

MONKEY ANTI-PLACENTAL SERUM AS AN ABORTIFACIENT

S.J. Behrman, M.D.
Y. Amano, Ph.D.

Department of Obstetrics and Gynecology
The University of Michigan Medical Center
Ann Arbor, Michigan

ABSTRACT

Pregnant Rhesus monkeys can be aborted by injection of heterologous (auto-logous) placental globulin without any notable harm to the mother. Presumably, anti-placental globulin reacts with trophoblastic tissue in the presence of complement, which then destroys tissues, and abortion follows. Within this experimental model, neither MPL nor MCG appear as responsible antigenic elements; but rather, a placental specific antigen(s) as yet unidentified.

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INTRODUCTION

While the antigenicity of mammalian placenta has been claimed by many investigators in the usual course of mammalian pregnancy, rejection of this foreign material does not normally occur (1). Presumably, therefore, the placenta is either antigenically weak or the maternal antibody response is in some way suppressed. Theoretically, then, if sufficient antibody could be produced and supplied to the mother, some rejection phenomenon might be expected.

In previous experiments (2-4) using rabbit anti-mouse placental serum, rejection (abortion and fetal death) was readily obtained in pregnant mice. To investigate this hypothesis in the primate, some preliminary experiments have been performed using a heterologous (autologous) system, and in this report, the abortifacient effect of anti-placental sera is confirmed using goat and rabbit anti-Rhesus monkey placental globulin.

METHODS AND MATERIALS

Placental antigen

Term placenta of the Rhesus monkey was minced and washed with phosphate buffered saline (PBS) several times to eliminate blood components and homogenized in an equal volume (V/W) of PBS for one minute by a Virtis 45 homogenizer. The homogenate was centrifuged at 10,000 g, for 30 min, and the clear supernatant was separated. The above extraction procedure was repeated and the supernatants were pooled. This crude extract of Rhesus monkey placenta (RMP), after lyophilization, was used as placental antigen throughout the experiment.

Anti-Rhesus monkey placental sera

Adult female goats and male rabbits were used to produce anti-placental sera. In both animals, 20 mg (in rabbit) to 200 mg (in goat) of RMP were injected intradermally with Freund's complete adjuvant at one week intervals for four weeks. Two to four weeks after the last injection, the same antigen was given intramuscularly without adjuvant as a booster injection. One week after the booster injection, blood was drawn. For the following several months, booster injections and bleedings were repeated. Separated sera were stored at -20° C until used.

Immunoglobulin purification

To minimize side effects of passive immunization, globulin fractions were isolated from anti-Rhesus monkey placental sera, using an ammonium sulfate precipitation technique and a DEAE cellulose column chromatography. In brief, immune sera were mixed with an equal volume of saturated ammonium sulfate and the resulting precipitates were dissolved in a small volume of water, and then dialyzed against

phosphate buffer, pH 8.0 and 0.015 M. Fifteen ml of dialyzed sample was applied on a DEAE cellulose column (2.5 x 40 cm) and eluted by the linear gradient buffer system, from 0.015 M to 0.3 M, pH 8.0, phosphate buffer. Eluted protein fractions were tested against RMP by immunodiffusion and satisfactory fractions were pooled.

The pooled fractions were dialyzed against PBS, sterilized using a Millipore filter, and lyophilized in plastic tubes, 100 mg each (labeled as R-IgG or G-IgG).

Induction of abortion

A total of 600 mg to 1000 mg of IgG were injected intravenously into nine pregnant Rhesus monkeys at 39 to 54 days of gestation (all pregnant monkeys were purchased from Bionetics Research Laboratories, Kensington, Maryland) on three consecutive days (250 mg, 250 mg and 150 mg). A similar dose of normal goat globulin was injected into three pregnant monkeys as one form of control. Following the injections, all clinical symptoms were carefully observed; such as, anaphylactic responses, vaginal bleeding, uterine size, appetite, etc. In case of abortion, the aborted materials (placenta and fetus) were recovered for histological study.

Immunodiffusion and immunoelectrophoresis

Immune sera or isolated IgG preparations were tested by immunodiffusion and immunoelectrophoresis, using 1% agarose in 0.05 M (pH 7.2) phosphate buffer. Also, human chorionic gonadotrophin (HCG), monkey chorionic gonadotrophin (MCG) and monkey placental lactogen (MPL) (kindly provided by Henry Friesen, M.D., Royal Victoria Hospital, Montreal, Canada) were tested as antigen.

