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Effects of Uterine Fluids and Immunoglobulins from Semen-Immunized Rabbits on Rabbit Embryos Cultured *In Vitro*[1] (37813)

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Embryo survival as well as fertilization was shown to be adversely affected in female rabbits isoimmunized with semen or testis (1, 2). The majority of the induced mortality appeared to occur among embryos after entering the uterus and before implantation (3). Generally, serum titers of sperm antibodies were not closely correlated with the incidence of embryo loss. This has suggested that local synthesis, secretion, and reactions of antibodies against spermatozoa in the female reproductive tract are largely responsible for the immunologic infertility. Indirect evidence for this was shown in that serum antibodies, although capable of inhibiting spermatozoa from participating in fertilization, had no consistent adverse effect on embryos (4). Two classes of immunoglobulins, IgG and secretory IgA, have been detected in secretions of the mucosal tissues of the female genital tract of rabbits (5). Recently, we have reported that uterine fluid and its two predominant types of immunoglobulins from semen-immunized rabbits exhibited specific antifertility effects against spermatozoa (6). The present study considers the effects of the fluids and immunoglobulins from the uterus of the immunized rabbit on the *in vitro* development of rabbit embryos.

Materials and Methods. New Zealand white rabbits were used throughout the study.

Uterine fluid induction and handling. The methods of immunization, induction, and collection of uterine fluids and immunoglobulin separation have been reported (6). Briefly, female rabbits were systemically isoimmunized with semen and Freunds complete adjuvant by weekly injections for 3 weeks followed by 2 weeks of transvaginal injections of washed sperm cells with polyadenylic (Poly A) and polyuridylic (Poly U) acids as adjuvant. One to two weeks later the rabbits, under sodium pentobarbital anesthesia, were injected with washed spermatozoa (10×10^6) and 0.1 mg each of Poly A and U in 0.4 ml saline into the ligated uterine horns. Control rabbits received intrauterine injections of hamster sperm and/or mineral oil. Ten to 14 days after injection the uterine fluids were removed by aspiration and the uteri reinjected with sperm and adjuvant to induce additional fluid. The volume of fluid collected ranged between 2 and 30 ml/rabbit, and the protein concentration varied between 1 and 20 mg/ml. Samples from individual rabbits were concentrated by ultrafiltration to contain approximately 15–20 mg protein/ml. Separation of immunoglobulins from uterine fluid was done by column chromatography of pooled control samples and pooled positive immune samples using Sephadex G-200 with a 0.1 *M* phosphate buffer system. The secretory IgA was further purified by anion-exchange chromatography using DEAE-cellulose and stepwise elution with increasing concentration of NaCl in a 0.01 *M* phosphate buffer. Control serum samples were obtained before immunization and immune serum near the time of uterine fluid recovery. Sperm antibody titers in the serum samples were determined by the sperm-immobilization test (7).

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Embryo donors and culture. Female rabbits were given sc injections of 0.5 U porcine follicle-stimulating hormone (FSH) suspended in 0.5 ml sesame oil containing 5% beeswax once daily for 3–4 days. On the day after the last FSH injection, the rabbits were injected iv with 100 IU human chorionic gonadotropin and mated with two fertile male rabbits. At approximately 66 hr after mating, the rabbits were killed and the oviducts and uterine horns removed and flushed with warm (37°) Hanks balanced salt solution containing 5% rabbit control serum (RCS) to obtain the embryos. Embryo recovery and handling were done in a warm room at 32–37°. After examination and counting under a dissecting microscope, the embryos appearing viable (morulas containing homogeneous blastomeres) were transferred by pipette to drops ($\simeq$0.1 ml) of culture media in 15 × 30 mm Falcon petri dishes. The dishes were filled with heavy mineral oil and incubated in a humid chamber at 37° and gassed with 5% CO_2 in air. Four to eight embryos were placed in each drop.

