Immunonephelometry of Specific Proteins in Human Seminal Plasma

Jorge Lizana and Eva Blad

Using immunonephelometry, we measured albumin, transferrin, α1-antitrypsin, orosomucoid, hemopexin, IgG, IgA, and secretory IgA in seminal plasma of men who were attending a fertility clinic. Seminal plasma, obtained by centrifugation of semen, is a turbid liquid producing high blank values that were not diminished by ultracentrifugation, filtration, or treatment with protamine, dextran sulfate, or various lipophilic gels. Pretreatment of seminal plasma with polyethylene glycol 6000 (final concentration, 40 g/L) considerably decreased the blank values without appreciably changing the relative concentrations of the eight proteins studied. Our investigations illustrate the feasibility of this application of immunonephelometry. This approach can be a valuable diagnostic tool in the laboratory evaluation of the male partner in infertile unions.

Additional Keyphrases: infertility; sample pretreatment with polyethylene glycol

Involuntary childlessness is a worldwide problem, affecting 15–18% of today's couples during their fertile years (1). Causes of infertility may be found and corrected primarily for the female partner, because diagnostic tools and treatment are far more advanced than is the case for the male.

Over the years, laboratory tests intended to explore the details of the functional status of the male genital secretions have been relatively limited. Traditional analyses of human semen include counting of normal and abnormal spermatozoa, and estimation of spermatozoa motility and survival. Biochemical analysis of human seminal plasma (HSP), obtained by centrifugation of semen, is often limited to zinc, citrate, fructose, and acid phosphatase activity (2, 3). With the exception of azoospermia, attempts to correlate these indexes of semen quality with infertility have been disappointing and suggest that the cause(s) of infertility may be better reflected by other compounds in the secretions of the male genital accessory glands.

The composition of the proteins (potential antigens) in HSP has not been fully explored, but with the help of high-resolution techniques such as two-dimensional electrophoresis a clearer picture is now emerging (4, 5). Some of the proteins are of plasma origin (e.g., albumin, transferrin, IgG, etc.), but most are believed to be produced by the seminal vesicles (e.g., lactoferrin, as well as proteins involved in the coagulation and liquefaction of semen) or by the prostate (e.g., acid phosphatase) (2, 6, 7). Thus, identification and quantification of proteins in seminal plasma may be important to the laboratory evaluation of male infertility.

In this paper we used immunonephelometry to quantify albumin, transferrin, orosomucoid, α1-antitrypsin, hemopexin, IgG, IgA, and secretory IgA in normal specimens of HSP. We also describe how to remove substances causing high blank readings from the naturally turbid HSP.

Materials and Methods

Semen samples were obtained from men 22 to 43 years old, living in barren unions and attending the fertility clinic. Samples were also obtained from healthy volunteers. All specimens were obtained in the laboratory by masturbation, after three to seven days of continence. The ejaculate was collected in a test tube and mixed by inversion 30 times per minute for 30 min, at room temperature, to allow the semen to liquify. Then an aliquot was removed for the common semen analysis (3). A normal semen sample was defined as follows: more than 20 × 10^8 spermatozoa per milliliter, or more than 40 × 10^6 spermatozoa per specimen; spermatozoa motility of 3 (arbitrary units 0–3); more than 40% spermatozoa with normal morphology; >70% live spermatozoa (vitality stain); ejaculate viscosity of 1–2 s; and absence of agglutination (3). Seminal plasma was obtained by ultracentrifugation (35 000 × g, 30 min) before or after mixing with polyethylene glycol (PEG) to give a final concentration of 40 g/L. When not immediately used, the HSP samples were stored at −20 °C.

Polyethylene glycol (PEG), average relative molecular mass 6000 (Union Carbide Corp., New York, NY 10017), was dissolved in isotonic saline (9 g/L) to give concentrations up to 400 g/L.

Antisera to human plasma proteins were purchased either from Dako-Immunoglobulins, Copenhagen, Denmark; Seward Laboratories, London SE19UG, U.K.; Behringwerke AG, Marburg/Lahn, F.R.G.; Pharmacia Fine Chemicals, Uppsala S-751 04, Sweden; Kallestad Laboratories Inc., Chaska, MN 55318; or Atlantic Antibodies, Westerbrook, ME 04992. For immunonephelometry, the different antisera were diluted 70- to 100-fold with a solution of PEG (40 g/L) and let stand at room temperature for 30 min. This PEG-antibody reagent was then filtered through a 0.22-nm pore size filter (Millipore Corp., Bedford, MA 01730) and used only during that day.

