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Estimation of Anti-Spermatozoa Antibody Concentrations by a [^{125}I]Protein-A Binding Assay in Sera of Infertile Patients

By *A. B. Czuppon* and *L. Mettler*

Abt. Frauenheilkunde, Biochem. Reprod. Lab. am Klinikum der Christian-Albrechts Universität Kiel

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Summary: A solid-phase radioimmunoassay was developed for measuring the concentration of antispermatozoa antibodies in the sera of sterile patients. Whole human spermatozoa fixed in methanol and immobilized on plastic microtiter plates were used as antigens, and the spermatozoa-bound antibodies were quantitated with the aid of [^{125}I]protein-A. The assay is specific, simple to perform and up to 1000 tests can be carried out conveniently by one technician on a single working day. The new test was shown to correlate better with immunological sterility than the sperm microagglutination and immobilisation tests. An antispermatozoa antibody concentration of 210 µg/l and above was found be indicative of an immunological cause of infertility.

Bestimmung der Konzentration gegen Humanspermatozoen gerichteter Antikörper in Seren steriler Patienten durch einen [^{125}I]Protein-A Immunsorbent-Test

Zusammenfassung: Es wurde ein Festphasen Immunsorbent-Test für die Bestimmung der Konzentration gegen Humanspermatozoen gerichteter Antikörper in Seren steriler Patienten entwickelt. Fixierte intakte menschliche Spermatozoen, die auf Mikrotiterplatten aufgetragen sind, dienen als Antigene. Die an die Spermien gebundenen Antikörper werden durch ^{125}Iod-markiertes Protein-A indiziert. Dieses Testverfahren ist einfach durchzuführen, und es können bis zu 1000 Bestimmungen von einer technischen Assistentin an einem Tag durchgeführt werden. Der neue Test zeigt eine bessere Korrelation mit anhaltender Sterilität als die üblichen Mikroagglutinations- und Mikroimmobilisationstests für Spermien. Eine Konzentration gegen Humansperma gerichteter Antikörper von 210 µg/l deutet auf eine immunologisch bedingte Sterilitätsursache hin.

Introduction

Several methods have been described for the detection of antispermatozoa antibodies (1–5). Most of these tests rely on judgements of observation. In addition some are known to be adversely effected unspecifically by e. g. hormones or drugs present in the serum of the patient (6). None of the presently used tests can quantitate the antispermatozoa antibody concentrations in the sera.

In this paper we report the development of a new solid-phase protein-A binding assay that permits the estimation of antispermatozoa antibody concentrations in µg/l. Bacterial protein-A (*Staph. aureus*) is known to bind specifically to the Fc portion of IgG molecules in a number of species, including man (7). The binding to human IgG is restricted to the IgG_1, IgG_2 and IgG_4 subclasses.

This property of the protein-A molecule has been utilized by various investigatiors to quantitate immunoglobulins.

Materials and Methods

Protein A

Protein-A (*Staph. aureus*, Cowan strain) with an IgG binding capacity of 11 mg/mg was obtained from Sigma Chem., Munich FRG.

Radioiodination of protein-A

The radioiodinating reagent N-3-(4-hydroxy-3,5-diiodophenyl)propionyloxy succinimide (148 TBq/mmol) (*Bolton-Hunter* Reagent) was purchased from New England Nuclear, Dreieich FRG. In a typical labelling experiment 11.1 MBq of the *Bolton-Hunter* reagent and 5 µg of the protein-A were incubated in 0.01 mol/l phosphate buffer, pH 8.0 for 30 min at 0 °C and for 24 h at 4 °C. The prolonged incubation ensures that all the ester groups are either conjugated or hydrolysed (11). This obviates the need for inhibition by addition of an amino acid mixture. Following the incubation the [^{125}I]protein-A was purified on a Sephadex-G 25 column. The column was equilibrated and eluted with a 0.01 mol/l phosphate buffer pH 7.2, containing 1 g/l gelatin. One ml fractions were collected and the first radioactive peak fraction was aliquoted into 100 µl portions. The aliquots were kept at −20 °C until use.

Purification of antispermatozoa IgG

Different volumes of sera from sterile patients were pooled to give ca. 100 ml and dialyzed against 0.05 mol/l Tris-HCl buffer pH 6.5 for 72 hours at 4 °C. The spermagglutinating titer of the pooled serum was estimated before dialysis and was found to be positive at 1 : 512 dilution. The dialysed serum was then applied to a diethylaminoethylcellulose column (1.8 × 50 cm), which had been previously equilibrated with the above starting buffer. Elution commenced with a linear gradient of 0.1−1 mol/l NaCl with the same buffer. Ten ml fractions were collected at a flow rate of 1.5 ml/h. The fractions in the first peak were tested for purity by radial immunodiffusion and used in the solid-phase assay at a protein concentration of 100 mg/l (see below).

