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Tamm-Horsfall glycoprotein binds IgG with high affinity

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Tamm-Horsfall glycoprotein binds IgG with high affinity. Tamm-Horsfall protein (THP), a monomeric glycoprotein (M_r 80 to 100 kDa), is produced by the mammalian kidney's thick ascending limb of Henle cells and excreted into the urine. The function of THP is uncertain. Here we report that a high molecular weight contaminant in sheep THP (sTHP) preparations was identified as sheep IgG by its positive reaction with donkey anti-sheep IgG antibody and with protein G. To answer the question of whether sTHP and sheep IgG co-purified because of a physical interaction between the two proteins, an enzyme-linked immunosorbent assay (ELISA) using immobilized sTHP and soluble sheep IgG was performed. Analysis of the ELISA data identified the presence of two sets of binding sites: a high affinity site (K_d 10^{-12} to 10^{-13} M) and a lower affinity site (K_d 10^{-10} to 10^{-11} M). The ELISA detected a similar high affinity interaction between human THP (hTHP) and human IgG. The binding of sheep IgG to immobilized sTHP was inhibited by soluble sTHP. These observations suggest an additional factor to be considered in studies addressing THP's potential immunoregulatory function.

In 1950, Igor Tamm and Frank Horsfall isolated, by salt precipitation, a protein from human urine that inhibited hemagglutination by various viruses [1]. Over the last 40 years studies have elucidated many of the biochemical, physiological, and pathological properties of this protein, termed THP.

THP has an approximate molecular weight of 80 to 100 kDa [2–4] with roughly 28% of its mass being contributed by N-linked carbohydrates [5]. The primary amino acid sequence of human THP (hTHP) has been determined [6, 7] and shown to be identical to "uromodulin," an immunosuppressive protein originally purified from the urine of pregnant women [4]. The sole site of THP production is in the cells lining the thick ascending limb of Henle in the mammalian kidney [8]. Rindler et al [9] determined that THP was a glycosylphosphatidyl inositol-linked membrane protein for at least a portion of its life. However, large quantities of THP (20 to 200 mg) are excreted daily into human urine [10].

While the normal function of THP remains uncertain, THP has been hypothesized to be important in urine concentration [11, 12], immunoregulation, and as a host defense mechanism against bacterial urinary tract infections. Some of the potential immunoregulatory actions of THP involve the ability of THP to bind the cytokines IL-1 [13], IL-2 [14], and TNF [15]. Additionally, THP inhibited lymphocyte proliferation induced by antigen [4], IL-1 [16], and leucoagglutinin [17, 18] *in vitro*. Conversely, THP added to peripheral blood lymphocytes (PBL) in culture appeared to stimulate blastoid transformation [19, 20]. The potential role of THP as a host defense mechanism was suggested by the observation that *E. coli* exhibiting type 1 pili were bound and trapped by THP [21]. At concentrations of 100 μg/ml or more, THP greatly decreased adherence of type 1 fimbriated *E. coli* to cultured uroepithelial cells [22]. Additionally, THP stimulated polymorphonuclear leukocytes (PMN) under some conditions [23, 24].

The long-term goal of the current study was to elucidate the role of THP in the pathophysiology of *E. coli*-induced pyelonephritis, using sheep as the experimental model. Early in this project, however, samples of sheep THP (sTHP) were found to be contaminated with a protein which was subsequently shown to be sheep IgG by its reaction with donkey anti-sheep IgG antiserum and with protein G. To determine whether sTHP and sheep IgG simply co-purified because of similar chemical behaviors or whether they actually interacted with each other, subsequent studies utilized an enzyme-linked immunosorbent assay (ELISA) to quantify the binding between these two molecules. A comparable ELISA was used to test binding of human THP (hTHP) with human IgG.

Methods

Isolation of THP

Initial experiments were performed with THP isolated from the urine of a single, clinically healthy, adult Suffolk ewe (E-1). Subsequently, two mixed-breed adult ewes (E-2 and E-3) and two mixed breed wethers (W-1 and W-2), initially six to seven months old, were purchased. These sheep were healthy as assessed by physical examination, complete blood counts, serum chemistry profiles, and urinalyses.

Urine was collected from individual sheep by placing them in a raised, head-catch stanchion for 16 to 24 hours with access to food and water. Urine passed through a heavy gauge metal screen and then through a finer metal screen before being funneled by a plastic lined, slanted pan into a plastic bucket immersed in ice. Urine was removed from the bucket multiple times during the collection period and filtered through glass wool. Sodium azide, approximately 10 mg/liter, was added to the urine before storing the urine at 4°C.

Once the collection was completed, 0.58 mol NaCl/liter was dissolved in the urine [1]. After storing the urine at 4°C overnight, it was centrifuged at 13,900 × g for 75 minutes at 4°C. Pellets were resuspended in water and dialyzed against 6 liters water at 4°C overnight. After dialysis, an equal volume of 8 M urea was added to the sTHP-enriched solution and allowed to incubate at 4°C overnight to help solubilize the precipitated sTHP. This 4 M urea solution was ultracentrifuged at 85,000 × g at 4°C for 40 minutes. The supernatants were dialyzed extensively against water at 4°C, and then 4 M NaCl was added to this dialyzed sample to make the solution 0.58 M NaCl. The next day the pellets obtained by centrifugation at 85,000 × g

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were resuspended and dialyzed in water, before being frozen at −70°C either for future lyophilization or long-term storage.

hTHP was isolated from a pooled, 24-hour urine sample from two adult males. The techniques used were identical to those employed with sheep urine except no sodium azide was added to the urine during its storage at 4°C. Cat THP (cTHP) analyzed in these experiments was obtained from pooled urine samples from three castrated male cats as described previously [25].

