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CHARACTERIZATION AND ISOLATION OF A SPERM-COATING ANTIGEN FROM RABBIT SEMINAL PLASMA WITH CAPACITY TO BLOCK FERTILIZATION

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Summary. The relationship between the sperm-coating antigens of rabbit seminal plasma and the phenomenon of decapacitation was studied using agar-gel diffusion, immuno-electrophoresis, chromatography on Sephadex G-200, and polyacrylamide vertical gel electrophoresis.

Interpretation of data obtained with these techniques led to the conclusion that a sperm-coating antigen of seminal plasma origin possessed biological activity for blocking fertilization. The sperm-coating antigen was a glycoprotein of approximately 170,000 molecular weight, migrated in an electric field similar to a serum slow β-globulin and was still present in the seminal fluid of vasectomized males. This sperm-coating antigen was absent from the inactive upper supernatant fluid fraction of seminal plasma after 4 hr of ultracentrifugation at 105,000 ***g*** and was present in the active ultracentrifugal pellet.

INTRODUCTION

Chang (1951, 1958) and Austin (1951) have shown that rabbit spermatozoa must be present in the female reproductive tract for several hours before ovulation to acquire fertilizing ability. Austin (1952) termed this phenomenon capacitation. Subsequent evidence has suggested that a similar process may take place in the rat (Austin, 1951), ewe (Dauzier & Thibault, 1959), hamster (Chang & Sheaffer, 1957), mouse (Braden & Austin, 1954) and ferret (Chang & Yanagimachi, 1963).

Chang (1957) demonstrated that the fertilizing ability of capacitated rabbit spermatozoa was inhibited by mixing these spermatozoa with seminal plasma. The spermatozoa retained their motility, however, and if redeposited into the Fallopian tubes for a certain period of time before ovulation they regained their ability to fertilize ova. Seminal plasma from the bull, boar, stallion and primates will also decapacitate rabbit spermatozoa (Dukelow, Chernoff & Williams, 1967). Bedford & Chang (1962) showed that the detrimental material (termed decapacitation factor or DF) sedimented with ultracentrifugation. Weinman & Williams (1964) demonstrated that the DF pellet obtained by ultracentrifugation

could be resuspended in buffer and they also reported DF activity for epididymal fluid. Chernoff, Pinsker, Dukelow & Williams (1966) found that DF activity was not affected by proteolysis with pronase; however, the activity would no longer sediment at 100,000 *g*. Dukelow, Chernoff, Pinsker & Williams (1966) suggested that DF consisted of a protein–carbohydrate complex bound to the sperm head by sialic acid units. The active part of the molecule was in the carbohydrate portion since β-amylase destroyed activity (Dukelow, Chernoff & Williams, 1966a). The biochemistry and physiology of DF were summarized by Williams, Abney, Chernoff, Dukelow & Pinsker (1967).

In another area, Weil (1960) reported that rabbit spermatozoa were coated with seminal plasma antigens during ejaculation. Hunter & Hafs (1965) confirmed this and reported that antigenic coating also occurred upon passing through the epididymis. By immunofluorescent techniques, Weil & Rodenburg (1962) showed that sperm-coating antigens were present as a very thin coating on the surface of seminal spermatozoa. The first objective of the present research was to determine the relationship between the sperm-coating antigens of seminal plasma origin and the DF. A second objective was to characterize the DF electrophoretically and chromatographically.

MATERIALS AND METHODS

Biological materials

Semen was collected with an artificial vagina from New Zealand white rabbits and centrifuged at 12,000 *g* for 5 min. The seminal plasma was removed and recentrifuged before being frozen. Samples (8-ml) of pooled seminal plasma, diluted 1:1 with Ringer's sodium lactate solution, were spun at 105,000 *g* for 1 to 5 hr in a 40 rotor of a Spinco Model L centrifuge. The upper and lower supernatant fluids were each separated into 4-ml fractions. Each sedimentation pellet was also separated and made up to 4 ml with 0·005 M-phosphate-buffered saline (pH 7·4). This was essentially the technique of Bedford & Chang (1962) for the preparation of DF.

Immune sera and immunological tests

Procedures for the preparation of cow anti-rabbit spermatozoa or seminal plasma immune sera were identical to those described in a previous publication (Hunter & Hafs, 1965). Procedures for the Ouchterlony technique and immuno-electrophoresis were identical to those described by Hunter & Hafs (1964).