The basic culture medium was Ham's F-10 containing 100 U of penicillin and 100 μg streptomycin/ml. Control and immune serum and uterine fluid samples constituted 20% of the medium volume. Fractions from column chromatography were dialyzed against distilled water, lyophilized, and reconstituted in F-10 medium to approximately 5 mg/ml. Rabbit control serum was added to these media to a 10% level. For each trial the different treatments were done in replicates. The embryos were daily examined for blastocyst development and viability and measured by an ocular micrometer for size changes. The data presented in the tables represent values observed on the third day of culture. Statistical analysis of the data was by Chi-square and Duncans' new multiple range test. The percentage values were transferred to arcsin angles for analysis (8).

Immunofluorescent methods. Blastocysts recovered from the uterus on the fourth or fifth day of pregnancy were subjected to mechanical removal of the surrounding investments and fixed in cold acetone for 10 min. After washing in 3 changes of 0.05 *M* phosphate-buffered saline (PBS), (pH 7.5), the embryo tissues were treated with different samples of control and immune sera, uterine fluids, and uterine fluid immunoglobulins for 30 min. The tissues were rinsed in PBS and incubated for 15 min in fluorescein isothiocyanate conjugated goat anti-rabbit-7S globulin (Hyland, Inc.) and anti-rabbit SIgA.[2] The embryo tissues were again rinsed in PBS and mounted in glycerine on glass microslides. The slides were observed using a Zeiss microscope equipped with a halogen quartz lamp and a FITC-interference filter.

Results and Discussion. The embryos were in the morula stage when placed into culture. This stage of development was selected as earlier *in vivo* studies in semen-immunized rabbits indicated that degenerative changes

[2] Anti-SIgA serum kindly supplied by Dr. R. Freter, Department of Microbiology, University of Michigan.

TABLE I. Development of Rabbit Embryos from Morulas to Blastocysts in *In Vitro* Culture in Rabbit Sera and Uterine Fluids.

Treatment	No. samples	No. embryos cultured	Developing blastocysts (%) $\bar{X} \pm$ SE	Blastocyst diameter (μm) $\bar{X} \pm$ SE
Serum				
control	6	128	75.0 ± 8.9	213.3 ± 27.7
immune	6	99	78.8 ± 7.5	236.8 ± 34.5
Uterine fluid				
control	6	165	46.1 ± 10.8	234.8 ± 18.0
immune	14	283	19.4 ± 6.2[a]	155.8 ± 5.9[a]

[a] Differ significantly ($P < .05$) from control values.

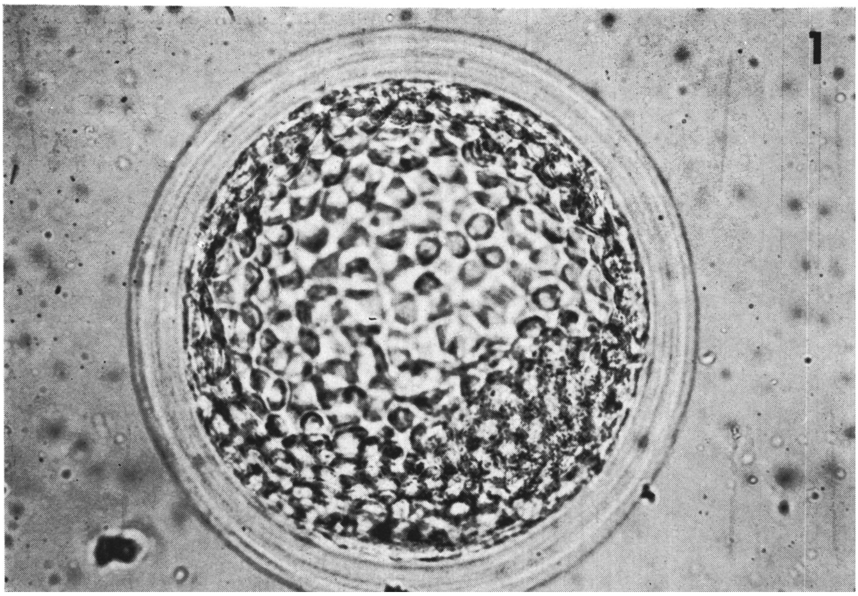

FIG. 1. Expanding rabbit blastocyst on third day of culture in control uterine fluid.