Cytotoxicity test

A technique, described by Wood (5), was used after minor modification. Forty- to fifty-day-old monkey placentae were purchased (Bionetics Research Laboratories) in ice cold Hank's solution under sterile conditions. Trophoblastic tissues were dissected from the basal plate and washed with PBS several times to eliminate blood cells, and then trypsinized in the usual manner and cultured in bottles using F-12 medium with 20% fetal calf serum. One to two weeks after the primary culture, cell sheets were trypsinized and subcultured on cover slips in tubes using the same medium as the primary. Forty-eight hours after subculture, cells were used for testing. Culture media of each tube were replaced with test samples (IgG diluted in Hank's solution) and incubated at 37° C for one hour. Incubated cells were then incubated with fresh guinea pig complement at 37° C for two hours. After rinsing complement, the cells were maintained overnight at 37° C with maintenance medium (medium F-12, 2% fetal calf serum). Cytopathologic effects were checked by phase contrast microscope without staining and the rate of damaged cells scored.

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Fluorescent antibody

To localize placental specific antibody, an indirect fluorescent antibody technique was employed. Cryostat sections of normal Rhesus monkey placenta were incubated with various concentrations of G-IgG absorbed with normal Rhesus monkey serum, and specific localization of goat globulin was determined by fluorescent rabbit anti-goat globulin (Hyland Laboratories) as the usual procedure. Routine controls were simultaneously run using normal goat globulin instead of G-IgG.

Monkey chorionic gonadotrophin neutralization test

Following Albert's procedure (6), MCG was prepared from pregnant Rhesus monkey urine. A neutralization test was performed to determine anti-MCG activity of G-IgG.

Five mg of MCG was added to 10 mg of G-IgG and incubated at 37°C for 30 min; two-fold dilutions were made and injected into immature mice of NIH strain, 18 days old, using three mice per each dilution. One hundred hours later, laparotomy was performed and the end point of ovulation induction was checked.

RESULTS

Induction of abortion

Results are summarized in Table I. Most abortions were observed within two to six days after the last injection. When these animals aborted following injection, the only clinical sign noted was vaginal bleeding prior to passage of tissue. Some monkeys who did not abort after the first series of injections, were given a second series of injections. Two monkeys, #967 and #97C were reinjected with the same amount and same lot of G-IgG as the first series, at an interval of four weeks. Monkey #97C then aborted 32 days after the second series of injections, but one cannot be sure that the abortion was the result of an immunoreaction. Monkey #268 also received two series of injections. The first series of injections were with G-IgG and the second series of injections were with R-IgG; one week after the second series of injections, abortion occurred.

After IgG injection, no anaphylaxis or harmful reactions were observed in these injected monkeys. Reduction of appetite was noted in two cases. Three out of six monkeys remated and are now pregnant and the uterus is growing normally. While only 12 monkeys are included in this report, existing studies include 12 more with similar data.

Immunodiffusion and immunoelectrophoresis

A typical pattern of immunodiffusion and immunoelectrophoresis is shown in Figure 1.

Table 1. INDUCTION OF ABORTION IN RHESUS MONKEYS

Monkey	Antibody	mg	Day Preg	Abortion	Remarks
405C	R-IgG	608	39	Yes	Remated (pregnant)
974	G-IgG	650	47	Yes	Remated (pregnant)
436C	G-IgG	644	46	Yes	Sacrificed
967	G-IgG	644 650	52 80	No	Sacrificed
599F	G-IgG	650	45	Yes	Remated (not pregnant)
97C	G-IgG	650 650	50 78	Yes	Remated (pregnant)
87C	G-IgG	1,000	45	Yes	Fetal death
210 A	G-IgG	650	47	No	
268	G-IgG R-IgG	650 650	43 54	Yes	
194C	Ng-Glob	650	48	No	Delivery at term (N)
941	Ng-Glob	644	53	No	Sacrificed
576F	Ng-Glob	650	42	No	Hysterotomy (105 days) Remated (pregnant)

