

Use of Polynucleotides with Seminal Antigens to Induce Isoantibodies and Infertility in Rabbits¹ (34999)

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Since the discovery of Freund's adjuvant, the material has been used widely with a variety of antigens to induce immune reactions (1). The reliable induction of immunoinfertility in mice (2), guinea pigs (3), rabbits (4), and cattle (5) has required the use of complete Freund's adjuvant with semen and spermatozoa. Complete Freund's adjuvant, however, has several disadvantages: the induction of abscesses and lesions at the site of injection (6), pathological changes in certain organs (7, 8), and the need to emulsify the antigen with the adjuvant. Recently, polynucleotides have been shown to stimulate antibody synthesis to soluble and cellular antigens in mice (9, 10) and in mouse macrophages cultured *in vitro* (11).

The present study was to determine the immune response and fertility effects in rabbits isoimmunized with seminal antigens using polynucleotides as adjuvant.

Materials and Methods. The rabbits used in the study were sexually mature females of the New Zealand Whites. The seminal antigens used were: rabbit seminal plasma (RSP), rabbit whole semen (RWS), and rabbit epididymal sperm (RES). The seminal plasma and semen were from pooled ejaculates collected with an artificial vagina from two vasectomized rabbits and six normal male rabbits of proved fertility, respectively. The epididymal sperm were obtained from the cauda epididymides of healthy males. The RES was washed three times with phosphate-buffered saline (PBS, pH 7.2) and diluted to contain approximately 2×10^8

sperm per ml of PBS. RWS was diluted to a similar concentration. All three antigenic materials were stored at -20° until used. The polynucleotides, polyadenylic acid (poly A), and polyuridylic acid (poly U) [Calbiochem Corporation] were used as the adjuvant. The injection schemes used were: (1) four weekly injections of 0.5 ml of antigen plus 0.5 mg each of poly A and U dissolved in 0.5 ml of PBS followed 3 weeks later by a fifth injection of the same amount of materials; and (2) three weekly injections of 0.4 ml of antigen plus 0.4 mg each of poly A and U in 0.4 ml of PBS followed 3 weeks later by a fourth injection consisting of 0.6 ml of antigen and 0.6 mg each of poly A and U. These injection schemes were designated as $5\times$ and $4\times$ respectively. Injections were given in multiple subcutaneous and intramuscular sites.

Serum samples were obtained before immunization and at the time of insemination or embryo transfer. Antibody titers in complement-inactivated sera (56° for 30 min) were evaluated by three methods: (1) passive hemagglutination (PHA), which involved coating tanned sheep red blood cells with supernatant fluid from freeze-thawed ($10\times$) semen; (2) sperm agglutination (SA) in a gelatin-saline solution (12); and (3) sperm-immobilization [SI] (13). Briefly, the SI test involved adding to 0.25-ml samples of serially diluted serum, 0.05 ml of a motile sperm suspension (30×10^6 sperm/ml saline) and 0.05 ml of guinea pig serum (diluted 1:20 in saline). The samples were incubated for 1 hr at 37° . Immobilization was recorded positive for an immune-serum sample if sperm motility was less than one-half that of the respective normal-serum sample.

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Seven to ten days after the last immunization the rabbits were artificially inseminated with approximately 5×10^7 sperm in 0.5 ml Tyrode's solution and injected intravenously with 75 IU of human chorionic gonadotropin (HCG). Rabbits used in fertilization studies were killed at 26–28 hr after insemination. Ova were checked for fertilization, as determined by normal cleavage, after being flushed from the excised oviducts. Embryo implantation rate was determined at laparotomy at 9 days after insemination or at 8 days after embryo transfer. Fetal survival rate was determined by comparing the number of viable young at 28 days *in utero* or at birth with the number of implantation sites at 9 days.

