

SUPPRESSION OF THE IMMUNE RESPONSE BY α -FETOPROTEIN

I. The Effect of Mouse α -Fetoprotein on the Primary and Secondary Antibody Response*

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The fetal circulation contains several embryo-specific proteins normally present in the serum of adult individuals in very small concentrations. Pedersen (1) described the first protein of this group in calf serum (fetuin) and since then several embryo-specific serum proteins have been demonstrated in a number of mammalian species (2, 3). Alpha-fetoprotein (AFP),¹ an embryo-specific protein synthesized by the liver, is the first alpha-protein to appear in mammalian sera during ontogenetic development and is the dominant serum protein in early embryonic life at a time when albumin and transferrin are present in trace amounts (4, 5). The physiological level of AFP in the fetus reaches milligram amounts (4 mg/ml in human) during early-mid gestation and then drops linearly as birth approaches and shortly thereafter falls to normal background adult levels which are on the order of 0.001% of the maximal fetal levels. It has been suggested that the appearance of AFP is due to the absence of a repressor which normally appears toward the end of embryonic life (6). This protein has been considered a tumor-associated embryonic antigen since Abelev and co-workers (7) originally observed the reappearance of high concentrations of AFP in the serum of patients with primary liver cancer. Elevated AFP levels have subsequently been shown to occur in other malignant (especially teratocarcinomas) as well as nonmalignant conditions, particularly those associated with liver regeneration (8). However, the relative specificity of markedly elevated AFP levels for primary liver cancer has been emphasized (9).

The function of AFP in the fetus is unknown, nor is it known why there is a re-expression of the protein in certain pathological conditions during postnatal life. One intriguing possibility is that AFP has immunoregulatory properties which are important for the exemption of the histoincompatible embryo from immunological attack by the maternal immune system. Furthermore, the demonstration of immunosuppressive activity by AFP in relation to its occurrence in certain malignant conditions would be consistent with the association known to exist between various forms of immune deficiency and neoplastic disease.

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¹ *Abbreviations used in this paper:* AFP, alpha-fetoprotein; MAF, mouse amniotic fluid; NMS, normal mouse serum; PBS, phosphate-buffered saline; PFC, plaque-forming cells; WMS, whole mouse serum.

During the course of a previous study (10) we found that mouse amniotic fluid administered *in vivo* significantly suppresses antibody formation. Since one of the major unique proteins in amniotic fluid is AFP, the present study was initiated to investigate the effect of AFP both as it occurs in amniotic fluid and in the isolated form on the primary and secondary antibody response *in vitro*. A subsequent paper will describe the effects of AFP on mixed lymphocyte reactivity and mitogen-induced lymphocyte transformation.²

Materials and Methods

Animals. CBA/J and BALB/c mice, purchased from Jackson Laboratories, Bar Harbor, Maine, were used in all experiments. Pregnant HA/ICR mice, bred locally at the Mayo Clinic, were used as a source of amniotic fluid.

Amniotic Fluid Collection. Mouse amniotic fluid (MAF) was collected from Swiss or HA/ICR mice in the late second and early third trimester of pregnancy. Pregnant animals were sacrificed by cervical dislocation and the embryonic sacs were exposed by dissecting away the placenta. The fluid was collected by puncturing the individual sacs with a 20 gauge needle and a 1 ml syringe. Special care was taken not to contaminate the MAF with blood. Approximately 1 ml of fluid was collected from each pregnant mouse. The fluids were pooled, centrifuged, and stored frozen for further use.

The total protein concentration of MAF was determined as the dry weight of a lyophilized aliquot of the fluid following exhaustive dialysis against water. The extinction coefficients of the isolated protein components of MAF were determined by lyophilizing 1-ml aliquots of the purified proteins after exhaustive dialysis against 0.1 M NH_4HCO_3 buffer. The dry material was weighed on an analytical balance, redissolved in a calibrated tube to 1 ml with phosphate-buffered saline (PBS), pH 7.2, and extinctions at 280 $\text{m}\mu$ determined in a Hitachi Perkin-Elmer, model 139 spectrophotometer.

Antisera. Antiserum to MAF was prepared in rabbits by subcutaneous injection of MAF supplemented with Freund's complete adjuvant. The injections, totaling 1 ml each, were given 2 wk apart. Serum obtained at various times after the second series of injections was used for the subsequent immunochemical characterization and purification of amniotic fluid protein components. The antiserum was rendered specific for AFP by absorption with lyophilized normal mouse serum (NMS). Rabbit antimouse transferrin and albumin were prepared by a similar immunization regimen using purified mouse serum, transferrin, and albumin. Monospecific antisera to mouse immunoglobulins were prepared in rabbits and goats as described in detail elsewhere (11). Antisera were prepared against the papain Fc fragment of MOPC-31C (γG_1), Adj-PC5 (γG_{2a}), and MOPC-195 (γG_{2b}) myeloma protein isolated from serum as previously described (12). Antimouse γG_2 was made in rabbits with J-606 (kindly supplied by Dr. Howard Grey, National Jewish Hospital, Denver, Colo.) with a mixture of purified MOPC-31C, Adj-PC5, and MOPC-195 myeloma proteins. Rabbit anti-MPC-1 myeloma γA antisera were similarly prepared and absorbed with newborn mouse serum. Multiple controls for the monospecificity of each antisera were done, including immunofluorescent staining of myeloma tumors representative of the major immunoglobulin classes.

