

FURTHER EVIDENCE CONCERNING THE AUTOANTIGENIC STATUS OF THE TROPHOBLAST*

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Interest in the possibility that the placenta contains organ-specific antigens was stimulated at the beginning of the century by the suggestion that some toxemias of pregnancy might result from maternal sensitization against placental antigens, leading secondarily to renal damage (1).

The general principle that an antiserum raised in one species against placental homogenates from a different species will interrupt pregnancy in individuals of the species that provided the antigen has been well established since Dobrowolski's (2) work with guinea pigs and rabbits (see reference 3). Contaminating erythrocytes have been exonerated as the effective antigens and the strong nephrotoxic activity of many anti-placental sera established (4-6). However, it seems fairly well documented that potent nephrotoxic anti-kidney sera are not necessarily placentotoxic (7, 8).

The nature and distribution of the antigens apparently shared in common by the placenta and kidney have been subjected to repeated investigations mainly of an *in vitro* nature (6, 8-13). These have strongly implicated trophoblast as the principal source of the shared antigens, but failed to produce unequivocal evidence of the existence of antigens restricted to trophoblast.

The findings of heterotopic transplantation studies on ectoplacental cones and blastocysts in mice by Hulka and Mohr (14) and particularly by Kirby (15), have recently strengthened belief in trophoblast-specific antigens. The experiments that form the subject matter of this communication, designed to evaluate the abortifacient activity, in pregnant rats, of antisera raised in rabbits against trophoblast, fetal, and various adult rat tissues, both before and after appropriate absorptions, have yielded results strongly favoring the existence of this antigen (or antigens).

Materials and Methods

Rats.—Rats of the isogenic Fischer (FI)¹ strain provided the subjects for most of the work described.

Rabbits.—The rabbits used as antibody producers were adult white New Zealand males.

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¹ Abbreviations used in this paper: ALS, anti-lymphocyte serum; FI, Fischer rats.

Trophoblast Cells.—Trophoblast cells for use as “antigen” were prepared from rat placentas of 12–14 days of gestation. After stripping the membranes from their fetal surfaces the placentas were thoroughly washed with Hanks’ solution, minced finely with scissors, and gently pressed through a 50-mesh stainless steel sieve. The resulting crude tissue suspension was washed twice in large volumes of Hanks’ solution containing penicillin, 10,000 units/ml, streptomycin, 10,000 $\mu\text{g/ml}$, and Fungizone, 25 $\mu\text{g/ml}$, (Grand Island Biological Co., Grand Island N. Y.), and resuspended at a concentration of approximately 1 organ equivalent/ml. Centrifugation at 1200 g for 10 min sedimented the principal cell types present in three distinct layers: leukocytes at the top, erythrocytes at the bottom, and a fairly homogeneous population of monodisperse trophoblast cells in the middle. The latter were removed, resuspended in Hanks’ solution, and counted in a hemocytometer, using the trypan blue exclusion method to assay viability. Examination of these suspensions by phase-contrast microscopy and of the stained smear preparations by light microscopy revealed that about 90% of the cells present were of trophoblastic origin. For immunization purposes, the concentration of all cell suspensions was adjusted so that they contained 50×10^6 viable cells/0.5 ml.

Suspensions of Viable Epidermal Cells.—Suspensions were prepared enzymically from tail skin by Silvers and Billingham’s (16) procedure.

Suspensions of Adult Spleen Cells, Thymocytes, and “Fetal” Cells.—Suspensions of cells (from whole fetuses of 10–12 days’ gestation and excluding all placental and umbilical cord material) were prepared by our standard methods (17). In all instances excised tissues and cell suspensions were prepared and kept at about 5°C.

Production of Heterologous Antisera.—Production followed an immunization protocol which was essentially that which has proved satisfactory in raising a biologically active anti-lymphocyte serum (ALS) (18).

