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Studies on Antigenicities of Sperm and Seminal Plasma, and Effects of Their Antibodies on Fertilization in Rabbit

Ⅰ. Antigenicities of sperm and seminal plasma

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家兎에 있어서 精子 및 精漿의 抗原性과 이의 抗體가 受精에 미치는 影響

Ⅰ. 精子 및 精漿의 抗原性

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국립보건원 혈청진단과

적 요

본 실험은 성숙가토의 정자와 정장의 항원성을 조사하여 면역적 불임원인 규명에 필요한 기초자료를 얻고자 시도하였다. 인공질로 채취한 정액을 원심분리하여 얻은 정자와 정장을 항원으로 사용하였으며 항원성의 측정방법은 크로마토그라프에 의한 단백질 분리, SDS-PAGE, HPLC, 한천확산법, 전기영동, 수동적혈구응집방법 및 부동화시험이었다. 얻어진 결과는 다음과 같다.

1. SDS-PAGE 전기영동시 정상가토 정장에서 약 23개의 단백질이 분리되었고, 그중 분자량이 약 20,000되는 단백질부분이 정관절제 수술한 정장에서 나타나지 않았다.
2. 정상가토 정장을 HPLC를 이용한 분석에서 3개의 peaks을 볼 수 있었고, 정관절제수술한 것에서는 peak 1에 해당하는 단백질이 소실되었다.
3. 한천확산시험에서 정상정장은 이종혈청과는 4개의 침강선을 나타냈고, 전기영동에서는 7개의 침강선을 나타냈다.
4. 이종면역에서 정자 및 정장은 항체가 상승이 용이하였지만, 동종면역시는 추가면역이 필요하였으며 개체간의 역가 차이를 보였다. 정자면역한 자성가토에서는 수동적혈구응집반응을 나타냈지만, 한천확산 및 전기영동반응은 보이지 않았고, 같은 처치를 받은 웅성가토에서도 역시 같은 반응양상을 나타냈다.
5. 동종 및 이종항혈청을 이용한 교차전기영동방법으로 정장의 항원적 구성요소를 구별할 수 있었다. 사출된 정액에서 분리된 정장은 일부 항원을 포함하고 있었으며 이는 성숙한 정자에서 기인한 것으로 사료되었다.

Introduction

It has long been recognized that male reproductive tissues are antigenic and their antigenicity implicates in both male and female infertility. Many conflicting results have, however, been reported on the source and origin of sperm specific and miscellaneous antigens in laboratory and farm animals. Most studies on the origin of semen antigens have been conducted through heteroimmunization in many species and partial characterization of some of these antigens has been achieved. It is obvious that many of these antigens detected by heteroimmunization are species specific and such antigens have the potential of eliciting the

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formation of antibodies which could either enhance or interfere with the normal reproductive process. Smith (1949) suggested that while some sperm antigens are found on the cell surface others are restricted to an intracellular localization. Within the class of sperm surface autoantigens, many can be detected by the immobilization reaction (Isojima et al., 1972). These antigens appear to be intrinsic to the sperm plasma membrane (O'Rand, 1977). In the rabbit, the isolation of sperm antigens was first reported by Menge (1971) and at least one large macromolecule present in the testis reduced fertility in female rabbits. More recent works on rabbit sperm membrane proteins (O'Rand and Metz, 1976) and sperm membrane isoantigens (O'Rand and Porter, 1979) have revealed several membrane proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Hunter (1969) reported that rabbit sperm were coated with two antigens origination in the testis, two from the epididymis and eight from above the level of the vas deferens. Barker and Amann (1970) showed that differences in the antigenic spectrum of bull sperm also arise during epididymal maturation. Katsh (1959) reported that appearance of antigen in guinea pig sperm occurred at the late spermatid. Voisin et al. (1974), therefore, isolated and characterized four antigens from guinea pig sperm, each with its own cellular and immunological properties. The three antigens of them are capable of inducing autoimmune orchitis and are acrosomal in antigen. Johnson and Hunter (1979) detected seminal antigens by Ouchterlony analysis using antisera against ejaculated rabbit sperm, seminal plasma and testis. One was a glycoprotein which was antigenically altered in ductuli efferentes-caput epididymis, two were detectable throughout the epididymis and in ejaculated semen, the fourth was only detected with testis, and four seminal antigens also originated in epididymis. Killian and Amann (1973) reported that some antigens present in the rate testis fluid were associated with testicular sperm, while others might be secretory products or enzymes originated in the seminiferous tubles. Olson and Hamilton (1978) revealed by labelling with radioactive markers that new proteins and glycoprotein are incorporated into the sperm plasma membrane during maturation. Moore (1980) suggested that some of these proteins clearly originate from epididymal epithelial cells.

Killian and Amann (1973) also detected the presence of certain antigens in the vesicular gland fluid on testicular as well as epididymal sperm. Bedford (1965) found that sperm show an increasing inclination to agglutinate as they pass down the epididymis, and that this agglutination is essentially head-to-tail agglutination when suspended in a medium containing heat inactivated serum. Immunodiffusion tests have revealed that under normal physiological conditions, the caput and cauda epididymis probably secrete antigens in addition to those secreted by the testis or caput epididymis (Barker and Amann, 1971). Mellad and Hines (1977) reported that bovine semen has at least four isoantigens. No visible precipitin reaction was observed when such sera were diffused against vesicular gland fluid, but all four antigens were present in the testicular fluid, cauda epididymal plasma, cauda epididymal sperm and caput epididymal fluid, indicating that they arise from the testis.

Weil and Finkler (1958) proved evidence that female rabbits responded similarly to males in antibody production when injected with pools of rabbit semen and/or seminal

plasma. A female rabbit responded in a manner quite similar to that of male animals (Shulman et al., 1968). Sperm may take up some antigenic materials from the seminal plasma at the time of ejaculation. This effect was discovered by Weil and Rodenburg (1960) in rabbits and human, and these materials have been termed the sperm-coating antigens. The seminal vesicle seems to be the source of these antigens. It is suggested that the sperm-coating antigen of rabbit is the active antigen in isoimmunization with either the male accessory glands extract or with seminal plasma. This antigen seems to dominate the immunologic behavior of ejaculated sperm. Recent experiments by Stites and Erickson (1975) proved that seminal plasma exerted a immunosuppressive effect. Prakash et al. (1976) reported that bovine seminal plasma also possed similar immunosuppressive activity. Lord et al. (1977) also supported the concept that a local immune response against sperm in the female reproductive tract is antively suppressed by a component in seminal plasma. Its presence in the semen of a vasectomized individual indicates that it is of prostatic or seminal vesicular origen.

This experiment was designed to investigate the antigenicities of sperm and seminal plasma of rabbit in heteroantisera and isoantisera as well as to analyse the protein profile of normal and vasectomized seminal plasma.

Materials and Methods

1. Animals

Rabbits: New Zealand white bucks and does weighing 3.0 to 4.5kg were allocated to this experiment. Four vasectomized bucks and seven normal ones were used for collection of semen. Female rabbits were randomly assigned to several groups to aid in the characterization of isoantigens of sperm and seminal plasma.

Guinea pigs and rats: Male guinea pigs weighing 300 gm and male rats weighing 200 gm were used for heteroimmunization against rabbit semen.

2. Antigens

Semen, collected with an artificial vagina from the ten bucks, was pooled. Seminal plasma was removed by refrigerated centrifugation at 10,000 rpm for 30 minutes and stored at -40°C. On the other hand, sonicated homologous sperm antigens were prepared by intermittent sonification of five times frozen and thawed rabbit sperm by a cell ultrasonic homogenzier. For the preparation of semen antigens, three-fold dilutions of semen were mixed an equal volume of Freund's complete or incomplete adjuvant, whereas for sperm and seminal plasma antigens, antigen dilutions were made in PBS (pH 7.2) of 2mg/ml and 4mg/ml, respectively, and mixed with Freund's complete adjuvant for the first two injections and with incomplete adjuvant for over third immunizations. Protein concentrations of the antigens were measured by Lowry's method (1951).