Affinity Chromatography. Antibody-agarose affinity chromatography was performed according to Cuatrecasas (13) by covalently linking at alkaline pH (10, footnote 2) the gammaglobulin fractions of the rabbit antisera to cyanogen-bromide-activated Sepharose 4B (CNBr-Sepharose from Pharmacia Fine Chemicals, Piscataway, N.J.). The γ -globulin fraction was obtained by sequential precipitation with 18%, 12%, and a second 12% Na_2SO_4 . Approximately 1×20 -cm columns were equilibrated in 0.5 M NaCl in PBS. Proteins not retained by the antibody-coated gel passed through the column in the buffer and were termed the fall-through preparations. The proteins bound by the gel were eluted with either 0.2 M glycine-HCl pH 2.8 or 4 M guanidine. These preparations, termed the acid washes, were immediately adjusted to pH 7.5 and extensively dialyzed against PBS.

²Murgita, R. A., and T. B. Tomasi, Jr. 1974. Suppression of the immune response by α -fetoprotein. II. The effect of α -fetoprotein on mixed lymphocyte reactivity and mitogen-induced lymphocyte transformation. *J. Exp. Med.* 141:440.

Polyacrylamide Gel Electrophoresis. Analytical disc electrophoresis was performed as described by Ornstein (14) and Davis (15) using a 5% concentration of monomer in the lower gel. The protein bands were stained with Coomassie blue (16) in 12% trichloroacetic acid. The gels were traced in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 610 $m\mu$. The scans were then carefully traced with a Keuffel and Esser 423 M compensating planimeter (Keuffel & Esser Co., Morristown, N. J.) and the relative amounts of protein in the AFP, albumin, and transferrin regions were calculated as a percent of the total area measured in arbitrary units. The position of the proteins in the polyacrylamide gels were determined by placing the unstained gels on agar contained in a petri dish with monospecific antisera in the lateral troughs. This technique has been previously described (17).

Preparative disc electrophoresis was carried out using a Shandon model SAE-2782 (Shandon Scientific Co., London). The buffer system used was identical to that used in the qualitative gels (pH 9.3 Tris-HCl, 0.06 M). A 10.0 cm 5% gel was used and the temperature maintained at 10°C with a circulating water bath. No stacking gel was used, instead the sample was applied to the top of the separating gel in stacking buffer (pH 6.9) made 30% in sucrose. Electrophoresis was carried out at 25-30 mA (350 V) with a pump rate of 25 ml/h and 2-ml fractions were collected over a period of 16 h. Electrophoresis with sodium dodecyl sulfate in 10% polyacrylamide gel was performed according to the method of Weber and Osborn (18).

Isoelectric Focusing. Sucrose density gradient isoelectric focusing was performed as previously described (12). An Ampholine column LKB 8102 (LKB Instruments, Inc., Rockville, Md.) with 2% carrier ampholytes of pH range 3-6 was used in most experiments. The isoelectrically separated proteins were eluted, optical density measured at 280 $m\mu$, and the pH was measured at 25°C to determine the isoelectric points of the separated protein components as defined by Ouchterlony gel diffusion with monospecific antisera.

Spleen Cell Cultures

PRIMARY IN VITRO ANTIBODY SYNTHESIS. Primary synthesis to sheep erythrocytes (SRBC) was measured using the method described by Marbrook (19). 20-30 $\times 10^6$ CBA spleen cells were cultured with 2 $\times 10^8$ SRBC in Medium 1066 (Grand Island Biochemical Co., Grand Island, N.Y.) containing 15% fetal calf serum and supplemented with glutamine (200 mM), streptomycin (10,000 γ /ml), and penicillin (10,000 U/ml). The cultures were incubated at 37°C for 5 days in an atmosphere of 10% CO₂ and 90% compressed air. The localized hemolysis-in-gel technique was used with the modifications of Wortis and Dresser (20) to detect and enumerate cellular synthesis of γ M antibody (direct plaque-forming cells [PFC]). The antibody responses are expressed as mean γ M PFC per culture \pm SE (standard error of the mean). Each preparation was cultured in triplicate. Preparations to be tested for immunosuppressive activity were added in 0.1 ml at the beginning of culture unless otherwise stated.

SECONDARY IN VITRO ANTIBODY SYNTHESIS. Secondary synthesis to SRBC was measured by a micro adaptation of the Mishell-Dutton system (21). Spleen cells from mice primed with 5 $\times 10^8$ SRBC 4 days previously were cultured for 5 days in flat-bottom Micro-Test tissue culture plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) without rocking and without daily supplemental feeding. Preliminary experiments showed that optimal conditions for cell density and antigen concentrations were 3.33 $\times 10^6$ cells/ml and 10 $\times 10^6$ SRBC respectively. Cultures were set up in a total vol of 0.3 ml in the following order: (a) 0.1 ml of antigen, (b) 0.1 ml of media, and (c) 1 $\times 10^6$ primed spleen cells in 0.1 ml. The media used was Hank's MEM made up with supplements in the following proportions: 90 ml H₂O, 10 ml Hank's MEM (10 \times), 1 ml nonessential amino acids (100 \times), 1 ml L-glutamine (200 mM), 0.5 ml penicillin and streptomycin (5,000 U/ml), 0.5 ml sodium pyruvate (20 \times), 10 ml fetal calf serum, 4 ml sodium bicarbonate (7.5%), and 1 ml Hepes buffer (1 M). Preparations to be tested for immunosuppressive activity were added in 0.02 ml at the beginning of culture unless otherwise stated. After the 5-day culture period in an atmosphere of 5% CO₂ and 95% compressed air, the cells were removed from the microplate wells by gentle aspiration with a Pasteur pipette. Usually 12, and sometimes 24, wells were pooled, washed and resuspended in 1.0 ml of M199 (GIBCO), and counted in a hemocytometer just before the direct and indirect PFC assays. Class-specific rabbit antimouse γ A, γ G, γ G_{2a}, γ G_{2b}, and γ G₃ immunoglobulins were titrated to determine the optimal dilutions for development of indirect PFC as previously described (11). The number of indirect plaques was calculated as the total plaques developed with facilitating antisera minus the plaques on identical

