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Antifertility Effect of Progesterone Antibodies in Mice

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Abstract□Early embryo development and implantation were arrested in ICR mice which were passively immunized with a mouse monoclonal progesterone antibody given as a single intraperitoneal injection at 12 hrs or 60 hrs post coitum (p.c.). Unimplanted embryos were recovered from the reproductive tract of the antibody-treated mice and none of these progressed to the blastocyst stage. The most pronounced effect was an arrest of embryonic development at a stage prior to cavitation. The plasma progesterone concentration in the blood taken by cardiac puncture increased greatly after the treatment by virtue of high affinity binding by the antibody in circulation. The results showed that passive immunization against progesterone shortly after mating interfered with early hormone dependent steps which were essential for normal embryonic development.

Keywords□Progesterone, antifertility, passive immunization, progesterone monoclonal antibodies, progesterone EIA.

Passive immunization of antibodies against steroid and protein hormones has been used extensively as a technique to investigate the endocrinology of pregnancy in various species of animals, and to study ways of enhancing or limiting fertility particularly in females[1]. However, little is known of the mechanisms by which progesterone antibodies block pregnancy when administered shortly after mating. The availability of a suitable monoclonal antibody enabled us to determine its effects on the initiation of implantation in ICR mice. The purpose of the present study was to investigate mechanisms by which a progesterone monoclonal antibody blocks pregnancy in mice.

EXPERIMENTAL METHODS

Animals

Mature virgin female ICR mice (average weight, 25g) and Balb/c mice, obtained from the animal breeding facility of the Bio-Potency Evaluation Program, Genetic Engineering Center, were housed in a light controlled (14 hr light: 10 hr darkness; lights off at 10 p.m.) and well-ventilated room (24-26 °C).

PMSG (pregnant mare's serum gonadotropin, Sigma Co.) was administered between 1 p.m. and 2 p.m. and hCG (human chorioic gonadotropin, Sigma) was administered 46-48 hours later (5 IU per mouse respectively). After the administration of hCG, each female mouse was placed in a cage with a stud male. Vaginal smears were taken to ensure that the mice completed regular oestrous cycles before mating. Pregnancy was dated from the morning when a vaginal plug was detected (day 1), and mating was presumed to have taken place at 2 a.m. (time 0.00 hr)[2].

Monoclonal antibodies

Progesterone monoclonal antibodies were prepared and characterized by the following procedures. Progesterone-11α-hemisuccinate-bovine serum albumin conjugate was prepared, using carbodiimide according to the method of Eshhar *et al.*[3] and used as an immunogen. After the spleen cells of Balb/c mice immunized with the immunogen were fused with P3-X63-Ag 8.653 cells, hybridoma cells were screened by radioimmunoassay using ^{3}H-progesterone. Ascitic fluid was collected from the mice injected with the cloned cells (051-01) and immunoglobulins were precipitated by adjusting the ammonium sulfate concentration to 40% saturation and then dialyzed against 0.9% NaCl (4 °C). The immunoglobulin G (IgG) concentration was measured by

Table I. Determination of total protein and total mouse IgG in ascitic fluids

Clone	Subtype	Total protein* (mg/ml ascites)	Total mouse IgG** (mg/ml ascites)	igG (%)
051-01	IgG_1	18.2	10.4	57.1
051-02	IgG_2b	23.6	4.5	19.0
051-03	IgG_1	20.0	6.3	31.5
051-04	IgM	17.4	1.1	6.3
mixed Ab***		20.6	5.3	25.7
2B7	IgG_1	27.4	2.4	8.8

*The total protein of ascitic fluids was determined by dye binding assay[12].
**The total amount of mouse immunoglobulin in ascitic fluids was determined by a single radial immunodiffusion method, using mouse IgG as a standard[4].
***The same volume of (051-01) and (051-04) ascitic fluids was mixed and the immunoglobulin fraction was prepared by ammonium sulfate precipitation.