Electrophoresis and Western blotting techniques

THP isolation from urine was monitored using discontinuous 7.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE) [26]. The molecular weight standards were reduced with β-mercaptoethanol, while the other gel samples were non-reduced. Subsequently, to assess the effect of disulfide bond reduction on the sTHP samples, SDS-PAGE using a 5% to 15% gradient was utilized to analyze non-reduced and reduced sTHP and sheep IgG. All gels were stained with silver nitrate [27] after fixation overnight in 50% methanol and 1.5% glutaraldehyde.

Western blot analyses characterized the sTHP isolate further. In the first blot, proteins from cat urine, cTHP, sTHP, and hTHP were separated by 7.5% SDS-PAGE and transferred electrophoretically to 0.45 μm nitrocellulose (NC) (Gelman, Ann Arbor, Michigan, USA) [28]. After incubating the resulting NC with 5% non-fat dry milk in TBS (50 mM Tris/150 mM NaCl, pH 7.4), one of the NC strips was incubated with affinity purified sheep anti-cTHP antiserum [25] diluted with 5% milk in TBS/0.1% Tween 20 (TBS-T) overnight, as part of a related study. Another NC strip was incubated with donkey anti-sheep IgG conjugated to horseradish peroxidase (HRP) (Sigma Chemical Company, St. Louis, Missouri, USA). The sheep anti-cTHP incubated blot was rinsed with TBS-T and then incubated for three hours with donkey anti-sheep IgG-HRP. Both strips were developed using 4-chloro-1-napthol.

In another experiment, electrophoresed samples of sheep serum, sheep IgG, and sTHP were transferred to NC. After blocking the NC with 3% gelatin in TBS, protein G-HRP (Bio-Rad, Richmond, California, USA) in 1% gelatin/TBS-T was incubated with the strips. As a control, a NC strip was incubated overnight in the same protein G-HRP solution with the addition of 2 mg of sheep IgG (Sigma Chemical Company). Bound protein G-HRP was detected with 4-chloro-1-napthol.

THP/IgG ELISA

Lyophilized sTHP and sheep IgG (Sigma Chemical Company) were dissolved in 0.05 M sodium carbonate buffer (pH 9.6). Samples were centrifuged for 10 minutes at 13,600 × g (4°C). The protein concentrations in the supernatants were determined spectrophotometrically [29]. Microtiter assay plates (Falcon Pro-Bind Assay Plate, Becton Dickinson, Lincoln Park, New Jersey, USA) were coated with 50 μl/well of 30 μg/ml sTHP. Similarly, wells to be used in converting the OD_{410} of test wells to "pM sheep IgG bound" were coated with 0.1 to 2.0 μg/ml sheep IgG. The plates were incubated at 4°C overnight with shaking. The next day, the plates were rinsed with 0.05 M sodium carbonate buffer (pH 9.6), before 150 μl of 1% bovine serum albumin (Calbiochem, LaJolla, California, USA) in 0.05 M sodium carbonate (pH 9.6) was added to all wells. After another overnight incubation, plates were rinsed with 19.8 mM Tris/20 mM NaCl/2.7 mM KCl/0.05% Tween 20 (pH 7.4) (Tris IgG assay buffer) and stored at −20°C.

The standard sTHP/sheep IgG ELISA was performed as follows. Sheep IgG was dissolved in Tris IgG assay buffer. Insoluble material was removed by centrifugation at 13,600 × g for 10 minutes at 4°C. The protein concentration of the supernatant was determined using the OD_{280} and an extinction coefficient of 13.6 [29]. Serial dilutions of sheep IgG were made in 1% albumin/Tris IgG assay buffer. There typically were twelve different sheep IgG dilutions ranging from 3 mg/ml to 1 μg/ml for each assay. Microtiter plates, previously coated with sTHP, were loaded with 50 μl of the various sIgG dilutions in triplicate wells and incubated overnight at 4°C. After rinsing plates in Tris IgG assay buffer, 50 μl rabbit anti-sheep IgG-alkaline phosphatase (Pel-Freez, Rogers, Arkansas, USA), diluted in 1% albumin/Tris IgG assay buffer, was incubated in wells at 37°C for two hours. Unbound enzyme-labeled antibody was pipetted from the wells and plates were washed, first with Tris IgG assay buffer, and then with 0.05 M sodium carbonate buffer (pH 9.6). The developing solution (4 mM p-nitrophenol phosphate/1 mM $MgCl_2$/0.05 M sodium carbonate buffer, pH 9.6), was added to all wells (100 μl/well) and the reaction proceeded at room temperature until the OD_{410} of the highest sIgG concentration wells reached approximately 2.3 (about 20 min). The reaction was stopped with 25 μl of 2 N NaOH/well. The absorbance of each well at 410 nm was measured with a manual microtiter plate reader (Dynatech, Chantilly, Virginia, USA). At least two sTHP samples from each of the five sheep were assayed.

Variations in this standard assay included competition experiments where the ability of soluble sTHP (30 μg/ml) to inhibit the binding of sheep IgG to sTHP coated plates was examined. Additionally, the ability of hTHP to bind human IgG (Sigma Chemical Co.) was tested using procedures analogous to sTHP binding studies.

Each ELISA plate had control wells, including wells coated with THP and incubated with the enzyme-conjugated anti-IgG antibody to detect background reactions. When the absorbance in these wells was above the usual background levels ($OD_{410} \approx 0.060$), this absorbance was subtracted from the OD_{410} of each well where IgG had been added. This was only necessary with THP from sheep E-1. Additionally, some wells were not coated with THP, but were blocked with albumin prior to incubation with 3 mg/ml IgG and the enzyme-conjugated anti-IgG. Higher than normal background readings were present only in the hTHP/human IgG assay. Thus, for each human IgG concentration, three wells coated with hTHP and three wells not coated with THP were analyzed and the OD_{410} from "no hTHP wells" were subtracted from the corresponding absorbances of the hTHP coated wells.