plates with added complement only (direct PFC). The numbers of PFC of each of the six mouse immunoglobulin classes are expressed as PFC/10⁶ spleen cells.

Results

Effect of MAF on the γ M PFC Response In Vitro. Cultures of CBA/J spleen cells were stimulated with SRBC in the presence of various dilutions of MAF. γ M PFC responses were determined after 5 days of incubation and compared to responses in control cultures (Table I). There was profound suppression of the PFC response when MAF was present in the cultures at a 1:12 final dilution and

TABLE I
Dose-Response Effect of MAF on the In Vitro Primary Antibody Response

Preparation	Mean % viability of recovered cells \pm SE	Mean γ M PFC \pm SE
Media	62 \pm 4	318 \pm 74
MAF (1/12)*	55 \pm 3	13 \pm 6
MAF (1/22)	58 \pm 1	44 \pm 17
MAF (1/110)	51 \pm 2	101 \pm 25
MAF (1/550)	55 \pm 2	188 \pm 12
MAF (1/1,100)	57 \pm 3	248 \pm 61

* Final dilution of MAF in the culture.

the suppressive effect gradually diminished as the MAF was further diluted. However, a significant degree of suppression was still evident at a 1/550 dilution of MAF. Viability, as measured by trypan blue dye exclusion of the recovered cells from each group of cultures containing the various dilutions of MAF did not differ from control cultures. Thus it was evident that MAF contained a substance(s) which was highly suppressive on primary in vitro antibody synthesis. Additional studies showed that the suppressive effect of MAF was not abolished by exhaustive dialysis or by absorption with SRBC; and, furthermore, it was clear from the viability studies that this activity was not due to a nonspecific cytotoxic effect on the cultured mouse lymphocytes.

Immunochemical Analysis of the Protein Components of MAF. An analysis of the protein content of MAF by immunoelectrophoresis and analytical polyacrylamide gel electrophoresis (Fig. 1 and 2) revealed three major protein components which were defined immunologically with monospecific antisera as albumin, AFP, and transferrin. The relative amounts of each of these proteins in MAF, as determined by polyacrylamide gel densitometry and planimetry, was 36%, 50%, and 14% respectively. Since the total protein concentration of pooled MAF was approximately 2 mg/ml (see methods section), it was estimated that the concentration of albumin in MAF was 0.72 mg/ml, AFP was 1.0 mg/ml and transferrin was 0.28 mg/ml. Isoelectric focusing of MAF in the pH range of 3-6 revealed two major peaks with pI's of 4.22 and 4.55 which were defined by

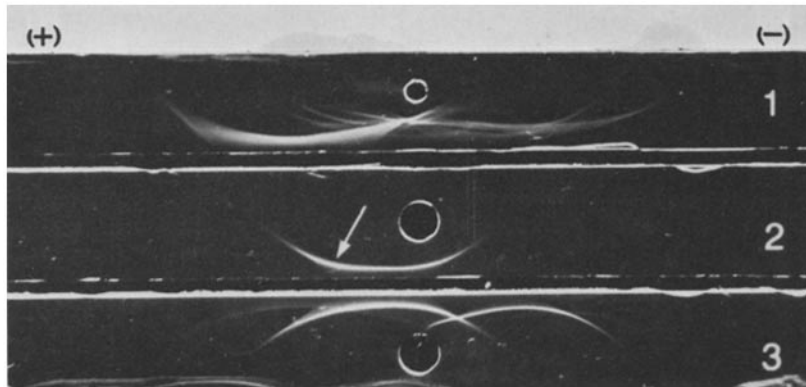


FIG. 1. Immunoelectrophoresis of (1) NMS, (2) pure AFP (indicated by the arrow) isolated by preparative disc electrophoresis, and (3) MAF. Upper trough contained rabbit anti-WMS. Lower trough contained rabbit anti-MAF.