Table II. The effect of various progesterone monoclonal antibodies on pregnancy in BALB/c and ICR mice

Treatment	Dose (nmole IgG)	Day of autopsy	No. pregnant / No. mated	plasma progesterone (ng/ml) non-pregnant	pregnant
Ab-treated					
Ab (051-01)					
ICR	6	13	0/4 (0%)	212	
Balb/c	6	15	0/2 (0%)	109	
Ab (051-03)					
ICR	6	12	0/3 (0%)	217	
Balb/c	6	12	0/2 (0%)	115	
Ab (051-04)					
ICR	1	12	0/3 (0%)	45	
mixed Ab*					
ICR	6	14	0/3 (0%)	210	
Controls					
saline treated					
Balb/c		15	2/2 (100%)		30
myeloma treated					
Balb/c		16	1/2 (50%)	4	29
myeloma treated					
ICR		14	2/3 (67%)	4	37

*The same volume of (051-01) and (051-04) ascitic fluids was mixed and the immunglobulin fraction was prepared by the ammonium sulfate precipitation method.
Antibodies were given as double intraperitoneal injections, 48 and 96 hrs after mating. The control females received a volume of saline (100 ul), or ammonium sulfate fractionated IgG, prepared from mouse myeloma (P3-x63-Ag8.653) ascitic fluid.

a single radial immunodiffusion method[4] using mouse IgG as a standard. The immunoglobulin subclasses of the monoclonal antibodies were determined by the double immunodiffusion method[5], using concentrated cell culture supernatant (20x-50x) and polyclonal antisera against the different mouse IgG subtypes. Aliquots of the dialyzed suspensions were stored at -20 °C. Ammonium sulfate

Table III. Effect of monoclonal progesterone antibodies on pregnancy in ICR mice

	Dose (nmole IgG)	Day of autopsy (p.c.)	No. pregnant / No. mated	Plasma progesterone (ng/ml) non-pregant	pregnant	Preg./Non-preg.
Antibody-treated	0.15	9	1/6 (17%)	16	31	189%
	0.75	9	2/13 (15%)	55	98	178%
	1.5	9	4/14 (29%)	58	59	102%
	2.0	11	0/5 (0%)	120		
	3.0	9	1/12 (8%)	97	132	137%
	4.0	11	0/5 (0%)	173		
	6.0	11	0/5 (0%)	212		
Controls						
myeloma		9	5/6 (83%)	18	56	310%
saline		10	5/10 (50%)	4	32	805%

Antibodies were given as a single i.p. injection (IgG in 100 u*l* saline) at day 3 (60 hr) after mating. The control females received a same volume of saline, or ammonium sulfate fractionated IgG, prepared from mouse myeloma (P3-x63-Ag8.653) ascitic fluid.

saturation (40%)-fractionated IgG from the non-immune ascitic fluid which was prepared from mouse myeloma P3-X63-Ag8.653, or 0.9% (w/v) NaCl was used as control.

Effect of the progesterone monoclonal antibody on tubal transport and embryo development

Mice were injected with a dose of 3 nmole IgG of the antibody intraperitoneally 12 hr p.c. At the autopsy, the number of corpora lutea was recorded and fallopian tubes and uterine horns were flushed with 0.9% NaCl to recover unimplanted ova.

Progesterone assay

Blood samples were taken by cardiac puncture under ether anesthesia and the plasma separated by centrifugation at 4°C was stored at -20°C until assay. Steroid-binding globulin in the serum was inactivated with 8-anilino-1-naphthalenesulphonic acid (ANS)[6], and total progesterone was assayed. The conjugate was prepared by reacting progesterone 3(O-carboxylmethyl) oxime N-hydroxy succinimide ester and horseradish peroxidase according to the method of Eshhar *et al.*[3] This direct competitive enzyme immunoassay (EIA) was reproducible over a broad operating range (0-100 ng/m*l*). The sensitivity of the assay was 0.4 ng/m*l* (calculated from $\overline{X} \pm 2SD$ at zero concentration), and the coefficient of variation of interassay was 7.6%. This competitive EIA showed good dilution linearity ($r = 0.997$).

RESULTS AND DISCUSSION

Determination of total mouse IgG in ascitic fluid

As shown in Table I, the total mouse IgG content of the 051-01 clone was much greater than those of other clones. This was presumably due to the fact that the 051-01 clone showed a strong and rapid growth in the culture and had a better morphology than any other clones. The total protein amounts of the ascitic fluids prepared from individual clones were almost same.

The effect of various progesterone monoclonal antibodies on pregnancy in Balb/c and ICR mice

When various antibodies were given double i.p. injections after mating, the pregnancy was blocked in all mice (Table II). It was noticed that the plasma progesterone concentrations of the antibody-treated ICR mice were twice as high as those of the antibody-treated Balb/c mice. From this fact, it was assumed that the anti-fertility effect by the passive immunization of antibodies against progesterone was influenced by genotypes. We used the 051-01 clone in the antifertility study because it showed a rapid growth in culture and produced a very high content of mouse IgG (57.1%) in ascitic fluid (Tables I and II).

