NOTE *Internal Medicine*

Peripheral Lymphocyte Subsets as a Prognostic Indicator of Mortality and Morbidity in Healthy Dogs

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(Received 14 December 2011/Accepted 17 February 2012/Published online in J-STAGE 2 March 2012)

ABSTRACT. To evaluate the relationship among immune status and increased morbidity and mortality, peripheral blood lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$ and $CD21^+$ cells) from 32 healthy dogs over 8 years of age were analyzed. Twenty-five of the 32 dogs were followed-up for 3 years after the analysis; and 14 dogs were found to be diseased, and nine dogs died. There was no notable difference between the ages of the dogs that died compared with the ones that survived. The relative percentage of $CD4^+$ and the $CD4^+$:$CD8^+$ ratio decreased notably in dogs falling ill compared with healthy dogs. The relative percentage of $CD3^+$ lymphocytes showed a notable decrease in dogs that died within 3 years in comparison with dogs that survived. In a discriminant analysis of morbidity and mortality, most patients were correctly classified as diseased or not and surviving or dead, respectively. These results indicate that the immunophenotypes of peripheral blood lymphocytes in older dogs offer promise as parameters for evaluating mortality and morbidity.
KEY WORDS: aging, canine, lymphocyte subset, morbidity, mortality.

doi: 10.1292/jvms.11-0554; *J. Vet. Med. Sci.* 74(7): 937–943, 2012

Changes in immune status are considered major contributing factors towards morbidity and mortality in aging humans. Many studies performed in human subjects and in experimental animals have suggested a correlation between immune function and age-related risk of morbidity and mortality. Immunosenescence has been associated with a numerical change in T-cell number [5, 22]. Previous studies suggested that a lower total lymphocyte count was associated with an increased risk of mortality [2, 7, 12]. Similarly, an association of high numbers of $CD8^+$ and low numbers of $CD4^+$ cells or low $CD4^+$:$CD8^+$ cell ratios with increased mortality has also been demonstrated [1, 3–5, 13, 19, 22]. Additionally, other evidence has been reported on the association of higher mortality with decreased lymphocyte proliferation to mitogens [14, 21].

Such immunological changes observed in aging humans have also been studied in dogs [9–11, 17, 18]. Our previous study demonstrated age-related changes in lymphocyte subsets (CD3, CD4, CD8, and CD21) in healthy dogs and a notable decrease in the absolute number and relative percentages of lymphocyte subsets in dogs with tumors compared with healthy controls. Furthermore, the relative percentages of particular lymphocyte subsets were significantly decreased in dogs with distant metastatic tumor in comparison with dogs without metastases [20].

Despite extensive research in humans, few studies evaluating the relationship among age-related change in immune status and higher morbidity and mortality have been performed in dogs. In this study, we analyzed whether the immune status in clinically healthy dogs over 8 years of age that tumor incidence becomes higher [20] could predict future morbidity and mortality.

Peripheral blood was obtained from 32 (11 males and 21 females) dogs over 8 years of age that were evaluated as healthy by physical examination and blood tests at private veterinary hospitals. The blood drawing was conducted between May and June in 2007. None of the animals had chronic diseases or had received any immunosuppressive treatment, including corticosteroids. All blood samples were drawn into ethylenediaminetetraacetic acid (EDTA) tubes and complete blood count (CBC) tests were performed immediately. Retrospective analysis of mortality and morbidity was performed in June 2010, 3 years after the blood sample collection.

Two-color flow cytometry was used to calculate relative percentages of $CD3^+$ (T cells), $CD21^+$-like (B cells), $CD4^+$ (T-helper cells) and $CD8^+$ (T-cytotoxic cells) cells using canine-specific monoclonal antibodies (mAbs). Details for all mAbs used for flow cytometry assays are listed in Table 1. Each blood sample was prepared with the protocol that was described in our previous study [20]. Samples were analyzed using a Coulter Epics XL flow cytometer (Beckman-Coulter, Marseille, France) and the Expo32 ADC software (ver. 1.1c; Beckman-Coulter, Marseille, France). Absolute values for each subset were calculated using counts ob-

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Table 1. (A) Monoclonal antibodies used for flow cytometry and (B) staining protocol used

(A) Phenotype	Specificity	Clone	Conjugate	Manufacturer
CD3	Pan T cells	CA17.2A12	FITC	Serotec, Oxford, UK
CD4	Helper T cells	YKIX302.9	FITC	Serotec, Oxford, UK
CD8a	Cytotoxic T cells	YCATE55.9	RPE	Serotec, Oxford, UK
CD21-like	Pan B cells	CA2.1D6	RPE	Serotec, Oxford, UK

(B) Tube number	FL-1	FL-2
1	CD4 FITC	CD8α RPE
2	CD3 FITC	CD21 RPE

tained from white blood cell (WBC) analysis in combination with flow cytometry data. WBC count was analyzed using a veterinary automatic blood cell calculator (MICROS abc LC-152, Horiba, Tokyo, Japan).