3. Analysis of seminal plasma protein

Fractionation of seminal plasma: Macromolecules of pooled seminal plasma were separated by gel-filteration on Sepadlex G-200. The buffer system was 0.1M Tris-HCl, pH 8.0, with 0.15M sodium chloride. Separation was achieved at 4°C on a 2.5 x 25cm column with a flow rate of 30 ml per hour. The effluent was collected in 4 ml increments, and relative protein concentration was determined by ultraviolet light spectrophotometry at 280 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis: The primary structure of proteins of seminal plasma obtained from both normal and vasectomized bucks was compared by the SDS-polyacrylamide gel electrophoresis with discontinuous buffer system, what is called Laemmli's modification of Davis (1964). To determine the molecular weight ranges of them, standard protein mixture was included. Solutions for electrode buffer (pH 8.3), separating (pH 8.8) and stacking gel (pH 6.8) were prepared indluding 0.1 (w/v) % SDS. Samples for electrophoresis were incubated in boiling water bath for 2 to 3 min. After addition of equal volume of double strength sample buffer. One to 2 mg of proteins were loaded and electrophoresis was carried out at 15°C. After electrophoresis, the gels were fixed and stained in a solution of 0.025% coomassie brilliant blue R in methanolic solution. The dried gel was then scanned with the LKB laser densitometer at 632.8 nm.

Separation and identification of seminal plasma proteins by high performance liquid chromatography (HPLC): The liquid chromatograph consisted of the following components; Model 6000 A pump (Waters Associated, Milford, MA); Model U6K injector (Water); a prepacked 4.4mm id x 30cm stainless steel column; ·u-Bondapak C-18 (Water); Model 440 UV detector at 254nm. The combination of 720 system controler and 730 Data Model (Water) was employed. The solvent used in the experiment consists of 0.1% trifluoracetic acid (TFA) in 80% CH_3CH water. After filtering and degassing the solution through a Milliopore 0.45μm filter (Millipore Corp., Bedfore, MA) the analytical profile of a 20μl sample in the solution was determined on u-Bondapak C-18 column. The samples included normal and vasectomized seminal plasma of rabbits which had been ultracentrifuged.

4. Immunization

Sperm antigen: For primary immunization, sonicated sperm antigen dilutions (2mg/ml) were mixed with equal volume of Freund's complete adjuvant (Difco) and administered twice in multiple subcutaneous injections into rabbits. Two weeks later, subsequent multiple subcutaneous injections of incomplete adjuvant plus sonicated sperm antigens, were given twice weekly to each animal. After an interval of 2 months, each animal received a booster injection by the same scheme for the primary immunization. But incomplete Freund's adjuvant was substituted for complete Freund's one. For each immunization course, each animal received 2ml of 2mg/ml of sonicated sperm.

Seminal plasma antigen: Seminal plasma dilutions (4mg/ml) plus the equal volume of Freund's complete adjuvant were injected subcutaneously four times weekly into female rabbits. The rabbits were given one booster injection after two months of the last injection. Among these, two rabbits were, in addition to that, immunized with the same homologous seminal plasma to maintain high antibody titers.

Semen antigen: Subcutaneous immunization was accomplished as mentioned above. Semen pooled from 10 bucks were diluted three-fold in PBS (pH 7.2) and homogenized with a cell ultrasonic homogenizer. The homogenized semen suspension was mixed with equal volume of Freund's complete adjuvant and homogenized again. For primary immunization the antigen injections were subcutaneously performed and followed by incomplete adjuvant complex twice weekly. Booster immunization courses

with two injections were given in guinea pigs and rats, and rabbits. The amounts of the antigen solutions given to the aminals were 2 ml of the adjuvant complex for a rabbit, and 1 ml for a guinea pig and a rat, respectively.

5. Determination of specific antibodies

Agglutination test: Agglutinating antibodies to sperm were determined by a modification of the tube-slide agglutination test (Friberg, 1974). Serial twofold dilutions of samples were made: a 50μl of each dilution was mixed with 10μl of the sperm suspension (10×10^7/ cells/ml) diluted in warm Baker buffer solution (pH 7.2) in a microplate well and incubated on a slide warmer at 37°C for 60 minutes. After the incubation, the reciprocal value of the highest dilution showing the approximately 50% agglutination of sperm cells examined under an inverted microscope was designated as agglutinating antibody titer.

Sperm immobilization test: The sperm immobilization antibody test was performed according to a modification of Isojima et al. Rabbit sperm of at least 50% motility and good forward progress were diluted to 10×10^7 cells per ml in prewarmed Barker buffer solution (pH 7.2). To microplate wells were added 50μl dilutions of inactivated test sera, 10μl of the rabbit semen dilution. As a mixture for detecting any non-specific sperm immobilizing activity of the test semen dilution, this consists of 60μl of the test serum plus the semen dilution. The mixtures were incubated at 37°C for 1 hr. Each mixture in the well was examined on the inverted microscope (200x). Then the percentage of motility was measured with positive results having motilities of one half or less that of the negative control mixture.

Passive haemagglutination test: The passive haemagglutination test, as described by Stavitsky (1954), was utilized to detect the presence of antibodies in the test sera. The test was modified in that the fresh sheep RBC were formalized for preservation before coating. Serial dilutions of the test sera were prepared with PBS (pH 7.2) containing the 1/100 (v/v) inactivated normal rabbit serum absorbed with sheep RBC. The tests were performed in the microplates containing both 25μl of the test serum dilution and the sheep RBC coated with antigen by tannic acid. The antigenic components consisted of seminal plasma (2mg/ml) and sperm homogenized suspension (2mg/ml).

Agar gel diffusion test: The agar gel diffusion slide tests were performed as described by Lazear (1958). Noble agar (Difco) was dissolved in veronal buffer (pH 8.6) at a 1% agar concentration. The test serum (10μl) in all cases was placed in the central well, and the fluids examined for antigenicity (10μl) were distributed in the peripheral wells. The fluids tested for antigencity were pooled seminal plasma, sonicated sperm and the fluids harvested from different areas of the male reproductive tract plus liver and kidney. Guinea pig antirabbit semen serum was absorbed with washed sperm (7×10^8 cells/ml) and with the suspension of testis, ductus deferens, head and tail epididymis. Antisera were mixed with antigens in the ratio of 1:1 incubated for 30 min at 37°C and centrifuged (2,300 g) to remove particulate matters. As control, unabsorbed antisera were diluted 1:1 with saline. Incubation was allowed to occur in a humid chamber at 4°C, and readings were recorded after 3 days.

Crossed immunoelectrophoresis: The crossed immunoelectrophoresis tests were performed as described by Clarke and Freeman (1968).

Seminal plasma was separated electrophoresically in an agarose gel. The Separated proteins were then run, at a right angle to the first direction, into a gel containing antibodies to the proteins. A 15 ml agarose tube in the water bath was poured onto the plate. Seminal plasma antigens were applied into the punched wells and the plate placed on the colling surface of the electrophoresis apparatus (LKB). To 9 ml agarose tubes cooled down to 55°C in the water bath, 250μl guinea pig antirabbit serum and rabbit antiseminal plasma serum each was added and mixed well without bubbles and the antibody containing gel was poured on the cleaned 10x7 cm glass plate. The plates were placed on the apparatus and connected with buffer reservoir. The potential gradient was adjusted to 2.0 V/cm overnight. The plates were cleaned and pressed twice under 5 layers of filter paper for 5 min. The plates were dried by warm air and stained for 10 min in Coomassie R, and detained and dried. Then the plates were examined for the number of precipitates.

