The Effects of Prednisone and Azathioprine on Circulating Immunoglobulin Levels and Lymphocyte Subpopulations in Normal Dogs

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ABSTRACT

This study investigates serum immunoglobulin (SIg) levels and lymphocyte subpopulations in normal dogs in response to putative immunosuppressive doses of prednisone and/or azathioprine. The objectives were to quantify SIg levels and lymphocyte subpopulations, including Thy-1+, CD4+, CD8+ and B cells, in normal dogs both before and after the administration of prednisone and/or azathioprine at 2 mg/kg, PO, each. Eighteen beagles were divided into 3 groups of 6 dogs each. Blood samples for radial immunodiffusion assay of IgG, IgM and IgA, complete blood count (CBC)and flow cytometry were collected prior to the administration of any drugs and again after 14 d of azathioprine, prednisone or azathioprine and prednisone. Peripheral blood mononuclear cells were isolated using density centrifugation and were incubated with monoclonal antibodies reacting with CD4+, CD8+, Thy-1+ and membrane immunoglobulin. Lymphocyte subsets were quantified using flow cytometry. Azathioprine-treated dogs had no significant changes in SIg levels or lymphocyte subpopulations. Prednisone-treated dogs had significant ($P < 0.05$) decreases in all SIg levels, all lymphocyte subpopulations and erythrocyte numbers, and had an increase in neutrophil counts. Prednisone and azathioprine-treated dogs had significant ($P < 0.05$) decreases in serum IgG levels and Thy-1+ and CD8+ lymphocyte subpopulations, with an increase in the CD4:CD8. These dogs also had a significant decrease in erythrocyte number and a significant increase in the monocyte count. These findings suggest that azathioprine and prednisone in combination or prednisone alone may be useful for the treatment of T cell-mediated diseases since decreased circulating T cell levels were demonstrated following treatment. The combination of drugs or azathioprine alone may not be appropriate for treatment of acute or autoantibody-mediated immune disease, because SIg levels were minimally affected by treatment.

RÉSUMÉ

Afin d'évaluer le rôle immunosuppresseur de la prednisone et de l'azathioprine, les niveaux d'immunoglobulines sériques (SIg) et les quantités de sous-populations lymphocytaires, incluant les cellules Thy-1+, CD4+, CD8+ et les lymphocytes B, ont été mesurés avant et après administration de prednisone et/ou azathioprine à un dosage de 2 mg/kg donné oralement à des chiens en santé. À partir des prélèvements sanguins effectués avant administration des médicaments et après 14 j de traitement, les niveaux de IgG, IgM, et IgA ont été mesurés par immunodiffusion radiale, une formule sanguine complète a été déterminée et une analyse par cytométrie de flux a été effectuée. Les mononucléaires du sang périphérique, isolés par centrifugation sur gradient de densité, ont été caractérisés à l'aide d'anticorps monoclonaux réagissant avec Thy-1+, CD4+, CD8+ et les immunoglobulines membranaires. Les sous-populations lymphocytaires ont été quantifiées par cytométrie de flux. Aucun changement dans les niveaux de SIg et des sous-populations lymphocytaires n'a été observé chez les chiens traités à l'azathioprine. Chez les chiens traités à la prednisone, des diminutions significatives ($P < 0,05$) du niveau de toutes les SIg, de toutes les sous-populations lymphocytaires et du nombre d'érythrocytes, ainsi qu'une augmentation du nombre de neutrophiles ont été observées. Chez les chiens recevant de la prednisone et de l'azathioprine, des diminutions significatives ($P < 0,05$) des niveaux sériques d'IgG et des sous-populations lymphocytaires de type Thy-1+ et CD8+ ont été notées, avec une augmentation du ratio de cellules CD4:CD8. Chez ces chiens on pouvait noter également une diminution significative du nombre d'érythrocytes et une augmentation significative du nombre de monocytes. Les résultats obtenus suggèrent que la combinaison azathioprine et prednisone ou la prednisone seule peuvent être utiles pour traiter des maladies causées par les lymphocytes T du fait de la diminution des lymphocytes T circulants suite à ces traitements. Étant donné l'effet mitigé sur les niveaux de SIg, la combinaison de médicaments ou l'azathioprine seule ne seraient pas appropriées pour le traitement de maladies aiguës ou auto-immunes.

(Traduit par le docteur Serge Messier)

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INTRODUCTION

Immune-mediated anemia, thrombocytopenia, arthritis and skin disorders are relatively common in dogs. The immune-mediated cytopenias are often associated with a high mortality rate. These conditions are described as primary or secondary in origin. Primary immune-mediated disease implies the development of autoimmunity through the loss of self-tolerance. Several theories have attempted to explain the mechanisms causing the development of systemic autoimmune disorders (1). Secondary immune-mediated disease is usually caused by an underlying condition such as neoplasia or infection, or is associated with the administration of a drug or vaccine (2).