Preparation of the microtiter plates with target cell antigens

Spermatozoa from healthy donors, with normal spermiogram and an average sperm count of 2.5 × 10^{10}/l, were washed three times in 0.06 mol/l phosphate buffer in 0.15 mol/l NaCl pH 7.2 at 600 *g* for 10 min at 4 °C.

After the final centrifugation the cells were resuspended in the above buffer at a concentration of 3 × 10^9/l. The cell suspension was then distributed in 100 µl portions into the U-bottom wells of microtiter plates (Dynatech Laboratories Inc., Massachusetts, U.S.A.). The plates were then centrifuged at 150 *g* for 10 min, 4 °C and the supernatants decanted. Approximately 100 µl of methanol was added to each well and the plates were centrifuged for another 10 min. After removal of the supernatants the rest of the methanol was allowed to evaporate. The attached cells were washed three times with 200 µl of cold phosphate-buffered saline. The plates were then incubated for 15 min at room temperature in a phosphate buffered saline, containing 1 g/l bovine serum albumin and 0.5 ml/l Tween- 20 and stored at −20 °C until use. Only wells with monolayers of spermatozoa were used for the assays.

Solid-phase-protein-A binding assay

In the routine assays of patient sera the sera were diluted 1 : 4 with 0.06 mol/l phosphate buffer pH 7.2 in 0.15 mol/l NaCl. One hundred microliters of the diluted sera were pipetted into the microtiter wells and the plates were incubated for 60 min at 25 °C and for 60 min at 4 °C. Following the incubation the wells were rinsed three times with the ice cold buffer, and 100 µl of [^{125}I]protein-A (1.11 kBq) was added to each well. This was followed by another incubation of 60 min at 4 °C. At the end of the incubation the wells were rinsed again with the same buffer and the plastic wells were cut out. Each of the cut out wells was put into a plastic tube (20 × 160 mm) and these were counted in a BF 5000 61 γ-counter for 1 min each.

Assay validation

Coating the microtiter plates at different target cell concentrations

Various concentrations of spermatozoa were tried in order to establish optimal assay conditions. The wells were coated with 3 × 10^2, 3 × 10^3, 3 × 10^4, 3 × 10^5 and 3 × 10^6 spermatozoa. The solid-phase assay was performed as described, using the purified antispermatozoal IgG fraction at a concentration of 200 µg/l. Nonspecific binding was calculated by substituting the IgG with 0.06 mol/l phosphate buffer pH 7.2 in 0.15 mol/l NaCl.

Estimation of spermatozoa-bound antibody concentration with the purified antispermatozoa IgG

The peak IgG fraction (number 19) was diluted to contain 2000, 200, 20 and 2 µg/l IgG with 0.06 mol/l phosphate buffer pH 7.2 in 0.15 mol/l NaCl, and assayed in the solid-phase assay. The conditions for the solid-phase assay were the same as described above.

The specific activity of [^{125}I]protein-A was calculated from the inhibition assay (see below). For the estimation of spermatozoa-bound IgG, the quantity of bound [^{125}I]protein-A (in ng) was multiplied by the binding capacity e.g. 11. Various phosphate buffers with the same ionic strength but different pH (6.5, 7.0, 7.2, 7.5) were tested in order to achieve optimal [^{125}I]protein-A binding.

Inhibition of [^{125}I]protein-A binding with cold protein-A in the solid-phase assay

A 200 µg/l solution of the purified antispermatozoa IgG fraction was used in the assay. Increasing concentrations of cold protein-A, from 0.25, 50, 100, 150, and 200 µg/l were added to [^{125}I]protein-A (5.38 KBq), and the amount (ng) of bound [^{125}I]protein-A on the spermatozoa-antibody complex was estimated at 0.5 inhibition of total binding.