0.5 ml aliquots of the appropriate type of cell suspension were mixed with equal volumes of Freund’s complete adjuvant and injected into the hind footpads of rabbits. Booster injections of 100×10^6 viable cells in Hanks’ solution were injected intravenously 21 and 28 days later and the animals exsanguinated on day 35. The blood was allowed to stand for 2 hr at room temperature for clotting to occur, followed by 24 hr at 4°C before separation and heat inactivation of the serum. The latter was twice absorbed with 1 volume of washed Fischer rat erythrocytes, first at 4°C and second at 37°C, to remove all hemagglutinins. All anti-lymphocyte activity, determined by cytotoxicity testing using the trypan blue exclusion technique (19), was removed when necessary by subsequent absorption with suspensions of washed lymphoid cells prepared from lymph nodes and spleens.

Assay of Titers.—Assay and determination of the characteristics of the antibodies were accomplished by the gel immunodiffusion method of Ouchterlony (20). A saline extract of the antigen was prepared by homogenization of the trophoblast cells. After hemagglutinin absorption, the standard anti-trophoblast antibody displayed a precipitating band at a dilution of 1:256.

Bioassay of Antisera.—Groups of about six time-mated and putatively pregnant females were segregated and treatment with the various antisera initiated at various stages of gestation. The standard treatment comprised a total of 3 ml of antiserum, injected intramuscularly in 1 ml subdoses on alternate days. The recipients were observed for vaginal bleeding and palpated under ether anesthesia on alternate days to assay fetal growth. Representative animals were killed at selected stages for gross and histological examination of fetal and maternal organs.

OBSERVATIONS

Influence of Rabbit Anti-Rat Antisera on Pregnant Rats and their Conceptions.—The basic tests performed, together with their results, are summarized

in Table I. Whereas normal rabbit serum had no discernible adverse effect on either the mothers or their fetuses, anti-trophoblast serum, unabsorbed with lymphoid cells, aborted 12 out of 12 subjects and caused the deaths of two of them.

Kirby (21) has reported that rabbit anti-mouse lymphocyte serum is abortifacient in mice. He attributed this to its luteolytic activity on the grounds that the ALS did not cause abortion if progesterone was administered concom-

TABLE I
Effect of Rabbit Antisera Against Various Types of Rat Cells on Gravid Females and Their Conceptuses

Type of rabbit antiserum*	Absorbed with*	Type		Pregnant female recipients			Status and No. of progeny			
		♀	♂	No. tested	No. aborted	No. which died				
Nonimmune	—	Rat: FI†	×	FI	8	0	0	Normal		
Anti-trophoblast	—	Rat: FI	×	FI	12	12	2	None		
Anti-lymphocyte	—	Rat: FI	×	FI	12	3	8	None		
Anti-trophoblast	Lymphoid cells	Rat: FI	×	FI	12	12	0	None		
		Rat: DA	×	DA	7	7	0	None		
		Rat: FI	×	DA	6	6	0	None		
		Rat: BH	×	BH	4	46	4	46	0	None
		Rat: LE§	×	LE	5	5	5	0	None	
		Rat: BN	×	BN	4	4	4	0	None	
Anti-trophoblast	Lymphoid cells and trophoblast cells	Rat: S-D	×	S-D	8	8	0	None		
		Rat: FI	×	FI	10	0	0	Normal		
Anti-fetal	—	Rat: FI	×	FI	6	0	0	Normal		
Anti-epidermal	—	Rat: FI	×	FI	6	0	0	Normal		
Anti-kidney	—	Rat: FI	×	FI	7	0	0	Normal		
Anti-decidual	—	Rat: FI	×	FI	6	0	0	Normal		
Anti-trophoblast	Lymphoid cells	Mouse: C57 BL/6 × C57 BL/6			4	0	0	Normal		
		Mouse: A × A			4	0	0	Normal		
		Hamster: MHA × MHA			4	0	0	Normal		
		Hamster: CB × CB			4	0	0	Normal		

* All antisera absorbed with rat erythrocytes to remove hemagglutinins.

† FI = Fischer.

