Detection of Anti-Leishmania Immunoglobulin G Antibodies in Urine Specimens of Dogs with Leishmaniasis

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For years, anti-Leishmania immunoglobulin G (IgG) antibodies have been detected in the sera of dogs living in areas of leishmaniasis endemicity. They have also been found in the aqueous humor and cerebrospinal fluid. In contrast, a review of the literature failed to identify the detection of anti-Leishmania antibodies in urine samples from dogs with leishmaniasis. Ninety-five dog urine samples were examined for the presence of anti-Leishmania antibodies by using a protein A enzyme-linked immunosorbent assay (ELISA). Twenty additional urine samples were collected from healthy dogs as controls. An IgG2 ELISA was performed on 26 urine samples found positive by the protein A ELISA. Twenty-three urine samples found positive to anti-Leishmania antibodies were tested for the local production of anti-Leishmania antibodies in the urinary tract by means of the urine antibody coefficient. Ten urine samples (and the corresponding serum samples) were compared by Western blot (WB) analysis. Thirty-five out of the 95 urine samples were found positive, 57 were found negative, and 3 were found inconclusive for antibody detection by the protein A ELISA. A high correlation between protein A and IgG2 levels was found in positive urine samples. Anti-Leishmania antibodies were present in the urine of dogs that had leishmaniasis, urinary protein/creatinine (U P/C) ratios of greater than one, and normal urinary sediment. A statistically significant correlation was observed between the U P/C ratios and the levels of anti-Leishmania antibodies in positive urine samples. In general, WB analysis and the urine antibody coefficient suggested that the presence of anti-Leishmania antibodies in urine was the consequence of an impairment of filtration of the glomerular barrier. However, in some dogs, WB analysis could be interpreted as suggesting that the presence of anti-Leishmania antibodies was caused, to a lesser extent, by local antibody production in the urinary tract. Antibody detection in urine could be a noninvasive method for leishmaniasis diagnosis and prognosis in dogs with glomerulonephropathies.

Canine leishmaniasis, caused by the protozoan parasite Leishmania infantum, is a severe systemic disease highly prevalent in the Mediterranean basin. Clinical manifestations of the disease include nonpruritic skin lesions, such as exfoliative dermatitis and ulcerations; local or generalized lymphadenopathy; loss of weight; poor appetite; ocular lesions; epistaxis; lameness; renal failure; and diarrhea (8, 12, 21, 36). Dogs with leishmaniasis have high anti-Leishmania antibody levels in sera (23), and clinicopathological findings include anemia, hypoalbuminemia, hyperglobulinemia, hypercreatinemia, and proteinuria (22).

In the vast majority of cases, the fatal course of canine leishmaniasis is due to renal involvement (5, 32). The major renal lesion in canine (32) and human (7, 11) leishmaniasis is glomerulonephritis. However, interstitial nephritis, tubular nephropathy, and glomerular amyloidosis in conjunction with glomerulonephritis have also been found in dogs with leishmaniasis (30). The pathogenesis of renal lesions in both human (7, 40) and canine (38) visceral leishmaniasis is mainly attributed to immune complex deposition and subsequent glomerular injury. There have been reports of immunoglobulins and immune complex deposition in the glomerular capillaries and mesangial matrix of human (7) and canine (28, 32) patients with leishmaniasis. Moreover, immune complexes have been found in the sera of human (19) and canine (26, 32) patients.

Because of the importance of glomerular injury in canine leishmaniasis, several tests, such as protein/creatinine (U P/C) ratios (31) and enzymuria (30), have been used to detect early renal damage in canine leishmaniasis to establish a prognosis and appropriate treatment. A recent study demonstrated that a large number of dogs with leishmaniasis (46%) have U P/C ratios of greater than one (22).

For years, anti-Leishmania IgG antibodies have been detected in the sera of dogs living in areas of leishmaniasis endemicity (6). They have also been found, but less frequently, in the aqueous humor (14) and cerebrospinal fluid (39). Moreover, investigators have detected anti-Leishmania antibodies in the urine of human patients with visceral leishmaniasis (20) and in the urine of Leishmania donovani-infected hamsters in association with glomerulonephritis (34). Nevertheless, a review of the literature failed to identify the detection of anti-Leishmania antibodies in urine samples from dogs with leishmaniasis.

