CELLULAR AND GENETIC RESTRICTIONS IN THE IMMUNOREGULATORY ACTIVITY OF ALPHA-FETOPROTEIN

I. Selective Inhibition of Anti-Ia-Associated Proliferative Reactions

AMMON B. PECK, ROBERT A. MURGITA, and HANS WIGZELL
(From the Department of Immunology, Biomedical Center, Uppsala University, Uppsala, Sweden)

The mammalian maternal fetal relationship appears to contradict basic principles of transplantation immunology in that the implanted fetus represents a histoincompatible allograft exposed to, but well tolerated by, a potentially harmful maternal-immune system (1). Available information suggests that exemption of the fetus from premature-immune attack depends in part on the cumulative action of specific and nonspecific immunoregulatory factors (2–6). Recent studies (7–9) indicate that one of the contributing regulatory factors may be alpha-fetoprotein (AFP), a major alpha-globulin component of fetal and newborn sera. The implication that AFP may have important immunoregulatory function in vivo is based on its demonstrated ability to selectively suppress human and murine immune responses in vitro (7–14), and is further supported by the frequent association of elevated levels of AFP with the immunological hyporeactivity present in some normal (15, 16) and pathological (17–22) conditions.

Evidence from both the human and murine systems indicate that the effects of AFP in vitro are restricted to certain functions of thymus-derived (T) lymphocytes, with no apparent direct effects on B cells (10, 23). For example, in the mouse, T-cell-dependent antibody synthesis, T-cell mitogenic responsiveness, and T-cell-mediated allogeneic reactivity are suppressed by AFP (8, 9), whereas polyclonal B-cell activation and T-cell-independent antibody responses are not affected (10). Recently it has been reported (24) that murine AFP does not inhibit the in vitro induction of antigen-specific helper T cells directly, but instead, induces the formation of suppressor T cells which can effectively interfere with the co-operative interaction between B and T cells, yet have no influence on B-cell responses to T-independent antigens. Similarly, in the human system, AFP, at concentrations which significantly inhibit alloantigen-induced and T-mitogen-induced lymphocyte transformation, fails to affect the mitogenic responses to the human B-cell mitogen Protein A of Staphylococcus aureus.

Controversy concerning the immunosuppressive activity of AFP on T-dependent responses has surfaced after a number of reports (25–29) indicating that AFP exhibits

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 Abbreviations used in this paper: AFP, alpha-fetoprotein; CML, cell-mediated lympholysis; FBS, fetal bovine serum; MHC, major histocompatibility complex; MLC, mixed leukocyte culture; PBS, phosphate-buffered saline; SD, serologically-defined (in reference to the K and D regions of MHC); [3H]Tdr, tritiated-thymidine.

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only weak or variable suppressive activity on in vitro T-cell responses. Furthermore, Parmely and Thompson (30) have suggested that the immunosuppressive activity associated with AFP is not due to AFP per se but to contaminants in the preparation. Similarly, it has also been claimed (31) that AFP must first bind estrogen before obtaining the capacity to elicit suppressive activity.

We have undertaken a systematic study of the possible regulatory influence of AFP on the proliferative and effector phases of the T-cell-mediated-immune reaction utilizing the in vitro mixed leukocyte culture (MLC) and cell-mediated lympholysis (CML) assays. In this first report, we present evidence that highly purified fetal-derived AFP can exert differential effects on both the primary and secondary MLC responses of purified T-cell populations ranging from strong inhibition to occasional enhancement. Data shown here indicate that proliferative responses of T lymphocytes towards major histocompatibility complex (MHC) I region determinants are markedly suppressed by AFP, whereas responses against MHC serologically-defined (SD) region alloantigens are generally unaffected. In addition, AFP is shown to exhibit a differential effect on MLC activity induced by non-MHC alloantigens, with suppression occurring only in those antigenic systems whose recognition is dependent on a concomitant recognition of I region gene products. The regulatory effects of AFP on T-cell proliferation noted in this study, therefore, appear to be restricted by the genetic relationship between responding and stimulating cells and/or a differential activity on subclasses of T lymphocytes.

Materials and Methods

**Mice.** Inbred strains of mice used in this study and maintained in our laboratory are A.CA, A.TH, A.TL, B10.A, B10.AKM, B10.BR, B10.D2/n, B10.G, B10.HTT, B10.M, B10.S, B10.S(7R), B10.T(6R), CBA/H, CBA-H-2<sup>b</sup>, CBA/J, C3H/HeJ, C3H/Tif, C57BL/6Jom, DBA/2, and the hybrid (A.SW x CBA/H)F<sub>1</sub>. The congenic resistant partner strains of C57BL/10Sn, were established from breeding pairs provided by Dr. F. H. Bach (Immunobiology Research Center, University of Wisconsin, Madison, Wis.). Strains A.TH and A.TL were provided by Dr. P. Petersson (Medical Chemistry, Biomedical Center, Uppsala, Sweden) and the mutant strain, CBA-H-2<sup>b</sup>, by Dr. L. C. Andersson (4th Department of Pathology, University of Helsinki, Finland). Both male and female mice were utilized and ranged in age from 4 to 24 wk.