Bio-assay procedures

Decapacitation activity was assayed following the method outlined in Table 1.

Column chromatography

Sephadex G-200 powder was suspended in 0·005 M-phosphate-buffered saline at pH 7·4. A column was poured with an internal diameter of 2·5 cm and length of 35 cm. The seminal plasma sample (0·5 ml or 18 mg protein) was eluted at

room temperature with 0·005 M-phosphate-buffered saline using a flow rate of 3 ml/cm^2/hr. Fractions (2-ml) were collected volumetrically. The void volume of a column was determined by chromatography of blue dextran. The elution volumes of three proteins of known molecular weight (bovine albumin, egg albumin and cytochrome C) were determined and plotted against the logarithm of their molecular weight. The molecular weights of the seminal plasma components were determined from the plotted curve according to the techniques of Leach & O'Shea (1965).

Zone electrophoresis

Rabbit seminal products (30 mg/ml) were submitted to zone electrophoresis in vertical polyacrylamide gels following the procedure of Raymond (1962).

TABLE 1

ASSAY PROCEDURE FOR DECAPACITATION FACTOR

Day	*Time*	
1	22.00 hours	Inseminate capacitator doe with semen from four bucks and inject three ova donors with 3·2 mg Armour LH
2	09.00 hours	Recover capacitated spermatozoa by flushing each uterus with 5 ml KRP*, centrifuge at 600 *g*, suspend in two portions of 0·3 ml KRP, pool
	09.15 hours	Add 0·2 ml of an ultracentrifugal fraction of an unknown or control solution to 0·2 ml of capacitated spermatozoa, incubate 30 min at 37° C
	10.00 hours	Inseminate 0·10 ml (0·60 to 12×10^4 spermatozoa) of treated sperm suspension or untreated capacitated spermatozoa into each upper uterine horn of three ovulating does
3	10.00 to 12.00 hours	Recover ova by washing oviducts with 5 ml KRP and examine under microscope

* KRP = Krebs-Ringer phosphate buffer.

The buffer system was tris-Na_2 EDTA-boric acid (0·1 M), pH 9·2. At the termination of the initial electrophoretic run, slabs were cut from the gel and stained with amido black for protein or developed with periodic acid–Schiff reagent for glycoprotein (Keyser, 1964).

RESULTS

Decapacitation activity in rabbit seminal plasma

Data in Table 2 show that spermatozoa incubated in the uterus for 11 hr yielded an 85% fertilization rate and hence were capacitated. Treatment of capacitated spermatozoa with rabbit seminal plasma interfered with fertilization as shown by the 38% fertilization rate. Bedford & Chang (1962) have termed this inhibition of fertilizing ability of capacitated spermatozoa by mixing with seminal plasma, 'decapacitation'. However, following ultracentrifugation of seminal plasma for 4 hr or more at 105,000 *g*, the decapacitation activity disappeared from the upper supernatant fluid (90% fertilization) and was concentrated in the 5-hr ultracentrifugal pellet (28% fertilization). The decapacitation activity of this pellet was equivalent to that of untreated seminal plasma (28% versus 38%).

TABLE 2

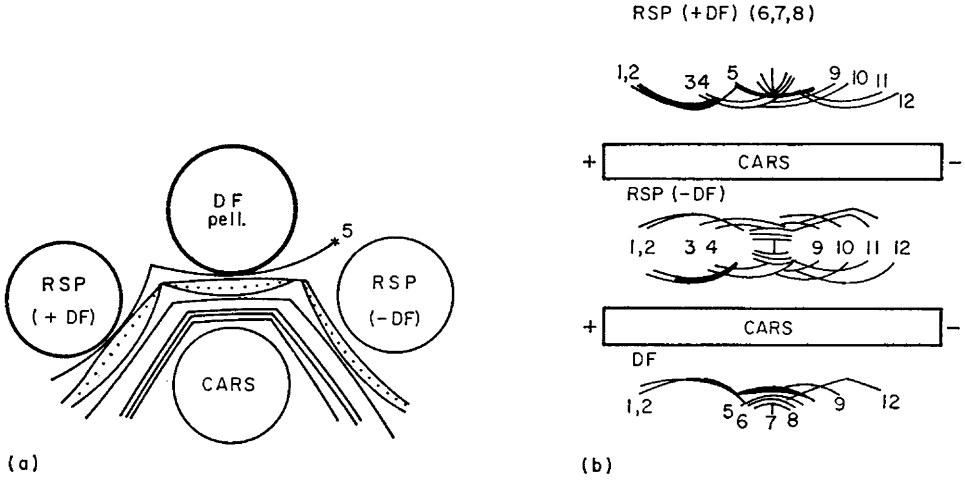
DECAPACITATION ACTIVITY WITH ULTRACENTRIFUGAL FRACTIONS OF RABBIT SEMINAL PLASMA