Immunoelectrophoretic analysis: Immunoelectrophoretic studies were conducted on almost the same antigenic preparations as used in the agar gel diffusion tests. Sepadex G-200 column fractions of seminal plasma were placed in each well and resolved in an electric field for 100 min at 4 mA per frame. Twenty μl of the antiserum including guinea pig antirabbit semen or rabbit antiseminal plasma sera was added to the respective trough following electrophoresis, and diffusion was allowed to happen for 3 days in the refrigerator.

Results

1. Macromolecular fractionation

Rabbit seminal plasma components were separated on a Sephadex G-200 into four fractions designaed I to IV. A typical elution pattern is presented in Fig. 1. Fraction 1 contained predorminantly void material, which had molecular weights of 50,000. Fractions II and III had molecular weights of 48,000 and 18,000, respectively. Fraction IV was composed of a weak component, the molecular weight of which was about 20,000. Seminal plasma heteroantigens, identified by four gel diffusion precipitin lines, were found in three separate column effluent areas. There was clear-cut separation of individual precipitin lines into 2, 5 and 1 for Fraction I, II and III against heteroantiserum, respectively, by immunoelectrophoresis, while Fraction I, II and III produced 1, 2 and 1 precipitin lines, respectively, against isoantiserum (Fig. 10). Polyacrylamide slab gel electrophoresis revealed that the first one of the four fractions by Sepadex G-200

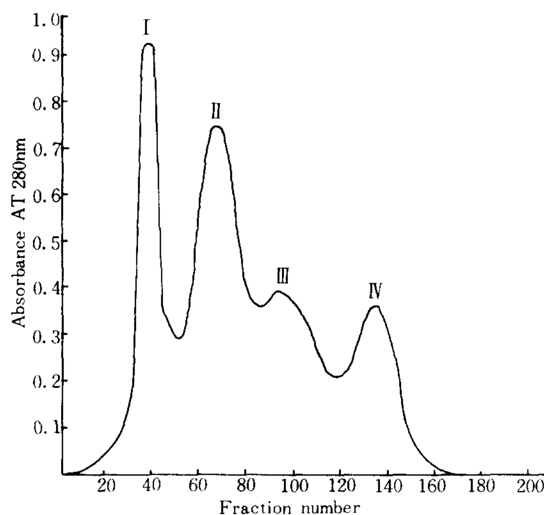

Fig. 1. Fractionation of rabbit seminal plasma by gel filtration. Pooled seminal plasma (2ml) was characterized on a column (2.5×25cm) of Sephadex G-200 at 4℃ with the use of 0.1M Tris-HCl as the eluting buffer. UV-absorbing material was monitored at 280mm.

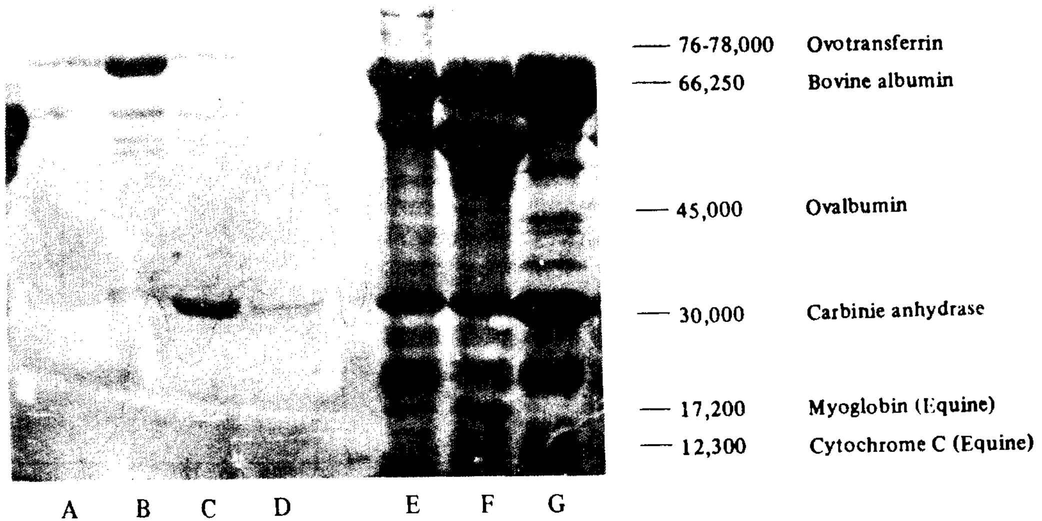

Fig. 2. SDS-polyacrylamide gel electrophoresis of rabbit seminal plasma with discontineous buffer system.
Lane A: Fraction I of elutes from Sephadex G-200. B: Fraction II of elutes from Sephadex G-200, C: Fraction III of elutes from Sephadex G-200, D: Fraction Ⅳ of elutes from Sephadex G-200, E: Normal rabbit seminal plasma, F: Normal rabbit seminal plasma, G: Vasectomized seminal plasma,

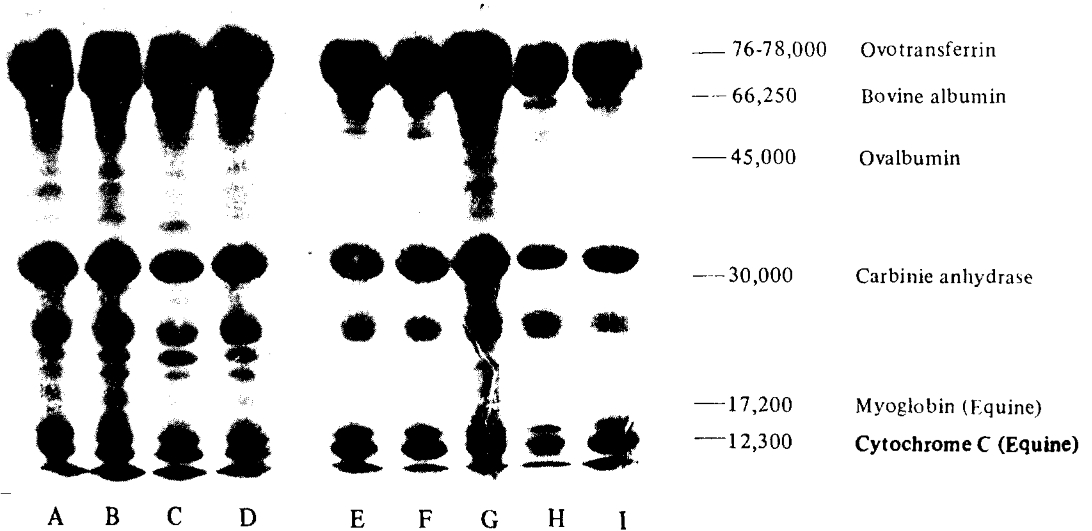

Fig. 3. SDS-polyacrylamide gel electrophoresis of rabbit semen with discontinuous buffer system.
Lane A: Normal rabbit seminal plasma 1, B: Normal rabbit seminal plasma 2, C: Normal rabbit seminal plasma 3, D: Normal rabbit seminal plasma (pool of 3), E: Vasectomized seminal plasma (pool of 3), F: Vasectomized seminal plasma (pool of 3), G: Normal rabbit seminal plasma, H: Vasectomized seminal plasma, I: Vasectomized seminal plasma,

produced 8 proteins, with the second one producing the highest number of 10 proteins; whereas the third of the fractions produced 5 proteins, with the last one producing the