were first observed near the time of embryo entry into the uterus and blastocyst formation (3). The results of Table I confirm earlier reports that serum with high titers of sperm antibodies (256–1024), in comparison to control serum, did not adversely affect embryo development *in vitro* (4). In the presence of immune uterine fluid (IUF), however, embryo development, in terms of blastocyst viability and size of the viable blastocysts, was significantly depressed overall compared with values for embryos in control uterine fluid (CUF) cultures (Figs. 1 and 2). Of the 14 different IUF samples tested, 11 samples gave positive results. The poorer rate of blastocyst for-

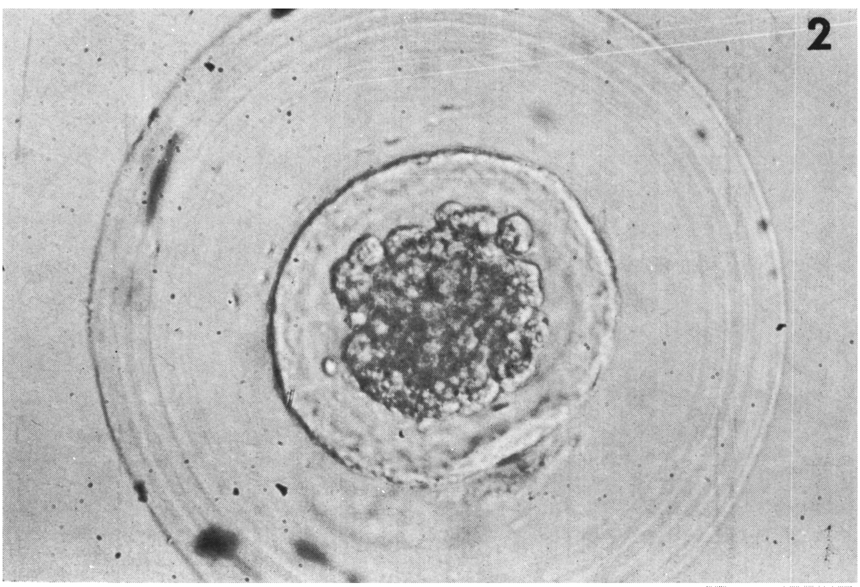

FIG. 2. Degenerating rabbit embryo on third day of culture in uterine fluid from semen-immunized rabbits.

TABLE II. Blastocyst Development of Rabbit Embryos Cultured *In Vitro* in Immunoglobulins and other Fractions of Control and Immune Rabbit Uterine Fluids.

Factor	Control samples		Immune samples			
	SIgA	IgG	SIgA	IgG	Fract. 3	Fract. 4
No. embryos cultured	48	50	58	47	43	38
Developing blastocysts (%) $\bar{X} \pm SE$	88.0 ± 8.8^a	92.0 ± 6.8^a	8.1 ± 6.4^b	61.4 ± 14.3^a	85.1 ± 8.2^a	69.4 ± 9.9^a
Blastocyst diameter (μm) $\bar{X} \pm SE$	$231.6 \pm 30.0^{a,b}$	$229.0 \pm 29.2^{a,b}$	161.0 ± 4.5^c	246.4 ± 24.2^a	203.8 ± 20.4^b	198.6 ± 19.3^b

[a,b,c] Values with different letters differ significantly ($P < .05$).

mation and maintenance in CUF compared with serum may have been due to the low protein concentration in the media; thus, in subsequent trials, 10% RCS was included in the basic medium.