§ LE = Lewis.

|| Sprague-Dawley rats of an outbred strain.

itantly. The influence of rabbit anti-rat ALS was therefore tested in pregnant Fischer rats. As shown in Table I, not only did this abort 25% of the subjects but it also killed 67% of them. This observation raised the question whether the abortifacient potency of the anti-trophoblast serum resided in an anti-lymphocyte moiety (22). To investigate this possibility, anti-trophoblast serum was repeatedly absorbed with suspensions of lymphoid cells until cytotoxicity tests for the presence of anti-lymphocyte activity gave negative results. Administration of this absorbed antiserum to pregnant Fischer rats aborted 12/12 but did not harm them or interfere with their subsequent reproductive performance.

Irrespective of the stage of gestation at which lymphocyte-absorbed anti-trophoblast serum treatment was initiated, the first injection prompted vaginal bleeding from the pregnant recipients within 24 hr. In an additional group of 15 treated animals killed and dissected after they had passed beyond their expected date of delivery, fetuses at various stages of resorption were identified.

Histological examination of placentas obtained from animals receiving anti-trophoblast serum absorbed with lymphoid cells revealed areas of hemorrhage and necrosis as well as cellular infiltration of these organs by plasma cells, small lymphocytes, and polymorphs. The fetuses were edematous and macerated. No abnormalities were observed either grossly or microscopically in the uteri, deciduae, corpora lutea, kidneys, lungs, or livers of the mothers, indicating a high degree of tissue specificity.

If the abortifacient activity of anti-trophoblast serum is due to an antibody corresponding to a specific determinant(s) associated with trophoblast, then this activity should be removable by repeated absorptions with viable trophoblast cells. Accordingly lymphoid cell-absorbed anti-trophoblast serum of proven potency was repeatedly absorbed with trophoblast cells until it no longer produced precipitation lines against a saline extract of trophoblast cells in immunodiffusion tests.

On bioassay, this antiserum proved to be completely innocuous in 10 out of 10 pregnant females, none of which showed vaginal bleeding and all of which delivered healthy litters of normal size.

The following additional findings (Table I) support the premise that an antibody specifically reactive with a distinctive component of trophoblast, rather than with an antigenic determinant shared in common by a variety of cell types, is responsible for the abortifacient potency of anti-trophoblast serum: neither (a) rabbit anti-rat epidermal cell serum nor (b) rabbit anti-rat fetus serum harmed either pregnant females or their fetuses. Unlike most previous investigators, however, we have been unable to demonstrate that our anti-trophoblast serum had any nephrotoxic effect on female rats or reacted with kidney antigen in immunodiffusion tests possibly because of the prior absorption with lymphocytes. However, whereas rabbit antiserum of high titer prepared against renal tissue was devoid of abortifacient activity in a panel of 15 pregnant females and failed to react with trophoblast cell extracts in immunodiffusion tests, it did cause histologic lesions in the recipients' kidneys.

Since the trophoblast cell preparations employed as antigen in this study were contaminated by decidual cells, the possibility remained that the active component of the anti-trophoblast antiserum was in fact an anti-decidual or anti-endometrial cell antibody. To test this possibility, an antiserum against decidual cells was raised in rabbits, absorbed with rat erythrocytes, and assayed in pregnant Fischer females. Its inability to harm them or their pregnancies dismissed the interpretation under consideration.

Influence of Timing of Administration and Dosage of Heterologous Anti-Pla-

central Serum on its Abortifacient Capacity.—To define the period during gestation when rat fetuses are susceptible to the activity of anti-trophoblast serum, the standard treatment with lymphocyte-absorbed antibody was initiated in panels of five to seven gravid females at 7–18 days postconception (Table II). Despite this great range of developmental age, all fetuses appeared to be at equal risk insofar as they were all aborted.

Although no attempt was made to determine the minimal dose of antiserum required to procure abortion, in many instances it was found that a single 1 ml inoculation sufficed to terminate a pregnancy.

Strain and Species Specificity.—Since all defined organ and tissue specific antigens are shared in common by all members of the same species, irrespective of their genetic constitutions, and frequently show cross-reactivity between related species, the influence of administering lymphoid cell-absorbed rabbit anti-Fischer rat trophoblast serum to panels of pregnant rats of the following strains, BN, DA, Lewis, Black Hooded, and outbred Sprague-Dawley, and to

TABLE II
Abortifacient Effect of Rabbit Anti-Trophoblast Serum at Varying Stages of Gestation in the Rat

Inception of antiserum therapy after conception	No. of gravid hosts tested	No. in which abortion or resorption procured
Day 7	6	6
Day 10	7	7
Day 14	6	6
Day 18	5	5

mice and hamsters was studied (Table I). Whereas the antiserum proved equally effective in aborting rats, irrespective of the genetic constitution of their fetuses, it was without any perceptible effect on pregnant mice and hamsters.