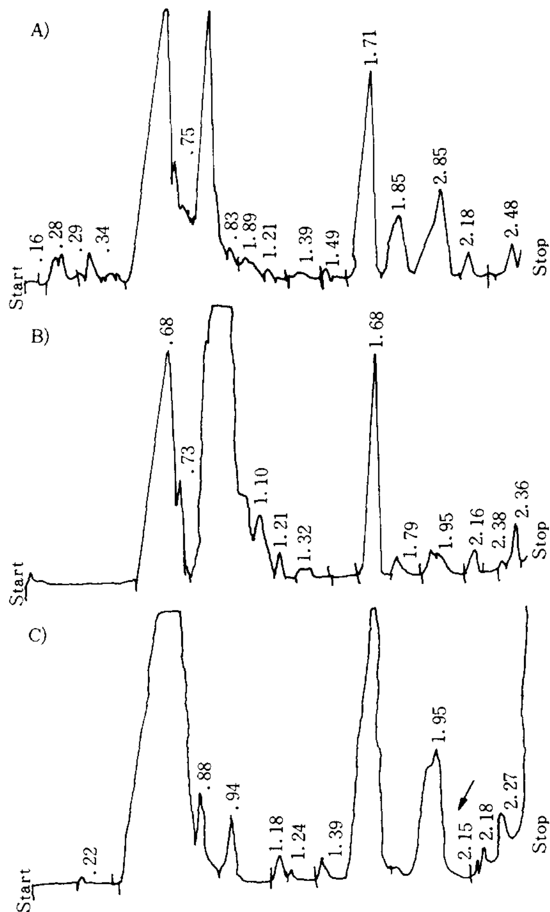

Fig. 4. Protein patterns of rabbit seminal plasma; A) Normal rabbit seminal plasma, B) Normal rabbit seminal plasma, C) Vasectomized seminal plasma. SDS-poly acrylamide gel electrophoresis, pH 8.8; Densitometric tracing ultroscan laser densitometer (LKB2202).

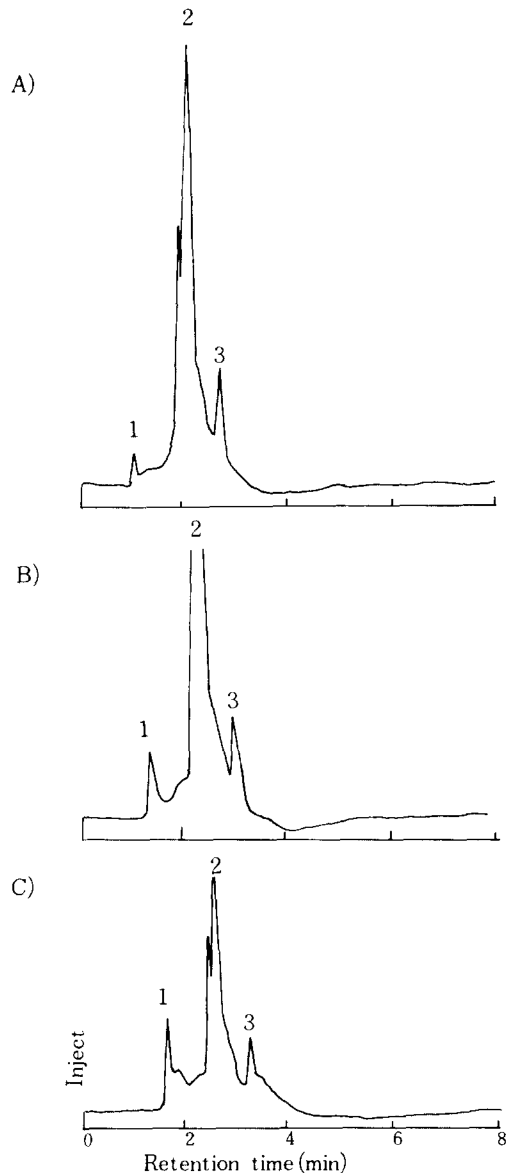

Fig. 5. Normal seminal plasma was analysed by HPLC on the μ-Bondapak C-18 (4×30cm) column. A 20μl sample was injected on to the column which had been equilibrated with 0.1% TFA in 80% CH_3CN water. The peptides were detected with a UV at 250nm. A), B), C): Normal seminal plasma.

profile of only a low molecular weight protein, which is common to Fraction II and III (Fig. 2). Gel electrophoretograms showing seminal plasma proteins for normal and vasectomized bucks are presented in Figures 2 and 3, together with the densitometer scans of them (Fig. 4). Both normal and vasectomized seminal plasma were generally capable of producing the seminal plasma specific protein profile except for only one of the protein bands (20,000 daltons) being absent in the vasectomized seminal plasma.

2. Seperation of proteins by HPLC

Figure 5 depicts the total protein chromatogram of seminal plasma. For the seminal plasma of normal rabbits injected on to the analytical column, 3 distinct large peaks were detected with peak splitting differences also observed, whereas the vasectomized seminal plasma and similar protein profile to the normal one except for lack or a trace of protein corresponding to the peak 1 (Fig. 6). This observation seems to coincide with that of SDS-PAGE electrophoresis, which showed that one of the protein was lost in case of vasectomized seminal plasma. This result indicated that normal seminal plasma was different in its protein composition from vasectomized one. It was also suggested the possibility that part of the proteins observed in normal seminal plasma may have originated from the testis region.

3. Agar gel diffusion tests

By means of agar gel diffusion test, four precipitin lines were formed by seminal plasma against guinea pig antirabbit semen (GPAPS) and rat antirabbit semen (RABS). These were designated 1 to 4 in order of their proximity to the antiserum well. With GPARS, two sperm antigens were detected with one precipitin line in the cauda epididymis, whereas with RARS no sperm antigen was approved instead of an identical precipitin line detected against ductus deferens, testis and cauda epididymis, respectively (Fig. 7). Two antigens originated in sperm and one in cauda deferens, testis and cauda epididymis, respectively. Two antigens originated in sperm and one in cauda epididymis with GPARS. Absorption of GPARS with sperm resulted in elimination of one of the four antigens and all the antigens of sperm and cauda epididymis. And adsorption by

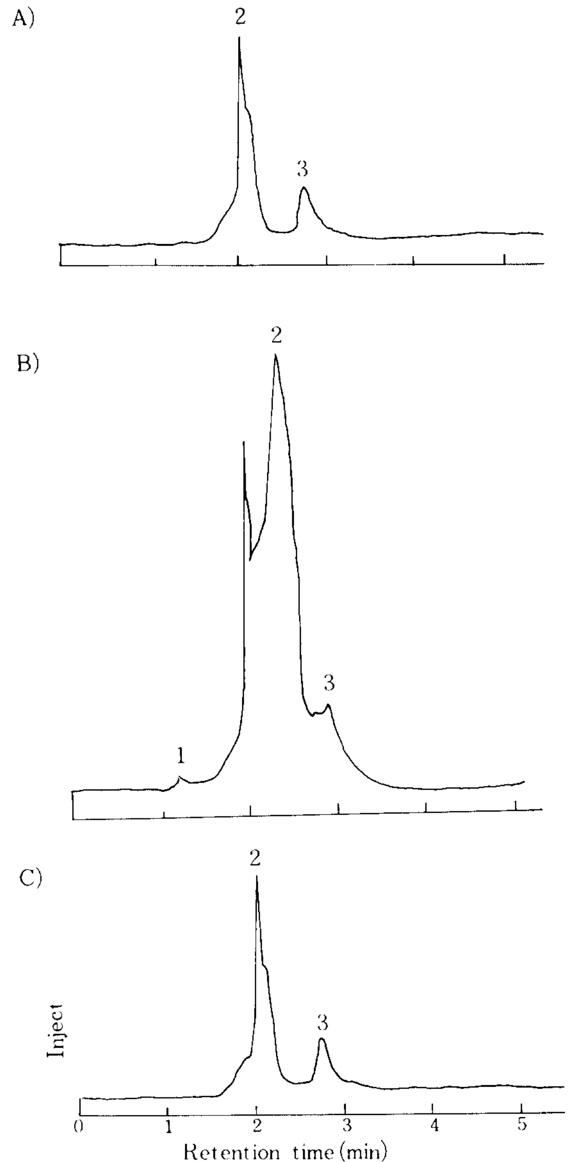

Fig. 6. Vasectomized seminal plasma was analysed by HPLC on the μ-Bondapak C-18 (4 × 30) column. A 20μl sample was injected on to the column which had been equilibrated with 0.1% TFA in 80% CH_3CN water. The peptides were detected with a UV at 250nm. A), B), C): Vasectomized seminal plasma.

