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Failure of Conception in Rabbits Inseminated with Nonagglutinating, Univalent Antibody-Treated Semen[1]

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Guinea pig anti-rabbit sperm and control γ globulin were digested to nonagglutinating, univalent (3.5S) fragments with papain. Samples of rabbit semen (0.3 ml) were incubated (30 min) with 1-ml samples of the univalent or undigested parent γ globulin preparations and then used to artificially inseminate female rabbits. After 6 days the females were examined for blastocysts, ova, and corpora lutea. In five such experiments, all females which received the digested, and four of the five which received the undigested control γ globulin, yielded blastocysts. In terms of corpora lutea, the percentages of blastocysts were 111 and 69%, respectively. In the antibody-treated series blastocysts were found in only one female with digested and none in those treated with undigested antibodies (16 and 0%, respectively).

Since sperm are not agglutinated by univalent antibody and their motility was not visibly affected, these two factors do not explain the conception failures in the univalent antibody-treated series. Therefore, the experimental procedure was repeated but with examination of flushings of the female tracts for sperm at approximately 12 hr. Flushings from females inseminated with digested control γ globulin-treated semen contained approximately 50 times more spermatozoa than those from females inseminated with digested antibody-treated semen. It is suggested that univalent antibody blocks some sperm (surface ?) antigen that has an important role in penetration of cervical mucus.

Considerable interest centers about inhibition of conception by antibodies. Treatment with normal, bivalent antibody inhibits the fertilizability of sea urchin sperm and eggs (Tyler, 1948; Metz, 1967, for review). In the frog, antisera against oocytes (Nace and Lavin, 1963) or oviducal products (egg jelly) can render the eggs unfertilizable (Shaver and Barch, 1960; Shaver, 1966). In mammals isoimmunization of the female with sperm can produce sterility (Menge, 1967; see Tyler and Bishop, 1963 for earlier literature) and artificial insemination with antibody-treated semen fails to result in conception. Unfortunately, the mechanism of the antibody action is incompletely known in these cases both with respect to the immunochemistry of the action and the stage or reproductive process that is blocked. At the immunochemical level, the action could result from direct blocking of an essential antigenic site ("Fertilization Antigen") through interaction with antibody, or it could result from secondary or tertiary steric factors including the cross-linking of neighboring antigens by bivalent antibody. Likewise, conception could be blocked at any one of several stages depending upon the particular material.

Certain of these possibilities, notably the more obvious, steric and mechanical effects (e.g., precipitation and agglutination) that depend upon cross-linking of neighboring

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antigens can be eliminated through the use of nonprecipitating and nonagglutinating, univalent antibody fragments such as the Fab (3.5S) fraction prepared by papain digestion of normal 7S antibody (see Putnam, 1969 for review of antibody structure). Accordingly, nonagglutinating, univalent antibody was examined for inhibiting action on rabbit semen in experiments comparable to earlier studies on sea urchin and amphibian material (Shivers and Metz, 1962; Metz *et al.*, 1964; Metz and Thompson, 1967). The results show that univalent antibody-treated semen does not produce conception and that the treated sperms fail to pass into the uterus and oviducts in normal numbers in intravaginal artificial insemination experiments.

MATERIAL AND METHODS

Ejaculated semen was collected by means of an improved type of artificial vagina (Walton, 1958).

Immunization procedure. Antisera were prepared in guinea pigs against rabbit "seminal solids" (washed centrifugate from fertile ejaculated semen, e.g., sperm plus seminal particles; Metz *et al.*, 1968). The immunizing antigen preparations were emulsified in an equal volume of Freund's complete adjuvant (Difco) and 0.25 ml of the emulsion was injected intramuscularly into each hind leg. Two such injections were given a week apart. Three weeks after the second injection and again at approximately 5–6 weeks, the guinea pigs were bled by cardiac puncture to obtain immune serum. Bleedings prior to the first injection provided control serum. Sera from several bleedings from the same or different animals were pooled to provide sufficient control and immune material, respectively, for several experiments.

Preparation of univalent antibody. Univalent (3.5S) antibody was prepared by the papain-digestion procedure of Porter (1959). The globulin fraction of the guinea pig serum was partially purified by precipitation with 18% (w/v)Na_2SO_4 or half saturated $(NH_4)_2SO_4$. The digestion mixture, pH 7.0, consisted of 0.01M KH_2PO_4, 0.01M cysteine, 0.002M EDTA, and papain (2× crystalized, Nutritional Biochemical Co., 1 mg/100 mg globulin). This digestion mixture was incubated at 37 C for 19–22 hr and the progress of digestion monitored by testing for sperm-agglutinating action. It was considered complete when the antibody failed to agglutinate rabbit sperm. In every digestion, control samples of undigested γ globulin from the parent stocks were subjected to the complete procedure except that saline was substituted for the papain. These undigested controls routinely included samples of the immune and preinjected γ globulin. To stop digestion, iodoacetamide (0.2M final concentration) was added to each sample, and the digestion mixtures were dialyzed against cold 0.85% NaCl overnight.