Amniotic fluid was obtained from pregnant female outbred mice which were purchased from Anticimex AB (Stockholm, Sweden).

**Culture Medium.** The serum-free medium as described by Click et al. (32) and Heber-Katz et al. (33) was supplemented with 0.5% normal mouse serum according to the protocol of Peck and Click (34). The mouse serum was homologous with the responding strain and pooled from a minimum of three animals.

**AFP.** AFP was purified from amniotic fluid by affinity chromatography and preparative gel electrophoresis according to procedures described elsewhere (8).

**MLC Combinations.** Primary MLCs were performed in microtiter plates (no. M 24A, Cooke Microtiter System, Novakemi AB, Farsta, Sweden) or in tissue culture flasks (no. 3013, Falcon Plastics, Div. of Bioquest, Oxnard, Calif.) according to procedures described elsewhere (35). Flask cultures were employed to generate alloantigen-activated T-cell blasts used in the secondary MLCs. Each flask contained 20–25 × 10<sup>6</sup> responding T lymphocytes cultured together with an equal number of X-irradiated (2,000 rads) stimulating cells in 12 ml of medium.

All secondary MLCs were performed in microtiter plate cultures as previously described by Peck et al. (36). Cultures consisted of 0.05 × 10<sup>6</sup> or 0.075 × 10<sup>6</sup> responding T-cell blasts cultured together with 0.5 × 10<sup>6</sup> X-irradiated stimulating spleen cells.

At appropriate times of primary or secondary culture, as indicated in the text and figure legends, 0.8 μCi tritiated-thymidine ([<sup>3</sup>H]TdR) (Amersham, London, England) in a vol of 0.02 ml
was added to each well for 4 h. Cells were filtered through glass-fiber filters with a multiple-sample harvester (Skatron, Flow Laboratories, Stockholm, Sweden) and total \(^{3}H\)TdtR incorporation was determined by liquid scintillation procedures. Data are expressed in counts per minute of the mean of either duplicate or triplicate cultures. Standard deviations of the means are generally included.

**Preparation of Splenic T Lymphocytes.** In the majority of cases, the responding cells in primary MLC were prepared by passage of whole spleen cell suspensions whose contaminating erythrocytes had been lysed in a 0.84% NH\(_4\)Cl treatment through Ig-anti-Ig glass bead columns, according to the protocol of Wigzell et al. (37). This procedure removes Ig-bearing B cells. After passage through the columns, the nonadherent cells are washed twice in phosphate-buffered saline (PBS) and resuspended to a density of 100 × 10\(^6\) cells per ml. The resulting cell population can be shown to be >98% theta antigen-bearing T cells and <0.5% Ig-bearing B cells.

**Separation of MLC-Reactive T-Blast Cells from Primary Cultures.** The MLC-reactive T lymphocytes were separated as T-blast cells from the nonresponsive populations using 1 g velocity sedimentation procedures as described by Miller and Phillips (38) and Andersson and Häyry (39). Primary MLC cells were collected from the culture flasks after maximum \(^{3}H\)TdtR incorporation, resuspended in PBS supplemented with 4% fetal bovine serum (FBS), then layered over a linear 15-30% FBS gradient. The cells were permitted to fall through the gradient for 2-4 h. Fractions of 12 ml each were collected and microscopically examined for numbers of blast and nonblast cells. Only those fractions containing >95% blast cells were kept. After two to four washes with PBS, the resulting pooled T-lymphocyte blast cell suspension was dispensed to microtiter plate wells and restimulated with appropriate stimulating cell populations.

**LY Antigen Phenotype Determinations.** Complement-dependent cytotoxic reactions were carried out according to the protocol of Shiku et al. (40). Ly antisera used in this study were generously provided by Dr. K. I. Welsh (McIndoe Research Unit, Queen Victoria Hospital, E. Grinstead, England).

**Results**

In the mouse, lymphocyte proliferation as measured by MLC can be induced by genetic differences associated with the MHC (reviewed in references 41 and 42) or with loci segregating independently of the MHC (43-46) (referred to as non-MHC loci in the present report). The various regions and subregions comprising the MHC contain genes which code for at least two distinct classes of membrane-bound MLC-activating products. The \(K\) and \(D\) regions encode for the classical SD determinants, whereas the \(I\) region genes encode for the \(Ia\) antigens. Both the \(SD\) and \(Ia\) molecules, besides initiating lymphocyte proliferation, appear to function as target antigens for cytotoxic T lymphocytes in CML (47, 48). Two MLC-stimulating, non-MHC antigenic systems of interest for the present investigation are the \(Mls\) locus (43, 44) and an as yet undefined system which became readily distinguishable from the \(Mls\) locus through lymphocyte-typing analysis (46). The non-MHC alloantigens apparently cannot function as target antigens for cytotoxic T lymphocytes. These four classes of determinants, together with the presently known requirements involved in their recognition, are listed in Table I. Note that \(Mls\) locus products induce lymphocyte proliferation only if there is concomitant recognition of MHC \(I\) region gene products (46). In the results to follow, the differential activity of AFP on responses directed towards these four classes of antigens further delineates the different nature of each system.