We hypothesized that in dogs with Leishmania-associated glomerular injury, anti-Leishmania antibodies can pass through the glomerular barrier and therefore that urine from these dogs may contain anti-Leishmania antibodies. The first objective of this study was to investigate the presence of anti-Leishmania antibodies in the urine of dogs with leishmaniasis. The
second objective was to determine whether the presence of anti-Leishmania antibodies in the urine was caused by an impairment of the charge and/or size selectivity of the glomerular capillary wall or whether the antibodies were locally produced in the urinary tract.

**MATERIALS AND METHODS**

**Urine and serum samples.** Ninety-five dog urine samples collected from patients examined for a variety of diseases or disorders at the Veterinary Teaching Hospital of the Universitat Autònoma de Barcelona (VTH-UAB) between the years 2000 and 2002 were obtained from the sample bank kept at the Veterinary Clinical Biochemistry Service of the Universitat Autònoma de Barcelona. The urine samples were analyzed at the Veterinary Clinical Biochemistry Service for U/P C ratios. For the 95 urine samples, we were able to obtain clinicopathological data for 30 dogs from complete records maintained at the VTH-UAB (30 dogs with leishmaniasis and 20 dogs with other disorders). Partial information for 6 of the remaining 45 dogs was obtained from a database of 30 dogs with leishmaniasis and 20 dogs with other disorders. Partial information for 6 of the remaining 45 dogs was obtained from a database.

**Demonstration of the local production of anti-Leishmania antibodies in the urinary tract.** Twenty-three urine samples found positive for anti-Leishmania antibodies by a protein A enzyme-linked immunosorbent assay (ELISA). Twenty urine samples were collected from healthy dogs as controls.

In order to ensure the detection of Leishmania-specific IgG by the protein A ELISA, 26 urine samples positive for protein A were screened for the presence of anti-Leishmania IgG2 antibodies. Twenty-three urine samples had anti-Leishmania antibodies, and their corresponding serum samples were used to assess the local production of anti-Leishmania antibodies in the urinary tract. Ten urine samples (and the corresponding serum samples) were used for immunoblot analysis.

All urine and serum samples used in this study were stored frozen at −20°C until assayed.

**ELISA for the detection of anti-Leishmania antibodies in urine samples.** All urine samples were tested for the presence of anti-Leishmania antibodies by the protein A ELISA. An ELISA that had been used for dog serum samples was adapted for urine samples. Microtiter plates were coated with 20 μg of L. infantum antigen ml⁻¹ in 0.1 ml of coating buffer (0.1 M carbonate-bicarbonate [pH 9.6]) and incubated overnight at 4°C. One hundred microliters of dog urine, diluted 1:100 in phosphate-buffered saline (PBS)-0.05% Tween 20 (PBST)-1% dried skim milk (PBST-M), was incubated in each well for 1 h at 37°C. After three washes with PBST and one wash with PBS, 100 μl of protein A (1:5,000 dilution in PBST-M) conjugated to horseradish peroxidase (HRPO) (Sigma, St. Louis, Mo.) was added to each well. The plates were incubated for 1 h at 37°C and then washed. The substrate solution (ortho-phenylenediamine [0.4 mg/ml] [Sigma]-H₂O₂ [0.05%] in 0.1 M phosphate-citrate buffer [pH 5.0]) was added at 200 μl/well and developed for 20 min at 24°C. The reaction was stopped with 50 μl of 3 M H₂SO₄. Absorbance values were read at 492 nm with an automatic microELISA reader (Anthos 2001; Anthos Labtec Instruments GmbH, Wies, Austria).