Adsorption of antispermatozoa antibodies with unfixed human spermatozoa

In order to check whether the spermatozoal membrane antigens are affected by methanol fixation, and to establish specificity, the purified antispermatozoa IgG was adsorbed with fresh human spermatozoa. One hundred microliters of diluted IgG, 1 : 5000 in 0.06 mol/l phosphate buffer pH 7.2 containing 0.15 mol/l NaCl, was incubated with 10^7 washed spermatozoa at a spermatozoa concentration of 10^{11}/l for 24 h at 4 °C. Following the incubation the mixture was centrifuged at 6000 *g* for 30 min and the supernatant was incubated again with new spermatozoa under the same conditions.

This process was repeated twice and finally the supernatant assayed in the solid-phase test.

Adsorption of the positive control serum (see quality control) was done similarly, except that the incubation was repeated only once.

Quality control

For an accurate calculation of the specific activities of [^{125}I]protein-A at a given time, the isotope decay chart of ^{125}I was used. The BF 5000 G 1 γ-counter was calibrated using a simulated ^{125}I radiation source standard (^{129}I nuclide in propylene RIA-tube, 370 Bq. New England Nuclear, Dreieich FRG). Rabbit antihuman-spermatozoa antiserum diluted 1 : 4 in 0.06 mol/l phosphate buffer (Behringwerke, Marburg/Lahn, FRG) was used routinely with each set of patient sera to maintain quality control of the coated plates, [^{125}I]protein-A binding and assay conditions.

Only control sera that showed a total [^{125}I]protein-A binding of 0.65 (± 0.05) were used for quality control.

Test sera

The sera of sterile patients were sent to our laboratory for routine diagnosis of antispermatozoa antibodies. All patients had wanted children for at least one year and no other clinical tests could explain their persistent infertility.

A group of fertile couples, i.e. last child born within the past two years, served as negative controls. The groups included husbands or wives or both.

Sperm-micro-agglutination and immobilisation tests

The microscopic tests of sperm-agglutination and sperm-immobilisation were carried out as described by *Friberg* (2) and *Hjusted & Hjort* (1) respectively.

Results

Radioiodination of protein-A resulted in specific activities of 222 to 296 kBq/ng protein-A. Keeping the labelled product at −20 °C for up to three weeks had no significant effect on the sensitivity of the immunoassay, when the aliquots were stored with gelatine carrier and the samples were thawed only once. In this study, protein-A with an IgG binding capacity of 11 mg/mg was used throughout. The assay sensitivity and reproducibility was not effected when protein-A with lower binding capacities were tried, but allowances had to be made in calculating the bound IgG concentrations.

The pH of the reaction mixture seems to be an important factor, probably because of the pH dependence of protein-A binding to the IgG subclasses (12). The assay seems to be optimal at pH 7.2 and this is achieved conveniently by use of phosphate buffered saline.

Various concentrations of the spermatozoa target cells (antigen) showed that under the conditions described maximal binding occurs at 3×10^5 spermatozoa per well (fig. 1). Higher cell concentrations resulted in multi layers of the coated cells without significant elevation of antibody binding. Therefore a cell concentration of 3×10^5 per well was chosen for all assays.

The linearity of the antibody concentration determined against spermatozoa with the $\log_2$ titer of rabbit antihuman spermatozoa antiserum is shown in figure 2. A correlation coefficient of $r = 0.989$ was obtained, while the C.V. of interassay variation was 0.08%. Day to day variation of the control serum was estimated to be ≤0.1 over a period of one year. Non specific binding with normal serum amounted to 0.05 and with phosphate buffered saline to 0.03.

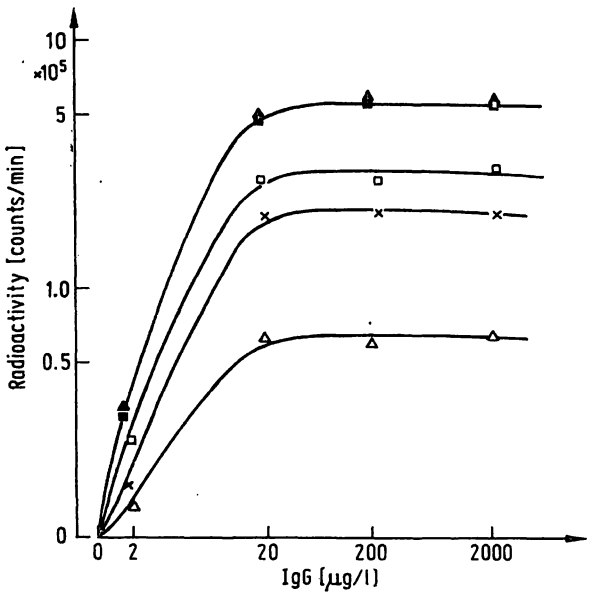

Fig. 1. Dependence of the concentration of bound [^{125}I]protein-A on the number of target cells. The plates were coated with increasing numbers of spermatozoa (△ $0.3 \cdot 10^3$; x $3 \cdot 10^3$; □ $30 \cdot 10^3$; ■ $300 \cdot 10^3$; ▲ $3000 \cdot 10^3$) and purified antispermazotoa IgG was used as antiserum. The assay was carried out as described in Materials and Methods. A spermatozoa concentration of $3 \cdot 10^5$ per well was chosen for the routine assays.