Calibration and control solutions: A 200 g/L solution of human serum albumin was from Kabi AB, Stockholm, Sweden. Chromatographically pure human secretory IgA (lot no. 15999) was purchased from Cappel Laboratories, Cochranville, PA 19320. The reference material for human secretory IgA (lot no. 1646A, Seward Laboratories) was used in recovery studies. Purified lactoferrin was kindly supplied by Dr. Nils Harboe (Protein Laboratory, Copenhagen Univ., Denmark). Stabilized standard human serum (Behringwerke) was used as calibrator for transferrin, α1-antitrypsin, orosomucoid, hemopexin, retinol-binding globulin, IgG, IgA, and IgM. The different calibration solutions were diluted in saline to give various concentrations of about 1 to 250 mg/L, divided into 0.5-mL aliquots, and stored at −20 °C. Once thawed, these were used only once.

Reference set A (Kallestad) for the nephelometric measurement of specific human proteins was diluted in saline, stored at −20 °C, and used as an immunonephelometric
control. Purified IgG (50 mg/L; Kabi AB) was also used as a control.

**Immunonephelometry.** We used the LSA-290 digital nephelometer (Kallestad). The procedure has been described earlier (8). Briefly, the PEG-antibody reagent (1 mL) was added to 10–20 μL of the calibration solutions, controls, and samples, in 10 × 75 mm disposable borosilicate tubes. All tubes were run in duplicates. Tube contents were mixed by inverting 20 times, then left at room temperature for 60 min. In the final assay the HSP samples were first mixed with an equal volume of PEG (80 g/L) at room temperature, and centrifuged at 35 000 × g for 30 min. We then used the supernatant liquid (referred to above as "sample") for immunonephelometry. For albumin, the supernate was further diluted 10-fold with saline before pipetting 20 μL into the tubes. Sample and reagent blanks made up with the PEG solution (40 g/L) were always run.

**Protein determination.** Total protein in seminal plasma was determined by the method of Doumas (15) with purified human serum albumin as the standard. Untreated seminal plasma specimens were used.

**Double immunodiffusion.** Agarose type A (Pharmacia), 10 g/L in Tris–barbitol buffer (pH 8.6, 25 mM/L), was used as matrix. Antisera against the α-chain of IgA (Behring), secretory IgA (Dako), and "secretory piece" (Dako and Seward) were used to demonstrate the presence of both serum-type IgA and secretory IgA in seminal plasma. Milk secretory IgA (Seward) was used as a control. We observed the precipitin arcs after 24 and 48 h of diffusion and again after washing with saline and staining with Coomassie Brilliant Blue (5).

**Gel filtration.** To a 0.7 × 27 cm column packed with Sephadex G-50 fine (Pharmacia) and equilibrated with saline, we applied 0.5-mL samples of seminal plasma and eluted with phosphate-buffered saline (50 mmol/L, pH 7.4). We collected 1.1-mL fractions and assayed them for albumin and blank-producing substances by immunonephelometry.

**Results**

**Attempts to Remove Substances Causing High Intrinsic Light-Scattering**

**Centrifugation.** Seminal plasma cannot be directly measured by immunonephelometry because blank values are very high. Assay of diluted centrifuged HSP proved useful only in determination of proteins present at high concentration, such as albumin. Use of low centrifugal forces (1500–3000 × g, 30 min) yielded a yellowish seminal plasma, which could be seen microscopically to contain spermatozoa tails, tail pieces, and other cellular debris. Ultra centrifugation (35 000 × g, 30 min) reduced the blanks by an average of 38% (n = 17). The combination of low and high centrifugal forces yielded a mean blank reduction of 79%, but the nephelometric values of the blanks still exceeded 70% of the total readings for the antigen–antibody complexes. Thus measurement of HSP proteins present in low concentration was unreliable.