An anti-dog IgG2–HRPO conjugate (1:2,500 dilution in PBST-M) (Bethyl Laboratories, Montgomery, Ala.) was also used with some of the urine samples to verify that the results were mainly due to IgG. The Bethyl anti-dog antiserum specific to IgG2 react with the four fractions of IgG so that the anti-dog IgG2–HRPO conjugate can be considered a reagent that measures total IgG. The reaction was quantified as units relative to a positive urine sample used as a calibrator and arbitrarily set at 100 U. The cutoff values were established at 10 U for protein A and 16 U for IgG2 (mean + 2 standard deviations for 20 urine samples from healthy dogs). Negative results were established at 6 U for protein A and 10 U for IgG2 (mean + 2 standard deviations for 20 urine samples from healthy dogs). Uncertain results were established between positive and negative ELISA results for both conjugates.

**Statistical analysis.** The Student t test of independence and the Pearson coefficient r analysis were performed by using the SPSS program.

**RESULTS**

**Detection of anti-Leishmania antibodies in urine samples by ELISA.** Of the 95 urine samples tested, 35 were found positive, 57 were found negative, and 3 were found inconclusive for antibody detection by the protein A ELISA. For the 35 positive urine samples, we had records from the VTH-UAB for 20 dogs (20 out of 30 dogs with leishmaniasis), and we had a diagnosis of leishmaniasis for six more dogs from the Serological Diagnostic Laboratory of Leishmaniasis of the Universitat Autònoma de Barcelona. The remaining nine dogs with positive urine samples were from the 39 dogs for which we had no information at all.

The levels of anti-Leishmania antibodies in positive urine samples ranged from 10.9 to 268.1 ELISA units, with a mean and standard deviation of 84.3 ± 81.5 ELISA units. The levels of anti-Leishmania antibodies in negative urine samples ranged from 0 to 5.6 ELISA units, with a mean and standard deviation of 0.8 ± 0.9 ELISA units.
deviation of 1.5 ± 1.5 ELISA units. The levels of anti-<i>Leishmania</i> antibodies in inconclusive urine samples ranged from 8.3 to 10 ELISA units, with a mean and standard deviation of 8.9 ± 0.9 ELISA units.

The IgG2 ELISA was performed for 26 out of 35 urine samples found positive by the protein A ELISA. All 26 urine samples were also found positive by the IgG2 ELISA. The levels of anti-<i>Leishmania</i> IgG2 in urine samples ranged from 22.6 to 324.0 ELISA units, with a mean and standard deviation of 149.0 ± 105.0 ELISA units. The levels of anti-<i>Leishmania</i> antibodies detected by the protein A ELISA in the 26 urine samples ranged from 13.6 to 300.0 ELISA units, with a mean and standard deviation of 22.6 to 324.0 ELISA units, with a mean and standard deviation of 87.6 ELISA units. A high correlation between protein A and IgG2 ELISA results (r = 0.72, P < 0.0001) was found for the 26 positive urine samples.

### Clinical pathological data for dogs

The records kept at the VTH-UAB showed that 30 urine samples were from leishmaniasis patients and that 20 were from patients with other disorders.

All 30 leishmaniasis patients showed high anti-<i>Leishmania</i> antibody levels in sera. Twenty out of these 30 leishmaniasis patients were also positive for antibody detection in urine (designated group 1), while the remaining 10 were negative for antibody detection in urine and positive for antibody detection in sera (designated group 2). All 20 patients with other disorders were negative for antibody detection in both urine and sera.

Dogs with leishmaniasis and with antibodies in urine had nonspecific clinical signs related to glomerulonephritis or renal involvement, such as loss of weight, lethargy, anorexia, polydipsia-polyuria, and vomiting. Dogs with leishmaniasis and without antibodies in urine had nonspecific clinical signs of leishmaniasis, such as loss of weight, cutaneous and ocular lesions, and lymphadenopathy.

The results of serum and urine analyses for dogs previously diagnosed with leishmaniasis are recorded in Table 1. Serum analysis showed that in both leishmaniasis groups, the altered parameters were higher concentrations of gamma globulin and beta globulin and a lower concentration of albumin. However, dogs in group 1 had a statistically significant higher concentration of gamma globulin and a lower concentration of albumin than those in group 2. In addition, dogs in group 1 had an altered albumin/globulin (A/G) ratio and a significantly lower A/G ratio than dogs in group 2. In the urine analysis, dogs in group 1 had a U P/C ratio of greater than one and normal urinary sediment. Dogs in group 1 had a statistically significant higher U P/C ratio than dogs in group 2. In summary, as shown in Table 1, dogs with antibodies in urine had clinicopathological abnormalities related to glomerulonephritis or renal involvement, such as hypoalbuminemia, hyperglobulinemia, and proteinuria (U/P/C ratio of greater than one).