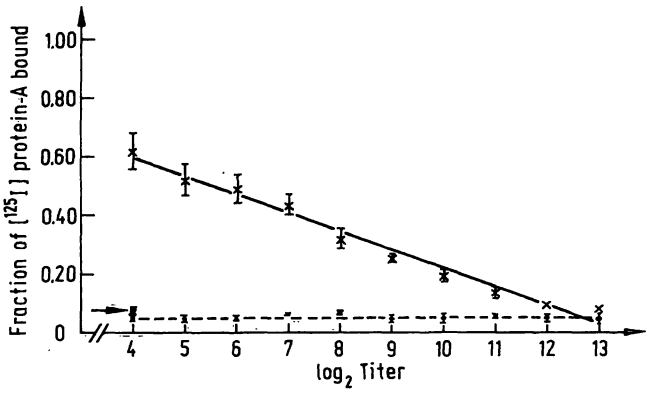

Fig. 2. A typical calibration curve of the solid-phase immunoassay with [^{125}I]protein-A and the positive control serum (Behring Werke). Values represent mean ± 2 × S.D. (x) serially diluted positive control serum; arrow at left = negative levels following adsorption of the positive control serum with fresh, unfixed human spermatozoa (□); non-immune rabbit serum (▲).

The specificity of the solid-phase immuno assay is demonstrated by the fact that absorption of the control serum with unfixed, fresh spermatozoa, prior to the assay, resulted in negative antibody levels (fig. 2). Similar results were obtained using normal rabbit serum.

DEAE-cellulose affinity chromatography of the pooled patient sera showed an IgG peak in fractions

13–21. Fraction 19 was found to contain the highest anti-spermatozoa antibody concentration. The solid-phase immunoassay with that fraction (fig. 3) indicated maximal protein-A binding at concentrations of 20 µg/l and above. The specificity of [^{125}I]protein-A binding is indicated by the fact that the reaction can be inhibited quantitatively by unlabelled protein-A (fig. 4). A 0.5 inhibition is obtained with 2.1 ng of cold protein-A. This was not significantly different from calculations based on the specific activity alone (1.95 ng).

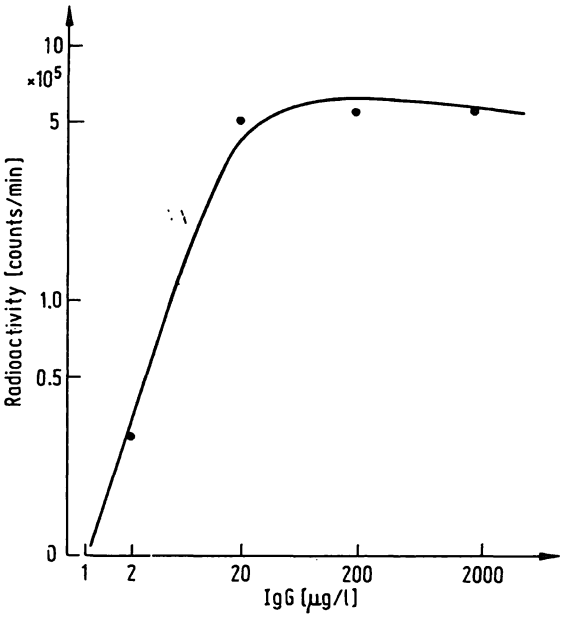

Fig. 3. Solid-phase immunoassay with purified antispermatozoa IgG from pooled sterile patient sera of various dilutions. The spermatozoa-bound IgG concentrations were calculated using the specific activity of the [^{125}I]protein-A and an IgG binding capacity of 11 mg/mg protein-A (see Material and Methods). Maximal protein-A binding was observed at IgG concentrations of 20 µg/l and above.