Treatment	*Ova recovered*	*% Ova fertilized*
Capacitated spermatozoa + buffer	13	85
Capacitated spermatozoa + RSP*	8	38
Capacitated spermatozoa + 3-hr upper‡	5	20
Capacitated spermatozoa + 4-hr upper	10	90
Capacitated spermatozoa + 5-hr pellet†	18	28

* RSP = Rabbit seminal plasma.
‡ Upper = Ultracentrifugal supernatant top fraction.
† Pellet = Ultracentrifugal sedimentation pellet.

Antigenic spectrum of rabbit seminal plasma

The Ouchterlony technique resolved seminal plasma into eight detectable antigens. One less antigen was noted in the upper supernatant fluid fraction of seminal plasma after 4 hr of ultracentrifugation (Text-fig. 1a). This fraction lacked DF activity (Table 2). The missing antigen (component 5 in Text-fig. 1a) normally formed nearest the antigen well, thus indicating a large molecular weight component. This antigen was present in the seminal fluid of vasectomized males and stained with periodic acid–phenylamine diamine as a glycoprotein. The 4- and 5-hr ultracentrifugal pellet fractions of seminal plasma contained this antigen in very high concentration. Analysis of each of the 5-hr ultracentrifugal pellets revealed that both the total number of antigens and their titres increased with time. After 5 hr, six to eight antigens were detected in

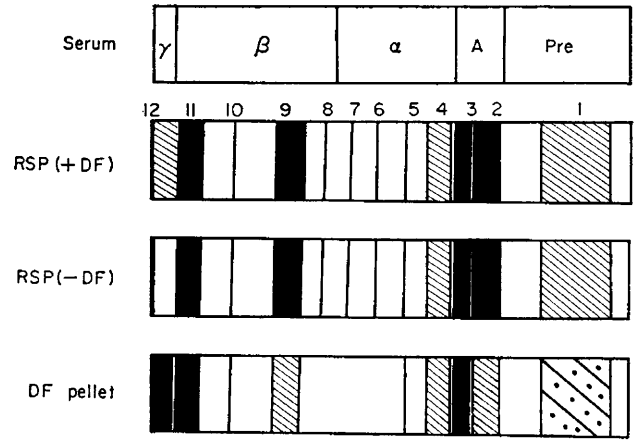


TEXT-FIG. 1. Precipitin reactions of bovine antisera with rabbit seminal materials. (a) Diagram of an agar gel diffusion plate. (b) Diagram of an immuno-electrophoresis plate. RSP (+DF) = rabbit seminal plasma with decapacitation factor activity; RSP (−DF) = 4-hr ultracentrifugal upper supernatant fluid fraction without DF activity; DF pell. = 5-hr ultracentrifugal pellet of RSP with decapacitation activity; CARS = cow anti-rabbit spermatozoa

the pellet and at least five of these were sperm-coating antigens based on their ability to react with antisera to washed ejaculated spermatozoa.

Immuno-electrophoretic data (Text-fig. 1b) revealed twelve antigens in seminal plasma. One less antigen was noted in the upper supernatant fluid fraction of seminal plasma after 4 hr of ultracentrifugation. The missing glycoprotein (component 5 in Text-fig. 1b) normally migrated with a mobility of a slow serum β-globulin. Immunologically, this glycoprotein did not cross-react with any of the blood serum proteins. This antigen and five to seven others were detected in the 5-hr ultracentrifugal pellet of seminal plasma.

Basically identical findings were obtained on the antigenic spectrum of all seminal products when antisera to rabbit seminal plasma were substituted for antisera to washed ejaculated rabbit spermatozoa. This agreed with the concept that sperm-coating antigens were of seminal plasma origin (Weil, 1960).



