Measurement of Immunoglobulin Concentrations in the Feces of Healthy Dogs

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Selective immunoglobulin A (IgA) deficiency is the most common primary immunodeficiency in humans and may be associated with chronic gastrointestinal disease. This observation has led to the suggestion that the susceptibility of German shepherd dogs (GSD) to chronic enteropathies is related to a deficiency in mucosal IgA production. Relative deficiencies of IgA have been reported in the serum, saliva, tears, and feces of GSD both with and without alimentary disease; however, the findings of different studies are not consistent. The aim of this study was to confirm whether a relative deficiency of IgA exists in the feces of GSD. Feces were collected from healthy GSD (n = 209), Labrador retrievers (n = 96), beagles (n = 19), and miniature schnauzers (n = 32). Fecal IgA, IgM, and IgG were measured by capture enzyme-linked immunosorbent assays. Fecal IgG concentrations in the four breed groups were not significantly different. IgA concentrations were significantly greater in miniature schnauzers than in GSD (P < 0.0003) and Labradors (P = 0.0004) but not significantly different from those in beagles. IgM concentrations were significantly greater in miniature schnauzers than in GSD (P < 0.0001), Labradors (P < 0.0001), and beagles (P = 0.0098). These findings do not support the hypothesis that GSD have a relative deficiency in fecal IgA. The differences in immunoglobulin concentrations measured from a single defection, between individuals of the same breed and between breeds, as well as the lack of an internal control molecule, make the determination of a normal reference range for all dogs impossible. Therefore, the usefulness of fecal immunoglobulin quantification for the assessment of intestinal immunoglobulin secretion in dogs is limited.

Selective immunoglobulin A (IgA) deficiency is the most common primary immunodeficiency in humans (7) and has been associated with chronic gastrointestinal disease (7, 25). This observation is one factor that has led to the suggestion that the susceptibility of German shepherd dogs (GSD) to chronic enteropathies is related to defective production of IgA by the intestinal mucosa. Relatively low serum IgA concentrations occur in GSD either with or without alimentary disease (2, 16, 30, 31), yet the concentration of IgA in serum correlates significantly with that in mucosal secretions. Therefore, a number of studies have measured the concentration of IgA in the tears (8), saliva (13), duodenal juice (2), 24-h duodenal explant culture supernatants (13), and feces (21) of GSD to determine whether they have a relative deficiency in mucosal IgA production. Indeed, lower concentrations of IgA have been found in the tears (8), duodenal juice (2), and 24-h duodenal mucosa explant culture supernatants (13) of GSD than in those of other breeds of dogs. These findings contrast with the observation that within the same populations, there are equivalent numbers of IgA+ plasma cells within the lamina propria of the duodenal mucosa (2, 13).

Humans with IgA deficiency have been found to have undetectable levels of IgA in their feces (17). One study of canine fecal immunoglobulins found lower concentrations of IgA in the feces of GSD than in those of a control group of Labrador-retrievers (21). For each individual, the fecal IgA concentration was found to be significantly correlated with the IgA concentration in supernatants taken from duodenal explant cultures.

The aim of the present study was to confirm whether a relative deficiency of IgA exists in the feces of GSD. Fecal samples were collected from a large number of GSD, Labrador-retrievers, beagles, and miniature schnauzers in order to allow comparison of IgA concentrations between multiple breed groups.

MATERIALS AND METHODS

Samples. A single fecal sample was obtained from the first spontaneous defecation of the day from each dog and was stored in a preweighed fecal collection tube (Sarstedt, Numbrecht, Germany). Samples were then stored at −70°C for as long as 3 months in order to allow batching of the samples for extraction.

Fecal samples were collected from two groups of dogs (Table 1). Group 1 consisted of 209 privately owned GSD that were kept either as household pets or in kennelled groups. The median kennel group size was 5 (range, 1 to 24). All dogs were healthy at the time of collection and had no history of diarrhea.