Rabbits used as embryo donors were injected subcutaneously once daily for 3 days with 0.5 mg equine follicle-stimulating hormone (FSH, Mann Laboratories) suspended in 0.5 ml sesame oil containing 5% beeswax. On the fourth day the rabbits were mated with two fertile males and both the embryo donors and recipients were injected with 75 IU of HCG. The donors were killed 28 hr after mating and the embryos were recovered by flushing the oviducts with Hanks' solution containing antibiotics and 20% rabbit normal serum. Generally, 30–50 two-cell embryos were recovered from each superovulated donor that had been successfully fertilized. Five embryos were transferred into

each oviduct of recipient rabbits through a midline laparotomy. All surgery was performed under pentobarbital anesthesia using antiseptic procedures. Data were analyzed by analysis of variance and Student's *t* test.

Results. Isoimmunization after the 5 \times schedule resulted in complete inhibition of fertility, as determined by the number of implantations in rabbits sensitized with RWS and RES, compared with an 86.7% implantation rate in the RSP group (Table I). After parturition, the young were removed from the RSP-immune rabbits and within 2 weeks the three groups of rabbits were reimmunized with the respective antigens and amounts previously injected. The rabbits were inseminated and killed to determine fertilization rates. As seen from Table I, 88% of the ova from the rabbits immunized with RSP were fertilized, whereas fertilization had occurred in none of the ova from the other two groups of immune rabbits. Isoimmunization of rabbits following the 4 \times schedule resulted in similar fertilization rates as the 5 \times schedule; with the exception that fertilization occurred in 13.3% of the ova from the RWS-immune rabbits.

Survival rates of embryos transferred into the immune rabbits did not vary significantly due to immunization schedule (Table II). Within both schedule groups, isoimmunization with RWS and RES resulted in highly significant ($p < .01$) reductions in embryo

TABLE I. Fertility in Rabbits Isoimmunized Against Seminal Components Using Poly A and Poly U as Adjuvant.

Immunization		No. of rabbits	No. corpora lutea	First breeding		Second breeding ^a	
				Implanted	Born	Recovered	Fertilized
5 \times	Seminal plasma	4	30	26 (86.7) ^b	23 (88.5)	25 ^c	22 (88.0)
	Semen	3	22	0	—	23	0
	Epididymal sperm	3	23	0	—	20	0
4 \times	Seminal plasma	3				27	25 (92.6)
	Semen	3				30	4 (13.3)
	Epididymal sperm	3				28	0

^a First breeding for rabbits on 4 \times schedule.

^b Figures in parentheses are percentages.

^c Three rabbits, one of original four rabbits died 4 days postpartum.

TABLE II. Survival Rates of Embryos Transferred to Rabbits Isoimmunized with Seminal Components Using Poly A and Poly U as Adjuvant.

Immunization		No. of rabbits	No. embryos transferred	Survival rates (%)	
Schedule	Material			9 days	28 days ^a
5×	Seminal plasma	4	40	60.0	88.9
	Semen	4	40	15.0	16.7
	Epididymal sperm	3	30	0.0	—
4×	Seminal plasma	3	30	73.3	77.3
	Semen	3	30	13.3	25.0
	Epididymal sperm	3	30	16.7	20.0

^a Percentage of implantation sites at 9 days surviving to 28 days.

survival rates at 9 days compared with RSP immunization. The overall 9-day survival rates were 65.7%, 14.3%, and 8.3% for the RSP, RWS, and RES groups, respectively. Because of the small number of rabbits and fetuses involved in the 28-day survival rates, an analysis was done on the pooled data. The analysis indicated that fetal survival rates, in comparison to RSP-immune rabbits (80.4%), were significantly less ($p < .05$) in the RWS-immune (20.0%) and RES-immune rabbits (20.0%).

Serum titers of isoantibodies as determined by the three methods are presented in Table III. There was an overall trend for serum titers of rabbits on the 4× schedule to be less than those of rabbits on the 5× schedule. Compared with anti-RWS sera that showed relatively high titers in each test, anti-RSP sera possessed similar PHA titers, significantly lower ($p < .01$) SA titers and no detectable SI antibodies; whereas, anti-RES

sera had significantly lower ($p < .01$) PHA titers and comparable SA and SI titers.

Correlations of titers between immunologic tests were calculated within each treatment subgroup and pooled overall after determining that correlation heterogeneity did not exist among the immunizing antigen X-injection schedule subgroups. The only significant correlation (0.87, $p < .01$) was between the SA and SI titers of serum samples from the rabbits immunized with semen and epididymal sperm. Correlations of PHA titers with those of the other two tests were small and nonsignificant.