Data analysis

For analysis of the THP/IgG ELISA binding data, the absorbance readings of the IgG coated wells that yielded values in the middle portion of the absorbance range were divided by the picomoles of IgG loaded into the wells. If more than one IgG concentration was utilized, this factor was averaged and then used to convert the absorbance readings in the THP coated wells into "pmoles of IgG bound". For the wells coated with THP, the [free IgG] was calculated using the equation: [free IgG] = [total IgG] − [bound IgG], where [total IgG] was the amount initially added to these wells. Scatchard plots [30] were drawn by plotting [bound IgG]/[free IgG] versus [bound IgG]. The affinity constants were determined using a non-linear least squares computer program (MINSQ, MicroMath Scientific Software, Salt Lake City, Utah, USA) and the following equations:

$$\text{One-site model: } A = \frac{A_1}{1 + (K_{d1}/x)}$$

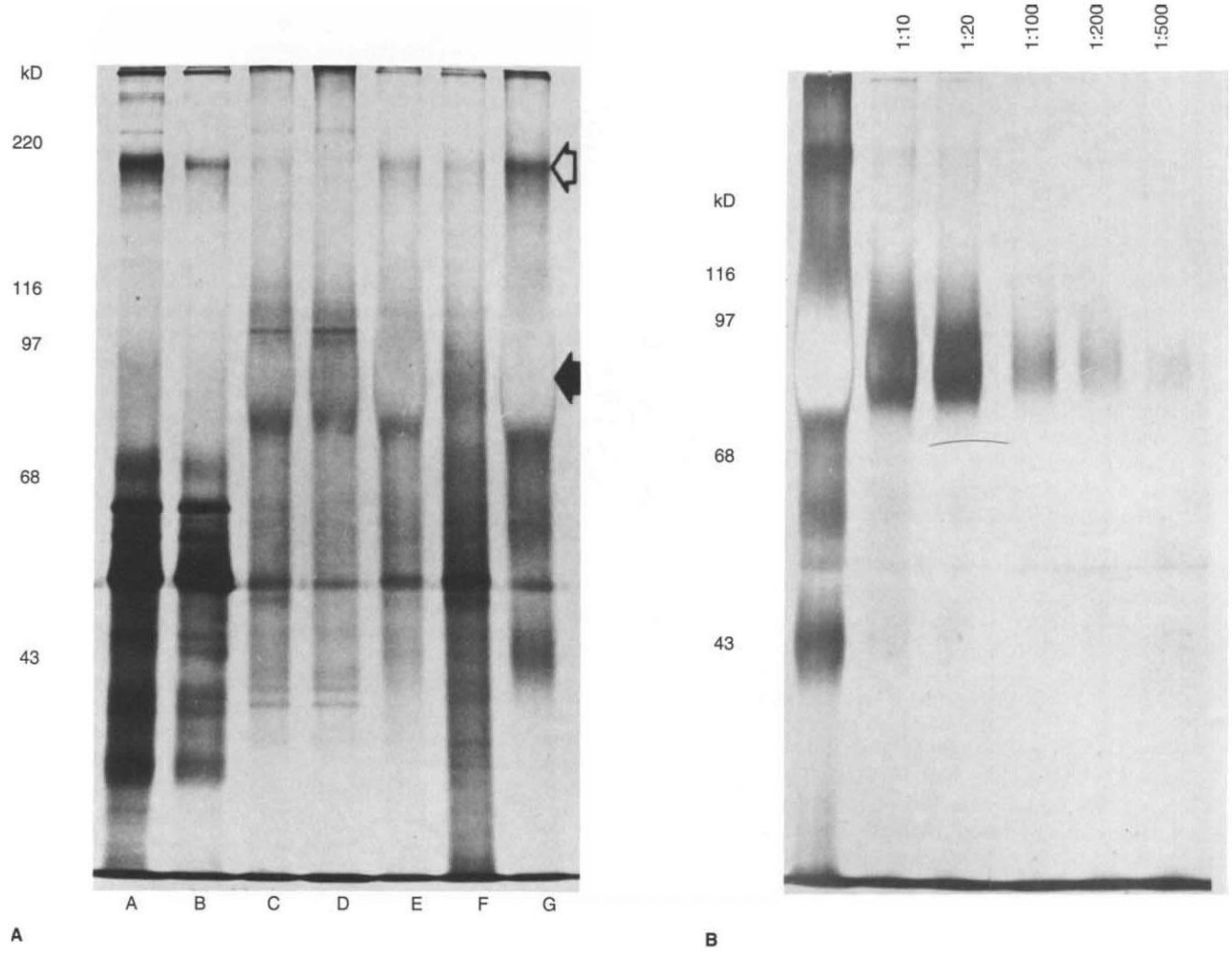

Fig. 1. A. *Steps in purification of sTHP from sheep urine analyzed by silver-stained 7.5% SDS-PAGE.* Except for the molecular weight standards (β-spectrin, β-galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin) which were reduced with 2% β-mercaptoethanol, the gel samples were not reduced. Approximate protein concentrations loaded in each well were determined by either dry weight measurements or Coomassie Brilliant Blue G-250 assays [33]. Lane A: sheep urine (3 μg protein); Lane B: supernatant from first centrifugation (3 μg protein); Lane C: pellet from first centrifugation (6 μg protein); Lane D: 4 M urea pellet (6 μg protein); Lane E: 4 M urea supernatant (6 μg protein); Lane F: supernatant after 0.58 M NaCl addition (6 μg protein); Lane G: final pellet after 0.58 M NaCl precipitation (10 μg protein). Solid arrow indicates negatively staining sTHP band. Open arrow indicates high molecular weight contaminating protein. **B.** Serial dilutions of the 10 μg of protein in lane G assessed by silver-stained 7.5% SDS-PAGE. All sTHP samples were non-reduced.

$$\text{Two-site model: } A = \frac{A_1}{1 + (K_{d1}/x)} + \frac{A_2}{1 + (K_{d2}/x)}$$

where A = total [bound IgG], x = [free IgG], K_{d1} and K_{d2} are the dissociation constants for the two binding sites, and A_1 and A_2 are the concentrations of each binding site [31]. The F-ratio test was used to determine if the two-site model predicted parameters significantly better ($P < 0.05$) than the one-site model [32].