The treatment of immune-mediated diseases in dogs is based upon the treatment of similar conditions in humans, and involves the use of immunosuppressive chemotherapy. The goals of therapy are to identify an underlying cause or causative agent (if secondary disease is present), to suppress the immune response, to control secondary inflammation, and to minimize the side effects of drug therapy (3). Immunosuppressive drugs downregulate the aberrant immune response, and in so doing, also suppress the normal protective mechanisms of the host. Patients undergoing immunosuppressive chemotherapy are susceptible to infection and sepsis. Nonetheless, several treatment protocols have been recommended in the veterinary literature, including the use of glucocorticoids and azathioprine (4–9). Although veterinarians consider this combination to be relatively safe, little is known about its efficacy.

Glucocorticoids are the most widely used drugs to induce immunosuppression in dogs, and are often used in combination with other immunosuppressive agents. The beneficial effects of glucocorticoids are numerous and include suppression of both the cell-mediated and humoral immune responses, as well as anti-inflammatory activity (3). Dosages of prednisone commonly recommended in the veterinary literature are 2 to 4 mg/kg daily. This dosage is continued until clinical remission is obtained, and is then gradually tapered over several months (5,7,8,10,11). The side effects associated with the use of glucocorticoids at immunosuppressive doses in dogs are numerous and include polyuria, polydipsia, polyphagia, weight gain, weakness, thin skin, alopecia, hyperpigmentation, hypertension, steroid hepatopathy and suppression of the hypothalamic-pituitary-adrenal axis (3).

Azathioprine is a thiopurine antimetabolite commonly used in the treatment of immune-mediated diseases in dogs, and it is usually used in combination with glucocorticoids. Although the immunosuppressive action of azathioprine is not well studied in dogs, its effects may be due to suppression of the T cell response, with little effect on the B cell response (12). The dose commonly recommended in the veterinary literature is 2 mg/kg daily, which is continued until clinical remission is obtained, and then is gradually tapered over weeks to months (6–9). Side effects of azathioprine in dogs include bone marrow suppression, pancreatitis, gastrointestinal irritation, infections and hepatotoxicity (4,13–15).

The immunosuppressive actions of azathioprine and glucocorticoids in normal dogs are not well described, and there is essentially no information on the activity of these drugs in dogs with immune-mediated diseases.

This study was designed to describe specific changes in selected cell and serum immunoglobulin levels in the normal canine in response to clinically relevant dosages of prednisone and azathioprine. The objectives were to quantify serum IgG, IgM and IgA levels and lymphocyte subpopulations (B cells, total T cells and CD4+ and CD8+ T cell subsets) both before and after the administration of prednisone and/or azathioprine, each at a dosage of 2 mg/kg daily for 14 d.

MATERIALS AND METHODS

Eighteen beagles were divided into 3 groups of 6 dogs each. The dogs were between 12 and 18 mo old and were either intact males or females. They were conditioned and acclimatized to the facilities for at least 1 wk prior to the study. The dogs were determined to be healthy on the basis of a physical examination, complete blood count (CBC), serum biochemical profile and urinalysis. Antigen tests for heartworm and fecal flotation analysis for gastrointestinal parasites were both negative. The dogs were vaccinated against canine distemper, adenovirus type 2, parainfluenza, parvovirus, *Bordetella bronchiseptica*, and rabies; none of the dogs had been vaccinated within the previous 6 mo.

The dogs were housed at the Animal Care Services facility at the University of Guelph during the study. All procedures met the guidelines set by the Canadian Council on Animal Care (Guide to the Care and Use of Experimental Animals, Volumes 1 and 2). Procedures were performed in accordance with the Animals for Research Act (Ontario, 1980) and were approved by the Animal Care Committee, University of Guelph.

Blood samples for radial immunodiffusion (RID) assay of IgG, IgM and IgA and flow cytometry were collected from the jugular vein prior to administration of the drugs under study. Serum for RID was assayed by standard procedures (Mancini method) established in the Clinical Immunology Laboratory at the Ontario Veterinary College, University of Guelph. Purified canine immunoglobulin protein (VMRD, Pullman, WA, USA) was used as a standard antigen, in addition to one control. At the same time, blood for mononuclear cell separation was collected into heparinized tubes (Monoject, St. Louis, MO, USA). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation at 300 g (at room temperature) for 30 min over Ficoll-Paque, specific gravity 1.077 ± 0.001 g/mL (Pharmacia Biotech, Baie d'Urfé, Quebec). The mononuclear cell layer was isolated and washed once with cold phosphate-buffered saline (PBS).

Following a second wash, the cells were centrifuged at $300 \times g$ and the supernatant was discarded. The cells were resuspended in a medium composed of 95% fetal bovine serum and 5% cell culture grade dimethyl sulfoxide (Sigma, St. Louis, MO, USA) at 3 to 5×10^6 cells per mL (personal communication, Kaushik A). Aliquots of cells for all 3 treatment groups (pre-treatment and post-treatment) were frozen slowly and stored at −70°C until used for flow cytometry.