**Filtration.** We next tried Millipore MF microfilters and Millex disposable filter units with pore sizes of 0.22 and 0.45 nm. The seminal plasma blanks decreased by 62% (n = 17) and 28% (n = 16), respectively. To test the possible loss of proteins due to binding to the microfilters, we assayed 11 samples for IgG by immunonephelometry, before and after filtration through a 0.22-nm pore size MF filter. No apparent decrease in the IgG content after filtration was found (Table 1). Similarly, analytical recoveries were high for a solution of purified IgG diluted with PEG (40 g/L). But when the IgG solution was diluted with saline or an albumin solution (18.6 g/L), all the IgG was lost. A recovery study of a seminal plasma pool containing 2.9, 7.3, 9.6, 16.1, and 27.6 mg of hemopexin per liter showed a loss of 11–34% of hemopexin content after filtration through a 0.22-nm pore size Millex unit.

**Protamine treatment.** Protamine has been used to clarify blood plasma samples (9). Fourteen seminal plasma samples showed an increase of about 90% in the blank values after such treatment.

**Removal of lipophilic material** by use of dextran sulfate (10) yielded a mean increase of 23% in the seminal plasma blanks (n = 23), whereas the same pretreatment of 21 serum samples gave an average blank reduction of 67%. Batch experiments with lipophilic gels such as Sephadex LH-20 (Pharmacia), Lipidex 1000 and 5000 (Packard Instruments Co.), and Frigen (Behringwerke) yielded a blank reduction of, at most, 9%.

**Gel filtration.** As shown in Figure 1, about 80% of the substances responsible for the intrinsic light-scattering of HSP followed the albumin peak. The remaining 20% was eluted after albumin in a low-molecular-mass fraction.

**Mixing with PEG followed by centrifugation** (11). We mixed semen with an equal volume of PEG (80 g/L) at room temperature and centrifuged this mixture at various gravi-

| Table 1. Recovery of IgG after Filtration through a 0.22-nm Pore-Size Filter |
|-----------------------------|-----------------|-----------------|
| Seminal plasma IgG          | 67–258 | 11  | 97 ± 4  |
| Purified IgG + NaCl (9 g/L)  | 30–300 | 8   | 0      |
| Purified IgG + albumin (18.6 g/L) | 30–300 | 8   | 0      |
| Purified IgG + PEG (40 g/L)  | 30–413 | 11  | 98 ± 3  |

*Kabi AB, Stockholm, Sweden.

**Fig. 1. Gel filtration of 0.5 mL of seminal plasma through a 0.7 × 27 cm column packed with Sephadex G-50 fine and eluted with phosphate-buffered saline (pH 7.4)**

Fractions of 1.1 mL were collected.
tational forces (1500–40 000 × g) for 30 min. At 35 000 × g the mean blank reduction was 95.6% (SD 2.7%) (n = 17). To determine the resistance of the different proteins tested to precipitation by PEG, we mixed semen or serum with an equal volume of PEG to make final PEG concentrations of 0, 20, 40, 60, 80, and 100 g/L. The mixture was then centrifuged (35 000 × g, 30 min) and the concentration of the specific protein in the supernate assayed by immunonephelometry (Figure 2). Except for lactoferrin and IgM, none of the other proteins precipitated appreciably with the 40 g/L solution of PEG. Electrophoretic studies of the precipitate formed with PEG (40 g/L) showed that lactoferrin was one of the main components, but further attempts to characterize the nature of the precipitate were not undertaken.

Immunonephelometry

Sensitivity. The sensitivity of the LSA-290 nephelometer is regulated by adjusting the "span" knob. The results obtained with transferrin in concentrations ranging from 2.5 to 82.5 mg/L and various sensitivity settings are shown in Figure 3. Amounts of 25 ng of transferrin (10 μL of a 2.5 mg/L solution) in the reaction tube can be easily determined.

The assay range for the proteins tested was as follows (mg/L): albumin, 3.8–250; α1-antitrypsin, 2.9–296; orosomucoid, 3.7–183; hemopexin, 1.0–77; IgG, 2.5–248; IgA, 1.6–103; and secretory IgA, 3.7–425.

Precision. The reproducibility of the immunonephelometry as determined from repeated measurements of control samples over a period of several months is shown in Table 2.

Quantification of proteins. Once the blank problem was solved, the setting up of immunonephelometric assays for the different proteins was straightforward. Secretory IgA and lactoferrin were the only exceptions to this.