Efficacy of the monoclonal antibody to block pregnancy

Increasing amounts of the progesterone anti-

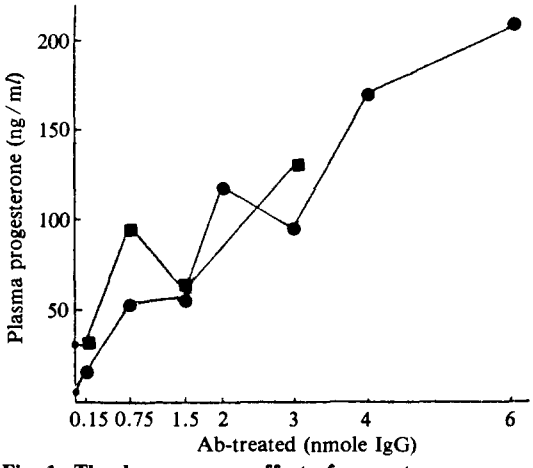

Fig. 1. The dose response effect of progesterone monoclonal antibodies on the plasma progesterone concentration.

—■ pregnant ●—● non-pregnant

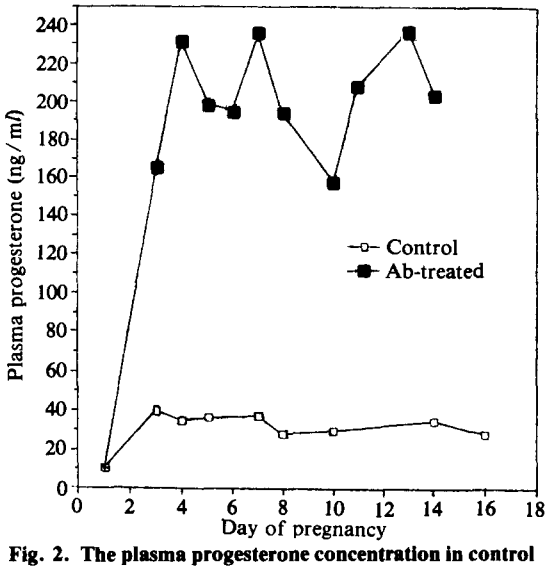

Fig. 2. The plasma progesterone concentration in control ICR mice (myeloma treated) and monoclonal antibody treated ICR mice (6 nmole IgG).

Antibodies were injected as a single i.p. at 12 hrs after mating, and control females received a volume (100 μl) of ammounium sulfate fractionated IgG, prepared from mouse myeloma (P3-x63-Ag8.653) ascitic fluid.

body were injected into ICR mice to determine an effective antifertility dose (Table III). A single dose of IgG greater than 2 nmoles blocked the pregnancy completely. At a dose of lower than 1.5 nmole IgG, pregnancy occurred in only 20% of the mouse population while the pregnancy rate in the control group was 80% with IgG of myeloma ascitic fluid and 50% with saline injection. When the plasma progesterone concentrations were compared between the antibody-treated mice and control mice, the concentration was 1.5 times higher in the pregnant mice than in the non-pregnant mice in the antibody-treated group. In the control group, the progesterone concentration was 3-8 times higher in the pregnant mice than in the non-pregnant mice. In both pregnant and non-pregnant mice in the antibody treated group, the plasma progesterone concentration increased gradually, depending on the amount of the antibody treated (Fig. 1). Also, the non-pregnant mice treated with a high dose of the antibody had a much higher circulating progesterone concentration than the pregnant mice in the control group.

It was assumed that the injected antibody bound the circulating progesterone and that the antibody-bound progesterone could not be taken up by its target receptors. Therefore, the free progesterone level in the plasma decreased and the pregnancy was blocked even if the total progesterone level, including the antibody-bound progesterone, increased in the plasma. To determine the most effective injection timing at which the monoclonal antibody blocked pregnancy, mature virgin ICR mice were given a single i.p. injection at 12 hr (1 day), 60 hr (3 days), 110 hr (5 days), and 150 hr (7 days) p.c.