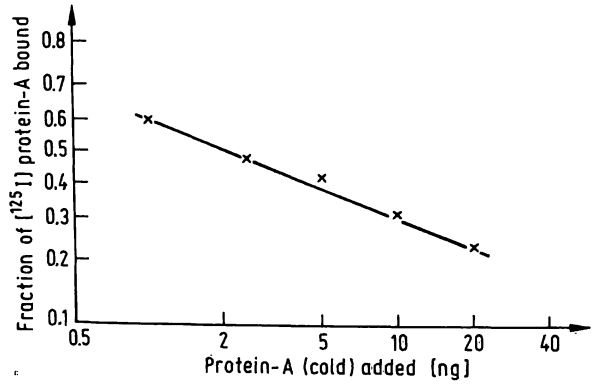

Fig. 4. Quantitative inhibition of the [^{125}I]protein-A by unlabelled protein-A in the solid phase immunoassay. The value obtained at 0.5 binding (2.1 ng) was not significantly different from that of 1.95 ng, calculated from the specific activity of [^{125}I]protein-A alone.

The positive control serum, rabbit antihuman spermatozoa antiserum was found to have an average antispermatozoa antibody concentration of 1122 µg/l (± 102). Normal rabbit serum showed antibody levels of 41 µg/l, (±12); and the antibody concentration of rabbit antiserum was reduced from 1122 µg/l to 57 µg/l (±16.6) by adsorption with human spermatozoa (tab. 1).

The routine assay of the sera of patients revealed a wide range of antispermatozoa antibody concentrations from as low as 54 µg/l to as high as 1098 µg/l. There was also an increasing tendency for an elevated antispermatozoa antibody concentration with progressive years of persistent infertility in these groups. The average antispermatozoa antibody concentration ranged from 472 µg/l with patients of one year of wanting pregnancy to 717 µg/l for one patient with a history of 16 years of sterility (tab. 2).

A comparison of the antibody concentrations in the solid-phase assay with the sperm microagglutination and immobilisation tests in sera of a randomly selected subpopulation of sterile patients (tab. 2) indicates a significant disagreement between the two type of tests. Only about 15% of the sera in the infertile groups was also positive in the sperm microagglutination/immobilisation tests. In these positive sera there was no correlation between the titers in the sperm agglutination/immobilisation and antispermatozoa antibody concentrations in the solid-phase assay. One serum, for example, with an antibody concentration of 1098 µg/l was still negative in the sperm microagglutination and immobilisation tests.

Tab. 1. Results of the routine quality control of the solid-phase [^{125}I]protein-A binding assay during evaluation of the patient sera. The positive control serum rabbit antihuman spermatozoa antiserum was included with each set of assays. Values represent the mean ± S.D. Each assay was done in triplicate.

Control sera (N)	Antispermatozoa IgG (µg/l) $\bar{x} \pm s$	Number of positives in microscopic tests
Rabbit-anti-human spermatozoa (168)	1122 (± 102)	168
Normal rabbit serum (168)	41 (± 12)	0
Rabbit-antihuman spermatozoa adsorbed with human spermatozoa (10)	57 (± 17)	0
Fertile couples (10)	[1])166 (54 – 204)	4

[1]) = mean and range of IgG concentrations indicated.

On the other hand two of the patients with proven fertility and an antispermatozoal antibody concentration of as low as 200 µg/l had a positive titer 1 : 16 in the sperm microagglutination test. Additional false positives of 1:4 to 1:8 were found in the fertile group (tab. 1). In the group of patients with proven fertility the average non-specific antibody concentration was found to be 166 µg/l by the solid-phase assay. This was significantly lower than the antibody concentrations in all the sterility groups (*Student*'s t-test, $p < 0.05$).

Discussion

Radioiodinated protein-A has been used previously to quantitate IgG in various solid and liquid phase immunoassays (8, 9). As shown previously, the predominant Ig class in sera of sterile patients is IgG (13). Furthermore, there is a subclass of antispermatozoa IgG antibodies, which is present naturally in sera, and which reacts with staphylococcal protein-A (10). Taking these facts into consideration, a solid-phase [^{125}I]protein-A binding assay was developed that is relatively simple to perform and is specific for detection of antispermatozoa antibodies in immune sera. In addition, under carefully controlled assay conditions, the direct estimation of antispermatozoa antibody concentration is possible, for which no other tests are available at present. For adequate quality control the specific activity of the radioiodinated protein-A should be accurately estimated and day to day corrections should be made with the aid of the ^{125}I decay chart.