Finally, we addressed ourselves to the important question as to whether rats are potentially capable of reacting against their own trophoblast antigens, i.e., whether trophoblast is potentially autoantigenic. Two experiments were performed. In the first, male Fischer rats were subjected to the standard immunization protocol, as described for rabbits, only in this case rats of the unrelated DA strain provided the trophoblast cells. Alien strain donors were selected in the belief that foreign transplantation antigens on the same cellular vehicle with which the putative trophoblast-specific antigen was associated might exert an adjuvant effect (see reference 23). Serum was harvested from these animals and administered to 10 pregnant Fischer females, following the standard regimen.

In the second experiment, eight virgin female Fischer rats were actively immunized with DA rat trophoblast cells as described above and then mated with Fischer males. In both experiments treatment of the females failed to influence the normal development to term and subsequent health of their fetuses.

DISCUSSION

The findings that rabbit anti-rat trophoblast serum, absorbed with rat erythrocytes and lymphocytes, has a powerful abortifacient influence in pregnant rats of a wide variety of strains, which can be removed completely by absorption with trophoblast cells greatly strengthens the thesis that there is a highly tissue-specific or unique antigen associated with trophoblast.

The observation that the abortifacient activity of anti-trophoblast serum can be removed by absorption with fairly pure suspensions of intact, viable trophoblast cells suggests that this antigen (or antigens) is present on cell surfaces. In the light of Bagshawe's (24) finding that high dilutions of rabbit antisera prepared against highly purified human chorionic gonadotrophin were rapidly lethal to both human choriocarcinoma and normal trophoblast cells in vitro, the possibility must be borne in mind that the putative trophoblast cell-associated antigen may be a hormone produced by these cells, or absorbed from the serum by them. Indeed it could even be a hormone receptor.

If subsequent experiments, using more sophisticated immunological procedures, substantiate and extend the present conclusions by defining the antibodies and the distribution and immunochemical characteristics of the antigen(s), heterologous anti-trophoblast serum will merit clinical evaluation. Firstly it may serve as an abortifacient effective from very early stages of pregnancy onwards, and secondly it may afford an ancillary therapeutic agent in patients with choriocarcinoma, whose tumors have proved refractory to treatment with chemotherapeutic agents such as methotrexate and actinomycin D. The clinical application of heterologous ALS as an immunosuppressant has provided considerable information about the possible dangers and probable systemic side effects of such an agent (25).

SUMMARY

Heterologous antisera were raised by inoculation of rabbits with fairly pure suspensions of trophoblast, lymphoid, fetal (excluding placental components), epidermal, decidual, and renal cells from Fischer rats. After absorption of hemagglutinins, these antisera were assayed for abortifacient activity by intramuscular inoculation into time-mated Fischer females.

The anti-trophoblast serum aborted all the recipients, but had some non-specific activity in that it caused the deaths of 12% of them. Anti-lymphocyte serum was more toxic and less potent as an abortifacient. None of the other sera harmed either the mothers or their fetuses. However, the anti-kidney cell serum caused kidney lesions.

Absorption of the anti-trophoblast serum with lymphoid cells completely removed its toxicity for the pregnant females without perceptible impairment of its abortifacient activity. However, the latter could be removed by absorption with trophoblast cells.

(a) The lymphocyte-absorbed anti-trophoblast serum was equally effective in aborting rats over the range 7–18 days postconception; (b) a single injection of 1 ml was sufficient in most cases, and (c) it was equally efficacious in terminating gestation in rats irrespective of their genetic constitution, and (d) its effect was highly species-specific.

These findings support the premise that a unique antigen (or antigens), of possible clinical significance, is associated with trophoblast cells.

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