Discussion. Isoimmunization with seminal antigens in combination with the polynucleotides poly A and U induced serum titers of specific isoantibodies and infertility in rabbits. Polynucleotides presumably enhance antibody synthesis by decreasing the interval to antibody induction and stimulating the RNA synthesis in cells involved in immuno-

TABLE III. Serum Titers of Isoantibodies.^a

Immunization			Test ^b		
Schedule	Material	No. of sera	PHA	SA	SI
5×	Seminal plasma	8	17.6 ± 0.7	4.0 ± 0.6	<1
	Semen	7	19.1 ± 1.0	10.6 ± 0.4	8.9 ± 0.7
	Epididymal sperm	5	10.2 ± 1.2	10.4 ± 0.5	8.8 ± 0.6
4×	Seminal plasma	6	17.5 ± 1.4	4.2 ± 0.9	<1
	Semen	6	16.8 ± 0.8	9.7 ± 0.9	7.8 ± 0.7
	Epididymal sperm	6	10.0 ± 0.7	7.3 ± 0.8	8.2 ± 0.5

^a Titers are presented as reciprocals of powers of 2 ± standard error of the mean.

^b PHA, passive hemagglutination; SA, sperm agglutination; SI, sperm immobilization.

globulin production (10, 14). In the present study, lesions and abscesses were not found at the sites of injection nor were any gross pathological changes noted in the kidneys, livers, and spleens of the rabbits. This contrasts to the deleterious side effects of Freund's adjuvant reported in other studies (6-8).

The infertility obtained, fertilization inhibition and embryo mortality, was of identical nature as that reported using complete Freund's adjuvant (4). Although a direct comparison between the two types of adjuvant was not made in this study, the results using poly A and U appear equivalent to those obtained with Freund's adjuvant. Recently, sodium alginate was reported to be an effective adjuvant in combination with epididymal sperm in inducing antibodies and infertility in rabbits (15). However, a series of weekly injections for 10 weeks was required. Fertilization was inhibited, but specific effects on embryo survival were not examined. The present study again indicates that only the sperm-specific antigens of semen are capable of inducing infertility. Sperm-immobilizing antibodies appear to be closely associated with the induced infertility as these were found only in the sera of rabbits immunized with semen and sperm. The sperm-agglutinating antibodies were induced by all three antigenic materials; although not to the same extent by seminal plasma as by the sperm-containing materials. The high correlation of sperm-agglutinating and immobilizing titers of rabbits immunized with semen and epididymal sperm suggests these antibodies were induced by a single antigen or closely related antigens. The antigenic activity inducing the two types of antibody actions on sperm are separable, however, as was shown by guinea pig antisera produced against rabbit sperm extract that had been hydrolyzed by the enzyme pronase (16). This antisera possessed sperm-immobilizing and antifertility activities but did not cause sperm agglutination. The lack of association of passive hemagglutination titers with titers of the other tests may be due to the fact that semen supernatant fluid was used to coat the red cells. Semen supernatant fluid contains large-

ly the soluble antigens of seminal plasma, and, since the agglutination and immobilization tests depend mainly on antibodies against sperm antigens, significant correlations would not necessarily be expected.

Summary. Injections of female rabbits with homologous seminal antigens in combination with polyadenylic and polyuridylic acids (0.4-0.5 mg of each per injection) induced antifertility effects and serum titers of specific antibodies. Rabbits immunized with seminal plasma had normal fertility, whereas rabbits immunized with semen and epididymal sperm showed fertilization inhibition after artificial insemination and low embryo and fetal survival after embryo transfer. Immunization with semen induced high serum titers of specific antibodies as determined by passive hemagglutination, sperm agglutination, and sperm immobilization. Epididymal sperm caused moderate hemagglutination titers and high titers of sperm-agglutinating and immobilizing antibodies, whereas seminal plasma induced high hemagglutination titers but low sperm-agglutination titers, and no immobilizing antibody. Sperm-agglutinating and immobilizing titers were highly correlated in rabbits immunized with semen and epididymal sperm.

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