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Immunosuppressive Properties of Mouse Amniotic Fluid[1] (39206)

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Mouse amniotic fluid (MAF)[2] has been shown to contain a noncytotoxic moiety that inhibits the *in vitro* responses of mouse spleen cells to SRBC, to allogeneic cells, and to B and T cell mitogens (1, 2). Observations made after the selective removal of transferrin, albumin, and alpha-fetoprotein from MAF suggested that AFP was the immunosuppressive agent. This conclusion was strengthened by the demonstration that in microgram quantities putatively pure murine AFP suppressed *in vitro* antibody synthesis whereas equivalent amounts of NMS, transferrin, or albumin did not.

Reported below are studies confirming the ability of MAF to suppress mouse immune responses *in vitro*. However, it will also be shown that MAF does not inhibit antigen- or mitogen-induced proliferation of human blood cells, and that human AF and cord sera, despite their relatively high concentrations of AFP, do not significantly impair the immune responses of cells from either species. These results suggested that the immunosuppressive agent in MAF may be biologically active in a limited number of species, and that mouse and human AFP either differ pharmacologically to a great degree and/or the immunosuppressive activity attributed to mouse AFP (1, 2) is actually produced by another agent(s) physically associated with it. Because of the questions raised by these experiments, several hormones associated with pregnancy were screened for their immunosuppressive activities.

Materials and methods. Mice. Male B6D2F$_1$ and CBA/J mice of both sexes were obtained from Jackson Laboratories, Bar Harbor, Maine.

Amniotic fluid. Pregnant CBA/J mice were killed between the 9th and 20th day of gestation; the age of the pregnancy was determined by considering the day the vaginal plug was observed as Day zero. The uterus was removed and washed in balanced salt solution. The intact gestations were carefully dissected from the uterine wall and washed free of maternal blood. The AF was aspirated through a 20 gauge needle, and care was taken to prevent contamination with placental or fetal blood. AF from a single pregnancy was pooled, centrifuged twice to remove cellular elements, filter-sterilized (0.22 μm), heat-inactivated at 56° for 15 min, and stored at −20°.

Human AF obtained from 14- to 18-week gestations were generously donated by Drs. A. Myron Johnson, University of North Carolina School of Medicine, and Hiram Mendenhall, University of South Alabama School of Medicine. These AF were secured for other studies that had been approved by the appropriate Human Rights Committees, and in no instance was a pregnancy disturbed for the purpose of obtaining AF. The HAF was processed and stored as noted above.

Cord sera. Fresh human newborn cord sera were obtained from the serology laboratory of the North Carolina Memorial Hospital after a sample had been taken for a VDRL test. The sera were processed and stored as noted above.

Sera. Human and mouse sera were taken from normals by standard methods, and they were processed and stored as noted above.

Aggregated human IgG. Human IgG (Miles Laboratories, Kankakee, Illinois) was dissolved in distilled water (10 mg/ml), heat-aggregated at 63° for 20 min (3), and stored at −20°. It was added to cultures at a concentration of 100 to 200 μg/ml to pro-

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[2] Abbreviations used in this paper: MAF, mouse amniotic fluid; AF, amniotic fluid; HAF, human amniotic fluid, AFP, alpha-fetoprotein; SRBC, sheep red blood cells; HCG, human chorionic gonadotropin; Con A, concanavalin A; KLH, keyhole limpet hemocyanin; NMS, normal mouse serum.

vide a nontoxic standard against which to measure immunosuppressive activity (4).

Hormones. HCG (Follutein, E. R. Squibb & Sons, Inc., Princeton, N.J.) was added to cultures at concentrations of 10–200 i.u./ml. Estriol, estrone, and 17 β-estradiol (Sigma Chemical Company, St. Louis, Missouri) were added to cultures at concentrations of 10 ng to 10 μg/ml.

Culture conditions: Mouse spleen cells. Primary responses to SRBC were elicited *in vitro* under conditions described previously (5). Briefly, an underlay of washed SRBC (2%) in 1.5 ml of agar (0.25%) was placed in 35 × 10 mm tissue culture dishes (Falcon Plastics, Oxnard, California), and 4 ml of CBA or B6D2F$_1$ spleen cell suspension (20 × 10^6 nucleated cells) were added after the agar had hardened. Sera, AF, mitogens, or hormones were added, and the cells were cultured at 37° in 5% CO_2 in air for 3 days. At this point the medium and cells were discarded, and 1 ml of complement (1:10) was added to each dish. After 1.5 hr at 37° the areas of focal hemolysis were counted. All cultures were done in triplicate, and replicate samples were generally within 5% of the mean.

The culture medium was Eagle's minimum essential suspension medium augmented with nonessential amino acids, L-glutamine, 10% heat-inactivated fetal calf serum, and penicillin and streptomycin (100 U and 100 μg/ml, respectively).