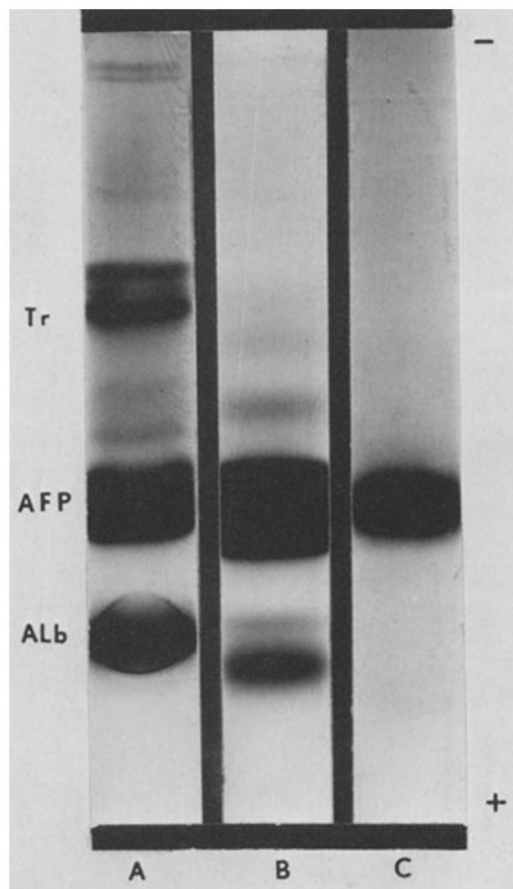


FIG. 2. Polyacrylamide gel electrophoresis of (A) MAF, (B) semi-purified AFP preparation from the fall-through of an anti-WMS affinity column, and (C) pure AFP isolated by preparative disc electrophoresis. The three major protein components of mouse amniotic fluid, albumin (Alb), α -fetoprotein (AFP), and transferrin (Tr) are indicated.

Ouchterlony analysis and analytical disc gels as α -fetoprotein and albumin respectively. Transferrin was detected in the fraction nearest the cathode with a pI of 6.0.

Separation of the Immunosuppressive Activity in MAF by Affinity Chromatography. In an attempt to determine whether the immunosuppressive activity was associated with one of the three major protein components of MAF, a series of antibody-agarose affinity columns were used to remove separately each of the three proteins. Selective removal of albumin, AFP, and transferrin was accomplished by passing MAF over antialbumin, anti- α -fetoprotein, and anti-transferrin columns. MAF was also passed over an anti-whole mouse serum (WMS) column in order to obtain an AFP preparation which had not been subjected to acid elution since this could have had a detrimental effect on the potential biological activity of the protein. All fall-throughs (nonretained protein) and acid washes (retained proteins) were dialyzed against PBS, pH 7.2, concentrated, and adjusted to similar protein concentrations on the basis of optical density at 280 m μ . The albumin and transferrin obtained from the acid washes of antialbumin and antitransferrin columns were not used because of insufficient recoveries. The results shown in Table II indicate that when 0.1 ml of each of the preparations from the affinity columns were added to spleen cell cultures with antigen, the suppressive activity resided in those preparations containing AFP and not in those which were depleted of AFP. These results strongly suggested that AFP was the immunosuppressive moiety in MAF. However, although the AFP preparation obtained by affinity chromatography appeared uncontaminated by Ouchterlony analysis and immunoelectrophoresis, analytical gels consistently revealed several components (see Fig. 2). Therefore, the possibility could not be excluded that one of these contaminating proteins also possessed immunosuppressive activity.

Suppression of the In Vitro Primary Antibody Response by Pure AFP. A pure preparation of AFP was obtained by first passing the MAF over an anti-WMS affinity column and then subjecting the semipurified AFP preparation to

TABLE II
Suppression of the In Vitro Primary Antibody Response by MAF Protein Components Fractionated by Affinity Chromatography

Preparation	γ M PFC/culture \pm SE
Media	687 \pm 167
AFP ^a	156 \pm 47
MAF-AFP ^b	702 \pm 97
AFP ^c	246 \pm 53
MAF-AFP ^d	692 \pm 163
MAF-Alb ^e	142 \pm 48
MAF-Tr ^f	115 \pm 62

Fall-through anti-WMS affinity column (a); acid wash anti-WMS affinity column (b); acid wash anti-AFP affinity column (c); fall-through anti-AFP affinity column (d); fall-through antialbumin affinity column (e); fall-through antitransferrin affinity column (f).

preparative polyacrylamide gel electrophoresis. The criteria of purity was a single band on the analytical disc gel (Fig. 2). The mol wt, as determined by sodium dodecyl sulfate acrylamide electrophoresis, was 77,500 unreduced and 72,400 when reduced with 1% (0.14 M) β -mercaptoethanol. A scan of the pure protein using the Cary Model 14 recording spectrophotometer revealed no absorption maxima in the 240–300 $m\mu$ region. An extension coefficient was calculated on the basis of dry weight to the $E_{280}^{1\%} = 2.0$, which suggests that AFP has a very low aromatic amino acid content.

The pure AFP was tested for immunosuppressive activity by adding it to spleen cell cultures at a final concentration of 200 $\mu\text{g/ml}$. As shown in Table III the preparation was highly suppressive compared with controls consisting of cultures to which just media, NMS, purified serum albumin, or transferrin were added in similar concentrations. The dose-response effect of the pure AFP shown in Table IV demonstrates a dilution effect which is strikingly similar to the dose-response effect for MAF (see Table I), assuming that the AFP concentration in undiluted MAF is 1 mg/ml .

Effect of Preincubation of Spleen Cells with MAF on the In Vitro Primary Antibody Responses. The duration of exposure of spleen cells to MAF in cultures without antigen necessary to achieve suppression of a subsequent primary immune response was determined. 30×10^6 spleen cells in a vol of 1.1 ml

TABLE III
Suppression of the In Vitro Primary Antibody Response by Purified Mouse α -Fetoprotein

Preparation	$\gamma\text{M PFC/culture} \pm \text{SE}$
Media	766 \pm 151
AFP (200 $\mu\text{g/ml}$)*	49 \pm 30
NMS (200 $\mu\text{g/ml}$)	635 \pm 85
Albumin (200 $\mu\text{g/ml}$)	491 \pm 60
Transferrin (200 $\mu\text{g/ml}$)	742 \pm 96

* Final concentration in the culture.

