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Effects of Isoimmunization and Isoantisera Against Seminal Antigens on Fertility Process in Female Rabbits[1]

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The numbers of sperm and polymorphonuclear leukocytes and the motility and progression of the sperm were determined in flushings from the reproductive tract at 7 and 15 hr postinsemination of 28 rabbits in two experimental trials. In Trial 1, isoimmunization with semen as compared with seminal plasma resulted in a trend for fewer sperm to be recovered from the oviducts, significant decreases in motility of sperm from the oviduct, and in progression of sperm recovered from the uterus and a prevention of fertilization (0.0 vs 90%). Insemination of nonimmune rabbits in Trial 2 with semen treated with isoantiserum against semen in comparison to isoantiserum against seminal plasma caused significant decreases in sperm numbers recovered from the oviduct, uterus, and vagina; a decrease in sperm motility in vaginal flushings; a reduced progression of uterine sperm, and an inhibition of fertilization (12.5 vs 95.6%). Leukocyte numbers were influenced by treatment only in vaginal samples of both groups. The range of values for number and motility of sperm recovered from the oviducts overlapped between treated and control groups within experiments suggesting that these factors alone were not responsible for the observed inhibition of fertilization. Correlation coefficients were calculated among the variables in both experimental trials.

Immunization of females with homologous testis, semen, and sperm has induced infertility in cattle (Menge, 1967), guinea pigs (Isojima *et al.*, 1959; Otani *et al.*, 1963), mice (Edwards, 1964; McLaren, 1964) and rabbits (Behrman and Nakayama, 1965; Menge, 1968, 1970b). Similarly, treatment of semen before insemination with specific antisera against these antigenic materials has also resulted in antifertility effects in rabbits (Kiddy *et al.*, 1959; Menge and Protzman, 1967) and cattle (Menge *et al.*, 1962). Inhibition of fertilization appears to be a major effect in the induced infertility. The following factors have been suggested as possible causes for the immunologic infertility; interference with sperm transport, immobilization of sperm, increased phagocytosis of sperm, and the blocking of fertilization sites on the sperm membrane. The present study was undertaken to investigate the effects on the transport, motility, and fertilizability of sperm in the reproductive tracts of female rabbits isoimmunized with semen and seminal plasma and of female rabbits artificially inseminated with semen treated with antisera against these two antigenic materials.

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MATERIALS AND METHODS

Sexually mature New Zealand white rabbits were used in the trials. Semen was collected with an artificial vagina from male rabbits.

Trial 1. Eight female rabbits were immunized with pooled ejaculates of rabbit whole semen (RWS) and eight rabbits with rabbit seminal plasma (RSP) from the pooled ejaculates of two vasectomized male rabbits. The rabbits were injected once weekly for 4 weeks followed by a fifth injection 3 weeks later. Each injection consisted of 0.5 ml of antigen plus 0.5 mg each of polyadenylic and polyuridylic acids in 0.5 ml of saline. The use of these polynucleotides as an adjuvant has

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been previously reported (Menge, 1970b). Seven to ten days after the final injection, the rabbits were bled, artificially inseminated (AI), and injected with 50–75 iu of human chorionic gonadotropin (HCG). The semen, 100×10^6 sperm in 0.5 ml of Tyrode's solution, had been treated prior to insemination with tetracycline following the method of Ericsson (1967). Examination of the semen samples at the time of insemination revealed motility estimates of 60% or greater and a progression value of 3 in each case. Sperm motility was the estimate of percentage of live sperm and progression of the motile sperm was rated from 0–3 on the following basis: 0, oscillatory motion; 1, poor forward or circulation motion; 2, fair forward motion; and 3, good forward motion.

At approximately 7 hr after AI the rabbits were anesthetized with sodium pentobarbital and the reproductive tract was exposed through a midline incision. Small arterial clamps were placed on one uterine horn at the cervix and uterotubal junction and on the infundibulum of the adjoining oviduct. This portion of the tract was excised after ligation of the connecting arterial vessels and cervix with nylon sutures. The incision was closed and the rabbits were returned to cages. At 15 hr after AI (approximately 8 hr after laparotomy) each rabbit was given a lethal dose of pentobarbital and the remaining oviduct and uterine horn, the cervices, and anterior vagina were clamped and removed. Immediately after removal the different portions of the tract were flushed with Tyrode's solution at 37C. The flushings were examined microscopically under white light to determine sperm motility, progression, and agglutination and under uv light to determine whether the sperm had retained the fluorescent tetracycline label. Ova were recovered from the 15-hr oviduct flushings and examined with a phase-interference microscope to determine sperm penetration and polar body extrusion. The oviduct flushings, after removal of ova, were centrifuged and the pellets resuspended in 0.2 ml of solution to aid in counting cellular contents. Sperm and polymorphonuclear leukocyte counts were made by the hemacytometer method on duplicate samples. In those cases of oviduct flushings with few sperm, the total number of sperm was estimated by counting cells in two or more 20-μl drops placed on a glass slide. Portions of the reproductive tissues were fixed in Bouin's solution, embedded in paraffin, and stained with hematoxylin and eosin for histological evaluation.