These *in vitro* results with uterine fluids duplicated for the first time the anti-embryo effects seen *in vivo*. As earlier reports had indicated that the uterus is capable of secreting SIgA and IgG and that both exerted antifertility effects on spermatozoa, the next step was to test the effects of these immunoglobulins in embryo culture (5, 6). Table II indicates that of the CUF immunoglobulins, the IUF immunoglobulins, and the IUF column fractions, only immune SIgA caused significant inhibition of blastocyst formation and development. Often, initial blastocyst formation would occur in some of the immune SIgA-treated embryos, but by the second or third day many of these would have collapsed and degenerated. Fractions 3 and 4 of the IUF chromatography appeared to cause some inhibition of blastocyst size. Gel filtration of uterine fluid resulted in four peaks of protein activity in the following elution order: SIgA, IgG, Fraction 3 and Fraction 4. Fraction 3 contained albumin and Fraction 4 consisted of apparently smaller-molecular-weight substances.

To ensure that the embryotoxic effect of IUF–SIgA was due to specific-antibody activity, aliquots of the immunoglobulin fractions were absorbed with an equal volume of packed, thrice-washed rabbit spermatozoa and goat anti-rabbit-SIgA antiserum. Absorption with goat antiserum prevented the SIgA from reacting in immunodiffusion. In comparison with the unabsorbed immune SIgA, which completely inhibited blastocyst development, absorptions with sperm and anti-SIgA serum inhibited the anti-embryo activity of the immune SIgA (Table III). The average size of embryos treated with unabsorbed SIgA represents the degenerated blastocysts as none persisted to the third day. The majority of blastocysts cultured in the sperm-absorbed media underwent hatching and greater expansion (Fig. 3) as a result, apparently, of digestive action on the zona pellucida by proteolytic enzymes from the spermatozoa used in absorption.

The immunofluorescent study provided additional verification of the specific-antibody activity of sperm-induced SIgA against embryos and the lack of this cross-activity by IgG.

Embryo tissue treated with IUF and immune SIgA gave bright fluorescence after reaction with the conjugated anti-SIgA (Fig. 4). CUF, RCS, RIS, immune IgG, and conjugated antiserum treatment of embryo tissue resulted in little or no fluorescence (Fig. 5). The specific fluorescence of IUF and immune IgA fractions was greatly reduced after absorption with washed rabbit spermatozoa.

Secretory IgA has been described as active in defense against certain pathogens in the organs and tissues of the body with access to the external environment. The reproductive tract of the human female was shown to contain the necessary components for production of SIgA (9), and tissues from the tract were observed to be capable of *in vitro* synthesis of secretory immunoglobulin (10). Human cervicovaginal secretions were found to contain specific antibodies, predominantly of the SIgA class, against *Candida albicans* (11). The majority of the IgA present in the secretions was of the secretory type. In cattle, also, the female reproductive tract responds to infection of *vibrio fetus* by local secretion of IgA with specific-antibody activity (12). Local immunization in the female reproductive tract can induce antibody detectable only in local secretions and not systematically (6). Also, the reverse has been shown to be true. In the present study, the results indicate that the SIgA system of the rabbit uterus responded to an antigenic determinant of rabbit spermatozoa to which there was no response by the IgG system, or the IgG antibodies induced were too few or of too low avidity to be detectable and effective. Indirectly, the results also suggest that the sperm-cell antigen inducing the SIgA anti-embryo antibody is not the same as the antigen(s) inducing the IgA and IgG antibodies which inhibit fertilization. Although some of the uterine IgG is probably derived from serum, little if any IgA is of serum origin as the ratios of IgG to IgA are approximately 60:1 in serum and as low as 1:1 in uterine fluid (unpublished data). If

TABLE III. Blastocyst Development of Rabbit Embryos Cultured in Immune Uterine Immunoglobulins Absorbed with Rabbit Spermatozoa and Goat Anti-SIgA Serum.