The presence of a mixture of serum-type IgA (7S) and secretory IgA (11S) in human seminal plasma (Figure 4) requires the use of two different antisera to quantify these two types of IgA. The concentration of secretory IgA in the

<table>
<thead>
<tr>
<th>Table 2. Precision of the Immunonephelometric Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>Albumin</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Haptoglobin</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>IgG</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>IgA (7S)</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Table 3. Content of Specific Proteins in Human Seminal Plasma* as Determined by Immunonephelometry

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total proteins</td>
<td>66</td>
<td>30.6</td>
<td>7.4</td>
<td>13.5–48.4</td>
</tr>
<tr>
<td>Albumin</td>
<td>66</td>
<td>1096</td>
<td>443</td>
<td>367–2208</td>
</tr>
<tr>
<td>Transferin</td>
<td>66</td>
<td>68.9</td>
<td>60.1</td>
<td>12.0–324</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>49</td>
<td>124.3</td>
<td>92.6</td>
<td>4.0–298</td>
</tr>
<tr>
<td>Orosomucoid</td>
<td>34</td>
<td>26.6</td>
<td>20.2</td>
<td>4.0–76.0</td>
</tr>
<tr>
<td>Hemopexin</td>
<td>34</td>
<td>7.8</td>
<td>5.4</td>
<td>1.0–22.0</td>
</tr>
<tr>
<td>IgG</td>
<td>49</td>
<td>94.9</td>
<td>55.7</td>
<td>21.0–573</td>
</tr>
<tr>
<td>IgA</td>
<td>49</td>
<td>33.7</td>
<td>18.5</td>
<td>4.0–79.5</td>
</tr>
<tr>
<td>Secretory IgA</td>
<td>34</td>
<td>191.2</td>
<td>75.9</td>
<td>17.0–452</td>
</tr>
</tbody>
</table>

*Specimens from men attending an infertility unit but with morphologically normal ejaculate findings.

The amount of protein present in HSP varies between 18 and 55 g/L (2, 12, 13), an impressive figure compared with the amount in other external secretions under physiological conditions. In our quantitative studies of HSP proteins, we have chosen immunonephelometry because this technique is simple to perform, fast, and sufficiently sensitive, as compared with other immunochemical assays (8, 14). The turbid and opalescent nature of seminal plasma yields very high intrinsic light scattering readings (blanks) for direct immunonephelometric assays. Dilution of HSP with saline to reduce the blank values proved feasible only for the proteins that were present in high concentrations. To measure the other proteins, present in low concentrations, the interfering substances in the blank had to be removed.

Ultracentrifugation of HSP reduced the blank values very little, indicating that the substances causing the high blank values are not particulate. Similar results were obtained with ultrafiltration through membranes of mixed cellulose and acetate esters. The situation is aggravated in the latter procedure by the fact that such filters have been reported to adsorb proteins, especially IgG (16). In our experiments (Table 1) neither the IgG present in seminal plasma nor purified IgG diluted with PEG (40 g/L) was lost. However, IgG diluted with saline or with an albumin solution was adsorbed by the filters. Seminal hemopexin was also lost after ultrafiltration. Because the cellulose esters in the filter membranes act as ion exchangers and adsorb different proteins in various unpredictable proportions, we decided not to use them in our work.

High blank values in serum have been related to lipoprotein–triglyceride complexes (11, 14). Lipoproteins have not been identified in HSP (13), but seminal plasma nevertheless contains lipophiliic material—phospholipids, cholesterol, and triglycerides arising mainly from the prostate. Removal of the seminal plasma lipids by treatment with dextran-sulfate, Frigen, or various lipophilic gels reduced the blank values very little, suggesting that the substances responsible for the high blank values in HSP are not lipids or lipid-like.

The gel filtration data (Figure 1) showed that the bulk of the blank-producing substances are of high molecular mass, and conceivably may be glycoproteins "specific" for seminal plasma, polysaccharides, and (or) mucopolysaccharides reported to be present in HSP (2, 17).

Hellsing and Enström (11) have reported a pretreatment schedule for serum and plasma samples with PEG. When we applied this procedure to seminal plasma samples, the removal of the high-blank-producing substances was almost complete (95%) and allowed direct immunonephelometry of...
specific proteins in HSP. The mechanism(s) by which PEG clarified seminal plasma is not well understood. Because PEG has been used as a precipitating agent in the fractionation of protein mixtures (18), we performed the experiments reported in Figure 2 to determine the stability of the various proteins in solutions of PEG. At a PEG concentration of 40 g/L only IgM and lactoferrin precipitated, whereas the other nine proteins remained essentially unchanged. These results confirmed the reported data on the solubility of various proteins in solutions of linear polymers (for a review, see 19). The resistance to precipitation by PEG of Cg-globulin, retinol-binding protein, secretory IgA, and lactoferrin has not been studied in detail before.