Serum samples, after complement inactivation (56C for 30 min), were tested for antibodies by three methods: (1) passive hemagglutination (PHA) which involved coating of tanned sheep red blood cells with supernatant fluid from freeze-thawed (10 ×) semen; (2) sperm agglutination (SA) in a gelatin solution (Kibrick *et al.*, 1952), and (3) sperm immobilization (SI) in the presence of guinea pig complement (Isojima *et al.*, 1968).

Trial 2. Six estrous rabbits were artificially inseminated with rabbit semen treated with antiserum against seminal plasma and six estrous rabbits with semen treated with antiserum against semen. The antisera were pools of decomplemented sera from three rabbits of each group in Trial 1. Semen samples of high quality (60% motility and progression of 3) were pooled and diluted with Tyrode's solution to contain 200×10^6 sperm/ml. Equal volumes of diluted semen and antisera diluted with equal parts of saline were incubated together for 20 min at 37C. After incubation the mixtures were centrifuged and the sperm was resuspended to 200×10^6 sperm/ml with Tyrode's solution. The rabbits were inseminated with 0.5 ml of treated semen and injected with 75 IU of HCG. The intervals and procedures for recovery and examination of the contents of the reproductive tract were identical to those outlined in Trial 1.

The sperm and leuckocyte recovery data were transformed to square root values and motility percentages to arcsin angles for statistical analysis (Snedecor, 1956). Transformation was necessary as a preliminary analysis of the original data revealed that the variances were related to the means within subgroups. The data were subjected to analysis of variance, paired t test (vaginal recovery data), and correlation calculations. The mean values presented in the tables have been reconverted from the transformed data.

RESULTS

Trial 1. The mean antibody titers, expressed as powers of the base 2 ± SE, in serum samples obtained at the time of insemination in Trial 1, and as determined by the PHA, SA, and SI tests were, respectively, 16.8 ± 1.02; 7.0 ± .71 and <1 for RSP-immunized rabbits; and 15.8 ± .84; 11.0 ± .64 and 7.8 ± .61 for RWS-immunized rabbits. Immunization with RWS in comparison to RSP caused significant increases ($p < 0.01$) in the SA and SI titers, but the PHA titers were not different.

There was a trend for fewer sperm to be recovered from the oviducts and uteri of RWS-immune rabbits than from RSP-immune rabbits, but the differences were not significant because of large within-group variation (Table 1). The range of values gives an indication of the extent of the variation. The recovery rate of sperm from

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TABLE 1

MEAN NUMBERS OF SPERM AND LEUKOCYTES AND MEAN MOTILITY AND PROGRESSION OF SPERM RECOVERED FROM THE REPRODUCTIVE TRACTS OF IMMUNIZED FEMALE RABBITS[a]

	Immunizing antigen				Effects[b]
	Seminal plasma		Whole semen		
	7 hr	15 hr	7 hr	15 hr	
No. of rabbits	8		8		
Oviduct					
Sperm no.	1722 (24–9100)	2430 (50–9840)	299 (30–3200)	1096 (80–7100)	I[c]
Sperm motility (%)	11.0 (2–30)	23.5 (5–40)	5.7 (2–20)	10.8 (5–25)	I*
Leukocyte no. (10^4)	14 (5–53)	18 (6–41)	8 (5–17)	11 (8–16)	
Uterus					
Sperm no. (10^3)	794 (624–1152)	503 (160–1862)	663 (180–1173)	250 (100–462)	T*
Sperm motility (%)	54.2 (40–60)	39.9 (30–50)	52.0 (40–60)	34.5 (20–55)	T[c]
Sperm progression	2.6 (2–3)	1.6 (1–3)	1.4 (0–3)	0.6 (0–1)	I** T*
Leukocyte no. (10^4)	137 (41–398)	324 (72–981)	165 (26–1217)	276 (76–1050)	
Vagina					
Sperm no. (10^3)		346 (180–638)		372 (160–748)	
Sperm motility (%)		21.6 (5–35)		8.4 (0–25)	
Sperm progression		1.4 (0–3)		0.4 (0–1)	I[c]
Leukocyte no. (10^4)		85 (38–207)		267 (70–1060)	I*