TEXT-FIG. 2. Diagram of protein separation by vertical gel electrophoresis with 7% polyacrylamide. Abbreviations as in Text-fig. 1. Components are numbered and plotted in relation to positions occupied by standard blood serum proteins.

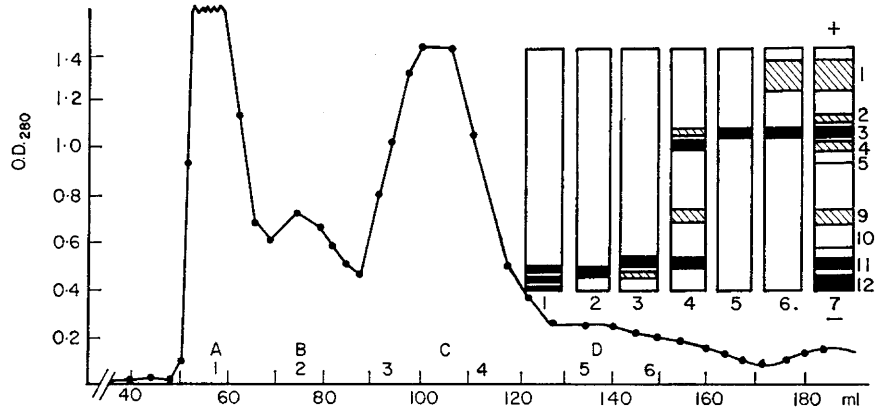
Electrophoresis

Text-fig. 2 presents the protein separation patterns of seminal products obtained by vertical gel electrophoresis with 7% polyacrylamide. Both rabbit seminal plasma and its 4-hr ultracentrifugal upper supernatant fraction lacking DF activity were separated into twelve protein components. However, broad components 11 and 12 were each resolved into two proteins after chromatography on Sephadex G-200 (Text-fig. 3). Component 11 migrated as a slow β-globulin when free of the molecular sieving effect of acrylamide.

The number of electrophoretically detectable proteins in the pellet increased with ultracentrifugal time and reached nine. Components 3 and 11 reached a maximum concentration in the pellet after 4 hr of ultracentrifugation or at about the time DF activity was lost from seminal plasma. Components 2 and 3 stained intensely with periodic acid–Schiff reagent for glycoprotein while components 11 and 12 stained faintly. The concentration of components 1, 2, 6, 7, 8 and 9 in the pellet was considerably less than that in seminal plasma.

Chromatography

Text-fig. 3 presents the elution diagram of the effluent obtained after chromatographing the 5-hr ultracentrifugal pellet (DF) of rabbit seminal plasma on Sephadex G-200. Four peaks were observed corresponding respectively to protein components with molecular weights of over 200,000; 170,000; 87,000; and 28,000. Electrophoretic analysis of the effluent revealed that components 11 and 12 were each composed of double protein bands. The effluent of peak B contained only a single major protein constituent (Text-fig. 3). This protein formed an immunological arc identical with immuno-component 5 of Text-fig. 1 when subjected to immuno-electrophoresis. Since this was the only protein antigen absent from the inactive 4-hr ultracentrifugal upper supernatant fluid, the effluent of peak B was tested for DF activity. Mixing capacitated spermatozoa with the peak-B effluent resulted in a 10·6% fertiliza-



TEXT-FIG. 3. Chromatography of 5-hr ultracentrifugal pellet (DF) of rabbit seminal plasma on Sephadex G-200 with subsequent analysis of peak fractions by means of 7% acrylamide vertical gel electrophoresis. Average molecular weights of peaks: A = >200,000; B = 170,000; C = 87,000; D = 28,000. Vertical gel electrophoretograms: 1, fraction of peak A; 2, fraction of peak B; 3, ascending arm fraction of peak C; 4, descending arm fraction of peak C; 5, ascending arm fraction of peak D; 6, descending arm fraction of peak D; 7, whole DF.

tion rate (2/19) as compared with an 85% fertilization rate (11/13) in the control. Hence, DF activity was associated with one of the components in the 170,000 size range. Immuno-component 5 was presumed to be the biologically active glycoprotein.