Where a uniformity of the labelling procedure with regard to specific activity, radiochemical purity etc., cannot be safeguarded, a simple inhibition assay with cold protein-A can be set up to estimate bound [^{125}I]protein-A in ng. Alternatively, radioiodinated protein-A preparations that are available commercially can also be used.

There is no need to use purified human antispermatozoal IgG for a calibration standard. It was used in the present work only for initial assay validation tests. A rigorous quality control can also be maintained with a polyvalent positive control serum, which is also available commercially. However, since there may be variations in the antispermatozoa antibody concentrations in the various batches, either the same lot numbers should be used or adjustments made with regard to total protein-A binding. A good positive control serum should give 0.6—0.7 of total binding of [^{125}I]protein-A under the assay conditions.

Similarly there could be some variations in the (hydrophobic) properties of the plastic surface of the microtiter plates that are supplied by the different manufacturers. In our laboratory the plates from Dynatech Co. proved to be of best quality, with regard to reproducibility. The coated plates with spermatozoa can be stored desiccated for prolonged periods at −20 °C, without affecting the assay reproducibility. The quality of spermatozoa that are used for coating seems to be of lesser importance. In these tests fresh spermatozoa from healthy donors with a normal spermiogram were used throughout. However we also tried coating the plates with spermatozoa that were stored at −20 °C for up to six months, without adverse effects on the assay. The possibility of using soluble spermatozoal antigens in the solid-phase assay is being investigated at present. The correlation of the antispermatozoa antibody concentration, estimated by the solid-phase assay, with unexplained persistent infertility could provide a useful diagnostic test in cases of functional disturbances of fertility.

Tab. 2. Solid-phase [^{125}I]protein-A binding assay of antispermatozoa IgG in sera of patients with progressive years of persistent infertility. The comparison with sperm microagglutination and sperm microimmobilisation is shown in the last column. The mean antispermatozoa IgG concentration of the infertile group was estimated to be 494 µg/l compared to the fertile group of 166 µg/l (see tab. 1). This was found to be significant at the $p < 0.05$ level (*Student*'s t-test).

Infertility (a)	Number of patients	Antispermatozoa IgG µg/l[1])	Number of positives in microscopic tests
1	17	472 (289 – 1098)	5
2	17	455 (224 – 761)	3
3	17	387 (204 – 537)	3
4	17	500 (258 – 897)	5
5	17	377 (265 – 615)	2
6	17	564 (323 – 1054)	1
7	10	404 (272 – 819)	0
8	8	414 (346 – 618)	0
9	8	482 (318 – 1071)	0
10	10	550 (380 – 683)	1
11	10	510 (380 – 595)	1
12	1	595 –	1
16	1	717 –	0

[1]) = Values represent mean IgG concentration and range. All assay were done in triplicate.

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References

1. Husted, S. & Hjort, T. (1975) Clin. Exp. Immunol. *22*, 256–260.
2. Friberg, J. (1974) Acta. Obstet. Gynaec. Scand. Suppl. *36*, 3–21.
3. Hansen, K. B. & Hjort, T. (1971) Clin. Exp. Immunol. *9*, 21–30.
4. Kremer, J. (1965) Int. J. Fertil. *10*, 209–215.
5. Kibrick, S., Belding, D. L. & Merrill, B. (1952) Fertil. Steril. *3*, 430–438.
6. Boettcher, B. (1974) J. Reprod. Fertil. Suppl. *21*, 151–161.
7. Forsgren, A. & Sjoquist, J. (1966) J. Immunol. *97*, 822–827
8. Werblin, T. & Mage, R. (1977) J. Immunol. Meth. *16*, 337–350.
9. Ansari, A. A., Bahuguna, L. M. & Malling, H. V. (1978) J. Immunol. Meth. *23*, 219–226.
10. Skrabei, H., Czuppon, A. B. & Mettler, L. (1980) Arch. Androl. *5*, 13–15.
11. Rudiger, J. & Ruegg, U. (1973) Biochem. J. *133*, 529–539.
12. Duhamel, R. C., Shur, P. H., Brendel, K. & Mezzan, E. (1979) J. Immunol. Meth. *31*, 211–217.
13. Czuppon, A. B., Mettler, L., Schauer, R. & Pawassarat, V. (1981) Hoppe-Seyler Z. Physiol. Chem. *362*, 963–968.

Dr. Dipl.-Biochem. A. B. Czuppon
Klinikum der Christian-Albrechts-Universität Kiel
Abt. Frauenheilkunde
Hegewischstr. 4
D-2300 Kiel