The Kruskal-Wallis rank test with a Dunn's post test and discriminant analysis were used to identify lymphocyte subset patterns that differentiated healthy dogs from dogs that died or became diseased during the follow-up period of 3 years. Analyses were carried out using StatMate III for Windows (Atomusu, Tokyo, Japan). Differences were considered significant where $P<0.05$. All values are presented as means ± SD, medians, or ranges.

Sixteen dogs were alive at the 3-year follow-up; nine of the 25 dogs had died during the 3-year period. Seven were lost to follow-up. Among the dogs that died during the follow-up period, one dog died in the 1st year after blood sample collection, 2 dogs died in the second year, and 6 dogs died in the third year. The causes of death included cancer (n=5), infectious diseases (n=1), cardiovascular diseases (n=1), and unknown causes (n=2). There was no notable difference among the ages of the dogs that died and those that did not. Thirteen of the 25 dogs developed 1 or some diseases during the period after examination. These diseases included cancer (n=5), immune diseases (n=3), neuropathic diseases (n=1), cardiovascular diseases (n=2), disc hernia (n=1), diabetes mellitus (n=1), hepatic disease (n=1) and pyometora (n=1).

To investigate whether lymphocyte subsets correlated with future mortality, dogs were categorized into dogs that survived and those that died (Table 2). Of all the lymphocyte subsets analyzed in the present study, only the relative percentage of $CD3^+$ lymphocytes showed any notable decrease in dogs that died in comparison with dogs that survived ($P<0.05$) (Table 3). The relative percentages of $CD4^+$, $CD8^+$ and $CD21^+$ lymphocytes and the absolute cell counts of $CD21^+$ lymphocytes were decreased in dead dogs compared with the surviving dogs, but there were no statistical differences.

To investigate whether lymphocyte subsets correlated with future morbidity, dogs were categorized into dogs that remained healthy and those that were diseased (Table 2), and the lymphocyte subsets were compared (Table 4). The relative percentage of $CD4^+$ lymphocytes demonstrated a significant decrease in dogs with onset of a disease compared with dogs without disease ($P<0.01$). The relative percentages of $CD3^+$ and $CD21^+$ lymphocytes were decreased in diseased dogs compared with healthy dogs, but there were no statistical differences. On the other hand, the relative percentage of $CD8^+$ lymphocytes was increased in diseased dogs, but there was no statistical difference. The $CD4^+$:$CD8^+$ ratio notably decreased in dogs with onset of a disease ($P<0.05$).

White blood cell count, absolute cell counts and relative percentages of all lymphocyte subsets (total lymphocyte, $CD3^+$, $CD4^+$, $CD8^+$ and $CD21^+$), $CD4^+$:$CD8^+$ ratio and $CD3^+$:$CD21^+$ ratio were included in a discriminant analysis to identify the relationship between lymphocyte subsets and increased mortality and morbidity. In a discriminant analysis of mortality, 92.0% of all patients were correctly classified as dead or surviving, as predicted by their immunophenotype. The linear discriminat function was $y = -100.6 + 2.4 \times$ [lymphocytes (%)] $+ 0.35 \times$ [$CD3^+$ T cells (%)] $+ 0.37 \times$ [$CD4^+$ T cells (%)] $+ 0.74 \times$ [$CD8^+$ T cells (%)] $- 3.0 \times$ [$CD21^+$ B cells (%)] $+ 0.0050 \times$ [WBC (cells/μl)] $+ 0.019 \times$ [lymphocytes (cells/μl)] $-0.040 \times$ [$CD3^+$ T cells (cells/μl)] $-0.060 \times$ [$CD4^+$ T cells (cells/μl)] $-0.039 \times$ [$CD8^+$ T cells (cells/μl)] $+ 0.18 \times$ [$CD21^+$ B cells (cells/μl)] $+ 11.1 \times$ [$CD4^+$:$CD8^+$] $+ 0.51 \times$ [$CD3^+$:$CD21^+$]. This linear discriminate function estimated dogs to be surviving if $y<0$ (dependent variable: dead=16; surviving=–9). In a discriminant analysis of morbidity, 100% of the patients were correctly classified as diseased or healthy. Linear discriminat function was $y= -16.7 + 0.42 \times$ [lymphocytes (%)] $-0.75 \times$ [$CD3^+$ T cells (%)] $+ 2.0 \times$ [CD4 + T cells (%)] $+ 0.37 \times$ [$CD8^+$ T cells (%)] $-2.9 \times$ [$CD21^+$ B cells (%)] $+ 0.0027 \times$ [WBC (cells/μl)] $+ 0.018 \times$ [lymphocytes (cells/μl)] $+ 0.045 \times$ [$CD3^+$ T cells (cells/μl)] $-0.14 \times$ [$CD4^+$ T cells (cells/μl)] $-0.061 \times$ [$CD8^+$ T cells (cells/μl)] $+ 0.15 \times$ [$CD21^+$ B cells (cells/μl)] $- 2.2 \times$ [$CD4^+$:$CD8^+$] $-0.15 \times$ [$CD3^+$:$CD21^+$]. This linear discriminated function estimated dogs to be healthy if $y<0$ (dependent variable: diseased=10; healthy=-13). The largest coefficient of linear discriminant function to classify dogs as surviving or dead and healthy or diseased was for the $CD4^+$:$CD8^+$ ratio.