Results

sTHP isolation

As illustrated in Figure 1, sTHP was purified progressively from whole urine (Lane A) where sTHP was a minor component, to the final stage (Lane G) where sTHP was the major component, staining negatively with a molecular weight of approximately 90 kDa. Besides sTHP in this final preparation the primary contaminating proteins were seen as a discrete dark band of approximately 200 kDa and two more diffuse bands (M_r 62 kDa and 43 kDa). By serially diluting the sTHP sample (Fig. 1B), it was estimated that each contaminating band represented less than 2% of the total protein in the final precipitant.

Identity of the 200 kDa contaminating protein

While the identity of the upper molecular weight contaminating protein in these non-reducing gels initially was unknown, three sets of experiments demonstrated that this protein was sheep IgG. First, in a Western blot where the reactivity of sheep anti-cTHP antibody was tested against cTHP, sTHP, and hTHP (Fig. 2), this antibody appeared to strongly recognize not only cTHP, but also the 200 kDa protein in the sTHP sample (panel B). However, in the negative control (panel C) where the NC was reacted only with HRP-conjugated donkey anti-sheep IgG

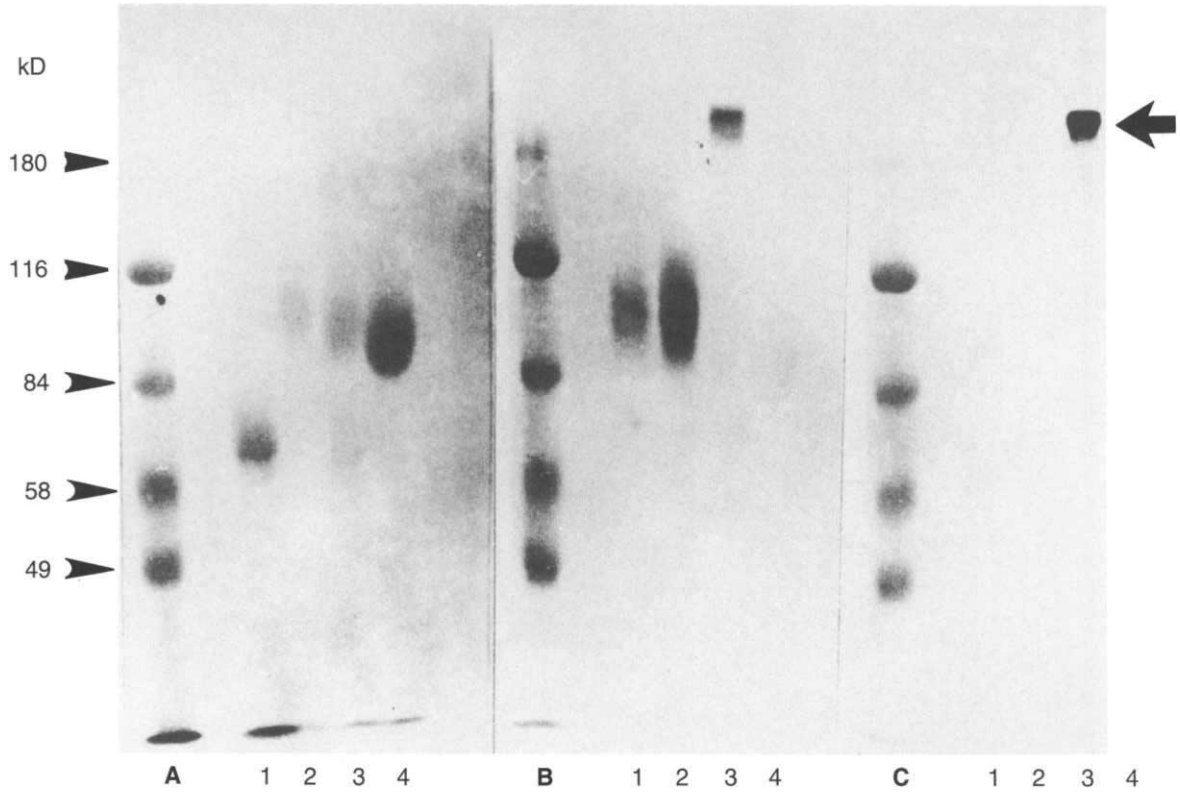

Fig. 2. *Western blot demonstrating reaction of upper molecular weight contaminating protein with anti-sheep IgG antiserum.* THP samples were non-reduced, while pre-stained molecular weight standards (α_2-macroglobulin, β-galactosidase, fructose-6-phosphate kinase, pyruvate kinase, and fumarase) were reduced. **A** Stained with amido black; **B** Reacted with sheep anti-cTHP serum and HRP-conjugated donkey anti-sheep IgG; **C** Reacted with HRP-conjugated donkey anti-sheep IgG only. Lane 1: dialyzed cat urine, Lane 2: cTHP final pellet; Lane 3: sTHP final pellet; Lane 4: hTHP final pellet. The solid arrow marks the upper molecular weight contaminating protein band.

antiserum, the upper molecular weight contaminant in the sTHP sample reacted strongly, suggesting that this protein was sheep IgG.