Factor	SIgA absorbed with			IgG absorbed with		
	Saline	Sperm	Anti-SIgA	Saline	Sperm	Anti-SIgA
No. embryos cultured	27	27	32	22	32	30
Developing blastocysts (%) $\bar{X} \pm SE$	0.0^a	91.4 ± 4.3^b	69.4 ± 7.5^b	74.7 ± 11.6^b	88.0 ± 6.8^b	62.9 ± 19.0^b
Blastocyst diameter (μm) $\bar{X} \pm SE$	113.0 ± 4.7^a	414.8 ± 33^b	158.3 ± 7.2^c	167.3 ± 11.5^c	447.3 ± 21.8^b	168.8 ± 10.4^c

[a,b,c] Values with different letters differ significantly ($P < .05$).

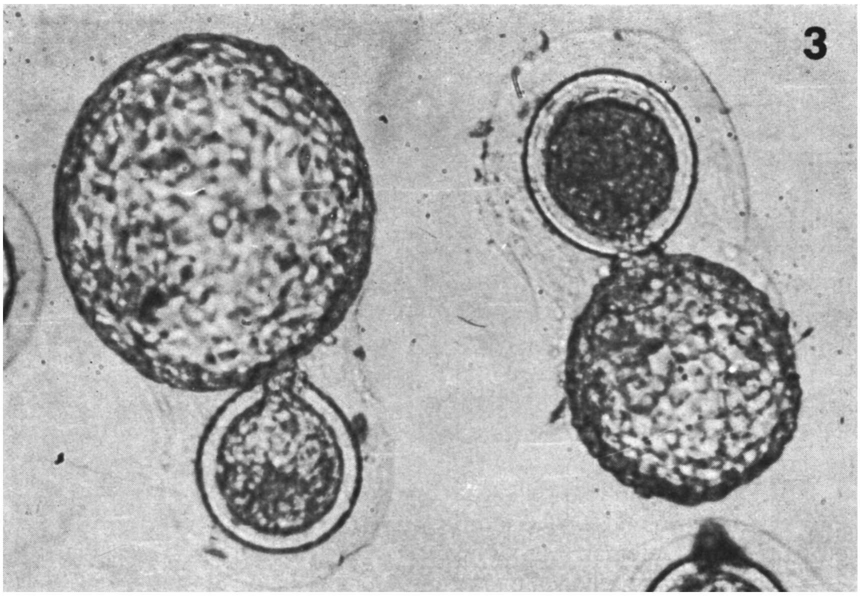

FIG. 3. Hatching rabbit blastocysts on third day of culture in sperm-absorbed secretory IgA fraction of immune uterine fluid.

SIgA is the factor responsible for embryo death *in utero* in rabbits systemically immunized with semen, this would suggest that either the antigen in some form or information concerning it reaches and sensitizes antibody-producing cells in the uterus or sensitized cells from the lymphocytic system settle in the uterus and then produce antibody. Transplantation antigens do not appear to be involved in this system as absorption of immune uterine fluid with paternal lymphocytes failed to remove the anti-embryo