The data presented in the current study suggested that the immunophenotype of peripheral blood lymphocytes in older dogs can be a useful predictor for evaluating immunosenescence, mortality and morbidity in dogs. Age-related immunological changes are mainly represented by alteration in T-cell phenotype and function [15]. Immunosenescence is characterized by alterations in T cell subsets characterized by

Table 3. Differences in immunophenotypes of peripheral blood lymphocytes from dogs that died compared with age-matched controls

(%)	Lymphocytes	Phenotype			
		$CD3^+$	$CD4^+$	$CD8^+$	$CD21^+$
Survived (n=16)					
Median	20.3	70.1	34.3	30.0	12.0
Range	6.4–27.6	49.8–84.7	14.5–53.7	11.6–51.6	7.3–31.1
Dead (n=9)					
Median	24.4	59.0[a)]	27.5	25.1	8.1
Range	14.1–30.7	32.4–83.5	12.6–44.2	11.7–41.4	1.7–36.2

(cells/μl)	WBCs	Lymphocytes	Phenotype			
			$CD3^+$	$CD4^+$	$CD8^+$	$CD21^+$
Survived (n=16)						
Median	8500	1621	1115	512	510	199
Range	3200–14700	511–2426	382–2038	181–1457	120–1189	45–468
Dead (n=9)						
Median	8400	1772	1069	537	453	154
Range	6100–9200	1182–2428	575–1476	172–863	209–971	21–692

	$CD4^+$:$CD8^+$	$CD3^+$:$CD21^+$
Survived (n=16)		
Median	1.1	6.2
Range	0.4–3.5	2.3–8.8
Dead (n=9)		
Median	1.1	6.9
Range	0.3–3.8	1.0–41.2

a) Significant difference vs. survival group.

Table 4. Differences in immunophenotypes of peripheral blood lymphocytes from dogs with onset of a disease compared with age-matched controls

(%)	Lymphocytes	Phenotype			
		$CD3^+$	$CD4^+$	$CD8^+$	$CD21^+$
Healthy (n=10)					
Median	20.6	70.5	35.8	27.2	14.4
Range	6.4–27.6	62.3–84.7	25.7–53.7	11.6–39.2	7.3–31.1
Diseased (n=13)					
Median	22.7	61.2	25.2[a)]	29.2	8.2
Range	11.9–25.5	32.4–83.5	12.6–48.4	13.3–51.6	1.7–36.2

(cells/μl)	WBCs	Lymphocytes	Phenotype			
			$CD3^+$	$CD4^+$	$CD8^+$	$CD21^+$
Healthy (n=10)						
Median	8100	1616	1082	523	517	259
Range	3200–14700	511–2426	382–2038	181–1457	120–668	45–468
Diseased (n=13)						
Median	8500	1655	1069	452	500	154
Range	6100–12700	1182–2251	575–1487	172–758	209–1189	21–692

	$CD4^+$:$CD8^+$	$CD3^+$:$CD21^+$
Healthy (n=10)		
Median	1.2	5.1
Range	0.7–3.5	2.3–8.8
Diseased (n=13)		
Median	1.0[a)]	7.0
Range	0.3–2.1	1.0–41.2

a) Significant difference vs. dogs that had no diseases.