Treatment of Semen and Artificial Insemination

To compare the effects of bivalent (undigested) and univalent (papain-digested) antibody, 0.3-ml aliquots of pooled undiluted, freshly ejaculated semen were mixed with 1.0-ml samples of papain-digested and undigested antibody γ globulin and with papain-digested and undigested preinjection (control) γ globulin. These mixtures were incubated at 37 C for 30 min. The four semen samples were then scored for motility and agglutination. Four female rabbits were inseminated intravaginally with these samples (1 ml). The four females were injected intravenously with an ovulatory dose (2.5 mg) of purified pituitary hormone (PLH-Armour) at the time of artificial insemination. Six days after insemination, the animals were sacrificed, and the uteri and oviducts were flushed. These flushings were examined for blastocysts and ova. Finally, the ovaries were examined for corpora lutea. Other technical details are given in the text and tables.

RESULTS

Female rabbits were artificially inseminated with bivalent and univalent guinea pig anti-rabbit semen antibody as described in the previous section. The experiment was performed five times and therefore involved a total of 20 female rabbits. The results are summarized in Table 1. Experiments 1, 2, 3, and 4, 5 were triplicates and duplicates, respectively, to the extent that the same γ globulin preparations were used in each. No blastocysts were found in any of the five females inseminated with bivalent antibody-treated semen. The ovaries of all of these females contained corpora lutea and a total of 11 atretic ova were recovered from three of the females.

Blastocysts were found in all but one (Expt. 5) of the females inseminated with control γ globulin. The total number of blastocysts (27) constitutes 69% of the total corpora lutea. This becomes 82% if Expt. 5 is disregarded. It is concluded from these results

TABLE 1

BLASTOCYSTS RECOVERED AFTER INTRAVAGINAL INSEMINATION WITH PAPAIN-DIGESTED ANTIBODY-PRETREATED SEMEN

	Digested antibody[a]			Digested control[a]			Undigested antibody[a]			Undigested control[a]		
Experiment	Blast.	C.L.	Ova	Blast.	C.L.	Ova	Blast.	C.L.	Ova	Blast.	C.L.	Ova
1	0	10	9	10	1	0	0	6	3	3	4	0
2	0	11	0	1	8	0	0	8	0	6	12	0
3	8	8	0	9	8	0	0	8	6	9	9	0
4	0	9	4	11	10	0	0	14	0	9	8	0
5	0	10	7	9	9	0	0	7	2	0	6	0
Total	8	48	20	40	36	0	0	43	11	27	39	0
Blast./C.L. ×100		16			111			0			69	

[a] Blast. = blastocysts; C.L. = corpora lutea.

that the untreated, bivalent antibody inhibited conception in the experiments.

In the papain-digested antibody and control γ globulin-treated series comparable results were obtained. With one exception (Expt. 3) no blastocysts were recovered from females inseminated with papain-digested antibody-treated semen. A total of 48 corpora lutea and 20 atretic ova were found in this series. In terms of corpora lutea, and including Expt. 3, this gives only 16% blastocysts. As expected, the recovery of blastocysts was high in the series inseminated with digested control γ globulin-treated semen. Experiment 1 is anomalous in that 10 blastocysts but only one corpus luteum was found in this control rabbit inseminated with papain-digested antisemen γ globulin-treated semen. It is concluded that the papain-digested anti-semen γ globulin, like the undigested material, inhibits the capacity of semen to effect fertilization in the intravaginal artificial insemination experiments.

Effect of univalent antibody on spermatozoan motility. It seems clear, then, that univalent anti-sperm antibody can inhibit conception by some mechanism other than sperm agglutination. Menge and Protzman (1967) reached the same conclusion from other evidence. To explore the mechanism of this inhibition it seemed essential to determine if the motility of the treated sperms was affected. In the artificial insemination experiments, residual samples of treated sperm were examined microscopically for motility after the insemination. No significant differences in sperm motility were noted between control γ globulin and antibody-treated samples used. As an additional test for an effect of univalent antibody on the motility and longevity of rabbit sperm, γ globulin–semen mixtures (0.2 ml semen + 0.66 ml globulin in 0.85% NaCl) were incubated at 37 C and samples examined at intervals in seven experiments involving four different antibody preparations as shown in Table 2. It seems clear from this data that the papain-digested antibody had no obvious, consistent effect on either the degree or duration of spermatozoan motility. The same antibody preparation was used in Expts. 4 and 5 of Table 1 and Expt. 1, Table 2.