TABLE I. Pre- and posttreatment serum immunoglobulin level

	Azathioprine[a]		Prednisone[a]		Azathioprine & Prednisone[a]	
g/L	Pre	Post	Pre	Post	Pre	Post
IgG[b]	13.16 ± 3.9	11.81 ± 3.5	15.93 ± 3.6	9.98 ± 4.91[c]	16.43 ± 8.2	10.36 ± 3.8[c]
IgM[b]	1.78 ± 0.26	1.68 ± 0.46	1.93 ± 0.84	1.19 ± 0.73[c]	1.51 ± 0.49	1.52 ± 0.49
IgA[b]	0.63 ± 0.31	0.47 ± 0.12	0.41 ± 0.08	0.36 ± 0.1[c]	0.54 ± 0.20	0.30 ± 0.13

[a] See text for dosages
[b] Established laboratory reference values (g/L), Clinical Immunology Laboratory, Ontario Veterinary College, IgG = 15.0 ± 5.0, IgM = 1.5 ± 0.5, IgA = 1.0 ± 0.6
[c] $P < 0.05$
For all groups $n = 6$
All data expressed as Mean ± SD

TABLE II. Pre- and posttreatment complete blood counts

	Azathioprine[a]		Prednisone[a]		Prednisone & Azathioprine	
	Pre	Post	Pre	Post	Pre	Post
Leukocytes (6.1–17.4)	9.36 ± 2.4	9.18 ± 1.2	11 ± 1.5	14.6 ± 4.8	9.6 ± 1.4	11.8 ± 2.4
Neutrophils (3.9–12.0)	6.63 ± 2.3	5.94 ± 1.1	7.3 ± 0.86	12.8 ± 5.2[b]	7.2 ± 1.3	10.0 ± 2.7
Lymphocytes (0.8–5.6)	2.08 ± 0.36	1.94 ± 0.41	2.63 ± 0.66	1.1 ± 0.78[b]	1.86 ± 0.62	0.98 ± 0.8[b]
Monocytes (0.1–1.8)	0.36 ± 0.21	0.53 ± 0.25	0.77 ± 0.61	0.75 ± 0.48	0.32 ± 0.16	0.67 ± 0.20[b]
Eosinophils (0.0–1.9)	0.34 ± 0.14	0.77 ± 0.40	0.20 ± 0.19	0.03 ± 0.06	0.23 ± 0.18	0.105 ± 0.13
Platelets (145–440)	269 ± 52	281 ± 41	274 ± 48	327 ± 72	301 ± 47	369 ± 48
Erythrocytes[c] (5.6–8.5)	6.93 ± 0.62	6.97 ± 0.26	7.03 ± 0.86	6.14 ± 0.50[b]	6.8 ± 0.56	5.97 ± 0.4[b]

Established reference intervals, Clinical Pathology, Ontario Veterinary College
[a] See text for dosages
[b] $P < 0.05$
[c] ($\times 10^{12}$/L)
For all groups $n = 6$
All data expressed as Mean ± SD

For assay, the cryovials were quickly, partially thawed at 37°C. The cells were transferred to 5 mL PBS and centrifuged at 300 × g at 4°C for 12 min, then resuspended in 0.5 mL PBS with 0.1% sodium azide (Sigma). The cells were counted using a hemocytometer and evaluated for viability using trypan blue. Cell viability after cryopreservation was approximately 80%.

FLOW CYTOMETRY

For lymphocyte identification, 1 × 10^6 cells/well were incubated on ice for 30 min with one of the following primary monoclonal antibodies: rat anti-canine CD8, rat anti-canine CD4, or rat anti-canine Thy-1 (Serotec Ltd., Oxford, England). The antibodies were diluted in PBS with 0.1% sodium azide to 1:20, 1:10 and 1:20, respectively. Cells for B cell assay were initially incubated with sheep serum to prevent nonspecific binding of monoclonal antibody and subsequently stained with 1:20 dilution of fluorescein isothiocyanate (FITC)-labeled sheep anti-canine IgG (heavy and light) (Serotec Ltd)(16). Cells incubated with sheep anti-rat IgG (1:50) in place of primary antibody served as a control. After staining with specific monoclonal antibodies, the cells were incubated on ice for 30 min with a 1:50 dilution of FITC-labeled sheep anti-rat IgG (Serotec Ltd). Following incubation, the cells were washed twice with PBS and analyzed immediately.

The cell preparations were analyzed on a Becton-Dickinson FacsScan (Becton-Dickinson, Mississauga, Ontario). Lymphocytes were gated and analyzed based on forward and side-scatter of light; 1 × 10^4 cells were analyzed for each sample. Analysis was performed using the Lysis II program, Version 1 (Becton-Dickinson).

After the initial samples were obtained, the dogs were medicated as follows: Group 1 received azathioprine (Imuran, Burroughs Wellcome Inc., Kirkland, Quebec) orally at a dosage of 2 mg/kg daily for 14 d, Group 2 received prednisone (Apotex Inc., Toronto, Ontario) orally at a dosage of 2 mg/kg for 14 d, and Group 3 received both prednisone and azathioprine orally at 2 mg/kg, each for 14 d. Twenty-four hours after the last dose of medication, blood was collected for RID assay, flow cytometry and complete blood count, as previously described. The dogs were observed for one additional week to monitor for any adverse effects of drug therapy.