A statistically significant correlation between U P/C ratios and levels of anti-<i>Leishmania</i> antibodies was found for the 20 positive urine samples (r = 0.72, P < 0.0001). The results are shown in Fig. 1.

### Local production of anti-<i>Leishmania</i> antibodies in the urinary tract

Twenty-three urine samples positive for anti-<i>Leishmania</i> antibodies were tested for local production in the urinary tract, and only one had a C of greater than 1 (1.036). As expected, the anti-<i>Leishmania</i> antibody titers and concentrations of IgG in the urine samples were much lower than the anti-<i>Leishmania</i> antibody titers and concentrations of IgG in the serum samples for all dogs (Table 2).

The IgG concentration in the urine of eight healthy dogs showed a mean and standard deviation of 0.0026 ± 0.0036 mg/ml. This IgG concentration in the urine of healthy dogs was significantly lower than that in the urine of dogs with leishmaniasis (P value determined by the t test, 0.05).

### Immunoblot analysis

All positive samples (urine and serum samples) recognized numerous polypeptide fractions of the <i>L. infantum</i> antigen with masses ranging from 12 to 85 kDa, with most bands at 16 to 69 kDa. The most frequent polypeptide fractions observed in both urine and serum samples were those of 14, 16, 20, 30, 31, 48, and 69 kDa. The immunoblot analysis did not reveal any polypeptide fractions of the <i>L. infantum</i> antigen in ELISA-negative urine samples.

The band pattern revealed by urine from dogs with high levels of specific anti-<i>Leishmania</i> antibodies detected by the ELISA was more intense than the pattern revealed by urine

### Table 1. Clinicopathological data for 30 leishmaniasis patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for the following group of dogs with leishmaniasis:</th>
<th>Normal range</th>
<th>t test value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (n = 20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>57.4 ± 46.0</td>
<td>53.6 ± 23.9</td>
<td>21.4–59.9</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.2 ± 0.8</td>
<td>1.2 ± 0.6</td>
<td>0.5–1.5</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>8 ± 1.6b</td>
<td>7.6 ± 1.3b</td>
<td>5.4–7.1</td>
</tr>
<tr>
<td>Gamma globulin (g/dl)</td>
<td>3.4 ± 1.5b</td>
<td>2.1 ± 1b</td>
<td>0.5–1.3</td>
</tr>
<tr>
<td>Beta globulin (g/dl)</td>
<td>2 ± 0.4b</td>
<td>1.9 ± 0.5b</td>
<td>0.9–1.6</td>
</tr>
<tr>
<td>Alpha 2 globulin (g/dl)</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>0.3–1.1</td>
</tr>
<tr>
<td>Alpha 1 globulin (g/dl)</td>
<td>0.3 ± 0.8</td>
<td>0.4 ± 0.1</td>
<td>0.2–0.5</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>1.6 ± 0.4b</td>
<td>2.3 ± 0.4</td>
<td>2.6–3.3</td>
</tr>
<tr>
<td>A/G ratio</td>
<td>0.26 ± 0.11b</td>
<td>0.5 ± 0.2</td>
<td>&gt;0.45</td>
</tr>
<tr>
<td>U P/C ratio</td>
<td>5.8 ± 5b</td>
<td>0.7 ± 0.6</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Density (g/ml)</td>
<td>1,031 ± 8.8</td>
<td>1,030 ± 9.8</td>
<td>&gt;1,020</td>
</tr>
<tr>
<td>pH</td>
<td>7 ± 0.6</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*Data are reported as means and standard deviations. NS, not significant; ND, not determined.

b Altered parameter.
from dogs with low levels of anti-Leishmania antibodies detected by the ELISA.