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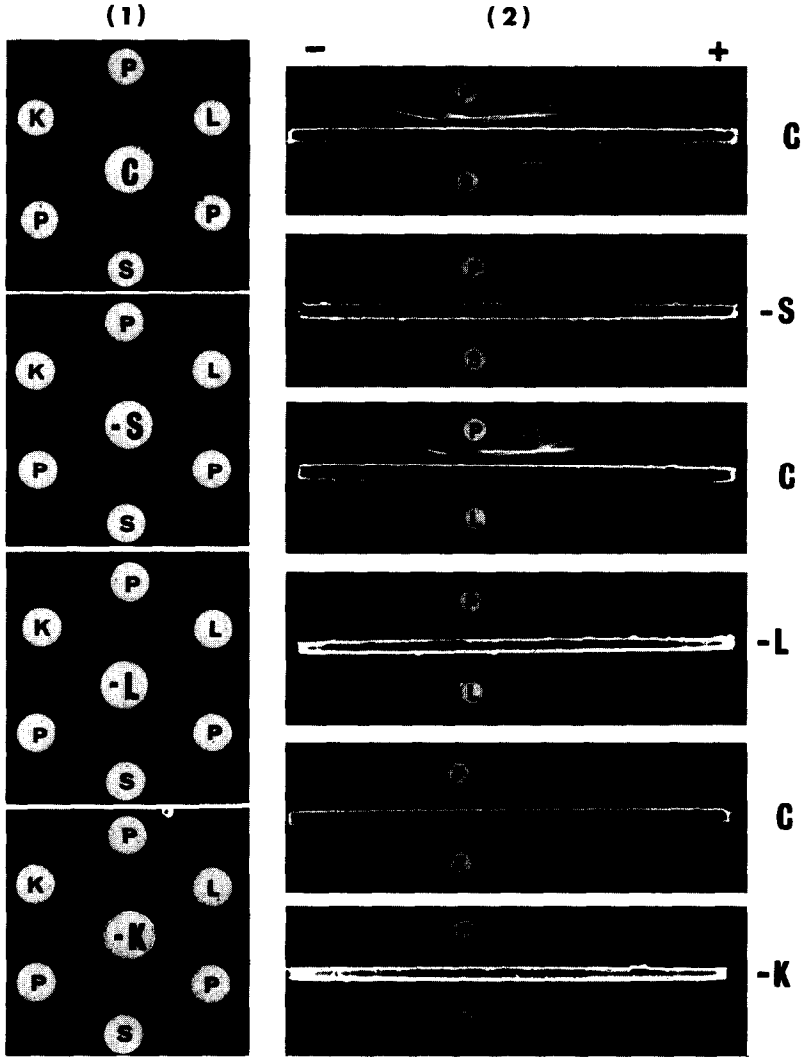


Figure 1. Immunodiffusion (1) and immunoelectrophoresis (2). Wells contain Rhesus monkey placental extract (P), normal monkey serum (S), monkey liver extract (L), kidney extract (K) and cross-reacted with rabbit anti-Rhesus monkey placental IgG (C), absorbed with serum (-S), absorbed with liver extract (-L) and absorbed with kidney extract (-K). Troughs also contain (C), (-S), (-L) and (-K). After absorption, only placental specific lines remain.

Unabsorbed anti-placental globulin reacts with monkey serum, liver and kidney extract, as well as placental extract; but absorbed IgG with organ extract and serum only reacts with RMP. By immunoelectrophoresis, two placental specific lines are formed toward the cathode from the origin and two or more lines are formed around the alpha and beta globulin regions of normal serum. These lines, formed at alpha or beta globulin regions, are constantly existent in any IgG preparation; however, one line migrates toward the cathode and is found only in selected IgG samples. MPL forms one line at the alpha globulin region. Neither HCG nor MCG produce any precipitin line by this method.

MCG neutralization test

As shown in Table 2, no neutralization was found. It thus appears that the absorbed anti-placental globulin reacts with placental antigen but not with monkey placental lactogen or monkey chorionic gonadotrophin.

Cytotoxicity test

Results are shown in Table 3.

The cytotoxicity titer varies with the IgG preparation. Thus, the effective IgG (producing abortion) shows a high cytotoxicity titer, whereas the non-effective IgG (failure to induce abortion) shows a very low titer. Normal control serum globulin shows no titer at all. It appears that the titers parallel effectiveness of the serum in producing abortion and is complement dependent. As shown in Figure 2, the results of the test are easily identified by phase contrast microscopy.

Immunofluorescent antibody

Using the effective IgG sufficiently absorbed with normal monkey serum, specific immunofluorescein localizes mostly on trophoblastic tissue (Figure 3).

DISCUSSION

The abortions described above are apparently caused by passive immunization. The exact mechanism or site of the reaction has not yet been completely explained. It is, therefore, possible to abort pregnant Rhesus monkeys with heterologous (auto-logous) anti-placental globulin without noticeable harm to the mother. It appears that the injected placental specific antibody(s) reacts with placental specific antigen, which localizes on the trophoblast, and then destroys the reacted tissue with participation of complement. This tissue destruction probably causes vaginal bleeding, fetal death and abortion. Anaphylaxis was not a feature in these experiments even when using large doses of antibody(s) which might react with normal monkey components. Furthermore, even those monkeys who received two series of injections showed no evidence of anaphylactic symptoms.