Subsequently, Western blots of sheep serum, sheep IgG, and sTHP were reacted with protein G. In all three of these samples (Fig. 3B), a band with the same electrophoretic mobility bound protein G-HRP. An identical NC strip was incubated with protein G-HRP and sheep IgG simultaneously as a negative control. As shown in Figure 3C, soluble sheep IgG did inhibit the reaction of protein G with bound IgG, however, the sTHP band showed a positive reaction under these conditions.

As a final confirmation of the 200 kDa protein's identity, purified sheep IgG and sTHP were analyzed simultaneously by gradient SDS-PAGE in both non-reduced and reduced states (Fig. 4). Non-reduced purified sheep IgG and the sheep IgG in the sTHP sample migrated similarly with an approximate molecular weight of 173 kDa. When the disulfide bonds in sheep IgG were reduced, major bands migrating at 62, 53, and 27 kDa were observed (lane 3). Similar bands were present in the sTHP reduced sample (lane 4).

THP/IgG binding assays

sTHP from the three ewes and two wethers were assayed for their ability to bind sheep IgG using an ELISA. sTHP from all five sheep bound sIgG in a dose dependent manner as illustrated by the data from E-2 in Figure 5. Transformation of the sTHP/sheep IgG binding data into a Scatchard plot resulted in graphs similar to the one shown in Figure 6 for all sheep. These curved Scatchard plots suggested that binding was occurring at sites with two distinct binding affinities [34, 35]. These binding affinities were estimated for two sTHP samples from each animal. The K_d of the high affinity sites ranged from 10^{-13} M to 10^{-12} M, while the low affinity site K_d was approximately 10^{-11} to 10^{-10} M for all samples. The F-ratio test showed that the two binding site model predicted the binding parameters significantly better than the one binding site model ($P < 0.05$).

Using a comparable ELISA, hTHP bound human IgG in a dose dependent manner. As in the sTHP studies, hTHP/human IgG binding data were best described by a two binding site model. The predicted K_d for the high and low affinity binding sites were approximately 10^{-12} M and 10^{-10} M, respectively.

To determine whether IgG was binding to THP at sites only exposed when THP molecules were bound to the solid phase, the ability of soluble sTHP to inhibit sIgG binding to immobilized sTHP was examined. As illustrated in Figure 7, soluble sTHP markedly inhibited sheep IgG binding to immobilized sTHP.

Discussion

The estimate of the molecular weight of non-reduced sTHP (90 kDa) predicted by SDS-PAGE was similar to the molecular weight of non-reduced hTHP previously reported [36–38]. In gels such as Figure 1A that were overloaded with THP (approximately 10 μg) in order to detect contaminants, THP migrated as a diffuse band that stained darker at its edges than in its "negatively" stained central region. This negatively stained center on heavily loaded gels has been noted with other silver-stained proteins and attributed to variability in the way different proteins react with silver stain [39, 40]. On gels where the sTHP protein concentration gradually was decreased (Fig.