In general, when the patterns obtained by immunoblotting for the urine and serum samples from the same dog were compared, we found that the number of bands revealed by serum antibodies and the intensity of those bands were higher than those of bands revealed by urine antibodies. The patterns of bands revealed by protein A and IgG2 conjugates showed slight differences. When the anti-IgG2 conjugate was used, some differences were observed in the patterns of bands revealed by urine and serum antibodies over the molecular mass range. In some cases, urine antibodies revealed bands that were not observed with the corresponding serum antibodies, and in other cases, some bands revealed by urine antibodies showed a higher intensity than the homologous bands in the serum strips (Fig. 2). When the protein A conjugate was used, some additional differences were observed, mainly in the region of low molecular mass (less than 30 kDa).

TABLE 2. Antibody detection and C for 23 dogs with leishmaniasis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Anti-Leishmania antibody titer in:</th>
<th>IgG concn (mg/ml) in:</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Serum</td>
<td>Urine</td>
</tr>
<tr>
<td>Mean</td>
<td>3,791</td>
<td>799,165</td>
<td>0.707</td>
</tr>
<tr>
<td>SD</td>
<td>5,956</td>
<td>616,092</td>
<td>0.913</td>
</tr>
</tbody>
</table>

DISCUSSION

Immunoglobulins have been found in the urine of human patients with glomerular disease in a variety of disorders (4, 3, 25, 29). Moreover, several infectious diseases are diagnosed by the finding of specific antibodies in urine, such as Helicobacter pylori infection (18), filariasis (17), or schistosomiasis (35). The presence of anti-Leishmania antibodies in the urine of human patients with visceral leishmaniasis (16, 20) and in the urine of L. donovani-infected hamsters (34) has also been described. In contrast, there is a lack of information about immunoglobulins in the urine of healthy and sick dogs. In this study, we report the presence of anti-Leishmania antibodies in the urine of dogs with leishmaniasis. In addition, we describe, for the first time, the detection of total IgG concentrations in the urine of healthy dogs and dogs with leishmaniasis.

One of the common features of canine leishmaniasis is the development of hyperglobulinemia, and the elevation occurs in the IgG fraction. Dogs with leishmaniasis have high Leishmania-specific IgG levels in sera (23). The diagnosis of canine leishmaniasis can be performed by serological tests, such as the dot ELISA (13), the ELISA (33), and WB (1), with protein A as the detection probe to detect Leishmania-specific immunoglobulins. The reactivity of protein A with immunoglobulins from dogs is quite strong. Protein A reacts with all subclasses of IgG and partially reacts with dog IgA and IgM (15). To verify that the results found for urine by the ELISA and WB...
were mainly due to the detection of IgG, an anti-dog IgG2-HRPO conjugate was also used with some of the urine samples. Previous studies demonstrated that the anti-dog IgG2-HRPO conjugate used (Bethyl) reacts with all subclasses of IgG (27) and that the levels of anti-Leishmania antibodies detected with this conjugate correlate with those detected with an anti-dog IgG-HRPO conjugate (37).

The correlation found between protein A and IgG2 ELISA units in positive urine samples was high. Therefore, we could consider that the anti-Leishmania antibodies found in urine were predominantly IgG. Immunofluorescence studies of pathological kidneys from dogs with leishmaniasis have described IgG and other immunoglobulins, such as IgM and IgA, in the affected glomeruli (28). Further studies are needed to determine the importance of other immunoglobulins in dogs with leishmaniasis.

