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RELATIONSHIP BETWEEN CANINE LYMPHOCYTE AgNOR COUNTS AND HAEMATOLOGICAL INDICES OF HEALTH

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ABSTRACT

A modified agyrophil technique was applied to peripheral blood smears to determine the mean AgNOR counts (MAC) of lymphocytes and ultimately assess the state of the lymphoid system in various clinical conditions of dogs. Fifty dogs, from clinically normal to pets with leukaemia, presented to the Veterinary Teaching Hospital, were recruited. Blood smears from each dog were stained with routine Romanowsky and modified agyrophil stains. Signalment, clinical diagnoses and hematologic parameters of the dogs were related to the MAC. An AgNOR proliferative index (AgPI) — percentage of lymphocytes with 3 or more AgNORs, was determined, and correlated with MAC. The statistical significance was determined at $P < 0.05$. MAC ranged from 1.17 in clinically healthy patients to 6.00 in leukaemic patients. The MAC was 2.00 in patients (n = 26) with lymphocyte counts within reference intervals (900—2400 per microliter); 2.23 in patients (n = 4) with lymphopenia; 2.18 in patients with lymphocytosis (n = 18) and 4.73 in patients (n = 4) with lymphocytic leukemia. Also, the MAC was 2.00 in non-anemic dogs while it was 2.47, 2.49 and 3.06 in patients with mild, moderate and severe anaemia, respectively. The MAC correlated strongly with AgPI (r = 0.91). The ancillary AgNOR technique provides a cheaper, more rapid and sensitive tool than routine lymphocyte counts in assessing the state of lymphoid proliferation in a variety of conditions in the dog.

Key words: AgNOR; canine; haematology; lymphocyte

INTRODUCTION

Nucleolar organizer regions (NORs) are loops of chromosomal DNA which contain clusters of ribosomal RNA genes and are transcribed by RNA polymerase I. NORs ultimately direct protein synthesis. NORs vary in size and number according to the degree of nucleolar transcription, and are intimately related to the cell cycle and degree of cellular proliferation [3]. Transcriptionally active NORs are selectively stained by a silver colloid technique and can be identified as black dots (AgNORs) under a microscope [16]. Several modifications to the original technique by Ploton et al. [16] have been made [6, 10, 14, 21].

Ever since the agyrophilic technique was introduced to detect NORs in human chromosomes [5], it has found extensive use in human histopathology [1], human haematology [7, 24] as well as veterinary histopathology [8, 22]. Detection of AgNORs has been seen to correlate with other markers of proliferation such as Ki67, PCNA BrDU, DNA flow cytometry and immunohistochemistry [3], and the mitotic index [9]. Enumerating AgNORs via counts [24], morphometry [2] or a combination of both methods of enumeration [4, 11] has led to the indication of AgNOR as a possible prognostic tool [1, 12, 13, 19]. Consequently, there has been discussion about the use of the AgNOR proliferative index, a derivative of the mean AgNOR counts, as a more sensitive tool to delineate among normal, hyperplastic and benign lesions or even to predict survival times [8, 12, 15, 18, 19].

Lymphocytes are unique among peripherally-circulating leukocytes in their ability to recirculate and undergo mitosis. The haemograms of canine patients presenting with diverse clinical conditions are varied and may be at times unspecific, non-diagnostic and inconclusive.

In this study, a modified agyrophil technique was applied to peripheral blood smears to determine the mean AgNOR counts (MAC) of lymphocytes and ultimately assess the state of the lymphoid system in various clinical conditions of dogs (*Canis familiaris*).

MATERIALS AND METHODS

Study population

Fifty dogs, whose health status ranged from apparently clinically-healthy to obviously clinically-ill, presented to the Veterinary Teaching Hospital, were included in the study. The pets comprised of 30 males and 20 females, aged between 3 months to 6 years.

Collection of blood and haematology

Two millilitres of blood obtained from the cephalic vein was collected into EDTA-containing vials. The complete blood counts and differential leukocyte counts were carried out manually. The blood smears from each dog were stained with routine Romanowsky and an adapted agyrophilic staining technique as described by Ogunsola and Antia [14]. We defined lymphopaenia as less than 700 per µl, and thrombocytopaenia as less than 200,000 per µl. The pets were grouped based on the erythrocytic, leukocytic, lymphocytic and thrombocytic values (Table 1).

Detection of AgNORs

After staining with the modified agyrophilic technique, the blood smears were observed under a microscope using the oil-immersion objective. AgNORs in lymphocytes were generally detected as brown dots in a golden yellow nucleus. Specifically, only AgNORs in lymphocytes were enumerated.