DISCUSSION

Rabbit spermatozoa are coated with proteins of seminal plasma origin as they pass along the male reproductive tract to the outside (Weil, 1960). The term, sperm-coating antigens, has been used to describe that phenomenon. The present data linked at least one of the sperm-coating antigens with the DF in seminal plasma. Bedford & Chang (1962) reported that washing did not decrease the time required for capacitation of ejaculated spermatozoa nor did it remove sperm-coating antigens.

Bedford & Chang (1962) reported that epididymal spermatozoa required as long a period for capacitation as ejaculated spermatozoa. Weinman & Williams (1964) showed that epididymal fluid was a potent source of DF. The glycoprotein sperm-coating antigen characterized in our work was present in the ejaculate of vasectomized males but not in the epididymal fluid. This would suggest that more than one glycoprotein can act as a decapacitation factor. Hunter & Hafs (1965) reported that rabbit epididymal spermatozoa were coated with at least four glycoproteins of seminal plasma origin, but no attempt has been made to study epididymal DF in this paper.

The ultracentrifugal pellet (DF) was resolved into nine electrophoretic components on polyacrylamide and DF activity was associated with a glycoprotein which has an electrophoretic mobility similar to a slow β-globulin (electrophoretic component 11 = immuno-component 5). Dukelow, Williams & Chernoff (1965) resolved DF into three protein components with starch gel electrophoresis, but no DF activity was detected in any of the three proteins. Their inability to detect DF activity might have been due to the inability of the active β-globulin to enter the starch gel due to its size of 170,000. Had the β-globulin entered the gel, it could have been bound irreversibly to the starch so that elution would not have been possible. Both conditions are common with starch gel electrophoresis.

The immuno-electrophoretic technique separated proteins on the basis of antigenic differences and mobility or net charge differences. At least four proteins in the DF pellet migrated with similar electrophoretic mobilities but possessed antigenic differences. The DF active glycoprotein could be resolved from this mixture but would have been missed if electrophoresis had been the only technique used.

In the present experiment, the fertilization rate of capacitated spermatozoa treated with seminal plasma was 38% which contrasted with the 0% fertilization rate for capacitated sperm treated with seminal plasma reported by Bedford & Chang (1962). This difference was due to the use of different sites, i.e. the Fallopian tubes of recipient does (Bedford & Chang, 1962; Weinman & Williams, 1964) compared with the uterine horns for surgically inseminating the 'treated' spermatozoa. Adams & Chang (1962) reported that the uterus had a greater ability to capacitate spermatozoa than the Fallopian tube. Their data demonstrated that rabbit semen deposited into the right uterine horn 12 hr after LH injection resulted in a fertilization rate of approximately 30% while tubal inseminations were unsuccessful. We conclude that, although seminal plasma had an adverse effect on fertilizing capacity, 'recapacitation' in the uterus was beginning under the conditions of our assay system. This would explain the 38% fertilization rate.

The present data confirm the findings of Bedford & Chang (1962) and Weinman & Williams (1964) that the inhibitory element to fertilization in seminal plasma could be removed by high-speed centrifugation. Dukelow, Chernoff & Williams, (1966 a, b) reported that amylase destroyed decapacitation activity. Kirton & Hafs (1965) reported that spermatozoa were capacitated *in vitro* by treatment with β-amylase. Both reports were compatible with the finding that a glycoprotein sperm-coating antigen had decapacitation activity.

Evidently, the reactive site on this molecule resided in the carbohydrate moiety rather than in the protein part of the molecule. Braden (1952) reported that, histochemically, the zona pellucida of the ovum was a mucoprotein-like substance. Since the present data demonstrated that a glycoprotein sperm-coating antigen functioned in the phenomenon of decapacitation, one might speculate that this absorbed glycoprotein-blocked reactive sites on the surface of the spermatozoa which are necessary for their attachment to the ovum. Chang (1955) reported that fertilization failure with ejaculated, epididymal or 2- to 4-hr uterine-incubated spermatozoa was due to inability of the sperm cells to penetrate into the zona pellucida. Bedford (1967) indicated that, in the rabbit, capacitation not only invested spermatozoa with the competence to penetrate the ovum, but also endowed them with the ability to establish contact with the surface of the zona pellucida during the fertile life of the ovum. Capacitation might, therefore, involve the alteration of the carbohydrate moiety of the sperm-coating glycoprotein. Unmasked sites on the spermatozoa could then combine with the mucoprotein-like substance of the zona pellucida. After this step, various other enzymes might function to complete the fertilization process.

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