testis removed one of the two antigens in sperm and cauda epididymis. One of the four antigens of seminal plasma showed no reaction with

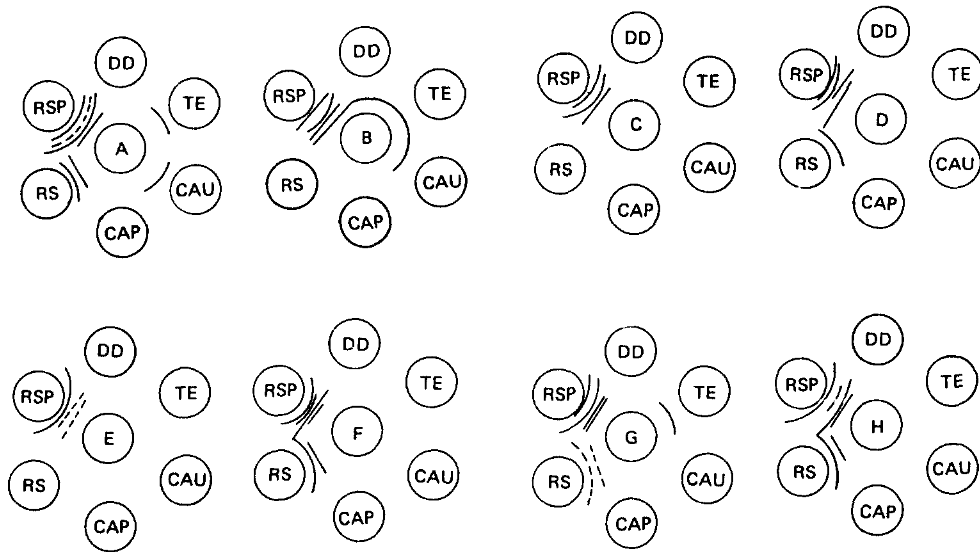

Fig. 7. Ouchterlony analysis of various antigens against guinea pig antirabbit semen (GPARS).
A : Ouchterlony plate of GPARS, B : Ouchterlony plate of rat antirabbit semen, C : Ouchterlony plate of GPARS adsorbed with sperm, D : Ouchterlony plate of GPARS adsorbed with testis, E : Ouchterlony plate of GPARS adsorbed with epididymis, F : Ouchterlony plate of GPARS adsorbed with caput epididymis, G : Ouchterlony plate of GPARS adsorbed with ductus deferens, H : Ouchterlony plate of GPARS adsorbed with kidney & liver, Symbols; RSP : rabbit seminal plasma, DD : ductus deferens, TE : testis, CAP : caput epididymis, CAU : cauda epididymis, RS : rabbit sperm, RSP : rabbit seminal plasma.
Dotted lines represent weak reactions. Lines next to a well in plates represent precipitin reactions.

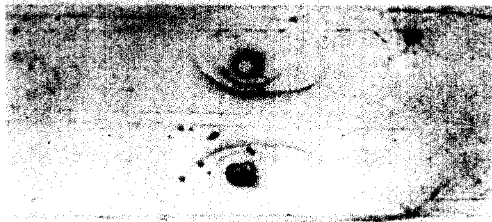

Fig. 8. Immunoelectrophoretic patterns of rabbit isoantiserum against seminal plasma. Well: Rabbit seminal plasma, Trough: Rabbit seminal plasma isoantiserum.

GPARS adsorbed with cauda epididymis; one of the two antigens of sperm did with the same GPARS. A caput originating line disappeared in GPARS adsorbed with the caput epididymis, having no effect on the seminal plasma antigens. GPARS adsorbed with the ductus deferens of rabbit showed no reaction but GPARS adsored with the caput epididymal antigens still showed precipitation reaction, whereas adsorption by kidney and liver had no influence on the seminal plasma and sperm antigens. This may indicate that liver and kidney have to some extent crossreacting common antigens with some parts of the reproductive organ. One of seminal plasma antigens originating from sperm and one antigen from the cauda epididymal portion seemed to bound to sperm in passage. Fig. 8 and 9 show the presence of two precipitin lines in the GPARS serum

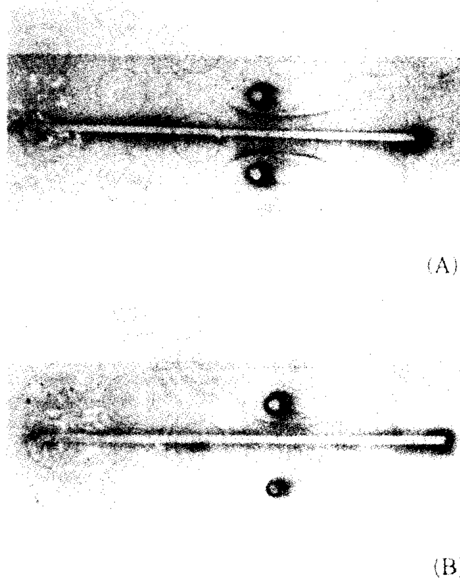

Fig. 9. Immunoelectrophoretic analysis of normal rabbit serum. Well: Normal rabbit serum, Trough: (A) Guinea pig antisera against rabbit seminal plasma, (B) Rat antisera against rabbit seminal plasma. Anode is on the right side.

against normal rabbit serum. Therefore, it was verified that many of the seminal plasma antigens detected by heteroimmunization are organ specific and still others are common to the serum.

4. Agglutination tests

Guinea pigs immunized with semen in Freund's complete adjuvant reacted well to produce higher heteroantibody titers which agglutinated in a dilution of 1:4,096 by passive haemagglutination test against both sperm and seminal plasma, which was compared to agglutination titers of 1,026 to the rat antisera under the same condition. On the other hand, in the rabbits under the same treatment, individual differences were shown in immune response, with some of them producing isoantibody titers of up to 512, but the average titer turned out to be 256 to sperm antigens and 128 to seminal plasma antigens. This finding

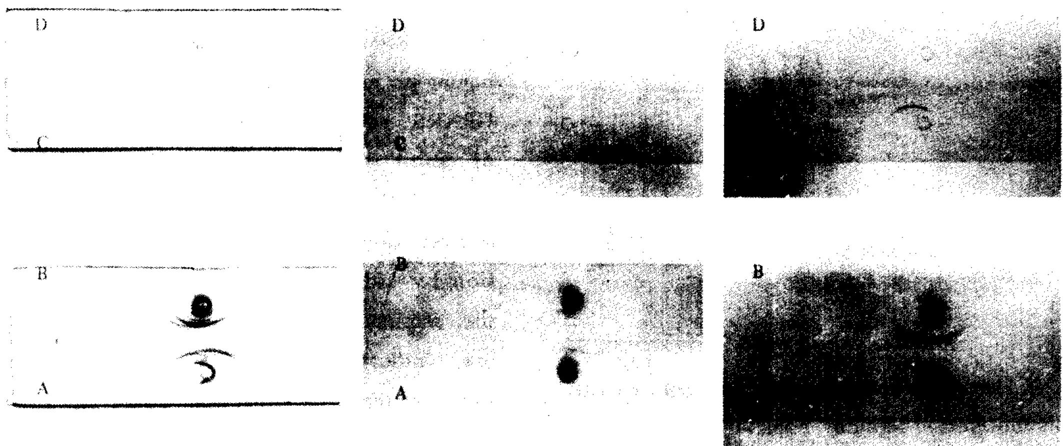

Fig. 10. Schematic diagram of immunoelectrophoretic patterns of rabbit seminal plasma and its four fractions by Sephadex G-200 chromatography. Well: A: Fraction Ⅰ, B: Fraction Ⅱ, C: Fraction Ⅲ, D: Fraction Ⅳ, Trough: (1) Guinea pig antirabbit semen serum, (2) Rat antirabbit semen serum, (3) Rabbit isoantiserum.