TABLE 2. MCG NEUTRALIZATION TEST

MCG dose (mg)	1.50	0.75	0.37	0.18	0.09
Treatment					
MCG + G-IgG	+	+	+	-	-
MCG + PBS	+	+	+	-	-

+ = positive ovulation
 - = negative ovulation

TABLE 3. CYTOTOXICITY TEST

CONCENTRATION mg protein/ml	2.0	1.0	0.5	0.25	0.125	0.065	0.031
SAMPLE							
EFFECTIVE G-IgG	100%	95%	90%	75%	50%	10%	0
NON-EFFECTIVE G-IgG	65%	50%	0	0	0	0	0
NORMAL GOAT GLOBULIN	0	0	0	0	0	0	0

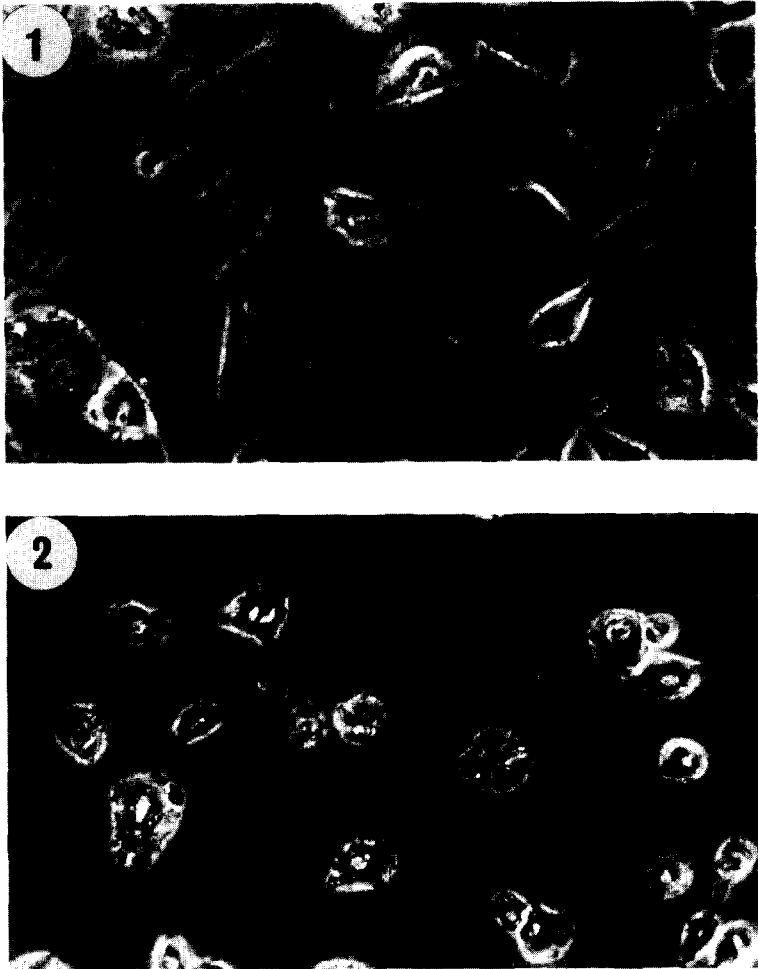


Figure 2. (1) Control Rhesus monkey trophoblast in culture.
(2) Demonstrating severe cell destruction after exposure to goat anti-Rhesus monkey placenta (G-IgG) for one hour and complement for two hours.