Table 2. Immunophenotypes of peripheral blood mononuclear cells and outcomes in>8-year-old healthy dogs

No.	Age (years)		Sex	Breed	Survival	Cause of death	Disease		WBCs	Lymphocytes	Phenotype					
	Collection	Death									$CD3^+$	$CD4^+$	$CD8^+$	$CD21^+$	$CD4^+$:$CD8^+$	$CD3^+$:$CD21^+$
1	8	-	F	Great pyrenees	Surviving	-	No	(%)		13.3	68	41.1	11.6	25.7		
								(cells/μl)	7900	1048	698	425	120	264		
															3.5	2.6
2	8	-	M	Mongrel	Surviving	-	No	(%)		6.4	77.7	53.7	21.5	31.1		
								(cells/μl)	14700	936	708	545	218	283		
															2.5	2.5
3	8	-	M	Shih tzu	Surviving	-	No	(%)		19.0	66	25.7	38.8	29.2		
								(cells/μl)	8300	1574	1057	403	609	468		
															0.7	2.3
4	9	-	F	Shih tzu	Surviving	-	No	(%)		27.6	77.7	53.4	17.5	17.2		
								(cells/μl)	8800	2426	2038	1457	477	451		
															3.1	4.5
5	10	-	F	Pug	Surviving	-	No	(%)		6.9	62.3	27.8	25.6	7.3		
								(cells/μl)	9900	680	382	223	205	45		
															1.1	8.5
6	10	-	F	Shih tzu	Surviving	-	No	(%)		27.3	68.9	33.3	35.7	7.8		
								(cells/μl)	6200	1695	1123	624	668	127		
															0.9	8.8
7	10	-	F	Shih tzu	Surviving	-	No	(%)		24.9	72.1	43.9	24.9	13.5		
								(cells/μl)	9000	2238	1596	1011	574	299		
															1.8	5.3
8	11	-	F	Chihuahua	Surviving	-	No	(%)		26.7	74.1	30.1	39.2	15.2		
								(cells/μl)	6200	1657	1236	502	654	254		
															0.8	4.9
9	13	-	M	Shetland sheepdog	Surviving	-	No	(%)		22.2	66.5	36.3	28.7	9.3		
								(cells/μl)	7500	1665	1107	705	557	155		
															1.3	7.2
10	14	-	F	Maltese	Surviving	-	No	(%)		16.0	84.7	35.3	31.3	9.9		
								(cells/μl)	3200	511	447	181	160	52		
															1.1	8.6
11	9	-	M	Maltese	Surviving	-	Cardiac disease	(%)		17.2	77.7	48.4	24	12.6		
								(cells/μl)	9000	1548	1196	758	376	194		
															2.0	6.2
12	11	-	M	Beagle	Surviving	-	Disc herniation	(%)		25.4	79.6	35.7	34.1	11.4		
								(cells/μl)	6900	1750	1423	618	591	204		
															1.0	7.0
13	8	-	M	Yorkshire terrier	Surviving	-	Immune disease	(%)		21.7	67.5	19.6	51.6	8.8		
								(cells/μl)	10200	2213	1487	452	1189	194		
															0.4	7.7