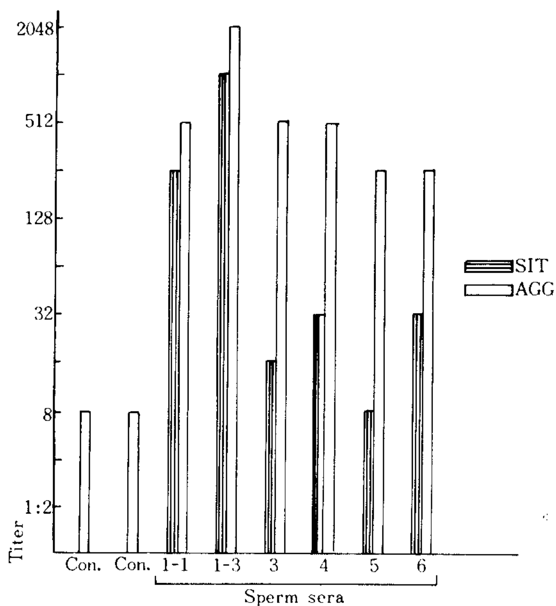

Fig. 11. Comparison of sperm agglutination and immobilization titers in sperm immune sera and control sera. SIT:Sperm immobilization titer. AGG:Sperm agglutination titer

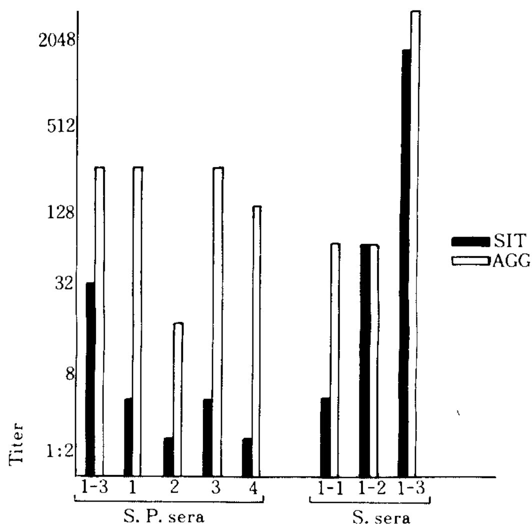

Fig. 12. Comparison of sperm agglutination and immobilization titers in seminal immune sera and seminal plasma immune sera. S. P.:Seminal plasma. S:Semen

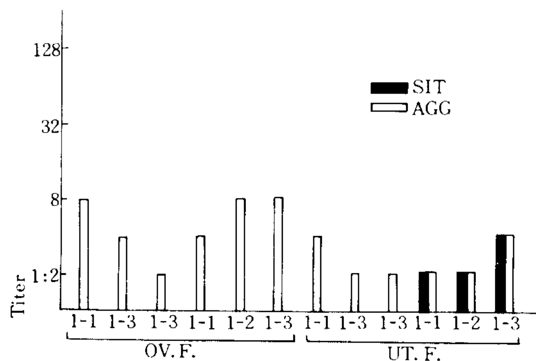

Fig. 13. Sperm agglutination and immobilization titers in the oviduct and uterine fluids from sperm, seminal plasma and semen immunized rabbits. The uterines and oviducts used here were washed with 6ml of saline solution and 6 times concentrated by PEG treatment. OV.F: Oviductal fluid. UT. F:Uterine fluid

confirmed individual differences in immune response. The incidence of sperm agglutinins remained generally high in the sperm treated group (Fig. 11). The control group also had sperm agglutinins, and the titers were low, 1:8. To confirm this finding, blood samples were twice tested at a different time. The results were about the same with both tests. For sperm immobilization a tendancy to decrease in the titers as compared to agglutination titers was observed. One of them had the highest titer of 1:1,024, showing individual differences. Fig. 12 shows that seminal plasma agglutinins were lower in titer compared with sperm ones and most of the sperm immobilization titers appeared only in the dilutions of below 1:4 except of a dilution of 1:32. On the other hand, comparative to the case of sperm, the seminal immune sera had low titers in both sperm agglutination and immobilization. Fig. 13 indicates the presence of sperm agglutinins in both the oviducts and uterine secretions from sperm, seminal plasma and semen immunized animals. But no sperm immobilization antibodies appeared in both secretions in case

of the sperm and seminal plasma treated animals, except for semen treated animals with low titers of below 1:4.

5. Identification of protein by crossed immunoelectrophoresis

Figure 14 shows the precipitation pattern obtained by crossed immunoelectrophoresis of normal rabbit seminal plasma against a guinea pig antirabbit semen serum. The agrose gel electrophoresis pattern corresponding to that of the first dimension electrophoresis was separated into three regions. Under this experimental conditions some proteins migrated towards the cathode. Twenty-one precipitates are seen in Fig. 5 but a few additional weak peaks could have been demonstrated using vairous mixture of antigens and antibodies. Further examination, therefore, should be done to get some more

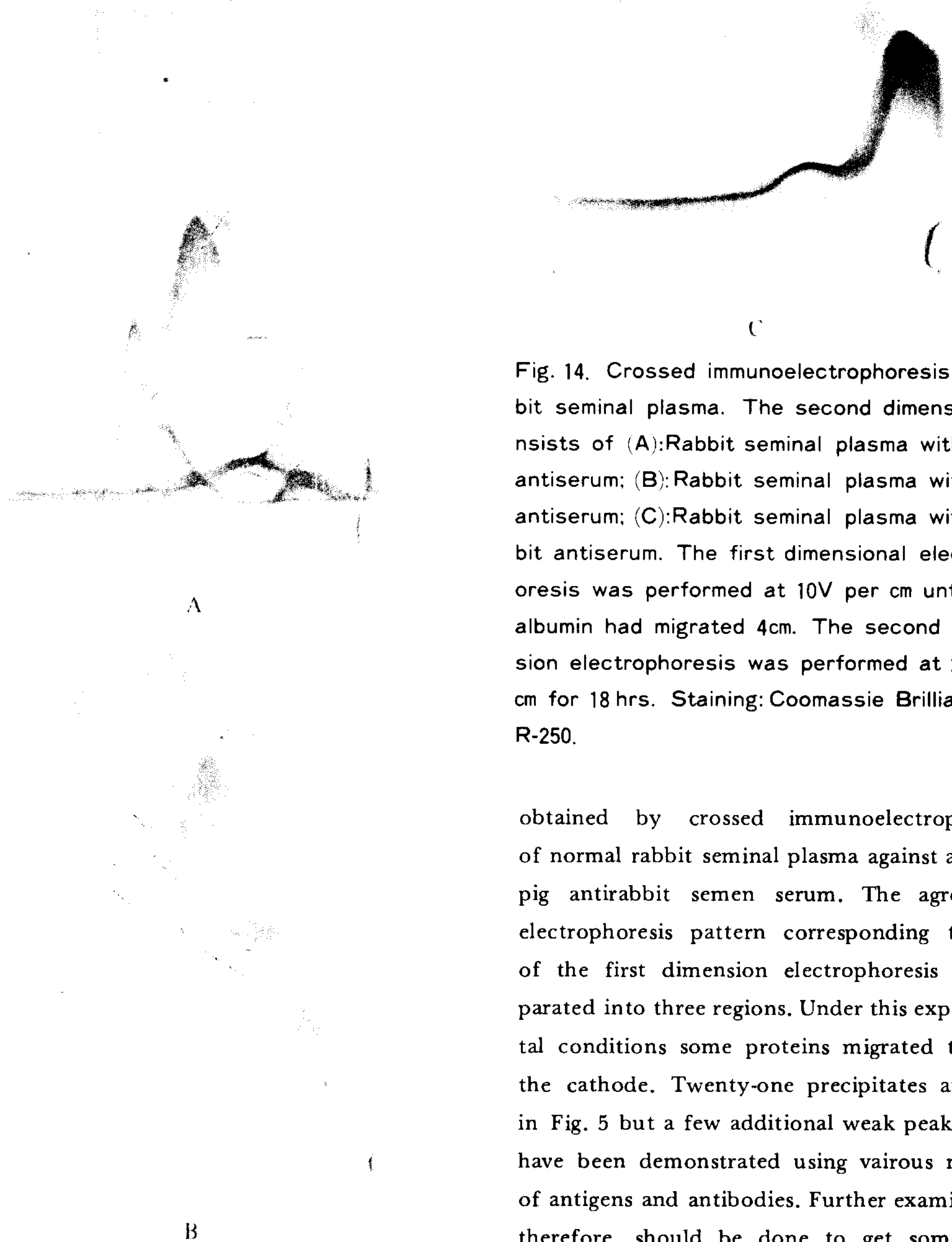

Fig. 14. Crossed immunoelectrophoresis of rabbit seminal plasma. The second dimension consists of (A):Rabbit seminal plasma with GP antiserum; (B): Rabbit seminal plasma with rat antiserum; (C):Rabbit seminal plasma with rabbit antiserum. The first dimensional electrophoresis was performed at 10V per cm until the albumin had migrated 4cm. The second dimension electrophoresis was performed at 2V per cm for 18 hrs. Staining: Coomassie Brilliant Blue R-250.