[a] Range of values given in parentheses.
[b] I, immunization effects; T, time effects.
[c] $p < 0.10$.
* $p < 0.05$.
** $p < 0.01$.

the uterus decreased significantly ($p < 0.05$) from 7 to 15 hr. Motility of sperm recovered from the oviducts of RWS-immune rabbits was less than that of sperm from the oviducts of RSP-immune rabbits ($p < 0.05$). Overall, sperm motility was significantly ($p < 0.01$) reduced in oviductal flushings in comparison to uterine samples. The degree of sperm progression was considerably lower in uterine ($p < 0.01$) and vaginal flushings ($p < 0.10$) from the RWS-sensitized rabbits. This may have been due in part to an apparently greater proportion of motile sperm adhering by their heads to leukocytes in these samples. Differences in type and degree of agglutination of sperm were not apparent in samples from the two groups of rabbits. Head-to-head agglutination was seen in all samples with no particular pattern evident. Leukocyte recovery rates were influenced significantly by immunization only in the vaginal flushings. Phagocytosis of sperm by leukocytes appeared minimal in both groups and at both intervals.

Correlation coefficients among the variables in Trial 1 were calculated on a within-subgroup basis and then pooled within immunization treatment if heterogeneity among subgroups was not significant (Table 2). The leukocyte and sperm recovery rates were correlated positively in the RSP-immune rabbits and unrelated in the RWS-immune group. In this latter group, however, increased leukocyte numbers were associated with a decreased sperm motility. The PHA and SA titers tended to be positively associated with sperm variables in RSP-immune rabbits and negatively associated in RWS-immune rabbits. Correlations of SI titers with sperm numbers and motility were

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TABLE 2

CORRELATIONS AMONG VARIABLES OF IMMUNIZED RABBITS[a]

Variables	Seminal-plasma immunized					Whole-semen immunized					
	Sperm			Leuk. no.	Titer SA	Sperm			Leuk. no.	Titer	
	No.	Motility	Progress			No.	Motility	Progress		SA	SI
Sperm no.	—	−0.30	0.19	0.92**,[b]	—	—	−0.36	0.16	0.05[b]	—	—
Leukocyte no.	—	−0.28	0.07	—	—	—	−0.64**	−0.45	—	—	—
PHA titer	0.40*,[b]	0.67**,[b]	0.29	0.52**	0.42	−0.70**,[b]	−0.37[b]	−0.20	0.16	0.56	−0.15
SA titer	0.47*,[b]	0.39,*,[b]	0.21	0.24	—	−0.27[b]	−0.53**,[b]	−0.38	0.41*	—	0.76*
SI titer	—	—	—	—	—	H[c]	H[c]	−0.49*	−0.09	—	—

[a] DF for correlations: among serum titers, 6; involving sperm progression, 15; and all others, 25.
[b] Correlations differed ($p < 0.05$) between immunized groups.
[c] Correlations heterogeneous among subgroups (see text for explanation).
* $p < 0.05$.
** $p < 0.01$.

heterogeneous among subgroups in the RWS-immune rabbits. SI titers and sperm numbers were negatively associated (−0.89, $p < 0.01$) in the 15-hr uterine flushings, whereas the correlations within the other subgroups were positive and small. Within oviduct flushings, high serum SI titers were correlated with low sperm motility, (−0.91, $p < 0.01$) in comparison to a nonsignificant positive correlation (0.24) in uterine flushings.

Of the 40 ova recovered from RSP-sensitized rabbits, 36 were fertilized (90.0%) compared with none of 37 ova from RWS-sensitized rabbits. The fertilized ova recovered from the former group averaged 3.5 sperm present in perivitelline space and were largely devoid of cumulus and corona radiata cells. None of the ova from the RWS-immune rabbits had sperm penetrating the intact corona radiata cells or the zona pellucida. A few motile sperm were observed among the peripheral cumulus cells which were still intact around all the ova from this group. Sperm recovered from the reproductive tracts of both groups of rabbits lacked fluorescence, whereas control sperm maintained in Tyrode's solution after tetracycline labeling showed bright fluorescence under uv microscopy.

Microscopic examination of the reproductive tissues revealed no apparent differences in the state of the inflammatory response due to immunizing antigen. However, there was an increase in the degree of tissue inflammation from 7 to 15 hr in both groups.