Table 2. Continu

No.	Age (years)		Sex	Breed	Survival	Cause of death	Disease		WBCs	Lymphocytes	Phenotype					
	Collection	Death									$CD3^+$	$CD4^+$	$CD8^+$	$CD21^+$	$CD4^+$:$CD8^+$	$CD3^+$:$CD21^+$
14	8	-	F	Shih tzu	Surviving	-	Immune disease	(%)		11.9	61.2	25.2	31.5	24.7		
								(cells/μl)	12700	1516	894	400	500	361		
															0.8	2.5
15	14	-	F	Mongrel	Surviving	-	Immune disease	(%)		18.2	71.2	32.8	32.7	8.2		
								(cells/μl)	8700	1586	1158	522	521	133		
															1.0	8.7
16	13	-	M	Beagle	Surviving	-	Nervous disease	(%)		25.5	49.8	14.5	27.9	8.1		
								(cells/μl)	6500	1655	812	246	473	132		
															0.5	6.1
17	8	10	F	Pomeranian	Dead	unknown	No	(%)		20.4	70	44.2	11.7	13.4		
								(cells/μl)	8700	1772	1285	800	212	246		
															3.8	5.2
18	11	14	F	Welsh corgi	Dead	unknown	No	(%)		30.7	55.6	36.8	41.4	8.8		
								(cells/μl)	7900	2428	1476	863	971	234		
															0.9	6.3
19	11	14	F	Shih tzu	Dead	Heart failure	Diabetes	(%)		22.7	59	22.3	35.6	36.2		
							/Cardic disease	(cells/μl)	8500	1932	1128	432	690	692		
							/Hepatic disease								0.6	1.6
20	8	11	F	Great pyrenees	Dead	Infection	Pyometra	(%)		24.5	46.7	28.1	13.7	3.4		
								(cells/μl)	9200	2251	1061	664	324	77		
															2.1	13.7
21	9	11	F	Golden retriever	Dead	Tumor	Tumor	(%)		24.9	32.4	30.9	29.2	30.9		
								(cells/μl)	6900	1716	575	539	510	548		
															1.1	1.0
22	11	13	M	Mongrel	Dead	Tumor	Tumor	(%)		15.1	59.1	12.6	40.3	8.1		
								(cells/μl)	8900	1347	773	172	549	106		
															0.3	7.3
23	11	11	M	Irish setter	Dead	Tumor	Tumor	(%)		14.1	70.1	24.1	25.1	1.7		
								(cells/μl)	8400	1182	871	281	293	21		
															1.0	41.2
24	11	14	F	Miniature schnauzer	Dead	Tumor	Tumor	(%)		24.7	83.5	14.5	13.3	8.1		
								(cells/μl)	6100	1509	1177	228	209	114		
															1.1	10.3
25	12	13	F	Golden retriever	Dead	Tumor	Tumor	(%)		24.4	53.5	27.5	23.2	7.7		
								(cells/μl)	7600	1852	1069	537	453	154		
															1.2	6.9

an increase in $CD8^+$ lymphocytes and $CD8^+CD28^-$ lymphocytes [5, 8] and a decrease in $CD4^+$:$CD8^+$ lymphocyte ratio in humans [5]. The increase in $CD8^+$ lymphocyte population and an associated decrease in the $CD4^+$:$CD8^+$ ratio appear to be characteristic of an ageing immune system. This typical effect of aging has been observed in our previous study and other canine studies [6, 10, 11, 20]. In addition, a lower total count of lymphocytes was associated with an increased risk of mortality in older humans [2, 12]. The association of high $CD8^+$ and low $CD4^+$ T cells, or a low $CD4^+$:$CD8^+$ lymphocyte ratio, with increased mortality was reported in some studies [1–5, 7, 12, 13, 19, 22]. Other studies have reported that the absolute number of $CD8^+$ memory T cells and decreased lymphocyte proliferation to mitogens correlated with increased mortality [14, 16, 21]. Our study demonstrated that the $CD4^+$:$CD8^+$ ratio significantly decreased in older dogs falling ill compared with dogs that remained healthy within the 3-year follow-up period. These data suggest a relationship between a low $CD4^+$:$CD8^+$ lymphocyte ratio and higher morbidity in older dogs.

In contrast, an association between T-cell subsets and higher mortality was not shown; however, this study may have the following major potential limitations. 1) The number of enrolled dogs was too small to clarify the statistical differences. 2) The cause of mortality was not selected. T-cell subset alterations and immunosenescence should be more relevant for certain conditions, such as mortality from sepsis, meningitis, other infectious diseases or tumors. 3) A follow-up period of 3 years was used to investigate the morbidity and mortality of older dogs, but there are no data as to whether a 3-year follow-up period is suitable to predict mortality and mobility from the lymphocyte subsets of dogs. 4) The immune system comprises various functions and consists of many types of cells, and it is therefore difficult to estimate immune status by one immunological parameter suitable for the assessment of immune functions in healthy dogs and patients suffering from various diseases.

The last limitation, however, may be resolved by the discriminant analysis performed in the current study. The discriminate analysis based upon the WBC count, absolute cell counts, relative percentages of all lymphocyte subsets (total lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$, and $CD21^+$), $CD4^+$:$CD8^+$ ratio and $CD3^+$:$CD21^+$ ratio identified a relationship between lymphocyte subsets and mortality or morbidity in older dogs. In a discriminant analysis of mortality, 92.0% of the patients were correctly classified by their immunophenotype as survived or dead. Similarly, in a discriminant analysis of morbidity, 100% of the patients were correctly classified as diseased or healthy. However, due to the small population of dogs used in this study, the linear discriminant functions developed here are a temporary. More cases would likely increase the reliability of these discriminant functions, and whether the prognoses of dogs can be estimated should be examined in a prospective study.

The current study suggests that evaluating the peripheral immunophenotype in older dogs may provide a prognostic value of survival perhaps by detecting early indicators of disease not detected by a clinician or owner.

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