Culture conditions: Human cells. Human blood was obtained by venipuncture, and the plasma was separated from the RBC by gravity sedimentation at 37°. The leukocyte-rich plasma was centrifuged, and the cells were suspended in the culture medium described above at a concentration of 1 × 10^6/ml. Two milliliters of the cell suspension were put in 12 x 75 mm polystyrene culture tubes. Sera, AF, Con A (5 μg/ml; Miles Laboratories, Kankakee, Ill.), or KLH (50 μg/ml; Calbiochem, La Jolla, California) were added, and the cells were cultured at 37° in 5% CO_2 in air for 3 days (Con A) or 5 days (KLH). The proliferative responses of the leukocytes were measured by [^{3}H]thymidine incorporation into deoxyribonucleic acid during an 18-hr period (2 μCi/tube). The cultures were processed for liquid scintillation counting as described previously (6). All cultures were done in triplicate.

Alpha-fetoprotein determinations. The levels of AFP in cord sera and HAF were determined in the laboratory of Dr. A. Myron Johnson using rocket immunoelectrophoresis as described by Norgaard-Pederson (7). HAF from normal 14- to 18-week human pregnancies has been found to contain 5 to 35 μg of AFP/ml; cord sera from newborn human infants has been reorted to contain approximately 100 μg of AFP/ml (7), and the results obtained in these studies are in agreement with this value. Mouse AFP did not cross-react with the antisera used to detect human AFP, and as a result, it was not possible to determine AFP levels in MAF.

Results. Inhibition of mouse primary responses by MAF (Table I). NMS (1:50) from pregnant and nonpregnant animals depressed the primary response of spleen cells from nonpregnant female mice 57% and 48%, respectively. At the same concentration, MAF from 9- to 20-day gestations inhibited the development of SRBC hemolytic foci up to 86% (mean, 77%); there was no apparent correlation between the age of the

TABLE I. INHIBITION OF THE *IN VITRO* PRIMARY RESPONSE OF MOUSE SPLEEN CELLS TO SRBC BY MOUSE AMNIOTIC FLUID.

Source of spleen	Percentage inhibition ± SD[a] (Number of samples)									
	Mouse serum (1:50)		Aggregated IgG (200 μg/ml)	Amniotic fluid (1:50) – Age of gestation						
	Normal	Pregnant		9–10	11–12	13–14	15–16	17–18	19–20	Mean
Normal female	48 ± 4.2 (9)	57 ± 9.8 (9)	73 ± 6.5 (9)	72 ± 7.3 (6)	61 (1)	79 ± 3.2 (3)	86 ± 4.1 (5)	72 ± 6.0 (4)	85 ± 3.3 (3)	77.0
Pregnant female	28 ± 6.5 (9)	22 ± 4.3 (9)	65 ± 7.0 (7)	45 ± 11.6 (4)	10 (1)	85 (1)	60 ± 8.9 (4)	55 ± 2.1 (5)		52.3

[a] Experimental results are compared with the number of hemolytic foci obtained in cultures to which nothing has been added. See the text for details of the culture conditions.

gestation and the inhibitory properties of the MAF. As the concentrations of NMS and MAF were decreased, the immunosuppression produced by these agents decreased proportionately, and no significant inhibition was produced by either at a titer higher than 1:200 (data not shown). Spleen cells from pregnant females were less suppressed by NMS and MAF than were cells from nonpregnant animals; aggregated IgG inhibited both to about the same degree.

Effects of HAF and cord sera on murine responses to SRBC (Table II). Eleven samples of HAF and 17 cord sera from human newborns proved less inhibitory to *in vitro* murine responses to SRBC than did comparable amounts of human serum. The final concentrations of AFP in these cultures were 0.2 to 0.3 μg/ml (HAF) and 1.6 to 2.2 μg/ml (cord serum). In the same experiments MAF was markedly inhibitory.

Effects of MAF and HAF on human blood cell responses to Con A and KLH (Table III). MAF and HAF (AFP, 0.9 to 1.3 μg/ml) clearly were less effective in inhibiting the proliferative responses of Con A- and KLH-stimulated human blood cells than were NMS or human serum. In this system, HCG (200 i.u./ml) was no more suppressive than were the control sera.

Suppression of murine responses to SRBC by HCG and estrogens (Table IV). HCG produced immunosuppression comparable to that achieved with MAF or aggregated IgG at a concentration of 200 i.u./ml. Estriol, estrone, and 17 β-estradiol had no effect on the response to SRBC at levels at least tenfold greater than those found in human fetal plasma (i.e., at 10 μg/ml; ref. 8).

Discussion. Previous work (1, 2) has demonstrated that mouse amniotic fluid contains a noncytotoxic agent able to inhibit the *in vitro* immune responses of mouse spleen cells. The immunosuppressive property of MAF was found to be abolished when AFP

TABLE II. FAILURE OF HUMAN AMNIOTIC FLUID OR CORD SERUM TO SIGNIFICANTLY INHIBIT THE *IN VITRO* PRIMARY RESPONSE OF MOUSE SPLEEN CELLS TO SRBC.

	Percentage inhibition ± SD[a] (Number of samples)			
Aggregated IgG (200 μg/ml)	Human serum (1:50)	Human AF (1:50)	Cord serum (1:50)	Mouse AF (1:50)
81 ± 3.1 (6)	51 ± 5.2 (6)	35 ± 6.4 (11)	40 ± 11.3 (17)	85.4 ± 3.6 (7)

[a] The experimental results are compared with the number of hemolytic foci obtained in cultures to which nothing has been added.