TABLE IV
Dose-Response Effect of Purified Mouse α -Fetoprotein on the In Vitro Primary Antibody Response

Preparation	$\gamma\text{M PFC/culture} \pm \text{SE}$
Media	423 \pm 12
AFP (200 $\mu\text{g/ml}$)*	29 \pm 4
AFP (100 $\mu\text{g/ml}$)	60 \pm 3
AFP (10 $\mu\text{g/ml}$)	78 \pm 11
AFP (1 $\mu\text{g/ml}$)	154 \pm 17
AFP (0.1 $\mu\text{g/ml}$)	323 \pm 23

* Final concentration of AFP in the culture.

were preincubated with 0.1 ml MAF at various intervals, excess MAF was removed by washing, and antigen was then added. The primary γ M PFC response was measured 5 days after addition of antigen. The percent viability of recovered spleen cells was equivalent in cultures preincubated in MAF or just media alone. Suppression was evident after 4 h of preincubation, but 8 h of exposure was required for a suppressive effect comparable to when MAF was present for the entire culture period (Table V).

Effect of Time of Addition of MAF on Primary Antibody Synthesis by Spleen Cell Cultures. MAF was added at various intervals after initiation of spleen cell cultures to determine whether the suppressive effect would occur only as an early

TABLE V
Effect of Preincubation of Spleen Cells with MAF on In Vitro Primary Antibody Synthesis

Preparation	γ M PFC/culture \pm SE
Controls (no preincubation)	394 \pm 27
MAF present throughout culture period*	31 \pm 10
Cells suspended in MAF and washed immediately*	341 \pm 12
Cells preincubated:*	
2 h in media	291 \pm 28
2 h in MAF	231 \pm 33
4 h in media	710 \pm 314
4 h in MAF	127 \pm 39
8 h in media	316 \pm 51
8 h in MAF	14 \pm 4
12 h in media	236 \pm 17
12 h in MAF	13 \pm 7
24 h in media	209 \pm 8
24 h in MAF	18 \pm 11

* 0.1 ml undiluted MAF was added to 30×10^6 spleen cells in a total vol of 1.2 ml.

event or at later stages of the immune response. As shown in Table VI, a sharp delineation occurred between 24 and 48 h; suppression was pronounced when added at 12 or 24 h after initiation, but no suppression occurred when MAF was added 48 h after initiation of culture. This is further evidence that inhibition is not the result of nonspecific cell death.

Dose-Response Effect of MAF on the In Vitro Secondary Antibody Response. Experiments were done to determine whether MAF and pure AFP could exert suppressive effects on the secondary antibody response and if so to compare the relative strength of suppression by a given amount of protein on the primary and secondary response. The PFC response in all six known classes of mouse immunoglobulins (Ig) were measured to determine whether there was any preferential or selective suppressive effects on the different Ig classes. The dose-response effect of MAF on the secondary response shown in Table VII is very

TABLE VI
*Effect of Time of Addition of MAF on Primary Antibody
 Synthesis by Spleen Cell Cultures*

Preparation	γ M PFC/culture \pm SE
Media	394 \pm 62
MAF added at zero time*	61 \pm 61
MAF added at 12 h*	39 \pm 10
MAF added at 24 h*	97 \pm 23
MAF added at 48 h*	385 \pm 95
MAF added at 72 h*	587 \pm 18
MAF added at 96 h*	497 \pm 230

* 0.1 ml of undiluted MAF was added to Marbrook cultures at times shown. All cultures carried out for 5 days.

TABLE VII
*Dose-Response Effect of MAF Secondary Antibody Response**

Preparation	PFC/10 ⁶ spleen cells					
	γ M	γ A	γ G ₁	γ G _{2a}	γ G _{2b}	γ G ₃
Control	83	116	433	125	150	416
MAF (1/12)‡	0	0	0	0	0	0
MAF (1/22)	16	0	0	0	0	0
MAF (1/110)	28	20	50	1	3	8
MAF (1/550)	90	100	140	60	110	160

* CBA spleen cells from mice primed with 10⁷ SRBC 4 days previously were cultured at 1 \times 10⁶/0.3 ml for 5 days with 10⁷ SRBC.

‡ Final dilution of MAF in culture.

similar to results obtained on the primary response (Table I). A partial suppression was obtained with a 1:110 dilution of MAF and cultures treated with a 1:550 dilution are approaching control values in some Ig classes. There is a suggestion that γ M PFC levels are less suppressed at the higher dilutions of MAF than are the other Ig classes.

Suppression of the In Vitro Secondary Antibody Response by MAF Protein Components Fractionated by Affinity Chromatography. MAF was selectively depleted of albumin, transferrin, and AFP by antibody-agarose affinity columns as described in experiments on the primary antibody response (Table II). It was shown that the inhibitory effect of MAF on the secondary response was abolished by the selective removal of AFP, whereas fractions from the affinity columns that are rich in AFP are strongly immunosuppressive (Table VIII). These experiments again demonstrate that most or all of the immunosuppressive activity in MAF is associated with the AFP component and not with the other proteins present in the fluid.