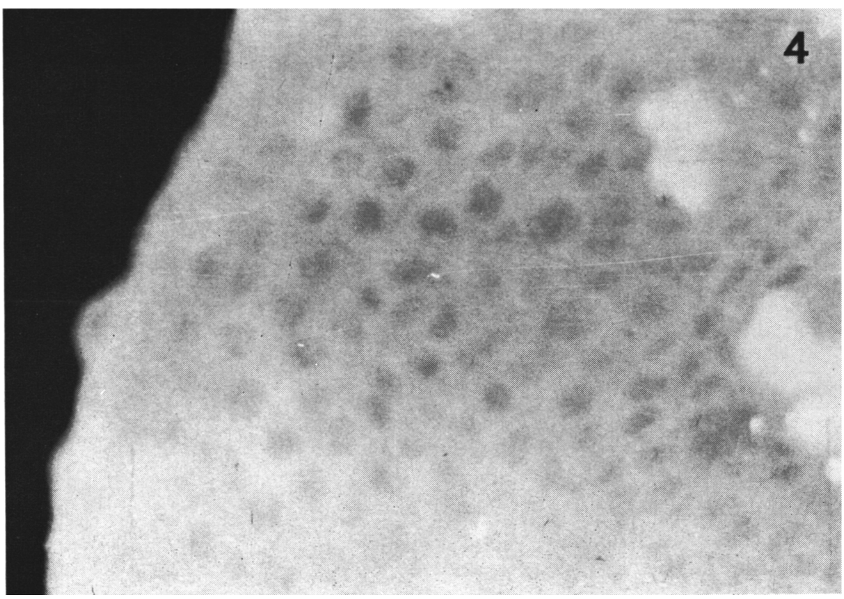

FIG. 4. Indirect immunofluorescence of rabbit blastocyst tissue treated with secretory IgA fraction of immune uterine fluid and fluorescein-labeled goat anti-rabbit-SIgA γ-globulin.

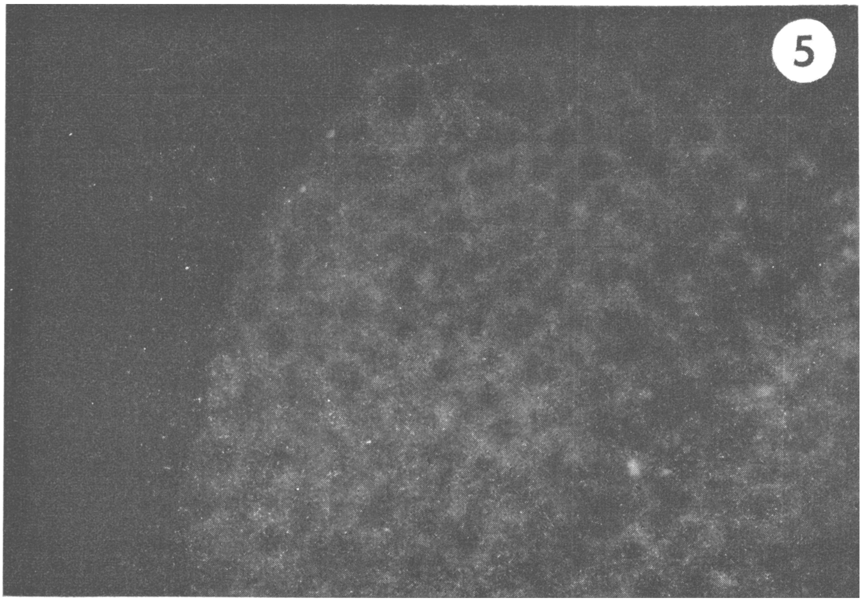

FIG. 5. Indirect immunofluorescence of rabbit blastocyst tissue treated with control uterine fluid and fluorescein-labeled goat anti-rabbit-SIgA γ-globulin.

effect (unpublished data).

The anti-embryo effect of immune SIgA observed under culture conditions needs verification *in utero* in the rabbit. We are currently determining survival rates of embryos treated *in vitro* with uterine immunoglobulins and transferred to uteri of recipient rabbits.

Summary. Rabbit embryos in the morula stage were cultured *in vitro* in Ham's F-10 medium containing different serum samples and uterine fluid samples from control and semen-immunized rabbits. The percentage of embryos that blastulated and developed and the average blastocyst size did not differ between control and immune serum samples, whereas immune uterine fluid significantly depressed these parameters in comparison to control uterine fluid. Control and immune uterine fluids were fractionated by gel filtration and ion exchange column chromatography and the two predominant immunoglobulin classes, SIgA and IgG, separated. Of the two classes of immunoglobulins and two other fractions obtained from the gel filtration column, only immune SIgA proved to be embryotoxic. This anti-embryo effect of the immune SIgA was removed by absorption with spermatozoa and goat anti-SIgA serum. Use of immunofluorescence also indicated that immune SIgA, but neither uterine nor serum IgG, cross-reacted with embryonic tissue. Thus, it appears that the immunologic-induced embryo mortality observed *in utero* in the rabbit may be due to a specific cross-reaction of the embryo with secretory IgA.

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