Trial 2. The results of Trial 2 in which rabbits were inseminated with semen treated with antisera are presented in Table 3. Recovery rates of sperm from the different portions of the reproductive tract were significantly reduced by pretreatment with anti-RWS serum. Sperm were not found in uterine and oviduct flushings at 7 hr from one and two rabbits, respectively, in this treatment group. Sperm motility was unaffected by antisera treatment except for vaginal flushings in which antiserum against RWS decreased the motility. Motility of sperm recovered from the oviducts and vagina was less ($p < 0.01$) than that from the uterus. Also, uterine sperm motility decreased from 7 to 15 hr ($p < 0.05$). Progressive motion of sperm was significantly depressed ($p < 0.05$) in the uterine flushings

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TABLE 3

MEAN NUMBERS OF SPERM AND LEUKOCYTES AND MEAN MOTILITY AND PROGRESSION OF SPERM RECOVERED FROM REPRODUCTIVE TRACTS OF RABBITS INSEMINATED WITH ANTISERA-TREATED SEMEN[a]

	Antisera against								Effects[b]
	Seminal plasma				Whole semen				
	7 hr		15 hr		7 hr		15 hr		
No. of rabbits	6				6				
Oviduct									
Sperm no.	826	(64–1620)	1076	(48–3240)	125	(0–360)	653	(32–3080)	A*
Sperm motility (%)	26.1	(10–40)	23.5	(15–35)	24.9	(20–30)	17.6	(10–25)	
Leukocyte no. (10^4)	3	(5–15)	3	(1–5)	3	(2–8)	3	(2–5)	
Uterus									
Sperm no. (10^3)	648	(280–1632)	797	(528–1134)	146	(0–550)	352	(176–704)	A**
Sperm motility (%)	54.1	(50–65)	36.8	(30–45)	48.8	(40–65)	32.9	(25–45)	T*
Sperm progression	2.8	(2–3)	2.0	(1–3)	1.6	(1–3)	1.4	(0–2)	A*
Leukocyte no. (10^4)	394	(62–2060)	318	(66–1210)	243	(14–1744)	330	(96–1458)	
Vagina									
Sperm no. (10^3)			1088	(400–2080)			334	(200–520)	A*
Sperm motility (%)			25.8	(20–35)			15.6	(10–25)	A*
Sperm progression			2.0	(2)			1.4	(1–2)	
Leukocyte no. (10^4)			529	(276–920)			267	(92–506)	A**

[a] Range of values given in parentheses.

[b] A, antiserum effects; T, time effects.

* $p < 0.05$.

** $p < 0.01$.

from rabbits inseminated with sperm after treatment with anti-RWS serum compared with anti-RSP serum. The number of leukocytes was affected by antiserum treatment of sperm in only the vaginal flushings with a greater number of leuckocytes recovered from rabbits inseminated with sperm treated with anti-RSP serum.

The associations among the variables were small and nonsignificant within both groups with the only exception being the correlation of leukocyte numbers with sperm motility ($-0.46, p < 0.10$) in flushings from rabbits inseminated with semen treated with anti-RWS serum.

Examination of the ova indicated that 22 of 23 ova (95.6%) recovered from rabbits inseminated with semen treated with antiserum against RSP were fertilized. Twenty of these ova were completely denuded of cumulus and corona radiata cells and averaged five sperm in the perivitelline space. From rabbits inseminated with sperm treated by antiserum against RWS, only 3 of 24 ova (12.5%) were fertilized. The ova from these rabbits still had intact cumulus masses and even the three fertilized ova (one rabbit) had intact corona radiata cells. After examination, all of the ova were cultured *in vitro* for 2 days at 37 C under 5% CO_2 in air in Ham's F10 media containing 20% rabbit normal serum. All of the previously designated fertilized ova underwent apparently normal cleavage, whereas none of the nonfertilized ova showed cleavage.

Again, as in Trial 1, histology of the tissues indicated an inflammatory reaction which increased with time, but was apparently unaffected by type of antiserum used to treat the inseminating sperm.