Suppression of the In Vitro Secondary Antibody Response by Pure AFP. Pure

AFP obtained by preparative disc electrophoresis (Fig. 2) was added to CBA spleen cells primed with SRBC in a final concentration of 200 $\mu\text{g/ml}$. The results shown in Table IX clearly demonstrate that AFP but not similar amounts of NMS, albumin, or transferrin can completely suppress the secondary antibody response. Shown in Table X is the dose-response effect of pure AFP on the secondary antibody response. A clear distinction can be seen between the effective doses needed for suppression of secondary γM compared to the other Ig classes. Secondary γM PFC were only partially suppressed at 100 $\mu\text{g/ml}$ of AFP whereas the other Ig classes are almost completely suppressed at this concentration. There was incomplete suppression of secondary γM PFC at 10 $\mu\text{g/ml}$ of AFP and no suppression with 1 $\mu\text{g/ml}$. These concentrations gave a much higher degree of suppression of primary γM PFC (see Table IV). Therefore, secondary γM PFC appear to be more resistant to suppression by AFP than are primary γM PFC. The secondary γA and γG -related PFC responses were more sensitive than the primary λM PFC response to suppression by AFP.

TABLE VIII
Suppression of In Vitro Secondary Antibody Synthesis by MAF Protein Components Fractionated by Affinity Chromatography

Preparation	PFC/ 10^6 spleen cells					
	γM	γA	γG_1	γG_{2a}	γG_{2b}	γG_3
Media	394	326	608	214	326	946
AFP ^a	82	18	5	0	0	46
MAF-AFP ^b	364	293	546	192	293	850
AFP ^c	122	0	0	0	0	19
MAF-AFP ^d	490	480	823	568	470	882

* CBA spleen cells from mice primed with 10^7 SRBC 4 days previously were cultured at 1×10^6 cells/0.3 ml for 5 days with 10^7 SRBC.

Fall-through anti-WMS affinity column (a); Acid wash anti-WMS affinity column (b); acid wash anti-AFP affinity column (c); fall-through anti-AFP affinity column (d).

TABLE IX
Suppression of the In Vitro Secondary Antibody Response by Purified Mouse AFP

Preparation	PFC/ 10^6 spleen cells*					
	γM	γA	γG_1	γG_{2a}	γG_{2b}	γG_3
Media	120	225	340	245	260	400
AFP (200 $\mu\text{g/ml}$) [‡]	2	0	8	0	2	0
NMS (200 $\mu\text{g/ml}$)	120	190	275	165	185	350
Albumin (200 $\mu\text{g/ml}$)	80	175	200	165	190	330
Transferrin (200 $\mu\text{g/ml}$)	135	220	365	230	215	420

* CBA spleen cells from mice primed with 10^7 SRBC 4 days previously were cultured at 1×10^6 cells/0.3 ml for 5 days with 10^7 SRBC.

[‡] Final concentration in the culture.

TABLE X
Dose-Response Effect of Purified Mouse AFP on In Vitro Secondary Antibody Response

Preparation	PFC/10 ⁶ spleen cells*					
	γ M	γ A	γ G ₁	γ G _{2a}	γ G _{2b}	γ G ₃
Control	220	280	460	320	270	560
AFP (100 μ g/ml)	87	0	22	0	0	54
AFP (10 μ g/ml)	161	26	56	0	0	81
AFP (1 μ g/ml)	200	41	57	7	21	86
AFP (0.1 μ g/ml)	177	51	101	18	31	131
AFP (0.01 μ g/ml)	227	118	181	132	116	367

* CBA spleen cells from mice primed with 10⁷ SRBC 4 days previously were cultured at 1 \times 10⁶ cells/0.3 ml with 10⁷ SRBC.

Discussion

The major purpose of this study was to determine whether AFP, both as it exists in amniotic fluid and in the isolated form, has immunosuppressive activity. Before discussing the results which directly focus on this problem, two observations in this study unrelated to immunosuppression by AFP bear mentioning. To our knowledge this is the first study in which indirect plaques were quantitated using an anti- γ G₃-specific antiserum. It is somewhat surprising, in view of the low serum concentrations of γ G₃ (0.1–0.2 mg/ml in normal mouse serum [22]), that this class is a major component of the antibody response to SRBC. In fact, an average of the control plaques in the various classes obtained in all of our experiments (see Tables VII, VIII, IX, and X) shows that γ G₃ is the most prevalent class representing approximately 30% of the total plaques developed from in vivo primed spleen cells cultured 5 days with antigen in vitro. The relative numbers of splenic γ G₃ PFC were also unexpectedly high in the primary in vivo response to SRBC (unpublished observation). This observation suggests the possibility that the synthetic rate of γ G₃ may be high and that the low serum levels could result from rapid catabolism. The second point concerns the very low extinction coefficient for AFP ($E_{280}^{1\%} = 2.0$). We have been unable to find an extinction coefficient for AFP in the literature and in those studies where the problem of protein quantitation is discussed, frequently serum albumin is used as a standard (23). This could potentially introduce serious errors into the quantitation of AFP by any of the currently employed techniques.