For clinical veterinarians, it is very important to check the renal status of dogs with leishmaniasis in order to provide appropriate prognosis and treatment. The U/P/C ratio has proven useful for detecting proteinuria as an index of renal failure in dogs (41). Furthermore, it has been demonstrated that the total urinary protein level and the U/P/C ratio have better diagnostic value than a serum biochemical renal profile for detecting early renal lesions in canine leishmaniasis (31). For these reasons, clinician veterinarian practitioners use the U/P/C ratio to detect proteinuria in dogs with leishmaniasis both at diagnosis and during follow-up treatment. However, few extensive studies are available concerning U/P/C ratios in dogs with leishmaniasis at diagnosis and during follow-up treatment (22). A recent study demonstrated that a large number of dogs with leishmaniasis (21 out of 45) had U/P/C ratios of greater than one. After 4 months of allopurinol treatment, most of the proteinuric dogs (15 of 17) not only did not experience any further deterioration of their renal function but also showed a decrease in (8 of 15) or even a disappearance of (4 of 15) proteinuria at the end of the trial (22). This previous study demonstrated the usefulness of assessing proteinuria at the time of diagnosis and during treatment to obtain an accurate prognosis for a patient with leishmaniasis. However, infection and marked blood contamination of urine samples could result in an abnormal U/P/C ratio in the absence of glomerular disease. Consequently, the U/P/C ratio alone does not distinguish between the types of proteinuria. Total serum protein, albumin, and urinary sediment evaluations would complete the screening profile to differentiate prerenal, renal, or postrenal proteinuria (2). Therefore, techniques that evaluate the renal status of dogs with leishmaniasis are of great value.

As we expected, dogs that had anti-Leishmania antibodies in their urine had U/P/C ratios of greater than one and urinary sediment findings compatible with renal disease. Moreover, we found a statistically significant correlation between U/P/C ratios and anti-Leishmania antibody levels in urine. In contrast, dogs that had leishmaniasis demonstrated by high anti-Leishmania antibody levels in sera but that were nonproteinuric were found negative for anti-Leishmania antibodies in urine. Because the detection of anti-Leishmania antibodies in urine is a more specific test than the U/P/C ratio, it might be a useful tool for establishing appropriate prognosis and treatment. Further studies are needed to investigate the usefulness of detec-
tion of anti-Leishmania antibodies in urine samples for the follow-up of treated dogs with leishmaniasis.

Due to the clinical and retrospective points of view of this study, we did not perform histopathological studies of the kidneys from any of the dogs. However, for 23 dogs, we tried to predict whether the presence of anti-Leishmania antibodies in the urine was caused by an inability of the glomeruli to properly filter or by local production in the urinary tract. In the majority of cases (22 out of 23), C was suggestive of passage from plasma through the glomerular barrier. None of the dogs had a C of greater than eight, which is considered strong evidence of local production (10). Therefore, our results compared favorably with those of previous histopathological studies. Because the major renal lesion in canine leishmaniasis is glomerulonephritis (32), most dogs appear to have glomerular injury. This nonselective proteinuria can explain the evidence of local production (10). Therefore, our results compared favorably with those of previous histopathological studies.

Studies that have compared the WB patterns in different samples from patients with Toxoplasma gondii infections (9) have considered the detection of specific oligoclonal IgG bands in cerebrospinal fluid to be indicative of a local immune response. In the present study, slight differences were observed in the patterns of polypeptide fractions in the Leishmania antigens recognized by urine and serum samples from dogs with leishmaniasis. The passage of IgM through the glomerular barrier may be restricted due to its high molecular weight, thus resulting in differences in the patterns revealed by urine and serum samples when protein A is used. Nevertheless, this restriction will not be evident when only the IgG2 isotype is investigated. In this situation, if all specific antibodies present in urine were of blood origin, the proportions of the different idiotypes in serum and urine would be the same. Consequently, the findings observed suggest that the presence of Leishmania-specific antibodies in urine is mainly caused by the passage into the urinary tract of blood-derived immunoglobulins and, to a lesser extent, by the local production of antibodies. This local production of anti-Leishmania antibodies in the urinary tract might be explained by the presence of a predominant tubulo-interstitial nephritis lesion in dogs (32) or by lesions in other urinary or genital organs, such as the bladder, urethra, or prostate.

In conclusion, this is the first study that describes the detection of anti-Leishmania IgG antibodies in urine samples from dogs with leishmaniasis. Our results showed that urine antibodies were found only in proteinuric dogs. Further studies are needed to evaluate the meaning and usefulness of the presence of anti-Leishmania IgG antibodies in urine. However, the findings suggest that anti-Leishmania antibody detection in urine is a more specific and more reliable noninvasive method for leishmaniasis diagnosis and prognosis than is the U/P C ratio for dogs with glomerulonephropathies.

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