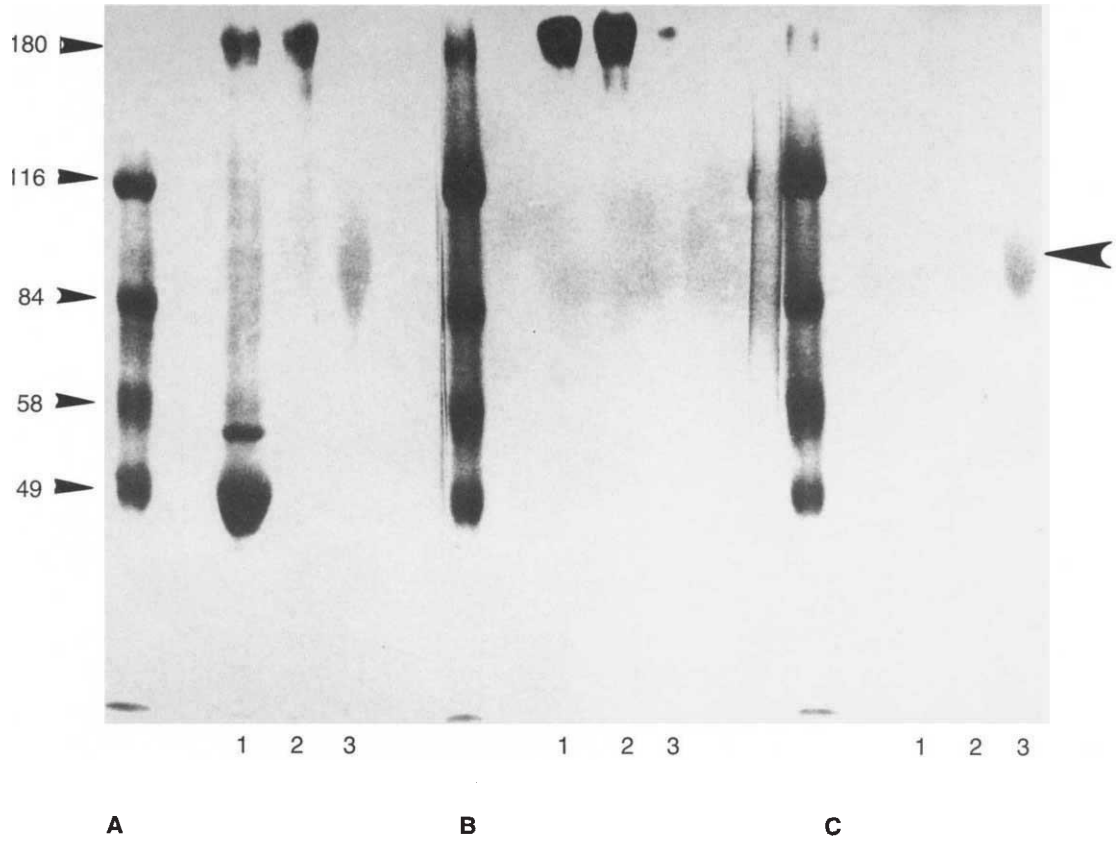

Fig. 3. *Western blot comparing the reaction of protein G with sheep serum (Lane 1), sheep IgG (Lane 2) and sTHP final pellet (Lane 3).* Except for the molecular weight standards which were the same as in Fig. 2, the protein samples were not reduced with β-mercaptoethanol. **A.** Stained with amido black. **B.** Incubated with protein G-HRP. **C.** Incubated with protein G-HRP and soluble sheep IgG. Arrowhead indicates positively stained sTHP band.

1B), the two densely stained borders and the negatively stained center of the sTHP band coalesced into a single band, with a mobility equal to the central region of the originally negatively stained band. This confirmed that only one protein was represented by the original broad band.

The identity of the two primary low molecular weight contaminating bands (62 kDa and 43 kDa) in the sTHP samples seen with 7.5% SDS-PAGE is uncertain. However, they may represent degradation products of sTHP since they migrate as diffuse bands typical of glycoproteins and since two glycoproteins with molecular weights of 66 kDa and 51 kDa were produced when calf THP was degraded partially by mild acid hydrolysis [41].

The identity of the higher molecular weight contaminating band in the sTHP samples as sheep IgG first was suspected when this protein reacted positively with donkey anti-sheep IgG-HRP (Fig. 2). The use of protein G not only helped confirm that this protein was sheep IgG, but also provided insight into the binding site of THP for IgG. The ability of sTHP to react positively to protein G-HRP when soluble sheep IgG was present (Fig. 3C) suggested that electrophoretically transferred sTHP was capable of binding soluble sheep IgG and that sTHP and protein G-HRP recognized different sites on sheep IgG.

Additional evidence that IgG was present in the sTHP sample was gathered from the gradient acrylamide gel. The high molecular weight protein migrated identically to sheep IgG in non-reduced samples, while similar bands representing the IgG heavy and light chains were present in both the sheep IgG and sTHP samples after disulfide bond reduction (Fig. 4). The predicted molecular weight of sheep IgG was 200 kDa in the 7.5% gels and 173 kDa in the 5 to 15% gels rather than the published value for IgG of 150 kDa [42] possibly because the SDS-PAGE molecular weight standards had disulfide bonds reduced, and thus more SDS/g of protein could bind these proteins than could bind the non-reduced IgG. In the reduced IgG sample, the heavy chain appeared as a doublet. This has been noted by others and attributed to heterogeneity in IgG's oligosaccharide moieties [43].

Previous researchers occasionally have noted an upper molecular weight band in hTHP preparations, but suggested that it represented a THP dimer [20, 44]. While IgG has not been identified previously in THP preparations, Muchmore and Decker [4], in their initial report on the isolation of uromodulin (THP isolated from pregnant women), noted that antibodies against uromodulin raised in rabbits appeared to recognize weakly an "area consistent with human IgG" in human sera by Western blot analysis, suggesting that the uromodulin (THP) used as the immunogen might have contained a small amount of human IgG.

Once the high molecular weight protein in the sTHP sample from E-1 was identified as sheep IgG, the question arose as to whether sTHP and sheep IgG bound each other. This was answered positively with the ELISA which demonstrated dose dependant binding between immobilized sTHP and soluble sheep IgG (Fig. 5). Scatchard plots from the sTHP/sheep IgG binding data produced non-linear curves (Fig. 6) indicative of reactions having more than one affinity constant. This can occur