The pregnancy was blocked in all of the 19 mice treated at 12 hr or 60 hr p.c. (Table IV). The pregnancy was also blocked in 4 out of 7 mice treated with the antibodies at 150 hr (7 days) p.c. But, 3 mice appeared to be pregnant. This finding indicated that a single injection of the antibody (450 μg/mouse, 18 mg/kg) between 12 and 60 hr p.c. prevented pregnancy and its effectiveness was slightly reduced when it was administered later.

This may be associated with the limited capacity of the antibodies to reduce circulating progesterone levels in the face of increasing luteal secretion[7], or with the fact that there are implanting blastocysts at 4.5 days p.c. and that the primitive endoderm is formed[8].

Plasma progesterone concentration after antibody treatment

As shown in Fig. 2, plasma concentrations of progesterone in the control mice were 5-10 ng/m*l* at day 1 p.c. At day 3 p.c. the concentration increased slowly to a peak of about 40 ng/m*l*, declined to a value of about 28 ng/m*l* at day 8 p.c., and then

remained at a value of about 35 ng/ml at day 14 p.c. After the antibody treatment (6 nmole IgG in 0.1 ml), plasma concentrations of progesterone at day 4 p.c. increased to a value at least six times higher than those in control females (Fig. 2). After day 4 p.c. the values declined to 159 ng/ml at day 10 p.c., and then remained at a somewhat higher concentration. As shown in Tables III, IV, and V, the postcoital administration of progesterone monoclonal antibodies resulted in high circulating concentrations of progesterone in the plasma. It has been reported that greater than 95% of progesterone in circulation was bound by monoclonal antibodies[9] and therefore was presumably not available for uptake by target receptors.

Effect of antibody on embryonic development

At day 3 after mating, embryos were recovered predominantly from the oviduct or the uterus in both antibody-treated and control mice. But, the developmental stages of the embryos from the two groups were very different (Tables V and VI).

As shown in Table VI, only 14% of the embryos had progressed into the morula stage in the antibody-treated mice, whereas 89% of the embryos had progressed into the morula stage in the control mice.

As shown in Fig. 3 and Table VI, the embryos of the antibody-treated mice were degenerated mostly in the oviduct and uterus and did not progress into further development. Therefore, the implantation did not take place in the uterus as shown in Table VII. In the control mice, 87% of the embryos were recovered in the oviduct and 13% in the uterus at day 3, but 100% of the embryos were found in the uterus at day 4. The combined results of Table IV and VII indicated that antibody injection was more effective in blocking pregnacy when the embryos

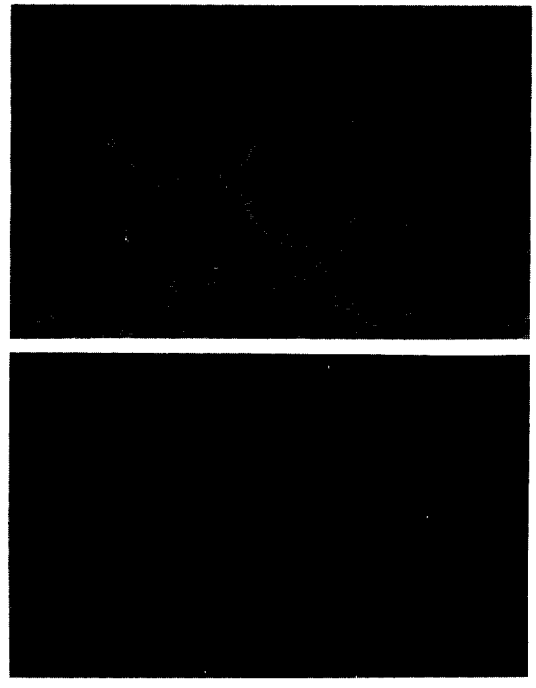

Fig. 3. Representative embryos flushed from the uteri of ICR mice on day 3 of pregnancy.

The mice had been passively immunized at 12 hrs after mating with a progesterone monoclonal antibody (3 nmole IgG). Control females received a same volume of saline.

A: A light micrograph of typical compact morula recovered from the uterus of a control female. Most of the embryos (92%) recovered from control animals were late morulae (magnification, X 250).

B: Embryos flushed from the antibody-treated females on day 3 of pregnancy. In contrast to the control group, most of the embryos (92.6%) recovered were degenerated and had not begun cavitation.