A paired t-test was used to compare levels of serum immunoglobulins (IgG, IgM, IgA) and lymphocyte subsets for Groups 1, 2 and 3 before and after the administration of azathioprine and/or prednisone. Statistical significance was defined as $P < 0.05$. An analysis of variance was performed to evaluate differences between Groups 1, 2 and 3 with respect to levels of serum immunoglobulins and lymphocyte subsets. Because pre-treatment cryopreserved cells for Group 1 were lost due to a freezer malfunction, the pre-treatment data for Groups 2 and 3 were pooled and compared to post-treatment data for Group 1 only. An F-test (2 sample for variances) was used to determine that Groups 2 and 3 pre-treatment values were similar. Since Groups 2 and 3 pre-treatment values were similar, they were then pooled and used as a pre-treatment value for Group 1. A t-test (2 sample assuming equal or unequal variances) was then done to determine significant differences between the pooled samples and Group 1 post-treatment values (17).

RESULTS

Serum immunoglobulin (SIg) levels (IgG, IgM, IgA) for Groups 1, 2 and 3 were within the established laboratory reference ranges prior to prednisone or azathioprine administration (Table I). There were no significant changes in SIg levels in Group 1 (azathioprine) after drug administration. All mean SIg levels in Group 2 (prednisone) were significantly decreased after drug administration. In Group 3 (prednisone and azathioprine), only mean serum IgG levels were significantly decreased after drug therapy. Mean serum immunoglobulin

concentrations for IgG were comparable in Groups 2 and 3 after treatment; the difference was not statistically significant.

All CBC values for leukocytes, erythrocytes, platelets and the white blood cell differential counts were within reference ranges prior to treatment with prednisone and/or azathioprine (Table II). There were no significant changes in Group 1 CBC values after azathioprine administration and the values remained within reference ranges. In Group 2 there was a significant increase in the neutrophil count (P = 0.04) and a significant decrease in the erythrocyte (P = 0.0009) and lymphocyte (P = 0.02) counts. In Group 3 there was a significant decrease in both erythrocytes (P = 0.0006) and lymphocytes (P = 0.04) with an increase in the monocyte (P = 0.02) count. Although the changes were significant, the mean values remained within the reference range.

Percentages of Thy-1+ lymphocytes ranged from 80.9 ± 5.8% to 83.8 ± 3.97% prior to drug therapy (Table III). Percentages of B cells ranged from 9.0 ± 6.53% to 9.81 ± 3.85% prior to drug therapy. Percentages of CD4+ and CD8+ lymphocyte subsets ranged from 43.35 ± 11.0% to 49.4 ± 7.48% and 17.7 ± 4.07% to 19.75 ± 7.2%, respectively, prior to drug administration. These values were considered to be within reference ranges. For statistical analysis, absolute lymphocyte numbers were calculated. There were no significant changes in any lymphocyte subsets (Thy-1+, CD4+, CD8+, B cells) in Group 1 after azathioprine administration. All lymphocyte subsets (Thy-1+, CD4+, CD8+, B cells) in Group 2 were significantly decreased after prednisone administration. In Group 3, only Thy-1+ and CD8+ subsets were significantly decreased after prednisone and azathioprine administration. Thy-1+ and CD8+ lymphocyte subsets were comparable in Groups 2 and 3; the difference was not statistically significant.

The ratio of CD4 to CD8 (CD4:CD8) cells was consistent with published values in all Groups prior to drug administration (Table IV). The CD4:CD8 decreased in Group 1 following drug administration, remained unchanged in Group 2, and was increased in Group 3, although none of the changes were statistically significant. No adverse clinical effects were observed in any of the dogs during or after drug administration.

TABLE III. Pretreatment percentages of peripheral blood lymphocytes

	Percent lymphocyte subsets in different treatment groups		
	Azathioprine[a,c]	Prednisone[a]	Prednisone & Azathioprine[a]
THY-1[b]	82.35 ± 4.97	80.9 ± 5.8	83.8 ± 3.97
CD4[b]	46.35 ± 9.5	43.35 ± 11.0	49.4 ± 7.48
CD8[b]	18.72 ± 5.7	19.75 ± 7.2	17.7 ± 4.07
B CELLS[b]	9.40 ± 5.13	9.81 ± 3.85	9.0 ± 6.53

[a] See text for dosages
[b] Previously published reference values (18–20): Thy-1 = 73%, CD4+ = 37.8 − 46.4%, CD8+ = 16.4 − 20%, B cells = 7 − 30%
[c] n = 12 (pooled samples from Groups 2 and 3, pre-treatment)
All other groups n = 6