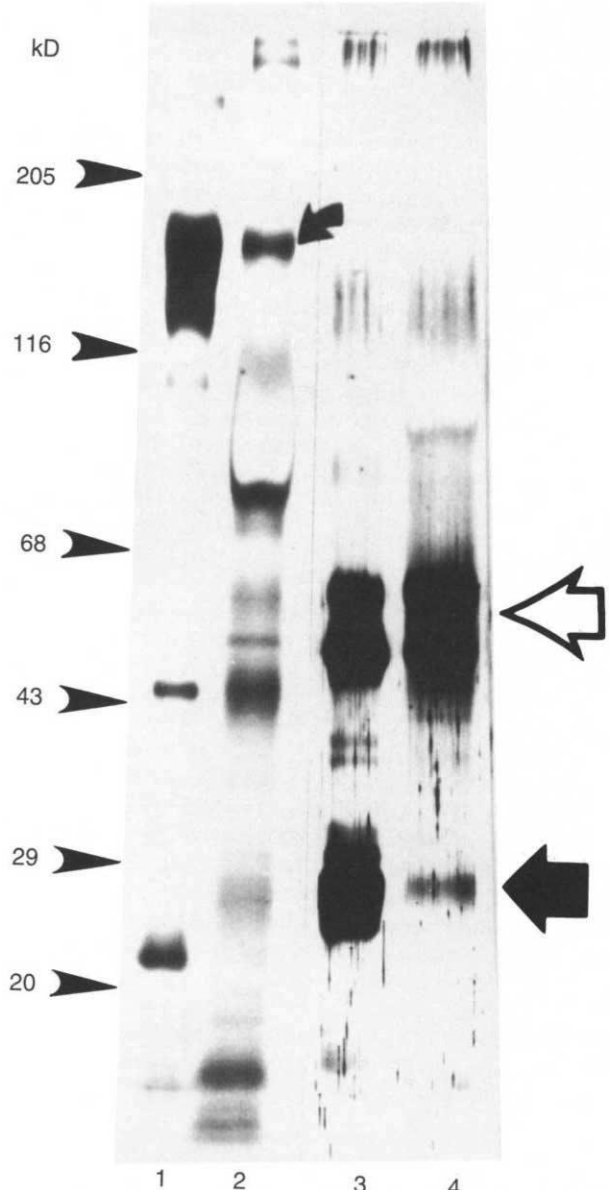

Fig. 4. *Silver-stained 5% to 15% SDS-PAGE comparing the electrophoretic mobility of purified sheep IgG to the sheep IgG in the sTHP sample in non-reduced and reduced states.* Lane 1: non-reduced sheep IgG; Lane 2: non-reduced sTHP (curved arrow indicates non-reduced sheep IgG); Lane 3: sheep IgG reduced with β-mercaptoethanol; Lane 4: sTHP reduced with β-mercaptoethanol (open arrow indicates IgG heavy chains and closed arrow indicates light chains). Molecular weight standards included myosin, β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

either when there are multiple binding sites with different and fixed affinities or when multiple binding sites exist that initially have the same affinity, but once ligand binds to one site, the affinity for ligand decreases at the unoccupied sites (negative cooperativity) [45]. Without further thermodynamic or structural data, the difference between these scenarios cannot be determined; however, the prediction of the binding constants is identical in either instance.

In the present study, the amount of IgG bound to immobilized THP was estimated by comparing the OD_{410} of these samples to that of wells in which IgG had been bound to the plastic substrate. In these calculations, it was assumed that all of the

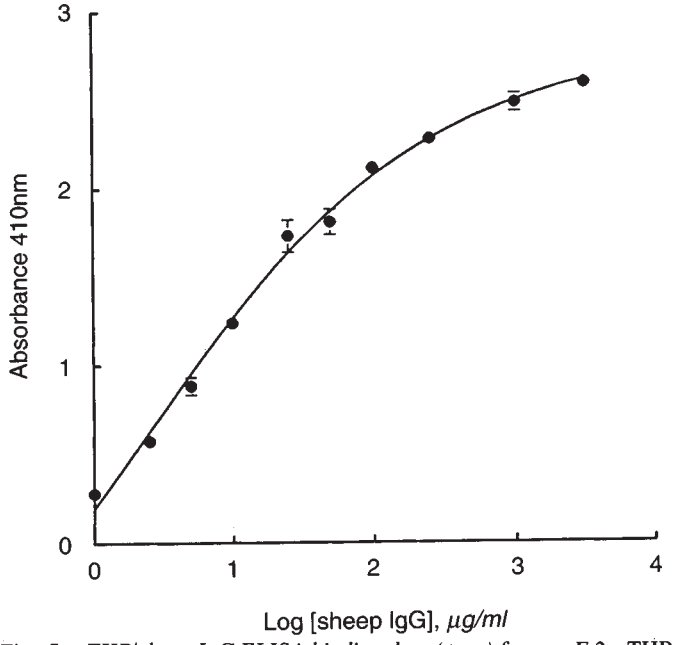

Fig. 5. *sTHP/sheep IgG ELISA binding data (± SE) for ewe E-2.* sTHP from each animal was bound to microtiter plates (1.5 μg/well). After incubating wells with 3 mg/ml to 1 μg/ml sheep IgG, the amount of IgG bound to sTHP was quantified using rabbit anti-sheep IgG conjugated to alkaline phosphatase and subsequently detected with p-nitrophenol phosphate.

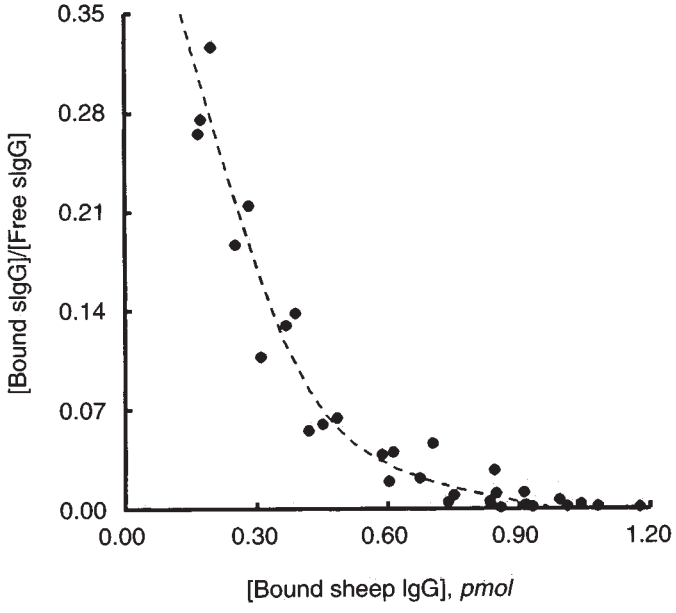

Fig. 6. *Scatchard plot of sTHP/sheep IgG ELISA binding data from ewe E-2.* Dotted line represents the least-squares best fit to the two-site model with $K_{d1} = 3.2 \times 10^{-11}$ M, $K_{d2} = 8.2 \times 10^{-13}$ M, $A_1 = 6.0 \times 10^{-13}$ M, and $A_2 = 4.0 \times 10^{-13}$ M.