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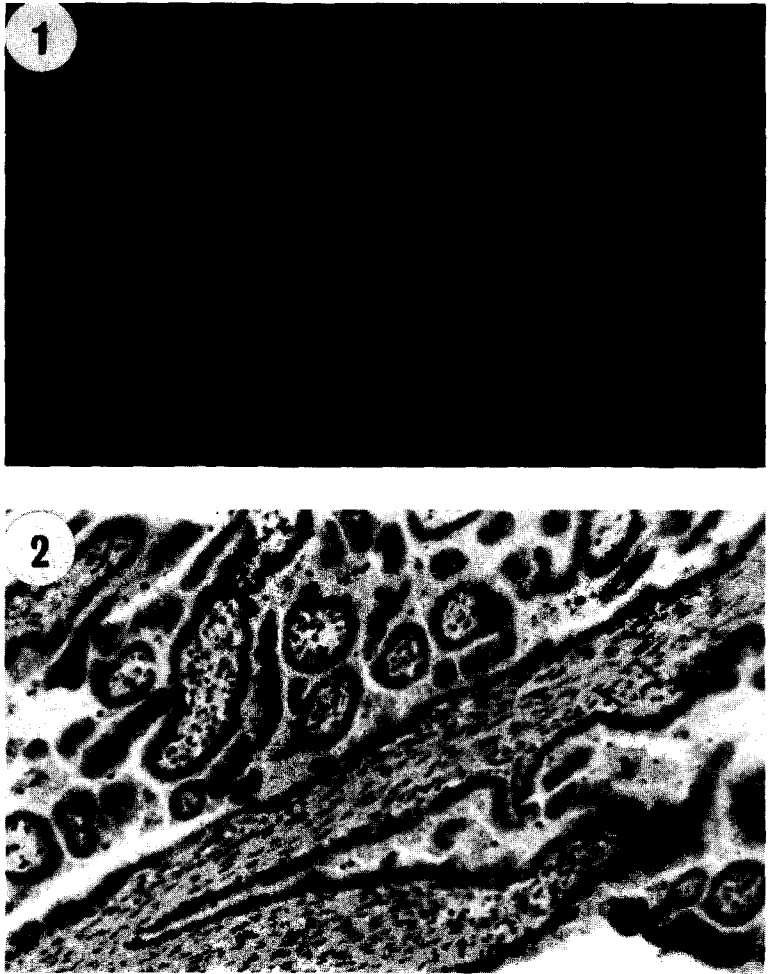


Figure 3. (2) H & E stain of term Rhesus monkey placenta; and (1) indirect fluorescence of adjacent section using goat anti-Rhesus monkey placenta (G-IgG) and FITC-labelled rabbit anti-goat globulin. Note heavy deposits of G-IgG in syncytio-trophoblast. Controls using normal goat globulin were negative.

Most abortions occurred within two to seven days after inoculation. This variation of abortion time might originate as a result of a difference in quality of each IgG preparation. The quality of IgG preparations was checked by immunodiffusion, immunoelectrophoresis, cytotoxicity and immunofluorescent antibody technique. However, it is difficult to standardize the titer of IgG. Using immunoelectrophoresis or immunodiffusion methods, placental specific precipitation lines are clearly identified; yet, the precise relationship between each component and abortion is obscure and needs clarification. Based on comparative experiments, the component which migrates toward the cathode seems to be the principal element. Anti-HPL does not appear to be a major factor.

While the results of the cytotoxicity titer tests suggest parallelism with induction of abortion, the test is largely dependent on the condition and constant availability of trophoblastic cells in culture. The immunofluorescent antibody technique appears to be a useful way to identify effective IgG, but is qualitative only. Furthermore, while most of the placental specific components presumably localize on trophoblastic tissue, not all of them necessarily participate in the abortion mechanism.

For further research, in order to adequately study mechanisms and develop a highly effective serum with high reproducibility, it is a most urgent matter to isolate and identify the principal antigen. Research is now progressing along these lines.

The antigenicity of placenta has been reviewed on many occasions, among them an exhaustive study on human placenta has been reported by Krieg (7) who reported a placental specific antigen localized in trophoblastic tissue composed of a protein polysaccharide or protein-lipid complex. In agarose electrophoresis, this antigen seems to be a single entity with a mobility similar to alpha-2 serum globulin.

While it is not conclusive that there is only one antigenic entity in placenta, presumably, Krieg's antigen is at least one of the placental specific substances. Using monkey placenta, our experiments show that when the placenta is vigorously washed at least one specific placental antigen is missing. Recently, a quantitative study of the rat placenta was reported by Tsuzuku *et al.* (9). Using radioimmunoassay method, they calculated that an anti-rat placental serum produced in rabbit contained placental specific antibody at a level of 30% of the total antibody production.

While Currie (8) using rabbit anti-HCG shows remarkable cytotoxicity against cultured human placental cells, and Morisada (10) showed the same toxicity *in vivo*, suggesting the antigenicity of these hormones, there was no detectable anti-monkey chorionic gonadotrophin in our antisera.

Rhesus monkey anti-placental serum produced abortion in Rhesus monkeys (40-80 days pregnant) about three to four days after injection. The placental antigen contains several placental specific fractions but the fraction specific for the abortion is yet to be identified. MCG and MPL do not appear involved in these experiments. Short and long term effects of immunization upon the monkeys will be the subject of another article.

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