TABLE III. FAILURE OF MOUSE AND HUMAN AMNIOTIC FLUID TO SIGNIFICANTLY INHIBIT THE *IN VITRO* PROLIFERATION OF HUMAN BLOOD CELLS IN RESPONSE TO CON A AND KLH.

	Percentage inhibition of [^{3}H]TdR incorporation[a]					
Response to	Aggregated IgG (100 μg/ml)	NMS (1:20)	Human serum (1:20)	Mouse AF (1:20)	Human AF (1:20)	HCG (200 i.u./ml)
Con A (5 μg/ml	62 ± 3.6	51 ± 2.4	47 ± 2.4	35 ± 5.1	28 ± 3.8	42 ± 3.4
KLH (50 μg/ml)	72 ± 2.1	50 ± 3.1	33 ± 3.2	24 ± 4.7	10 ± 2.1	47 ± 5.1

[a] Mean ± SD derived from five separate experiments.

TABLE IV. EFFECTS OF HCG AND ESTROGENS ON THE *IN VITRO* RESPONSE OF MOUSE SPLEEN CELLS TO SRBC[a]

	Percentage inhibition of hemolytic foci					
	HCG (i.u./ml)					
Aggregated IgG (100 μg/ml)	50	100	200	Estriol (10 μg/ml)	17 β-Estradiol (10 μg/ml)	Estrone (10 μg/ml)
76	34	55	80	22	16	7

[a] The data were compiled from five separate experiments. Each value represents the maximum suppression attained at the given dose level.

was removed, and a preparation of mouse AFP was clearly inhibitory at concentrations as low as 1 μg/ml. The AFP used in these studies was purified by first passing the MAF over an anti-whole mouse serum affinity column and then subjecting the semipurified preparation to polyacrylamide gel electrophoresis. This procedure, however, would not be expected to remove a substance not present in normal mouse serum from MAF, and if a moiety such as a hormone were physically associated with AFP, a clean separation of the two might not be achieved.

The studies reported here confirm the observation that MAF is an *in vitro* immunosuppressant for mouse spleen cells. However, it was also found that MAF impaired the responses of human blood cells to Con A and KLH to a lesser degree than did normal human or mouse serum (9, 10). Further, HAF and cord sera did not depress the immune responses of human or mouse cells when added to cultures in quantities sufficient to produce levels of AFP above 2 μg/ml. Taken together these observations suggested that the immunosuppressive agent in MAF might not be AFP itself, and therefore several other agents associated with pregnancy were screened for their inhibitory properties.

Studies with estrone, estriol, and 17β-estradiol failed to demonstrate significant immunosuppressive activity at concentrations considerably higher than those reported in normal pregnancies (8). HCG (11, 12) moderately depressed immune reactions by human and mouse cells at a level of 200 i.u./ml. However, very little HCG activity has been detected in fetal plasma or AF (11), and AF concentrations equivalent to 10,000 i.u./ml would be required to produce the degree of immunosuppression noted when MAF is added to cultures at a dilution of 1:50. Further, to date no HCG-like hormone has been detected in the serum or AF of pregnant rodents. While this does not rule out the possibility that HCG or an HCG-like hormone is the immunosuppressive agent in MAF, it would require that mouse and human HCG have widely different ranges of biological specificity and tissue distribution.

In addition to hormones, amniotic fluid contains many other components, some of which are derived from maternal serum (14). Among these may be substances present in normal (9, 10) and pregnant (15) sera that have been shown to inhibit the immune response. At the present time it is not possible to evaluate the potential contributions these or other substances make to the immunosuppressive activity of MAF. Thus, while it has been confirmed that MAF possesses the ability to suppress *in vitro* immune responses by mouse cells, no specific agent has been clearly identified as being responsible for this effect. The results do suggest, however, that the immunosuppressant in MAF may be biologically active in a limited number of species, and that if the activity is associated with AFP, then mouse and human AFP must differ pharmacologically and/or immunosuppression is produced by another agent bound to mouse AFP.

Summary. Previous reports have suggested that the alpha-fetoprotein present in mouse amniotic fluid is a potent nontoxic immunosuppressant. In the present studies mouse amniotic fluid (1:50) from 9- to 20-day gestations markedly inhibited the *in vitro* responses of mouse spleen cells to SRBC, and spleen cells from nonpregnant females were more affected than were cells from pregnant mice. On the other hand, MAF was less effective in depressing antigen- and mitogen-induced proliferation of human blood cells than were NMS or human serum. Human AF and cord sera did not significantly depress the immune responses of cells from mouse or man when added to cultures at concentrations sufficient to achieve levels of alpha-fetoprotein reported to be immunosuppressive if mouse AFP is used. While these studies do not identify the inhibitory agent(s) present in MAF, they do suggest that mouse AFP either is pharmacologically different from human AFP and/or that the immunosuppressive activity attributed to mouse AFP is actually produced by another agent physically associated with it.

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