Protein Content of HSP

Several groups of researchers have reported quantitative studies of HSP proteins in a search for compounds that could be diagnostically useful in the laboratory evaluation of infertility. We used immunonephelometric assay to quantify eight proteins in seminal plasma obtained from morphologically and biochemically normal human semen samples (Table 3). Results with abnormal samples and clinical correlations between groups will be published elsewhere.

The albumin values obtained by us are in accordance with those reported by Lindholmer et al. (7). Others have reported mean values between 590 and 830 mg/L (12, 20–22). Reported transferrin concentration in HSP varies between 10 and 300 mg/L (mean, about 80 mg/L) (12, 22), which is in agreement with our data. The alpha-, antitrypsin concentration in HSP has been measured by several investigators (12, 23, 24), who found mean values of 80 to 102 mg/L (range 57.5–146.5 mg/L) by immunodiffusion. Our mean value was 124.3 mg/L (range 4–298 mg/L). Blenk et al. (22) studied 30 normal ejaculates and found that orosomucoid was present in a mean concentration of 30 mg/L (range 1–100 mg/L), quite in accordance with our data (mean 26.6 mg/L, range 4–76 mg/L). Our results on seminal hemopexin constitute the first quantitative estimation of the concentration of this protein in human seminal plasma.

The suggestion that infertility may have an immunological cause prompted many researchers to study the immunoglobulins present in HSP, and today it is well established that IgG is present in normal HSP (25). Immunonephelometric assay of seminal IgG in our samples afforded values similar to those reported by others, i.e., about 1% of that found in the serum (12, 13, 22, 25).

The main immunoglobulin in external secretions is secretory IgA (11S), composed of two molecules of monomeric IgA (serum type, 7S) linked together by the J-chain and the secretory piece. Abundant evidence indicates that most of the secretory IgA is locally produced and protects the mucosal surfaces, and that it is resistant to the action of proteases (26). Seminal plasma contains a mixture of serum-type and secretory IgA (Figure 4). Various authors have reported values on the order of 0 to 60 mg of IgA per liter of HSP. These values should be regarded with caution, however, because the methods used were not able to distinguish between serum-type IgA and secretory IgA (12, 13, 22, 25). The report by Uehling (20) is the only one dealing with quantitative (radial immunodiffusion) studies of both types of IgA in seminal plasma from fertile and infertile patients. In a group of 54 fertile patients he found a mean value of 24 mg/L for IgA and 1550 mg/L for secretory IgA. We obtained a mean value of 33.7 mg/L for IgA and 191.2 mg/L for secretory IgA. The reason for this discrepancy is not clear to us. The standard used by Uehling was prepared from colostrum after precipitation with sodium sulfate, dialysis, and passage through a Sephadex G-200 column. The first protein peak eluted was used for immunization of rabbits and as a standard in radial immunodiffusion (27). The authors did not state that this purification scheme leads to pure secretory IgA, and we therefore believe that the high concentrations of secretory IgA in HSP obtained by Uehling are due to cross reaction with compounds related to large molecules in colostrum. Studies on the degradation of immunoglobulins by HSP (28) and our own analytical recovery experiments show that seminal plasma does not destroy the immunological activity of secretory IgA nor prevent its assay by immunonephelometry. Soliman and Olesen in a short communication (29) report measuring the concentrations of secretory IgA in HSP by nephelometry but, lacking an appropriate standard, give the values in arbitrary units, which cannot be compared with our data.

In conclusion, it is now possible to quantify specific proteins in human seminal plasma by immunonephelometry. The availability of such a reliable, fast, and relatively inexpensive method should soon find ready application in institutions dealing with the clinical and laboratory evaluation of the male partner in infertility unions.

The excellent technical assistance of Per Olof Sundh is fully recognized. Supported by the Swedish Medical Research Council (B-81-17x-06996-01) and Karolinska Institutets Forskningsfonder.

References