Table 1. Lymphocyte mean AgNOR counts (MAC) and patients' clinical and haematologic characteristics

	N	MAC ± SD
Total white cell counts		
Leukopaenia	2	2.63 ± 0.29*
Within reference rangea	23	1.94 ± 0.43
Leukocytosis	25	2.47 ± 1.18*
Lymphocyte counts		
Lymphopaenia	4	2.23 ± 0.51*
Within reference rangeb	25	2.00 ± 0.51
Lymphocytosis	18	2.13 ± 0.51*
Lymphocytic leukaemia	3	4.73 ± 2.19**
Packed cell volume (%)c		
Non-anaemic (35–57)	32	2.00 ± 0.49
Mild anaemia (30–34)	7	2.47 ±1.57
Moderate anaemia (20–29)	6	2.49 ± 0.28**
Severe anaemia (< 20)	4	3.06 ± 2.04*
Platelet counts		
Thrombocytopaenia	10	2.24 ± 0.46
Within reference ranged	34	2.02 ± 0.49
Thrombocytosis	6	3.54 ± 1.93**
Clinical health status		
Clinically healthy	5	1.36 ± 0.16
Clinically sick	45	2.33 ± 0.91**
Breed		
Alsatian	23	2.01 ± 0.54
Boerboel	4	2.31 ± 0.17
Rottweiler	4	2.21 ± 0.48
Others	19	2.48 ± 1.32
Sex		
Male	30	2.33 ± 1.09
Female	20	2.07 ± 0.54
Age		
< 1year	10	2.29 ± 0.52
1–3 years	23	2.01 ± 0.52
3 years	17	2.48 ± 1.38

N — number of patients; SD — standard deviation; * — $P < 0.05$
** — $P < 0.01$
[a] — 7–14 ($\times 10^3.\mu l^{-1}$); [b] — 0.7–2.9 ($\times 10^3.\mu l^{-1}$); [c] — N = 49
[1] — patient had PCV = 62%, MAC = 3.07, AgPI = 59%
[d] — 200–600 ($\times 10^3.\mu l^{-1}$)
[e] — Breeds include Pitbull, Caucasian, Mixed, Nigerian indigenous

Calculation of mean AgNOR counts (MAC) and AgNOR proliferative index (AgPI)

The MAC is defined as the total number of AgNORs detected in 100 lymphocytes divided by 100. AgPI was calculated as the percentage of lymphocytes that contained 3 or more AgNORs.

Statistical analysis

Mean AgNOR counts, calculated for each sub-type of haematologic diagnosis, were compared using the one-way ANOVA test. Following the ANOVA test, post hoc analysis was also carried out where necessary. The MAC was correlated with the AgPI. Statistical significance was determined at $P < 0.05$ using the SPSS software v.20.

Ethical consideration

The study was carried out according to set ethical guidelines approved by the University of Ibadan ACUREC. The informed consent of all clients who owned the pets employed was obtained.

RESULTS

Lymphocyte mean AgNOR counts (MAC)

Generally, MAC ranged from 1.17 in a clinically-normal patient to 6.00 in a leukaemic patient. Clinically healthy patients (n = 5) had MAC of 1.36 while clinically-sick animals (n = 45), irrespective of the presenting complaints and clinical diagnoses) had MAC of 2.33. When leukaemic patients with MAC of 4.73 are excluded from the list of clinically sick patients, the MAC of clinically-sick patients drops to 2.16.

The MAC of lymphocytes varied with the degree of anaemia. The counts were consistently higher in anaemic patients than in non-anaemic patients. MAC was 2.00 in non-anaemic dogs while it was 2.47, 2.49 and 3.06 in patients with mild, moderate and severe anaemia respectively. Also, the MAC of lymphocytes was higher in dogs with total white cell counts outside the reference intervals (leukopaenia = 2.63; leukocytosis = 2.46) than those with total white cell counts within the reference intervals (1.94). Specifically, MAC was 2.00 in patients with lymphocyte counts within reference intervals (700—2400 μl^{-1}); 2.23 in patients showing lymphopaenia; 2.18 in patients with lymphocytosis, and 4.73 in patients with lymphocytic leukaemia. Similarly, the MAC of lymphocytes was higher in dogs that had thrombocyte values outside the reference interval (200,000—600,000 μl^{-1}). Patients showing thrombocyte values within the reference interval had MAC of 2.02 while patients with thrombocytopaenia and thrombocytosis had MAC values of 2.24 and 3.54 respectively. Details are presented in Table 1.