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DISCUSSION

An antifertility effect was apparent both in rabbits immunized with semen and in those inseminated with semen treated with antisemen serum, although the exact nature of the effect was not evident. Immunization with seminal plasma from vasectomized rabbits was used as a treatment control because it does elicit sperm agglutinins but has no adverse effect on fertility (Menge, 1968). It also served as a control for the polynucleotide adjuvant as well as for those seminal antigens that are not sperm specific. Similar to reports for sperm-immunized mice (Edwards, 1964; McLaren, 1964) and rabbits (Sokolovskaya and Reshetnikova, 1969) and in rabbits inseminated with sperm treated with univalent antibody against semen (Metz and Anika, 1970), the numbers of sperm recovered from the oviducts of the two groups of treated rabbits were reduced in comparison to control rabbits. Inherent in this type of study is the inability to determine the total numbers of sperm that have actually traversed the different portions of the reproductive tract. Thus, we have assumed that the numbers of sperm recovered reflect the relative rate of sperm transport in the rabbits. Motility of sperm recovered from the oviducts of semen-immunized rabbits was also reduced. Within oviduct subgroups, however, the variation was large and the ranges of sperm numbers and sperm motility overlapped between the treated and control groups as illustrated in Tables 1 and 3. This suggests that a reduction in numbers and motility of sperm in the oviduct, unless absolute, does not necessarily result in fertilization failure. The numbers of sperm recovered from oviducts of the rabbits, especially the control groups, were within the ranges reported by Austin (1948) and Chang (1951) for rabbits after natural and artificial inseminations. These authors also found that fertilization could occur in rabbits inseminated with low numbers of sperm and consequently, with few sperm being recovered from the oviducts. A noticeable effect observed in the uterine and vaginal flushings of the semen-immunized rabbits and those inseminated with antiserum against semen was the reduced forward motion of the sperm. If sperm transport involves in part, the active participation of the sperm, the reduced progression could have accounted for the decreased sperm numbers in the upper tract. Fjällbrant, (1968) has reported that human sperm treated with antibodies and sperm from men with autoantibodies show a reduced penetration of cervical mucus. The subtle changes in sperm progression may have resulted from effects on the sperm cells by antibodies either directly or indirectly through interactions with leukocytes. The negative association of leukocyte numbers with sperm motility within the treated rabbits suggests that leukocytes had exerted some detrimental effect. It is known that antibody–antigen complexes can activate complement, which in turn, causes the release of destructive lysozymes by leukocytes. However, attempts to detect immobilizing antibodies in flushings and on sperm recovered from the uterus by the addition of complement to the samples failed in both groups. This is rather surprising, especially since sperm were treated prior to insemination with antiserum against semen that contained immobilizing activity. Treated sperm stored *in vitro* were immobilized by the addition of complement, suggesting that the antibodies were either removed or degraded in the reproductive tract. Edwards (1964) also failed to find immobilization of sperm recovered from the uteri of sperm-sensitized mice. The negative association of serum-immobilizing antibody with sperm motility in the oviducts, but not in the uteri of semen-immunized rabbits, suggests that serum antibodies may be entering the oviducts by transudation or through the infundibulum

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from the peritoneum. That this is probably a serum antibody of the IgG type and not a locally secreted antibody of the IgA type is further supported by the fact that IgA does not fix complement, which is a requirement for sperm immobilization. Metz and Anika (1970) found that the inhibition of passage in the female tract of rabbit sperm treated with antibodies was not complement dependent as they used univalent antibodies which do not activate complement. In the present study, specific agglutination of sperm was not seen in any of the reproductive tract flushings.

The apparent failure of the sperm in the oviducts of treated rabbits to penetrate and disperse the cumulus mass of the ovum was probably responsible for the lack of fertilization. This suggests an inhibition of normal sperm physiology. If, as according to Ericsson (1967), tetracycline removal from sperm is indicative of initiation of capacitation, it is apparent that the immune effects are not affecting this stage as the tetracycline label was removed in the reproductive tract of immune rabbits. Bedford (1968) indicated that capacitated sperm in the proximity of ova and cumulus cells undergo acrosomal membrane vesiculation as a prerequisite to fertilization. This may indicate release of acrosomal enzymes which disperse the cumulus and corona cells and aid the sperm in penetrating ova. The spontaneous adherence of rabbit sperm to ova observed *in vitro* can be prevented by both univalent and bivalent antibodies against sperm (Menge, 1970a). Sperm-specific antibodies may be blocking sperm adherence to ova and acrosome reactions by binding reactive sites on sperm membranes and consequently preventing fertilization. Peroxidase-labeled isoantiserum against sperm has indicated the presence of antigenic sites on the acrosome (unpublished data).

Apparently antibodies are acting at various levels of the reproductive tract and the fertility process, i.e., sperm transport and migration, sperm motility and progression, and sperm attachment and penetration of ova. The exact mechanisms involved, however, are still to be elucidated.

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