IgG incubated in the "IgG standard" wells had become immobilized on the plastic. Preliminary experiments suggest that the fraction of IgG adsorbed in these wells was closer to 75%. However, K_d values calculated from the ELISA were insensitive to variation in the estimate of the absolute amount of IgG bound. For instance, if instead of assuming that 100% of the IgG bound to the plastic in the "IgG standard" wells, it was assumed that only 0.1% bound, then the calculated values of K_d

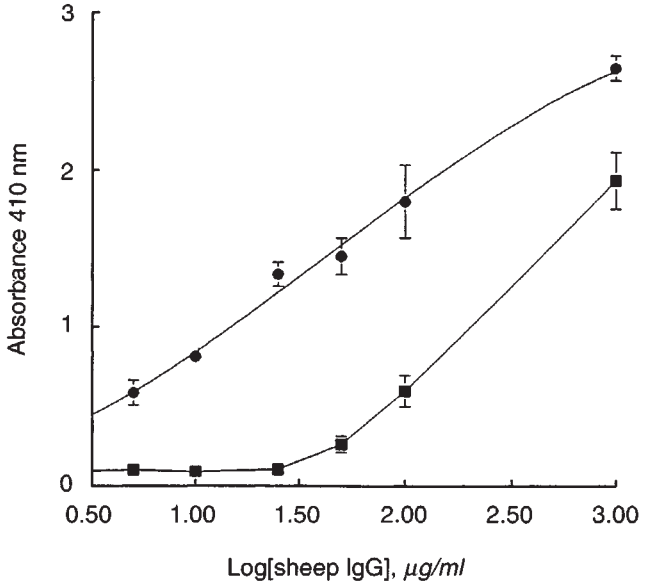

Fig. 7. *ELISA data demonstrating ability of soluble sTHP (30 μg/ml) to inhibit binding of added sheep IgG to immobilized sTHP.* Symbols are: (●) no soluble sTHP; (■) 30 μg/ml sTHP. "No soluble sTHP" denotes the standard ELISA technique with no soluble sTHP added during the sheep IgG incubation step. Absorbance at 410 nm ± SE for 1 mg/ml to 5 μg/ml sheep IgG samples.

for the high and low affinity sites remained virtually unchanged while the number of predicted binding sites, A_1 and A_2, decreased by a factor of 1000.

Previous studies demonstrating high affinity binding between immobilized TNF and soluble THP [15], and between immobilized IL-1 and soluble THP [13, 46] were questioned by Moonen, Gaffner and Wingfield [47], who suggested that these observations might be due to the binding of THP to denatured cytokines rather than THP having a true affinity for native cytokines. This apparently was not the case for the sTHP/sheep IgG interaction because soluble sTHP could inhibit this binding (Fig. 7).

The binding between hTHP and human IgG was significant in establishing that THP/IgG binding was not unique to sheep. The affinity binding constants of the one male hTHP sample tested was similar to the binding constants of sTHP. Much work remains in characterizing individual, sex, and species variations in THP/IgG binding affinities. Also, the ability of THP to bind the various subclasses of IgG and other classes of immunoglobulins (Ig) needs to be explored. Furthermore, the site on IgG involved in binding THP needs to be determined. It is possible that THP binds the IgG light chain since the ability of THP to aggregate with some Bence Jones proteins under variable ionic conditions has been documented [48–50]. However, the association of THP with Bence Jones protein occurred at NaCl concentrations [49] that typically aggregate THP alone [51, 52].

In light of this newly described ability of THP to bind IgG, re-evaluation of several previous studies which had yielded some puzzling results now produces some interesting hypotheses. For instance, B cells can be stimulated to proliferate and increase immunoglobulin production by some, but not all, hTHP preparations [19, 20]. Since THP binds IgG, perhaps B cells were stimulated by THP binding to surface Ig on these cells. Data from Hunt et al [20] demonstrated a correlation between the presence of a high molecular weight protein in hTHP samples and the sample's inability to stimulate B cells. If this high molecular weight protein was IgG, then in hTHP samples containing this contaminant, the THP/immunoglobulin binding sites would be occupied, greatly decreasing any potential interaction between THP and B cell surface Ig.

Besides the interaction of THP with B cells, experiments evaluating the interaction of PMN with hTHP warrant further examination. Kuriyama and Silverblatt [53] reported that the normal phagocytosis by PMN of nonopsonized, type 1 fimbriated *E. coli* was inhibited if hTHP was preincubated with these bacteria, presumably because hTHP and PMN competed for the same *E. coli* receptors. However, when 5% serum was added to the PMN/hTHP/*E. coli* system, PMN ingested *E. coli*. In a seemingly contradictory study, Horton et al [23] demonstrated PMN degranulation, respiratory burst, and leukotriene B_4 release initiated by particulate hTHP.

The differences in these two studies might be resolved by considering the possible contamination of the hTHP samples with human IgG. PMN have Fc receptors for IgG that, when occupied, produce the activation response [54] seen by Horton et al [23] upon THP addition. IgG bound to THP likely still has at least a portion of its Fc region available for binding to other proteins, as suggested in the present study, where protein G, which binds the Fc fragment [55, 56], appeared to recognize THP-IgG complexes (Fig. 3C). Therefore, a hTHP/human IgG complex may bind to PMN Fc receptors, thus explaining the findings of Horton et al [23]. Similar reasoning that THP samples were slightly contaminated with IgG could partially explain the results of Yu et al [24], who recently demonstrated that THP increased PMN phagocytosis and appeared to adhere to PMN cell surface. Additionally, if Kuriyama and Silverblatt's [53] hTHP samples were initially free of IgG, it is possible that no activation of PMN by hTHP coated *E. coli* would result until serum (with IgG) was added to the system.

While THP previously has been speculated to play a role in immunoregulation and in the immune defenses of the urinary tract [57], a definitive immunologic function has not been proven. The observation of high affinity binding between IgG and THP strengthens these previous suspicions. As research continues into the interaction between THP, immune cells, and pathogens, the significance of immunoglobulins in these interactions requires attention.

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