Group 2 consisted of 147 dogs kept in a single large colony. Breeds represented included Labradors (n = 96), miniature schnauzers (n = 32), and beagles (n = 19). The dogs were housed in small groups and were regularly walked outside the housing facility.

Fecal immunoglobulin extraction. The method used for saline extraction of fecal immunoglobulin was adapted from that used by Ferguson et al. (11). Approximately 1 g (wet weight) of feces was contained in the sample scoop of the fecal tube. The wet weight of feces extracted ranged from 0.83 to 2.16 g (median, 1.24 g). Extraction buffer (0.01 M phosphate-buffered saline [PBS] [pH 7.4], 0.5% Tween [Sigma-Aldrich, Poole, Dorset, United Kingdom], and 0.05% sodium azide) was added to each tube at a ratio of 10 ml of buffer to 1 g (wet weight) of feces, and samples were thoroughly homogenized by a combination of manual shaking and mechanical homogenization on a vortex mixer. The fecal suspensions were centrifuged at 1,500 × g for 20 min at 5°C.

A portion of the supernatant (2 ml) was transferred to a sterile Eppendorf tube containing 20 μl of protease inhibitor cocktail (Sigma-Aldrich), and the...
with PBST between steps. All incubations were carried out at room temperature to membranes at the dilutions given in Table 2, and the membranes were washed 5C.

ELISA. Capture enzyme-linked immunosorbent assays (ELISAs) were used to quantify the total concentrations of IgA, IgM, and IgG in feces. The optimum antibody dilutions were determined by checkerboard titration (Table 2). The IgG ELISA was the same as that used by German et al. (12) for quantification of canine IgG. Polyvinyl microtiter plates (Greiner Bio-One, Greene, United Kingdom) were coated overnight at 4°C with 100 μl of the primary antibody adsorbed in carbonate-bicarbonate buffer (0.05 M; pH 9.6; Sigma-Aldrich). Residual protein binding was blocked with 200 μl of 20% nonfat milk for 1 h. All incubations were carried out in a sealed humidified chamber, and plates were washed three times with PBST between incubations.

The transfer membranes were blocked with 20% (wt/vol) nonfat milk in 0.05% Tween 20°C. The samples were heated to 100°C for 10 min in a microcentrifuge, and the supernatants were transferred to clean Eppendorf tubes and stored at −20°C.

ELISA validation. The intraplate coefficients of variation were 8.6, 7.2, and 7.3% for the IgA, IgM, and IgG ELISAs, respectively. The interplate coefficients of variation were 11.6, 6.9, and 8.2% for the IgA, IgM, and IgG ELISAs, respectively. The specificity of each ELISA was determined by using purified canine IgA, IgG, or IgM (Fig. 1). No cross-reactivity was detected in either the IgA or the IgG ELISA against the other purified standards. The IgA standard did not produce a standard curve parallel to that produced with a pooled bile sample or a pooled serum sample. A pooled bile sample was therefore used to produce standard curves for all future quantifications. The IgM ELISA had minor cross-reactivity with the purified IgA standard.

All primary and secondary antibodies used in the immunoglobulin ELISAs were used in a Western blot against a pooled canine serum sample (dilution, 1:100) (Fig. 2). The goat anti-dog IgG (Fc), goat anti-dog IgM (Fc), and mouse anti-dog IgA (Fc) antibodies each detected a single protein, corresponding
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The same antibodies were used for ELISA and Western blotting at the dilutions indicated.

* a The same antibodies were used for ELISA and Western blotting at the dilutions indicated.
* b H/H11001 L, heavy and light chains; AP, alkaline phosphatase.
* c One percent goat serum was included with all tertiary antibodies.
* d Serotec Ltd., Kidlington, Oxford, United Kingdom.
* e Jackson ImmunoResearch Laboratories, West Grove, Pa.
* f The IgA concentration in the pooled bile sample was calculated by comparison with a pooled serum sample with a known IgA concentration.
* g Bethyl Laboratories.
* h Nordic Immunochemicals, Tilberg, The Netherlands.
* i Antibody used by German et al. (12).
* j Sigma-Aldrich.
to the γ, μ, and α chains, respectively, with molecular weights similar to those reported previously (12, 32). In addition to the γ chain, the goat anti-dog IgG (H+L) antibody detected a second protein corresponding to a light chain. Both the rabbit anti-dog IgM and the goat anti-dog IgA detected an additional protein of approximately 25 kDa, which was likely the canine J chain. This protein was the probable cause of the cross-reactivity of the IgM ELISA, since a similar faint band had been observed with the goat anti-dog IgM antibody, although this was not visible on the Western blot shown.