Passage of antibody-treated sperm through the reproductive tract. In view of the apparent failure of univalent antibody to affect the motility of sperm, it seemed appropriate to determine if treated sperm passed through the female reproductive tract and arrived at the site of fertilization, e.g., the upper oviduct, at approximately the time of ovulation. Accordingly, experiments were performed to examine for the presence of sperm in the uteri and oviducts of females artificially inseminated with antibody-treated semen. The initial experiment (Expt. 1, Table 3) duplicated the conception tests as nearly as possible except

TABLE 2

EFFECT OF PAPAIN-DIGESTED ANTI-RABBIT SEMEN GUINEA PIG ANTIBODY ON MOTILITY OF RABBIT SEMEN[a]

Time after mix	Expt. no. 1		2		3		4		5		6		7	
	I	C	I	C	I	C	I	C	I	C	I	C	I	C
30 min.	2+	2+	2+	2+	3+	3+	+	+	3+	2+	+	±	2+	3+
1 hr			+	+	+	2+	±	±	2+	+	+	±	+	2+
2 hr	±	2+			±	±	±	−	±	−	±	±	−	±
3 hr			−	−	−	−	−	−	−	−	−	−		
4 hr	±	±											−	−
5 hr	±	±												

[a] I = Papain-digested antibody-treated sperm. C = Papain-digested control γ globulin-treated sperm. −, ±, +, 2+, 3+, 4+ represent increasing degrees of sperm motility (−, immobile; 4+, maximum motility) as estimated from freshly prepared preparations examined with phase optics.

TABLE 3

TOTAL NUMBER (×100) OF SPERMATOZOA RECOVERED FROM OVIDUCTS AND UTERI AFTER ARTIFICIAL INSEMINATION WITH ANTIBODY-PRETREATED SEMEN

	Semen pretreated with[a]: UI	UC	DI					DC				
	Expt. no. 1	1	1	2	3	4	5	1	2	3	4	5
Left side												
Oviduct[b]												
Upper	0	0.9	0	0	4.4	12.8	0	0	0	0	0	0
Middle	0	0.9	0	9	0	0	0	0	0	0	0	0
Lower	8	40	0	0	0	0	0	42	1.2	16.8	0	100
Uterus	0	106	28	27.6	24	0	0	88	1310	15.9	390	4680
Right side												
Oviduct[b]												
Upper	0	0	0	0	1.8	0	0	8.4	0	0	0	0
Middle	0	0	0	0	0	0	0	0	4.7	0	0	0
Lower	2.2	55	0	43.8	17.5	0	0	0	22	0	1.1	14.4
Uterus	4.5	4.5	0	12	21	0	19.5	2085	1060	62.9	173.8	2100
Total, each rabbit	14.4	207.3	28	92.4	68.7	12.8	19.5	2223.4	2397.9	95.6	564.9	6894.4
Totals Expts. 1–5					211.4					12,176.2		
Average (1–5) Number of spermatozoa recovered from each female					42.3					2435.2		

[a] Semen pretreatment with antibody: UI = undigested antibody; UC = undigested control γ globulin; DI = papain-digested antibody; DC = papain-digested control γ globulin.

[b] Oviduct: oviducts were divided into thirds: upper; middle; lower oviducal segments.

that the females were sacrificed 11–12 hr after artificial insemination. The uteri and oviducts were then isolated, the oviducts were cut into three equal segments: ("upper," "middle," and "lower"), and these segments and the uteri were flushed with 0.85% saline and the volume of recovered flushing fluid measured. Approximately 0.3 ml of saline was

used to flush each oviducal segment. Uteri were flushed with 1.5 ml of saline. Sperm counts were then made on samples of all flushings, and the total numbers of sperm recovered per oviducal segment or uterus were calculated (Table 3).

In Expt. 1, control γ globulin (both undigested and digested)-treated sperms were found in considerable numbers, at least in the lower third of the oviducts. As expected, very few sperms were found in uterine or oviducal flushings of the rabbit inseminated with undigested (bivalent) antibody-treated semen. Likewise, very few sperms were found in flushings from the rabbit inseminated with nonagglutinating (univalent), papain-digested antibody-treated semen.