AgNOR proliferative index (AgPI)

The AgPI of lymphocytes ranged from 0 % in patient with MAC of 1.20 to 100 % in patients with MAC of 6.00. AgPI greater than 29 % was set as a benchmark for increased lymphocyte proliferation. All lymphopaenic patients and 70 % of patients presenting with lymphocytosis had AgPI greater than 29 %.

Correlation between total white cell counts, lymphocyte counts, MAC and AgPI of lymphocytes

There was a positive correlation between the MAC and lymphocyte counts (r = 0.71) as well as between MAC and AgPI of lymphocytes (r = 0.91). Details are presented in Table 2.

Table 2. Correlations between lymphocyte mean AgNOR counts (MAC), proliferative index (AgPI) and total white cell (TNCC) and lymphocyte (Lymphs) counts

	MAC	AgPI	TNCC	Lymphs
MAC	1	0.912**	0.586**	0.710**
AgPI	0.912**	1	0.485**	0.472**
TNCC	0.586**	0.485**	1	0.738**
Lymphs	0.710**	0.472**	0.738**	1

*P < 0.05; ** P < 0.01

DISCUSSION

Increasing lymphocyte counts were observed to correlate strongly with increasing MAC of lymphocytes as patients with lymphocytic leukaemia had the highest MAC of 6.00. This probably indicates that most causes of lymphocytosis were due to increased cellular proliferation of lymphocytes, and migration of this proliferating pool of lymphocytes into the peripheral vascular pool. It is noteworthy that 70 % of cases that presented with lymphocytosis had AgPI greater than 29 %.

However, there were a few cases where patients with lymphocytosis did not show a corresponding increase in AgPI. This finding was mostly observed in cases that presented with "leukaemoid" neutrophilic counts. This finding may be due to the fact that the absolute specific white cell counts are obtained from the product of differential counts and total white cell counts. The product will increase the lymphocyte counts without necessarily indicating a state of increased lymphocyte proliferation.

Also, patients with lymphopaenia had higher MAC than patients with lymphocyte counts within reference intervals. It is plausible that there is increased egress of proliferating/stimulated lymphocytes from the vascular pool into tissues or sites of injury. This is supported by the finding that all the patients that presented with lymphopaenia had AgPI greater than 29 %.

A rather puzzling but novel finding was that MAC of lymphocytes increased with increasing severity of anaemia. It is safe to assume that most of the causes of canine anaemia in the tropics are infectious agents. Indeed, about 80 % of the patients in this study had clinical diagnoses of some form of haemoparasitic infection. Commonly encountered infectious agents, such as *Babesia canis*, *Leptospira icterohaemorrhagiae*, *Ehrlichia canis*, canine parvovirus are antigenic and as such, they stimulate the immune system and ultimately induce lymphoid proliferation. It might also not be out of place to consider anaemia in itself, regardless of cause, as a stimulus for increased cellular proliferation of lymphocytes. In a previous study by VanBelle and Cocquyt [23], anaemia in itself has been considered as a prognostic and predictive factor in a number of neoplastic conditions. According to Yasunaga et al. [25], anaemia and AgNOR counts are the significant clinicopathologic prognosticators of survival in renal cell carcinoma. AgNOR counts have also been reported to increase with increasing severity of aplastic anaemia [17]. However, a decrease in AgNOR sites (compared with healthy controls) in haemopoietic cellular series in myelodysplastic syndrome was attributed to a decrease in the proliferative potential with disease progression [11].

The changes in platelet values and corresponding changes in MAC of lymphocytes in this study cannot be explained from the findings in this study. Breed, sex and age of the patients did not seem to significantly influence MAC of lymphocytes. This finding is in tandem with that reported by Sur et al. [20].

The strong positive relationship ($r = 0.91$) between MAC and AgPI of lymphocytes indicates that both indices may be used interchangeably. From the viewpoint of cellular kinetics, the AgPI may be a more sensitive index than the MAC. This is supported by the finding of a stronger correlation ($r = 0.71$) between lymphocyte absolute counts and AgPI, than the correlation ($r = 0.59$) between lymphocyte absolute counts and MAC. This corroborates the findings of Bukhari et al. [1] and Salehinejad et al. [19].

CONCLUSIONS

The agyrophilic technique is a relatively cheap and easy method of determining cellular proliferation, when compared with other cellular markers. Increasing lymphocytes' MAC correspond to increasing cellular proliferation of lymphocytes, and is associated with increasing severity of anaemia. The AgPI is an easier-to-estimate, more sensitive alternative to the MAC in determining the proliferative activity of lymphocytes in the dog.

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