Validation of collection technique. During the 7-day collection period, there was no significant difference in the amount of fecal IgA, IgM, or IgG. The average coefficients of variation for sampling of a single defecation were 25, 31, and 50% for IgA, IgM, and IgG, respectively.

The fecal samples were stored at −20°C at the time of collection prior to transport to the University of Bristol for storage and extraction. In order to assess the effect of delayed freezing of the samples, multiple samples were taken from a single defecation and three samples each were frozen at 0, 12, 24, 48, and 72 h after collection. There was no significant difference in the amount of IgA, IgM, or IgG following delayed freezing of the fecal samples.

Group comparisons. All immunoglobulin measurements were expressed as either ELISA units or micrograms of immunoglobulin per gram (wet weight) of feces. None of the feces examined were diarrheic, and they were all from healthy animals. Therefore, no correction was made for the percentage of dry matter in the feces. The groups were not age matched, because the GSD were significantly younger (P < 0.0001), and the beagles (P < 0.001) were significantly older, than the other three groups. There was no significant difference in age between Labradors and miniature schnauzers. Therefore, a general linear model was used to assess the effects of age, breed, and sex on the concentrations of immunoglobulins in feces. There was no significant effect of age or sex on the concentration of IgA, IgM, or IgG.

The relationship between the concentrations of the immunoglobulins in feces was assessed by examining the correlation between them in all of the samples analyzed. The concentration of IgA was significantly correlated with those of IgM and IgG (for IgM, r_p = 0.631 and P < 0.001; for IgG, r_p = 0.380 and P < 0.001), and the concentration of IgM was significantly correlated with that of IgG (r_p = 0.331; P < 0.001).

Fecal immunoglobulin concentrations were compared among the four breeds of dog sampled in this study (Fig. 3). GSD, Labradors, miniature schnauzers, and beagles were compared, and there was no significant difference in the concentration of IgG among the four breed groups. The concentration of IgA was significantly greater in miniature schnauzers than in GSD (P = 0.0005) and Labradors (P = 0.0004) but not significantly different from that in beagles. The concentration of IgM was significantly greater in miniature schnauzers than in GSD (P < 0.0001), Labradors (P < 0.0001), and beagles (P = 0.0098).

DISCUSSION

The work presented in this study demonstrates that it was possible to measure IgA, IgM, and IgG in the feces of healthy dogs. The IgG ELISA used in this study has been used previously for the quantitation of this immunoglobulin in mucosal secretions and serum (12). This ELISA demonstrated a high degree of specificity with no cross-reactivity with canine IgM or IgA, and the coefficient of variation reported for this assay by German et al. (12) was similar to that found here. The IgM ELISA showed a small degree of cross-reactivity with the purified IgA, which was also reported by German et al. (12), although their study used a different primary antibody. The second protein band detected by the anti-IgM antibody was likely the canine J chain, since it had a molecular weight lower than that of the light chains detected by the anti-IgG antibody and similar to those of the human (26) and mouse (22) J chains under reducing conditions. The cross-reactivity was considered minor, and therefore this ELISA was used for quantification of IgM in feces. Cross-reactivity with IgM was not detected with the IgA ELISA despite the detection of the J chain by the capture antibody. This was due to the use of a monoclonal secondary antibody which did not detect the J chain due to its affinity for a single epitope on the α heavy chain.