TABLE IV. Pre- and posttreatment lymphocyte subsets and CD4:CD8

	Absolute peripheral blood lymphocyte numbers					
	Azathioprine[a]		Prednisone[a]		Prednisone & Azathioprine[a]	
	Pre[b]	Post	Pre	Post	Pre	Post
Thy-1	1.85 ± 0 .60	1.63 ± 0.36	2.12 ± 0.52	0.72 ± 0.52[c]	1.57 ± 0.59	0.77 ± 0.7[c]
CD4	1.05 ± 0.45	0.67 ± 0.27	1.15 ± 0.48	0.42 ± 0.3[c]	0.95 ± 0.43	0.62 ± 0.59
CD8	0.41 ± 0.17	0.46 ± 0.16	0.51 ± 0.20	0.14 ± 0.09[c]	0.31 ± 0.06	0.09 ± 0.08[c]
B CELLS	0.21 ± 0.12	0.11 ± 0.07	0.26 ± 0.13	0.05 ± 0.06[c]	0.15 ± 0.09	0.06 ± 0.08
CD4:CD8	2.84 ± 1.33	1.58 ± 0.77	2.68 ± 1.62	2.72 ± 1.08	2.99 ± 1.10	8.23 ± 5.07

[a] See text for dosages
[b] n = 12 (pooled samples from Groups 2 and 3, pre-treatment)
All other groups n = 6
All data expressed as Mean ± SD
[c] $P < 0.05$

DISCUSSION

In the azathioprine-treated dogs, serum immunoglobulin levels remained unchanged from pre-treatment values. This may be due to a lack of effect of azathioprine with respect to antibody production or may be due to a decrease in synthesis concomitant with a decrease in immunoglobulin catabolism. Our findings are similar to those reported for dogs (12) and to those reported for humans (21,22). The B cell blastogenic response was not evaluated in the present study, although previous in vitro studies in humans indicate that B-cells exposed to azathioprine have a suppressed blastogenic response to mitogens (21). These findings suggest that, although B cell activity is suppressed in vitro, serum immunoglobulin levels in vivo do not decrease. The lack of decrease in serum immunoglobulin levels (despite a decrease in B cell activity) is assumed to be due to a decrease in production followed by a decrease in catabolism, thus, serum immunoglobulin levels remain constant. This mechanism has been described in both animals and humans, (23–25) and may be occurring in the dogs of this study as well.

In humans, the serum half-life of IgG, IgA and IgM are 23 d, 6 d and 5 d, respectively (1). To the best of our knowledge, half-lives of serum immunoglobulins have not been reported for adult dogs. However, reported serum immunoglobulin half-lives appear to be similar between domestic species (26). If we assume similar half-lives for immunoglobulins in normal dogs, it is possible that the duration of the present study was not long enough to demonstrate a significant decrease in serum immunoglobulin levels. Persistence of serum immunoglobulins may make azathioprine ineffective for the treatment of acute autoimmune diseases where autoantibodies are predominantly involved in immunopathogenesis.

Prior to azathioprine administration, lymphocyte subpopulations (B cells, CD4+ and CD8+) were similar to previously published ranges and did not change significantly after drug administration. Pre-treatment percentages of Thy-1+ lymphocytes were slightly higher than previously reported values and remained unchanged after azathioprine administration. In this study, the increased percentages of Thy-1+ lymphocytes may be due to differences in age,

breed and sex of dogs previously reported (19,27,28). Thy-1+ monoclonal antibodies have been reported to bind monocytes, as well as T cells (29). Different techniques in gating of lymphocytes during flow cytometry are utilized, and may or may not exclude monocytes in the population of cells counted. Therefore, monocytes binding Thy-1 may be included in the population of cells counted in some situations (30,31). In this study, however, lymphocyte gates were set to include only lymphocytes, thus, it is possible that the percentages of Thy-1+ lymphocytes are normal for these dogs, since reference ranges for lymphocyte subpopulations of normal adult dogs have not been published.

The lack of change in Thy-1+ lymphocyte levels after azathioprine administration was expected since CD4+, CD8+ and monocyte numbers did not change after drug administration. These findings indicate that azathioprine did not decrease circulating levels of Thy-1+, CD4+, CD8+ lymphocytes or B cells. It is not known whether or not lymphocyte function was affected by azathioprine in the present study (despite "normal" numbers of lymphocyte subpopulations), since lymphocyte blastogenesis was not performed. It is possible that the duration of the present study was not long enough to fully evaluate the lymphocyte subpopulations in response to azathioprine. However, for a drug to be clinically useful in the treatment of severe immune-mediated disease, one would hope to see a therapeutic response within a 14-day period (the duration of the present study). It is also possible that the dose of azathioprine used in this study, which is currently recommended for treatment of immune-mediated diseases in dogs, is inadequate to fully evaluate the response of lymphocyte subpopulations.

The CD4:CD8 ratio was slightly decreased after azathioprine administration, suggesting some degree of immunosuppression. This decrease was due to a slight decrease in the CD4+ population and a slight increase in the CD8+ population, although it was not statistically significant. The mean percentage of lymphocyte subpopulations remained within previously reported ranges, therefore, this change was not considered to be clinically significant.