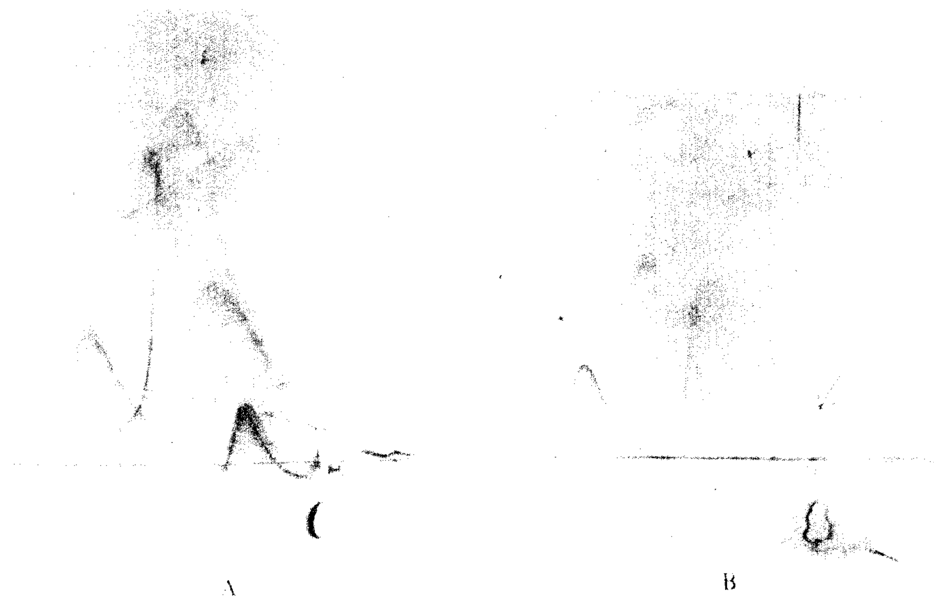

Fig. 15. Crossed immunoelectrophoresis of rabbit seminal plasma. The second dimension consists of (A):Rabbit seminal plasma with GP antiserum, (B): Vasectomized rabbit seminal plasma with GP antiserum.

information on involved antigens. On the other hand. Fig. 15 depicts the precipition patterns obtained by the same method of vasectomized seminal plasma against the same antiserum. For detection of the proteins moving towards the cathode, antibody containing agar portion was, in addition, set up in the cathodic side of the plate. In this case, seminal plasma specific proteins were distinguished in vasectomized ones from ejaculated semen by means of crossed immunoelectrophoresis with semen antiserum. A few antigens (arrow) were shown to be those of testis originated proteins. Although patterns of precipitates, obtained under the same working conditions, have different profile between the normal and vasectomized seminal plasma, the precipitates indicated above seem to be specific to the normal seminal plasma. In other words, it is confirmed that seminal plasma separated routinely from ejaculated semen contains these antigens which may have originated from mature sperm. Fig. 14-c indentifies the existing of two antigens in the seminal plasma against isoantiserum. The observation seems to coincide with the results obtained by immunolectrophoresis, even though it is unlikely that these antigens form separated distinct precipitins. In comparision of both normal and vasectomized seminal plasma antigens between heteroantiserum and isoantiserum one of the two antigens, which moves faster towards anode, is lost in vasectomized seminal plasma (Fig. 16). Contrary to some other earlier reports, the results give evidences that two antigens reactive to isoantiserum exist in the normal seminal plasma, whereas only one antigen, in larger amount, remains after vasectomization.

Discussion

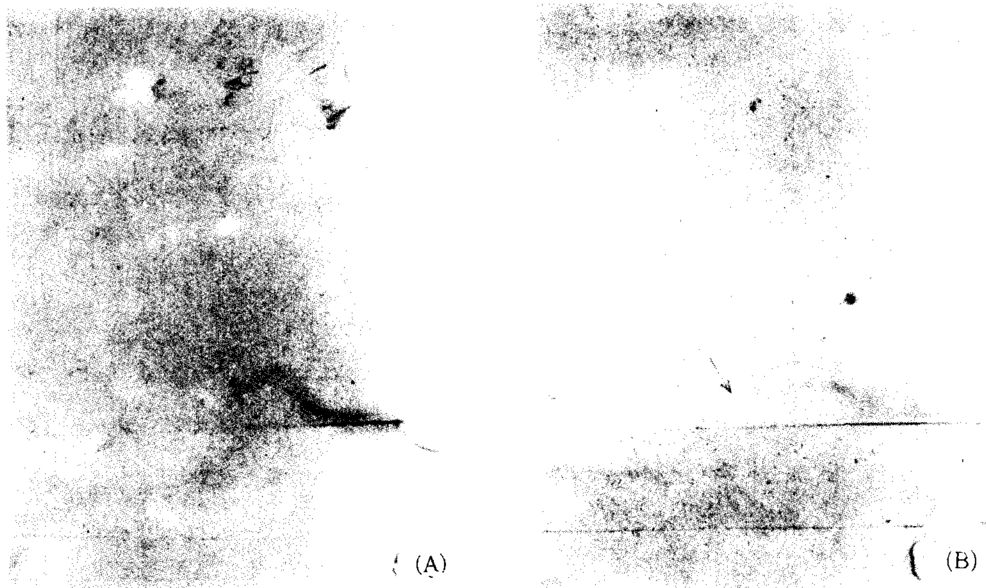

Fig. 16. Crossed immunoelectrophoresis with intermediate gel containing: (A): Rabbit normal serum, (B): Rabbit antiserum against rabbit sperm, Antigen: Rabbit seminal plasma, Antibodies: Rabbit seminal plasma isoantiserum

Okano et al. (1978) reported that Fraction I, II and III by Sephadex G-100 had 3, 2 and 1 precipitin lines with respectively rat antiserum. A difference however, was detected only in number between Fractiontion I and II by Sephadex G-200, but there was no change in total precipitin lines. Menge and Protzman (1967), Oliphant and Brackett (1973) found many precipitin lines by immunizing guinea pigs with rabbit seminal plasma. These results agreed well with those of the experiment. A single precipitin line was observed after isoimmunization by gel diffusion as well as immunoelectrophoresis. The single antigen was found to exist in the second fraction among the four fractions by Sephadex G-100 (Okano et al., 1978). Fractions I, II and III each by Sepadex G-200, however, turned out to have more protein antigens, resulting in 1, 2 and 1 precipitin lines respectively, with antiserum by immunoelectrophoresis. Therefore, increased numbers of seminal plasma antigens could result from 1) the fraction range for which the moecular weights of seminal plasma proteins were related to elution position or 2) the concentration of their antibodies in isoantiserum.