The purified IgA standard available from Bethyl Laboratories did not produce a dilution curve parallel to those of IgA in serum and bile in the IgA ELISA. This phenomenon has been

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**FIG. 1.** IgA, IgM, and IgG ELISA specificity. The specificities of the immunoglobulin ELISAs were tested by using the purified IgA, IgM, and IgG standards. There was no cross-reactivity between the IgA or IgG ELISA and the other immunoglobulin subclasses. The IgM ELISA showed some cross-reactivity with IgA, as also reported by German et al. (12), who used the same secondary antibody as that used here. The purified IgA standard, a myeloma-derived protein, did not produce a standard curve parallel to those of pooled bile or serum samples. A similar phenomenon was reported by German et al. (12). The pooled bile sample was used as a standard for all future quantification using this IgA ELISA. The standards used in the quantitation of fecal immunoglobulin concentrations produced dilution curves parallel to those of the fecal extract supernatants. (The concentrations shown in the dilution curve comparison do not reflect those found in the final study.)
reported previously by German et al. (12) and reflects the fact that this standard is a myeloma protein. The same study found that the molecular weight of this standard was lower than that of the “wild type” when it was examined by Western blotting. This standard, therefore, was not used for fecal IgA quantification, and a pooled bile sample was selected, because it contained secretory IgA, the same type expected in the fecal supernatants. The IgA ELISA based on the Bethyl standard overestimates the concentration of this protein. A study using this ELISA reported mean serum IgA concentrations between 2.1 and 6.1 mg/ml (4), values that compare poorly with those (0.22 to 1.0 mg/ml) reported by other studies using alternative reagents and standards (9, 12, 14, 19).

IgA plays an important role in gastrointestinal immune responses; it is present in mucosal secretions at concentrations far in excess of those of other immunoglobulin classes (18). The majority of this IgA is locally produced by IgA+ plasma cells within the lamina propria (29). Dimeric IgA and IgM are transported across the epithelial barrier by the polymeric immunoglobulin receptor, and a portion of the receptor, termed the secretory component, remains bound to the dimeric IgA or IgM after proteolytic cleavage from the apical membrane, forming secretory IgA and IgM (23). The secretory component increases the resistance of IgA to proteases present within mucosal secretions (5, 6, 20).

Poor correlation between the serum and mucosal concentrations of both IgA and IgM has been reported in dogs, reflecting the active transport of these immunoglobulins into mucosal secretions (12). The immunoglobulin present within feces is a combination of secretions from the gastrointestinal tract and the liver (as bile) and may thus provide a good indicator of mucosal immunoglobulin levels. The concentrations of all three immunoglobulin subclasses were significantly correlated with one another. The correlation of IgG with both IgA and IgM was unexpected, since IgG is not actively transported into mucosal secretions, unlike IgA and IgM. This correlation may have reflected the contribution of bile to the fecal immunoglobulins measured, as the concentrations of IgA and IgG have previously been found to be significantly correlated in canine bile (12).

There was day-to-day variation in the concentration of immunoglobulin measured in the serial samples collected from the two dogs examined, although this variation was not statistically significant. Diurnal and day-to-day variations in the concentrations of immunoglobulin in tears and saliva from dogs have been reported (12). Variations in the amount of bile contributing to fecal immunoglobulin levels would have a significant effect, since canine bile contains large amounts of IgA and IgG (10, 12). The quality of the data could have been improved by collection of samples on multiple occasions from a single dog, but this was impossible due to the numbers of animals and owners involved in the collections.
Some studies have indicated that the results of fecal immunoglobulin measurements must be interpreted with caution and can be misleading. Ferguson et al. (11) highlighted the difficulty of finding an internal control molecule for comparison with immunoglobulin concentrations. In our study, fecal immunoglobulin measurements were normalized to the wet weight of feces, since all samples were of a similar consistency. The inclusion of diarrheic animals would have presented problems due to the contribution of excess fecal water to the total volume of the supernatant, which would have affected immunoglobulin concentrations. In other studies of fecal immunoglobulin concentrations, investigators have freeze-dried the feces prior to extraction and therefore normalized the values to the dry weight of the feces (24, 27). The dry weight of the feces would be affected by the type of diet the dog was fed; dietary fiber, for example, would contribute to this value. The dogs in this study were fed a variety of diets due to the large number of individual owners. Feces were not freeze-dried due to the technical difficulties involved in handling the number of samples analyzed, and a previous study found no advantage in freeze-drying over saline extraction from wet feces (11). An ideal internal control molecule would not be affected by gut transit time, fecal dry matter, or water content and would not be degraded by proteolytic enzymes. No such internal control has been identified in dogs to date; therefore, the wet weight of feces was used for normalization, since only nondiarrheic feces were examined.