If azathioprine is not adequately absorbed following oral administration, a lack of response (unchanged serum immunoglobulin levels and lymphocyte subpopulations) could be observed. However, azathioprine is well-absorbed from the gastrointestinal tract in humans and absorption following oral administration is also suggested to be efficient in dogs (32,33). In the present study, serum levels of azathioprine were not measured, so its absorption is unknown.

Azathioprine may be useful in the treatment of immune-mediated diseases in which autoantibodies are produced, since it has been shown in humans to decrease antibody production (22). Similar immunoglobulin turn-over studies have not been done in dogs receiving azathioprine, therefore, it is not known whether a decrease in antibody production occurs in this species. Decreased production of antibodies alone may not be adequate for the initial treatment of some acute immune-mediated diseases. The use of serum plasmapheresis in addition to azathioprine may be indicated when a rapid decrease in antibody levels is required (34). Since the effect of azathioprine on the function of the T cell population was not elucidated in the present study, recommendations for the use of azathioprine for treatment of T cell-mediated diseases cannot be made. However, given the present data, it is difficult to recommend azathioprine at 2 mg/kg daily in normal dogs, when an immunosuppressive effect is needed quickly.

In prednisone-treated dogs, all serum immunoglobulin levels (IgG, IgM, and IgA) were significantly decreased, although they remained within established reference ranges. As previously discussed, the half-life of serum immunoglobulins in humans, and presumably in dogs, is such that the duration of this study may not have been long enough to demonstrate a decrease below reference values. The observed decrease in immunoglobulin levels is similar to that reported in humans receiving immunosuppressive doses of glucocorticoids (35–37). The mechanism of decreased antibody production is unknown, although it has been shown that glucocorticoids decrease IL-2 production and thus decrease T cell function (38). T-helper cells are necessary to stimulate mature B cells to produce antibody.

The total lymphocyte counts and all lymphocyte subpopulations assessed by flow cytometry were also significantly decreased in this group of dogs. The decrease in circulating levels of lymphocytes was most likely due to redistribution of lymphocytes to the bone marrow and lymphoid tissues, as discussed previously. The CD4+ and CD8+ lymphocytes decreased proportionately, as indicated by the unchanged CD4:CD8. The decrease in circulating B cells may be important in the decreased production of antibody, as previously discussed.

The decrease in erythrocyte count seen after prednisone administration was unexpected. Glucocorticoids stimulate red blood cell (RBC) production in the bone marrow, causing a mild increase in RBC numbers (39). The decreased erythrocyte count may have been due to decreased RBC production or increased RBC loss through gastrointestinal blood loss or RBC destruction, although there was no evidence of either. It is also possible that the erythrocyte count was slightly increased at the time of the initial sampling (due to splenic contraction), causing a mild increase in RBC numbers. It is unlikely that the decreased erythrocyte numbers were caused by plasma volume expansion, because the weight of the dogs remained unchanged during the study. The decrease in erythrocyte numbers was not severe enough (the numbers were still within the reference range) to warrant bone-marrow evaluation, so further assessment was not done.

The increase in neutrophils was likely due to demargination in blood vessels and decreased egress of neutrophils out of the circulation (due to inhibition of neutrophil adhesion to endothelial cells) (39). Other components of a "stress leukogram" were present in the prednisone-treated dogs, including a decrease in eosinophils and lymphocytes, although the values were still within the reference range.

In humans, glucocorticoids are useful for the treatment of immune-mediated disease when autoantibodies are produced (36). Although the decrease in serum immunoglobulin

levels was statistically significant in this study, the clinical significance of this decrease is unknown. It is possible however, that prednisone may be useful to decrease autoantibody production in dogs. Because prednisone causes lymphopenia (likely through sequestration of T and B cells), the drug may also be effective in treating diseases mediated by a T cell population.

Serum IgG levels were significantly decreased in dogs given prednisone and azathioprine in combination, although they were still within established reference limits. The magnitude of the decrease in IgG was not greater in the dogs given prednisone and azathioprine versus the dogs given only prednisone, indicating that azathioprine did not further decrease the IgG levels. In addition, because serum immunoglobulin levels were unchanged following azathioprine administration alone, it is possible that azathioprine may have a sparing effect on immunoglobulin catabolism. A change in the lymphocyte cytokine profile may also be responsible for causing a decrease in serum IgG levels. Cytokines, including IL-2 through IL-5, and interferon gamma stimulate antibody secretion from plasma cells (1). Perhaps the combination of azathioprine and prednisone induced a change in the cytokines secreted such that only the IgG isotype was affected.

The decrease of Thy-1+ lymphocytes in dogs given prednisone and azathioprine in combination is likely due to a decrease in CD8+ lymphocytes. As expected, the absolute lymphocyte count was decreased and the CD4:CD8 was increased. The decrease in Thy-1+ lymphocytes and CD8+ lymphocytes may be due to a glucocorticoid-induced redistribution of the cells, although it is unknown why CD4+ lymphocytes and B cells were not affected in a similar manner. It is possible that the addition of azathioprine may have prevented the sequestration of CD4+ and B lymphocytes normally caused by prednisone, or that the combination of prednisone and azathioprine caused preferential destruction of CD8+ cells. Recently, it has been shown that prednisone induces apoptosis of human peripheral T cells (CD8+ > CD4+) in vitro. These effects are time- and dose-dependent and were not counteracted by IL-2 administration (40). Perhaps the 2 mg/kg dose of prednisone used in the dog is not adequate to induce this response unless azathioprine is co-administered.