Table IV. The effect of the injection timing of the monoclonal antibodies on antifertility

	Day of injection after mating	Day of autopsy	No. pregnant / No. mated	Plasma progesterone(ng/ml) Non-pregnant	Pregnant
Antibody-treated	1	10	0/8 (22%)	62	
	3	10	0/11 (0%)	85	
	5	10	2/9 (22%)	144	130
	7	13	3/7 (43%)	115	158
Control (saline)					
	3	10	7/11 (64%)	3	62

The antibodies were given at days 1, 3, 5, and 7 after mating as a single i.p. injection (3 nmole IgG). The control females received 100 μl of saline at day 3 after mating.

Table V. Effects of anti-progesterone antibodies on tubal transport and embryo development in ICR mice

Day of autopsy (p.c.)	Antibody dose (nmole IgG)	No. of Mice	No. of Corpora lutea (mean per mouse)	Total No. recovered (mean per mouse)	Embryos				Plasma progesterone concentration (ng/ml)
					No. in oviduct		No. in uterus		
					(%)	degenerated (%)	normal (%)	degenerated (%)	
3	0*	4	44 (11)	38 (9.5)	30 (79)	3 (7.9)	5 (13.1)	0 (0)	14
	3	4	40 (10)	27 (6.8)	2 (7.4)	13 (48.2)	0 (0)	12 (44.4)	352
	4.5	6	55 (9.2)	39 (6.5)	3 (7.7)	16 (41)	0 (0)	20 (51.3)	367
4	0*	2	21 (10.5)	19 (9.5)	0	0	16 (84.2)	3 (15.8)	11
	4.5	1	9	8	0	0	5 (62.5)	3 (37.5)	225

A single i.p. injection was given 12 hrs. after mating.
*: 100 μl of saline was injected to the control group.

Table VI. Effects of anti-progesterone antibodies on embryo development in ICR mice

Antibody treated	No. of mice		No. of embryos		
			Total	in oviduct	in uterus
Control*	6	Total	57 (100%)	33	24
		Normal	51 (89%)	30	21
		degenerated	6 (11%)	3	3
Antibody-treated	11	Total	74 (100%)	34	38
		Normal	10 (14%)	5	3
		degenerated	64 (86%)	29	35

A single i.p. injection (3-4.5 nmole IgG/mice) was given at 12 hrs after mating.
*: 100 μl of saline was injected to the control group.

were in the oviduct rather than in the uterus. When embryo recovery sites between the control and antibody-treated mice at day 3 were compared, only 13% of the embryos were in the uterus in the control and 48% of the embryos were in the uterus in antibody-treated mice (Table VII). But at day 4, all embryos were recovered in the uterus for both control and antibody-treated mice. These results indicated that the tubal transportation of the fertilized eggs was accelerated in the early treatment of the antibodies and most of the eggs were recovered in degenerated forms at the oviduct and the uterus before the initiation of implantation. It has been reported that implantation failure arose from an enhanced estrogen effect[10]. The examination of the recovered eggs showed that embryonic development had been seriously retarded by the antibody treatment (Fig. 3). McLaren and Michie reported that asynchrony between the stage of embryonic development and the endometrium neutralized the effect of any embryo signal and caused the failure of implantation in the uterus.[11] By passive immunization against progesterone, the mechanism of impairing the rate of cell division in an early mouse embryo is a question that requires further investigation.

CONCLUSION

Passive immunization of mice against progesterone, administered shortly after mating, prevented the embryonic development at an earlier time be-

Table VII. The timing effect of antibody treatment on embryo development in ICR mice

Day of autopsy (p.c.)	Antibody treatment	No. of mice		No. of embryos		
				Total	in oviduct	in uterus
3	Control	4	Total	38 (100%)	33	5
			Normal	35 (92%)	30	5
			degenerated	3 (8%)	3	0
	Ab-treated	10	Total	66 (100%)	34	32
			Normal	5 (8%)	5	0
			degenerated	61 (92%)	29	32
4	Control	2	Total	19 (100%)	0	19
			Normal	16 (84%)	0	16
			degenerated	3 (16%)	0	3
	Ab-treated	1	Total	8 (100%)	0	8
			Normal	5 (63%)	0	5
			degenerated	3 (37%)	0	3

fore cavitation. Unimplanted embryos in the reproductive tract of monoclonal antibody-treated mice had not progressed to the molura or blastocyst stages. The postcoital administration of the antibodies interfered with early hormone dependent steps, which are essential for normal embryonic development.

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