The numbers of dogs examined in the four breed groups were not equal, and the relatively greater number of GSD presented problems for data analysis. There were further differences in the nature of the different breed groups. The majority of GSD were nonneutered animals, since they were kept for breeding purposes, whereas the dogs of the other breed groups were usually neutered. Most GSD were kept in a kennel environment, but the numbers in each location differed. The dogs of other breeds were kept as separate breed groups but were located within a single large colony with more uniform husbandry.

There was no significant difference in fecal IgG concentrations among the four breeds of dogs examined. The concentration of IgA was significantly greater in miniature schnauzers than in GSD and Labradors but was not significantly different from that in beagles. The results of this study do not support the previous finding of lower fecal IgA levels in GSD than in Labrador retrievers (21). The mean IgA concentration in Labradors was the lowest of all groups, although it was not significantly lower than that in GSD or beagles. The study by Littler et al. failed to detect IgA in many of the fecal samples; however, in that investigation, antisera and standards from the B ethyl Company were used to quantify IgA (R. Batt, personal communication). The fecal IgA deficiency in GSD reported by Littler et al. (21) was therefore likely related to the method used rather than indicative of the presence of a true deficiency.

The concentration of IgM was significantly greater in miniature schnauzers than in the other three breeds of dogs, with no differences between the other breed groups. The higher fecal IgA and IgM levels in miniature schnauzers may reflect a degree of immunological challenge to this breed group. The Labradors, beagles, and miniature schnauzers were housed in separate groups on the basis of breed. Therefore, the separate groups may have been exposed to different antigenic materials. This difference in immunoglobulin concentrations may also reflect the dogs’ diets at the time of sampling. The effect of diet (e.g., tinned versus dry food; protein and carbohydrate sources) could not be assessed due to insufficient information provided by the owners. Increased fecal IgA concentrations in dogs have been associated with diets supplemented with fructo-oligosaccharides and mannan oligosaccharides (27). Our finding may have reflected a phenomenon common to all dogs of the miniature schnauzer breed, but study of further dogs of this breed from different sources would be required to confirm that. Alternatively, a relative IgA deficiency may be present in Labradors and beagles, as well as GSD, which could result in the significantly lower fecal IgA levels relative to those of miniature schnauzers. A relative deficiency in serum IgA levels has been reported in a single colony of research beagles, but the same study did not identify this deficiency in other beagle groups (15), and IgA deficiency has not been reported in dogs of the Labrador breed. Therefore, we consider it unlikely that the higher concentration of IgA in the feces of miniature schnauzers is normal and that the lower concentrations observed in the feces of the GSDs, beagles, and Labradors reflects IgA deficiency in all of these dogs.

The findings presented in this study do not support the hypothesis that GSD have a relative fecal IgA deficiency. Their fecal immunoglobulin concentrations were comparable to those of Labradors and beagles but significantly different from those of miniature schnauzers. The variation in immunoglobulin concentrations measured from a single defecation, between individuals of the same breed and between breeds, as well as the lack of an internal control molecule, makes the determination of a normal reference range for all dogs impossible. These factors limit the usefulness of fecal immunoglobulin quantification for the diagnosis of immunoglobulin deficiency in dogs.

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system. Dogs, but not rats and rabbits, are suitable models for human studies. Hepatology 3:980–988.