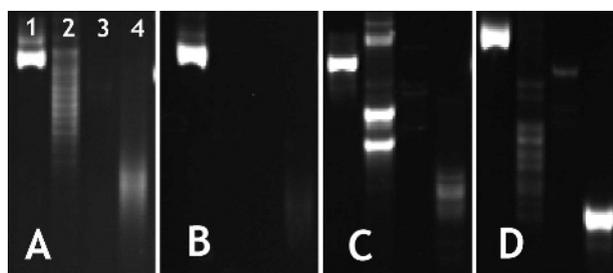
Our results are consistent with studies in human patients. For example, Sakuraba et al<sup>8</sup> found that the percentage of CD4+CD8+ lymphocytes in thymomas

ranged between 34 and 90, whereas in carcinomas it was between 1 and 12%. This group used the presence of CD4+CD8+ cells as diagnostic for thymoma. Gorczyka et al<sup>5</sup> showed that flow cytometry could be used to distinguish lymphoma from thymoma or thymic hyperplasia in 40 of 45 cases. In this same study, flow cytometry identified 7/8 cases of carcinoma as non-hematopoietic tumors, by the presence of cells that did not express CD45. In our study, the nonhematopoietic tumors (3 carcinomas) were characterized by a low proportion of lymphocytes, and the presence of T cells with a phenotypic distribution resembling peripheral blood. We did not consistently find a CD45-negative population in these tumors. Therefore, although flow cytometry is a useful tool for ruling out thymoma or lymphoma, it will not always help make a definitive diagnosis in the case of a nonhematopoietic tumor.

Although we had no histologically confirmed cases of thymic hyperplasia, there are no immunophenotypic features that would separate thymic hyperplasia from thymoma, because normal thymocyte development is found in each. Thymic hyperplasia appears to be a rare finding in dogs. Day reported no cases of thymic hyperplasia in a review of thymic pathology in 36 dogs.<sup>2</sup>

The phenotype of the mediastinal lymphomas in this series was unusual compared to published surveys of canine multicentric lymphoma. Five of 7 were T-cell lymphomas, whereas B-cell lymphomas predominate in most studies.<sup>10-12</sup> The sole B-cell lymphoma was unusual in that it co-expressed CD3, a T-cell marker, and CD21, a B-cell marker. Wilkerson et al reported a similar phenotype in 7% of lymphomas examined by flow cytometry.<sup>13</sup> One of the T-cell lymphomas coexpressed CD4 and CD8, a rare finding in multicentric lymphoma.<sup>11</sup> Finally, 1 of the 7 mediastinal lymphomas expressed CD34 and no other markers. Although the expression of CD34 on canine lymphomas has not been reported in the literature, anecdotal experience<sup>14</sup> (and our own unpublished observations) suggests that this marker is rarely expressed on lymphomas, whereas it is commonly found on acute leukemias.<sup>15</sup> Taken together, our results suggest that mediastinal lymphomas are phenotypically heterogeneous, and differ considerably from multicentric lymphoma. Precursor T cell acute lymphoblastic leukemia/lymphoma in people shares immunophenotypic features with some of the mediastinal lymphomas that we describe. This constellation of neoplasms is derived from precursor T cells at various stages of development in the thymus.<sup>16</sup> It is called leukemia when there are  $>25\%$  blasts in the marrow, but may be restricted to a mediastinal mass or mediastinal mass and lymphadenopathy. These tumors are often CD4+8+, or CD4-8-, and can also be CD34+.<sup>6</sup> Canine mediastinal lymphoma may be an equivalent disease.

Two situations could negatively impact the use of flow cytometry for the diagnosis of thymoma. The first is that peripheral blood contamination may decrease the relative proportion of CD4+CD8+ lymphocytes, potentially giving a false negative result. One way to establish peripheral blood contamination is the presence of



**Fig 2.** PARR assay showing results for two patients with thymoma (A) and (B) and two patients with lymphoma (C) and (D). Each sample was analyzed in four reactions. Lane 1 is the positive control for DNA. Lanes 2 and 3 show amplification with primers recognizing immunoglobulin genes. Lane 4 shows amplification with primers for T cell receptor genes. A and B show either polyclonal amplification of immunoglobulin and T cell receptor genes, or no amplification. C shows clonal amplification of immunoglobulin genes resulting the three discreet products, and polyclonal amplification of T cell receptor genes. This is case 1 from Table 3. D shows a single T cell receptor PCR product. This is case 7 from Table 3.

neutrophils (which can be detected by their size and scatter properties, and by staining with anti-CD4 and anti-CD14). The second limitation is that 20% (5/25) of the samples submitted could not be evaluated because of insufficient sample or a high percentage of dead cells in the sample. In order for this technique to be routinely useful in the clinical setting, clinicians should be encouraged to retrieve several aspirates when possible, and to rinse the syringe in media to obtain the most cellular sample possible. Samples from tumors other than thymoma or lymphoma were more likely to be nondiagnostic, probably because these tumors do not exfoliate as well when aspirated.

Overall, however, when samples had sufficient cellularity, we found that flow cytometry was an extremely useful tool for distinguishing thymoma, lymphoma and nonhematologic malignancies of the mediastinum. The presence of  $\geq 10\%$  CD4+CD8+ cells in the small lymphocyte population was 100% specific for thymoma, and thymoma could be ruled out in all cases where  $< 10\%$  of the small lymphocytes were CD4+CD8+. Therefore, flow cytometric assessment of mediastinal mass aspirates can be a very valuable means of diagnosis without the need for more invasive procedures.

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### Footnotes

<sup>a</sup> Purchased from Serotec, 3200 Atlantic Avenue, Suite 105, Raleigh, NC 27604

<sup>b</sup> Purchased from B-D Biosciences, BD Biosciences, 2350 Qume Drive, San Jose, CA 95131

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### Acknowledgment

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