In this investigation we have demonstrated that MAF contains a nondialyzable substance which is capable of exerting a noncytotoxic immunosuppressive effect on primary and secondary antibody production in vitro. Polyacrylamide gel analysis showed that dialyzed MAF is composed of approximately 14% transferrin, 36% albumin, and 50% AFP. The selective removal of each of these proteins from MAF by affinity chromatography showed that suppression of antibody synthesis was retained when albumin or transferrin was removed but that there was no suppressive effect in MAF which had been selectively depleted of AFP. The conclusion implied from these experiments that AFP was the immunosup-

pressive substance in MAF was verified by the subsequent demonstration that pure AFP suppressed *in vitro* antibody synthesis whereas equivalent amounts of normal mouse serum, transferrin, or albumin did not. Dose-response studies showed that the suppressive effect of AFP in the isolated form was equivalent to the suppressive effect of comparable amounts of AFP in MAF. These results suggest that AFP was responsible for essentially all of the suppressive activity of amniotic fluid. Suppression with purified AFP occurs at concentrations of 1 $\mu\text{g}/\text{ml}$ which is approximately 1,000-fold less than the levels present in mouse serum at birth. Thus, if the *in vitro* results are applicable *in vivo*, the concentration of AFP in the newborn is sufficient to provide significant immunosuppression at birth and the disappearance of AFP may be a factor in the gradual maturation of antibody responsiveness which occurs during the first few weeks of life (24, 25). Studies from this laboratory (10) involving the parenteral administration of MAF from birth to young adulthood suggest that AFP is capable of suppressing antibody synthesis *in vivo*.

The degree of suppression was dependent on the time at which AFP was added to the cultures; MAF added to antigen-stimulated cultures up to 24 h after the initiation of cultures was immunosuppressive whereas similar additions of MAF at 48, 72, and 96 h after initiation of cultures did not suppress. In fact, a slight augmentation of the PFC response was observed when MAF was added at 72 and 96 h after initiation of the cultures. These experiments also demonstrate the lack of a nonspecific cytotoxic effect of AFP or other constituents of MAF on the cells involved in *in vitro* antibody synthesis. The synthetic capacity of the antibody-secreting (B) cell is apparently not affected by AFP since the addition to cultures late in the antibody response did not suppress. However, this does not rule out the possibility that AFP is inhibiting an earlier event in the B-cell contribution to antibody synthesis. In fact, one or more of the early phases in antibody formation such as antigen processing by macrophages, B-T cell interactions, or clonal proliferation of antigen sensitive cells could be susceptible to the AFP-induced suppression. Preincubation experiments (Table V) suggested that AFP must interact with one or more of the cells involved in antibody production for approximately 8 h for effective suppression. Briefer exposures resulted in little or no suppression which suggests that the mechanism(s) involved in AFP-induced suppression are more complex than simply blocking Ig receptors and prevention of antigenic stimulation.

Although the exact mechanism(s) of immunosuppression induced by AFP cannot be defined from the available data, an interesting speculation which merits consideration is that a helper population of thymus-derived T cells for antibody synthesis may be inhibited. There is some evidence to suggest that the cellular events leading to the synthesis of γA and γG antibodies to some antigens are more dependent on thymus-derived cells than are the cellular systems which lead to the synthesis of γM antibodies (26-29). Dose-response experiments using pure AFP (Table X) demonstrated that secondary γA and γG PFC responses were suppressed by greater than a 100-fold lower concentration of AFP than were secondary γM PFC. A 10-fold higher concentration of AFP was required to suppress the secondary γM compared with the primary γM PFC response (Table IV). This difference in sensitivity to inhibition between direct and indirect PFC

responses is consistent with our recent findings (10) that animals injected with MAF from birth through young adulthood had greater suppression of γ A and γ G responses to a primary injection of SRBC compared with the γ M response which was considerably less affected. Our observations therefore are consistent with the concept that AFP is suppressing thymus-derived T cells which are involved in the mechanism of antibody synthesis. Data in a subsequent paper will show that AFP also suppressed certain T-cell-dependent functions such as allogeneic and mitogen-induced lymphocyte transformation.² In this regard, it is also interesting to note that in one report (30) all patients studied with ataxia telangiectasia, a disease involving an abnormal thymus and an immune deficiency, had raised serum AFP concentrations. However, despite the suggestion that the T cell may be involved, it has not been excluded that macrophages and/or B cells may participate in the suppression rather than or in addition to T cells. It is also conceivable that AFP stimulates a suppressor cell rather than inhibits a helper cell. Obviously, further studies are needed on the cellular site(s) of action of AFP using both thymus-dependent and independent antigens.

Substances present in normal (31, 32) and pregnant (33) sera have been shown to nonspecifically inhibit the immune response. A factor in NMS has been reported (34, 35) which is immunosuppressive for T-dependent but not T-independent antigens. The level of this substance increases substantially following immunization. The relationship of this factor to the α -regulatory globulin described by Mowbray (36), discussed briefly below, as well as the various suppressor substances reported to be present in pathological conditions (37) is unknown. It has not been excluded that one or more of the factors mentioned above are AFP. An α -globulin fraction from human, bovine, and rat serum has immunosuppressive activity both in vivo and in vitro (38, 39). α -globulin-rich fractions from normal human plasma suppresses primary and secondary antibody responses in vivo (40), and it has been suggested that the active moiety is an immunoregulatory peptide which is noncovalently bound to proteins with an electrophoretic mobility in the α -region (41). Amniotic fluid contains components which are derived from the maternal serum and, in fact, it has been recently reported (42) that the adult-type plasma proteins found in amniotic fluid are predominantly of maternal origin. Therefore, it must be considered that at least part of the immunosuppressive activity of amniotic fluid may be due to a maternal serum factor. However, as already discussed, essentially all of the suppressive activity of MAF can be explained by its AFP content. Another possibility in analogy with immunoregulatory α -globulin is that the suppressive moiety of AFP is a peptide bound to this glycoprotein. Although it cannot be completely excluded, this possibility is made less likely by the fact that one of the AFP preparations from an affinity column was subjected to low pH (2.8) and high salt (0.5 M NaCl) followed by extensive dialysis without loss of suppressive activity. These conditions have been shown to favor elution of the suppressive peptide from its carrier α -globulin (44). Therefore, we believe that AFP is probably not serving as a carrier protein for an immunosuppressive peptide and that the suppressive activity is an intrinsic property of the AFP molecule.