The use of Ficoll-Hypaque for the separation of lymphocytes from whole blood may lead to the selective loss of CD8+ cells, causing an increase in the CD4:CD8 (31,41,42). It is unlikely that this occurred in Group 3 dogs only, since cells from all dogs were processed the same way. In Group 2, although there was a decrease in CD8+ cells, the CD4:CD8 remained unchanged, and the decrease in CD4+ cells was proportional to the decrease in CD8+ cells, indicating that there was not a selective loss of CD8+ lymphocytes.

Overall, these findings suggest that prednisone alone may be an effective immunosuppressive drug in normal dogs, affecting both T and B cell-mediated processes. At the current dosage and time frame studied, azathioprine may not be an effective immunosuppressive drug in normal dogs. A synergistic effect between prednisone and azathioprine was not demonstrated. Other drugs with immunosuppressive activity, such as cyclophosphamide and cyclosporine, warrant investigation.

Lymphocyte blastogenesis is a useful test to evaluate lymphocyte function in response to mitogens (43). Although changes in lymphocyte subpopulation numbers are useful for determining which subpopulations are affected, it is also important to establish the function of those lymphocytes in response to prednisone and/or azathioprine. For example, although there were no significant changes in lymphocyte subpopulation numbers in the azathioprine-treated group, the lymphocytes may demonstrate a suppressed or increased blastogenic response. A suppressed response would indicate some degree of immunosuppression, and perhaps some benefit in using azathioprine for treatment of immune-mediated diseases.

The mechanisms causing autoimmunity are not fully understood. It is possible that a dog with autoreactive T or B cells may not respond to prednisone and azathioprine in the same manner as the clinically normal dogs evaluated in this study. Therefore, it may be important to evaluate the responses to these drugs in dogs with immune-mediated hemolytic anemia or thrombocytopenia.

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REFERENCES

1. **KUBY J.** Autoimmunity. In: Immunology. 2nd ed. New York: Freeman, 1994: 445–466.
2. **GORMAN NT.** Immunology. In: Textbook of Veterinary Internal Medicine. 4th ed. Philadelphia: Ettinger, Feldman, 1995: 1978–2029.
3. **MILLER E.** Immunosuppressive therapy in the treatment of immune-mediated disease. J Vet Intern Med 1992; 6: 206–213.
4. **BEALE KM.** Azathioprine for the treatment of immune-mediated diseases of dogs and cats. J Am Vet Med Assoc 1988; 192: 1316–1318.
5. **MACKIN A.** Canine immune-mediated thrombocytopenia part II. Compend Cont Ed Pract Vet 1995; 17: 515–535.
6. **DOUGHERTY SA.** Hemolytic anemia. Proceed of 12th ACVIM 1994: 152–154.
7. **KRUTH SA.** Immunosuppressive drug therapy in general practice. Proceed 14th ACVIM 1996: 283–284.
8. **GORMAN NT, WERNER LL.** Immune-mediated diseases of the dog and cat. Part IV. Br Vet J 1986; 142: 498–505.
9. **KINTZER PP.** Glucocorticoid therapy in nonendocrine disease. Proceed 15th ACVIM 1997: 105.
10. **LEWIS DC.** Management of refractory immune thrombocytopenia. Proceed 15th ACVIM 1997: 94–96.
11. **STEWART AF, FELDMAN BF.** Immune-mediated hemolytic anemia. Part II. Clinical entity, diagnosis and treatment theory. Compend Cont Ed Pract Vet 1993; 15: 1479–1491.
12. **OGILVIE GK, FELSBURG PJ, HARRIS CW.** Short-term effect of cyclophosphamide and azathioprine on selected aspects of the canine blastogenic response. Vet Immunol Immunopathol 1988; 18: 119–127.
13. **HOUSTON DM, TAYLOR JA.** Acute pancreatitis and bone marrow suppression in a dog given azathioprine. Can Vet J 1991; 32: 496–497.