Shulman et al. (1968) obtained an isoantiserum through immunization of extracts of accessory sexual gland and seminal plasma. The antiserum was confirmed to form a precipitin line against the gland extracts. Immune serum to bovine semen was recognized to posses three distinct precipitin lines either by gel diffusion or by immunoelectrophoresis (Menge, 1970). But the results of this investigation confirmed the fact that rabbit seminal plasma has two antigens capable of inducing isiommunization (Shulman et al., 1968), even if one of them seemed like a kind of splitting antigen. However, the antigen was detected only when high titer antiserum was available. Two kinds of the pooled seminal plasma (Fig.

3-A,B,H and I), in addition, furnished more information regarding the difference between normal and vasectomized animals in protein components. Three samples of vasectomized seminal plasma used in the study came from two bucks that had been vasectomized three months ago and the last one six months age. But they showed similiar of proteins different from normal seminal plasma. Since the present study revealed proteins in rabbit, perhaps part of proteins resulting from sperm maturation may have additionally been involved in the production of the complete seminal plasma specific protein profile. This idea seems logical since the seminal plasma specific proteins was generally found in the normal bucks (Menge, 1970).

For rabbit epididymis subdivided into 8 regions based on histological studies, it was determined that many rabbit seminal antigens were present in each epididymal region with goat antirabbit reproductive tract antiserum (Johnson and Hunter, 1979). Six seminal plasma antigens, therefore, were detected to be derived from the major accessory glands. There were no cross reactions of accessory gland antigens with antiserum from either testis or cauda epididymis. This observation was confirmed in the experiment with no absorption occurring in the testis absorbed GPARS. Seminal antigens are antigens that can react specifically with antibodies to ejaculated semen and can under appropriate conditions incite an animal to form antibodies (Schellpfeffer and Hunter, 1976). Barker and Amann (1970) with bull sperm and Hekman and Shulman (1971) with guinea pig, reported that antigens bind to ejaculated sperm much more tightly than to epididymal sperm. Orgebin-Crist and Tichenor (1972) suggest that maturation in epididymis may involve sperm coating antigen alterations. Figure 7 illustrates that one of the epididymal originating coating antigens was losted in the cauda epididymis absorbed GPARS. This observation supported the fact that sperm storage occurs in the terminal segment of the epididymis (Glover and Nicander, 1971). Female rabbits immunized with rabbit seminal plasma plus Freund's complete adjuvant required four injections of isoantigens given weekly and several booster injections after the fifth injection after two months for condensely much production of antibody. The isoantiserum used in this experiment was found to form two precipitin lines against the rabbit seminal plasma. One of the two lines detected in the experiment remained weaker in reaction and near the antigen well (Fig. 8 and Fig. 9). Such a difference has been shown earlier (Shulman et al., 1968; Okano et al, 1978). They had been able to acquire higher titers in the homologous animals against the seminal plasma antigens after several booster injections and the intervals of immunization had been extended from two weeks to 2 months. They agreed well with the observation that in order to produce a high titer antiserum for agar-gel diffusion and immunoelectrophoresis, animals have to be immunized with the same antigens several times after the routine courses of immunization.

The sperm microagglutination test has now been shown to be of uncertain immunological significance, as compared with the more respectable sperm immobilization test (Jones, 1974). It is confirmed in the test that there was no remarkable evidence on an association of positive sperm microagglutination and fertility status. On the other hand, positive sperm immobilization test was associated with an appreciable lowering of the pregnancy rate (Jones

and Ing, 1974). Cervical secretions from 20 women who had each received 3 intramuscular injections of fresh human semen, exhibited sperm cytotoxicity in about 50% of cases. But this showed no consistant relationship to semen reactivity (Baskin, 1932). It is also suggested in infertile and fertile individual that some sperm antigens may be uniquitous and associated with the formation of natural antibodies which may cross react with other antigens. Other appears to provoke only immune antibody formation and more closely associated with infertility (Tung, 1975). The immobilization was observed only with sera and some fluids which also contained sperm agglutinins detectable in the agglutimation test. However, the oviduct and uterine fluids with high titers of sperm agglutinins but without immobilization were also observed (Fig. 13). This finding seems to have coincidence with the report of Ansbacher (1973). It was observed that undiluted normal sera were involved in sperm agglutination and immobilization. Such effects could not be regarded as specific to the action of antibodies. Especially guinea pig serum used as a source of complement also seemed to have substances to interfere or kill fresh motile sperm, because up to 1:10 dilution, the complement serum still had a capacity of killing of some extent a dilution of motile sperm added for the immobilization test. As with other systems, it is necessary that guinea pig serum to be used should first be screened for the presence of natural cytotoxins against rabbit spermatozoa and titrated for complement activity. Any correlation between the two tests was not detected in both oviduct and uterine fluids of the animals that had been immunized with sperm and seminal plasma antigens each. On the other hand, semen treated animals showed the presence of agglutinins and immobilization in the uterine fluids, suggesting that different from sperm or seminal plasma antigens, some of many antigens demonstrated in semen appear to provoke only immune antibody formation and are more closely associated with immobilization in the uterine fluid. The discovery that fresh rabbit serum is source of highly-active complement for human isoantibody systems (Walford et al., 1964) has been of great importance for the study of HLA antigens. However, it appears that the rabbit complements either acted very feebly or not at all with most rabbit seminal plasma and sperm antisera. There is, however, a possibility that a trace of blood still reactive for agglutination and immobilization, may have been containated into the fluids during the removal of female reproductive tracts. It was found that some of seminal antigens are unique to sperm or seminal plasma but others are shared with semen, milk, saliva, nasal secretion, gastric juice, urine, vagina, cervical mucus, cervix, endometrium, fallopian tube, ovary and kidney-liver (Li and Behrman, 1970). In comparison of immobilization with tube slide agglutination, Vaidya and Glass (1971) were of the opinion that two methods detected different immunological responses. The results obtained in Fig. 14 were considered to convince that opinion. The two sperm coating antigens in caput regions are suggested to alter the surface charge of sperm outer membrane, and it is proposed that they are associated with the acquistion of fertilizing ability (Crabo and Hunter, 1975). Rabbit sperm that were retained in the epididymis by ligation for 12 days prior to a part of the capit regions never developed fertilizing ability (Orgebin-Crist, 1969). Alterations of sperm coating antigens

are necessary before fertilization can occur in the female rabbit (Johnson and Hunter, 1972). Although rabbit sperm from the distal corpus can fertilize, significantly fewer of them establish contact with ova compared to more mature sperm (Orgebin-Crist and Tichenor, 1972). This fact suggests that maturation in epididymis, involving sperm coating antigens, may need the addition of some of seminal protein antigens.

Summary

Antigenicities of sperm and seminal plasma of rabbit were studied in normal and immunized rabbits. The results obtained were summarized as follows:

1. By means of SDS-PAGE electrophoresis, normal seminal plasma produced about 23 protein bands and one of them, having a molecular weight of 20,000, was lost in the vasectomized seminal plasma.
2. On the basis of high performance liquid chromatography analysis, the normal seminal plasma showed 3 distinct large peaks on the analytical column, whereas vasectomized seminal plasma had lack or only a trace of the protein corresponding to the peak 1.
3. Seminal plasma formed 4 distinct precipitin lines with heteroantiserum in the agar-gel diffusion test, while 7 precipitin lines were shown in the immunoelectrophoresis.
4. Heteroantibodies against sperm or seminal plasma were easily produced by immunization, but isoantibodies required more booster injections than the former, and individual immune responses differed remarkably. Does immunized with sperm plus adjuvants showed immunological reaction to sperm antigens in the passive haemagglutination test, but not in both the agargel diffusion and immunoelectrophroesis; whereas bucks under the same treatment also followed the same reaction.
5. Antigenic components of seminal plasma were clearly detected and distinguished by the crossed immunoelectrophoresis using isoantiserum and heteroantiserum. A few antigens involved in the seminal plasma which had been separated from the ejaculated semen were able to be identified. It is strongly suggested that those antigens may have originated from the mature sperm.

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