A number of polypeptide hormones have been reported to be immunosuppres-

sive (43). In our experiments there was no difference between native MAF and MAF which had been exhaustively dialyzed and as mentioned above, affinity chromatography at low pH and high salt followed by dialysis might be expected to remove a noncovalently bound hormone. However, these findings do not completely exclude the possibility of a suppressive hormone tightly bound to AFP. AFP has recently been shown to have estrophilic activity (44), although it is reported that estradiol does not directly influence the immune response (45).

Although a considerable amount of information is now available concerning the isolation, pathological distribution, and diagnostic significance of AFP, very little is known about its biological function(s). No physiological explanation for its presence during embryonic development has yet been determined. However, it may not be fortuitous that a state of immunological hyporeactivity is often a common element associated with conditions where AFP is prevalent. The demonstration in this and a subsequent study² that AFP is a potent inhibitor of the immune response leads us to hypothesize that this protein has as one of its functions immunoregulatory activity. We speculate that AFP may have significance as an immunoregulatory protein in three areas. Firstly, there is some evidence that the maternal immune response is depressed at certain stages of pregnancy (46, 47) and the elevated levels of AFP in maternal sera could be one of the responsible factors. Maternal immunosuppression in turn could contribute to the delayed rejection of the developing histoincompatible fetus. It would be interesting in this regard to know whether higher concentrations of AFP exist locally in the uterine tissues surrounding the placenta than in the serum. Secondly, numerous studies have demonstrated a reduced capacity of the fetus and newborn to respond to antigenic stimuli (48). It should be emphasized that this is not complete unresponsiveness and when the fetus does respond to experimental inoculation of antigens or congenital infections there is usually a high ratio of γ M to γ G production (49). This is consistent with the significantly smaller suppressive effect of AFP on γ M production compared with other classes. Two reasons come to mind why there might be a survival advantage to fetal immunosuppression. Potentially antigenic substances of benefit to the fetus pass from mother across the placenta and were they to provoke an immune response in the fetus they would be rapidly inactivated. For example, Warner et al. (50), in discussing the acquisition by the fetus of foreign allotypes of immunoglobulin present in the mother (either across the placenta or during nursing), point out that the survival advantage of maternal antibodies would be seriously compromised "unless the offspring were unable to respond to (were tolerant of) the incompatible maternal globulins since an antibody response to maternal immunoglobulins would lead very rapidly to their elimination." Another area where fetal suppression may be important and perhaps a biological necessity is in preventing reactivity against self-antigens. Cells with the capacity for self-recognition have been shown to exist in both the adult (51, 52) and fetus (53). In fetal and early newborn life, AFP could be important in suppressing autoimmunity while later in development, after the disappearance of AFP, other factors including the postulated blocking antibodies (54) become of prime importance. Finally, AFP may have biological significance in later life when it appears as a pathological marker of embryo-specific differentiation in certain tissues undergo-

ing rapid regeneration or malignant transformation. AFP could, for example, be involved in the immunosuppression reported to be associated with liver disease (55, 57) and certain tumors (57). If so, then it may be an important factor in the failure to eliminate viruses (such as those presumed to cause hepatitis or tumors) and malignant cells.

Summary

Mouse amniotic fluid was shown to contain a noncytotoxic inhibitor of primary γ M and secondary γ M, γ A, and γ G subclass splenic plaque forming cells in vitro to SRBC. The suppressive effect was not abolished by exhaustive dialysis or by absorption of mouse amniotic fluid (MAF) with SRBC. Polyacrylamide gel analysis showed that dialyzed MAF was composed of three major protein components, transferrin, albumin, and alpha-fetoprotein (AFP). The selective removal of each of these proteins from MAF by affinity chromatography suggested that AFP was the immunosuppressive substance in MAF. This conclusion was verified by the demonstration that pure AFP suppressed in vitro antibody synthesis in microgram quantities whereas equivalent amounts of normal mouse serum, transferrin, or albumin did not. Dose-response studies showed that the effect of AFP in the isolated form was equivalent to the suppressive effect of comparable amounts of AFP in MAF. γ A and γ G plaque-forming cell (PFC) responses were suppressed by a significantly lower concentration of AFP than was the γ M PFC response. The degree of suppression was dependent on the time at which AFP was added to the cultures; MAF added to antigen-stimulated cultures up to 24 h after initiation of cultures was immunosuppressive whereas similar additions of MAF at 48 h after initiation or later did not suppress. The duration of exposure of spleen cells to MAF in cultures without antigen necessary to achieve suppression of a subsequent primary immune response was determined to be approximately 8 h.

The results suggest that AFP may have an immunoregulatory function. This has potentially important implications in the maternal-fetal relationship, the immune capabilities of the fetus and newborn, and in certain malignant and nonmalignant diseases in which AFP is elevated.

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