14. **RINKARDT NE, KRUTH SA.** Azathioprine-induced bone marrow toxicity in four dogs. Can Vet J 1996; 37: 612–613.
15. **MORIELLO KA, BOWEN D, MEYER DJ.** Acute pancreatitis in two dogs given azathioprine and prednisone. J Am Vet Med Assoc 1987; 191: 695–696.
16. **LANIER LL, JACKSON AL.** Monoclonal antibodies: Differentiation antigens expressed on leukocytes. Manual of Clinical Laboratory Immunology. 4th ed. Washington: American Society for Microbiology, 1992: 157–163.
17. **MENDENHALL W.** Introduction to Probability and Statistics. 7th ed. Boston: PWS-Kent Publishing Company, 1987.
18. **MOORE PF, ROSSITTO PV, DANILENKO DM, et al.** Monoclonal antibodies specific for canine CD4 and CD8 define functional T lymphocyte subsets and high-density expression of CD4 by canine neutrophils. Tissue Antigens 1992; 40: 75–85.
19. **GEBHARD DH, CARTER PB.** Identification of canine T lymphocyte subsets with monoclonal antibodies. Vet Immunol Immunopathol 1995; 45: 55–71.
20. **GUENTHER W, SCHUMM M, BUETTNER M, et al.** NK activity of canine blood and marrow cells. Tissue Antigens 1994; 43: 198–201.
21. **ABDOU NI, ZWEIMAN B, CASELLA SR.** Effects of azathioprine therapy on bone marrow-dependent and thymus dependent cells in man. Clin Exp Immunol 1973; 13: 55–64.
22. **LEVY J, BARNETT EV, MACDONALD NS, et al.** The effect of azathioprine on gammaglobulin synthesis in man. J Clin Invest 1972; 51: 2233–2238.
23. **SELL S.** Evidence for species' differences in the effect of serum gammaglobulin concentration on gammaglobulin catabolism. J Exp Med 1964; 120: 967–986.
24. **WALDMAN TA, SCHWAB PJ.** IgG (7 S gamma globulin) metabolism in hypogammaglobulinemia: Studies in patients with defective gamma globulin synthesis, gastrointestinal protein loss, or both. J Clin Invest 1965; 44: 1523–1532.
25. **FAHEY JL, ROBINSON AG.** Factors controlling serum gamma globulin concentration. J Exp Med 1963; 118: 845–867.
26. **TIZARD IR.** Veterinary Immunology. An Introduction. 3rd ed. Philadelphia: WB Saunders Co, 1987: 182.
27. **RIVAS AL, KIMBALL ES, QUIMBY FW, et al.** Functional and phenotypic analysis of in vitro stimulated peripheral blood mononuclear cells. Vet Immunol Immunopathol 1995; 45: 55–71.
28. **TIZARD IR.** Veterinary Immunology. An Introduction. 5th ed. Philadelphia: W.B. Saunders Co, 1996; 98–99.
29. **COBBOLD S, METCALFE S.** Monoclonal antibodies that define canine homologues of human CD antigens: Summary of the First International Canine Leukocyte Antigen Workshop (CLAW). Tissue Antigens 1994; 43: 137–154.
30. **VITALE M, PAPA S, MARIANI AR, et al.** Use of polygonal windows for physical discrimination among mononuclear subpopulations in flow cytometry. J Immunol Methods 1987; 96: 63–68.
31. **SHAPIRO HM.** Practical Flow Cytometry. 3rd ed. New York: Wiley-Liss Inc., 1995: 394.
32. **DELL S, ed.** Physicians Gen Rx. Mosby, 1996: II-180–II-182.
33. **PLUMB DC.** Veterinary Drug Handbook. 1st ed. White Bear Lake: Pharma Vet Publishing, 1991: 568–571.
34. **MATUS RE, GORDON BR, LEIFER CE, et al.** Plasmapheresis in five dogs with systemic immune-mediated disease. J Am Vet Med Assoc 1985; 6: 595–599.
35. **BOUMPAS DT, CHROUSOS GP, WILDER RL, et al.** Glucocorticoid therapy for immune-mediated diseases: basic and clinical correlates. Ann Intern Med 1993; 119: 1198–1208.
36. **FUJISAWA K, TANI P, PIRO L, et al.** The effect of therapy on platelet-associated autoantibody in chronic immune thrombocytopenic purpura. Blood 1993; 81: 2872–2877.
37. **BUTLER WT, ROSSEN RD.** Effects of corticosteroids on immunity in man. J Clin Invest 1973; 52: 2629–2640.
38. **PALIOGIANNI F, AHUJA SS, BALOW JP, et al.** Novel mechanism for inhibition of human T cells by glucocorticoids. J Immunol 1993; 151: 4081–4089.
39. **FELDMAN EC, NELSON RW.** Canine and Feline Endocrinology and Reproduction. 2nd ed. Philadelphia: WB Saunders Co, 1996: 207.
40. **LANZA L, SCUDELETTI M, PUPPO F, et al.** Prednisone increases apoptosis in in vitro activated human peripheral blood T lymphocytes. Clin Exp Immunol 1996; 103: 482–490.
41. **DE PAOLI P, VILLALTA D, BATTISTIN S, et al.** Selective loss of OKT8 lymphocytes on density gradient centrifugation separation of blood mononuclear cells. J Immunol Methods 1983; 61: 259–260.
42. **RENZI P, GINNS LC.** Analysis of T cell subsets in normal adults. J Immunol Methods 1987; 98: 53–56.
43. **KRISTENSEN F, KRISTENSEN B, LAZARY S.** The lymphocyte stimulation test in veterinary immunology. Vet Immunol Immunopathol 1982; 3: 203–277.