In view of this last result, the experiment was performed an additional four times using only papain-digested anti-semen and control γ globulin-treated semen as shown in Table 3 (Expts. 2, 3, 4, and 5). The same antibody preparation was used in Expts. 3, 4, and 5, Table 3, and Expts. 1 and 3, Table 2. Very few sperms were recovered from the upper two thirds of the oviducts in either group. Sperms were recovered from only two of the ten lower oviducal segments in the digested immune globulin-treated series. In the control series sperms were found in flushings from seven of the ten lower oviducal segments. Even more striking differences were found in the uterine flushings. Here no sperms were found in nearly half (four of ten) uteri from the digested immune series and the number of sperms recovered from the six positive uteri was small (< 3000/uterus). In contrast the flushings from the control series yielded numbers ranging from 1,590 to 468,000 per uterus. As seen in Table 3, averages of the total number of sperms recovered from the digested immune and the control groups, respectively, are 42.3×100 and 2435.2×100, a 57-fold difference.

It is concluded from these results that passage of spermatozoa into the uterus is markedly reduced (approximately 50-fold) by treatment with papain-digested (univalent) anti-sperm antibody. This, in fact, seems to be sufficient to explain the failures of conception by the papain-digested antibody-treated sperm in Table 1.

DISCUSSION

It seems clear from the results with univalent antibody reported here and from the experiments of Menge and Protzman (1967) that mechanical trapping of sperm by antibody agglutination is not in itself sufficient to explain the inhibition of conception by anti-sperm antibody. Menge and Protzman (1967) found that antisera against seminal plasma agglutinated sperm but failed to inhibit conception. However, the seminal plasma antigens and, especially, the spermatozoan coating antigen (SCA of Weil, 1960) may be relatively easily removed from the sperm surface under the conditions (e.g., ionic; enzymatic; pH) of the vagina and upper tract. This could cause reversal of agglutination and release of trapped sperm. In any event, Menge and Protzman's results would indicate that the SCA, and any other seminal plasma antigens shared with sperm surface, are not essential for sperm function, a result in agreement with the fact that epididymal sperm, presumably lacking SCA, can function in fertilization (Austin and Bishop, 1957; Bedford, 1965).

In addition to the above, Menge and Protzman (1967) found that anti-sperm or testis antibody did inhibit the ability of rabbit semen to produce conception in artificial insemination experiments. This indicates that some sperm-specific antigen(s) performs an essential function(s) in one or more steps in the reproductive process. The antigen(s) in question may be one or both of the two soluble sperm-specific antigens resolved by double-diffusion precipitin tests (Hunter and Hafs, 1965; Menge and Protzman, 1967). Alternatively, it may be an "insoluble antigen(s)" built into the sperm surface, or a constellation of both soluble and insoluble antigens. It is

reasonable to suppose that a sperm-specific antigen(s) is also involved in inhibition of conception by the univalent antibody in the present study. Blocking of this antigen(s) by univalent antibody does not appear to affect the degree or duration of rabbit sperm motility as determined by the usual phase optical examinations. A similar conclusion was reached with respect to the sea urchin (Metz *et al.*, 1964). However, such examination may not rule out the possibility of a subtle effect on the translational as opposed to "vibrational" component of sperm activity. A more sophisticated, quantitative study, perhaps involving cinéphotomicrography, would be required to examine this possibility in detail.

At least provisionally, then, some function other than sperm motility is assumed to be inhibited by blocking an essential antigen(s). The marked reduction in the number of sperms recovered from the uteri and oviducts suggests that the blocking interferes with passage of sperm through the cervix. Presumably, this results from action of the univalent antibody on the sperm, and not on the cervix or cervical mucus. Mechanisms that could explain this include inhibition of lytic enzymes of the sperm required to digest a passage through the cervical mucus. Hyaluronidase is one possibility. Hyaluronidase, at least from guinea pig semen, is reported to be antigenic (Katsh and Katsh, 1961). In addition, it is present in or on epididymal rabbit sperm (Stambaugh and Buckley, 1969). Proteolytic enzymes are another possibility.

Antibody (multivalent) inhibition of sperm passage through human cervical mucus has been reported by Fjällbrant (1968) who attributed the mechanism primarily to a reduction in sperm motility. Finally, additional steps in the mammalian reproductive process may be susceptible to antibody inhibition. Apart from sperm passage, possible targets include capacitation, the acrosomal reaction, penetration of the cumulus, corona radiata, and zona, and attachment and, finally, fusion of the sperm with the egg. These possibilities are currently under investigation.

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