**Effects of AFP on Primary and Secondary MLC Responses against MHC-Associated Alloantigens.** The effects of AFP on primary and secondary MLC reactivity induced by MHC-associated genetic differences were investigated using congenic resistant partner strains of both the C57BL/10 and A/Sn mouse.
TABLE I
A Listing of MLC-Stimulating Alloantigenic Systems

| MLC-Stimulating alloantigen* | Required recognition in MLC | Antigen target in CML |
|-----------------------------|-----------------------------|-----------------------|
|                             | MHC-Ia | MHC-SD | Mls locus | Non-MHC |           |
| MHC-Ia                     | +      | -      | -         | -       | +         |
| MHC-SD                     | -      | +      | -         | -       | +         |
| Mls Locus                  | +      | -/?    | +         | -       | -         |
| Undefined non-MHC          | -      | -      | -         | +       | -         |

* Reviewed in Klein (41) and Peck et al. (46).

**Fig. 1.** Effects of AFP on the primary MLC responses of splenic lymphocytes against MHC-associated histocompatibility alloantigens. The kinetics of primary MLC activation for B10.BR spleen cells and purified splenic T lymphocytes responding against X-irradiated B10.HTT spleen cells were carried out in the absence (●) or presence (○) of AFP (150 μg/ml). Syngeneic stimulated control cultures in the absence (■) and presence (□) of AFP are also shown. [3H]TdR (0.8 μCi) was added to each culture 4 h before harvesting the cells at the times indicated.

Thin summer. First, the kinetics of primary MLC activation in the presence and absence of AFP were examined for both B10.BR whole splenic leukocytes and purified splenic T lymphocytes responding against B10.HTT, a representative anti-MHC reaction. As shown in Fig. 1, strong primary MLC reactions are exhibited by both responding populations in the absence of AFP. In the presence of AFP, however, a suppressed [3H]TdR incorporation rate is observed for both splenic leukocytes and purified T cells throughout the time-course
TABLE II
The Effects of AFP on the In Vitro Stimulation of Splenic T Lymphocytes against MHC Alloantigens

| Group | Responding T lymphocytes* | Stimulating cells | MHC Derivation† | [3H]Tdr Incorporation (cpm ± SD) | NMS Control§ + AFP§ |
|-------|---------------------------|-------------------|-----------------|-------------------------------|---------------------|
| A     | B10.D2/n                  | B10.D2/n          | K I-A I-B I-C S G D | 1,028 ± 54 | 3,150 ± 59 |
|       |                           | B10.BR            | k k k k k k k k   | 41,466 ± 203 | 25,455 ± 695 |
| B10.S(7R) | B10.S(7R)            | s k s s s s d     | 9,899 ± 1,365 | 12,813 ± 2,636 |
|       |                           | B10.BR            | k k k k k k k k   | 38,268 ± 3,322 | 27,776 ± 2,047 |
|       |                           | A.SW × CBA/HF,   | 2,128 ± 350 | 4,422 ± 1,159 |
|       |                           | A.CA              | f f f f f f f f   | 22,436 ± 149 | 6,660 ± 1,205 |
| B     | B10.S(7R) anti-B10.BR   | B10.S(7R)         | s k s s s s d     | 4,608 ± 734 | 1,670 ± 611 |
|       |                           | B10.BR            | k k k k k k k k   | 112,390 ± 177 | 70,503 ± 9,002 |
| B10.A anti-B10.S(7R) | B10.A             | s s s s s s s s   | 10,856 ± 1,312 | 13,929 ± 6,012 |
|       |                           | B10.S (7R)        | k k k d d d d d   | 53,300 ± 2,339 | 25,617 ± 1,208 |
|       |                           | B19.S             | s s s s s s s s   | 99,005 ± 4,242 | 44,625 ± 2,131 |
|       |                           | B10.HTT           | s s s k k k k d   | 71,037 ± 878 | 34,430 ± 473 |

* T lymphocytes purified by passage through Ig-anti-Ig columns. Blast cells isolated on 1 g velocity sedimentation gradients after peak primary MLC activation.
† Underlined letters designate MHC regions against whose gene products the responding T-cell populations are sensitized.
§ AFP added at initiation of secondary MLC to a final concentration of 150 µg/ml. Control cultures received an equivalent concentration of normal mouse serum (NMS).

Effects of AFP on Secondary MLC Reactions Induced by Isolated I or SD.
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**Table III**

**Differential Effects of AFP on the In Vitro Stimulation of T Lymphocytes Sensitized against Either MHC I or SD Region Alloantigens**

| Responding T lymphocytes* | Primary and secondary MLC stimulating cells | MHC Derivation† | [3H]TdR Incorporation (cpm ± SD) +AFP |
|---------------------------|--------------------------------|-----------------|-----------------------------------|
|                           | K I-A I-B I-C S G D           |                 |                                   |
| A.TL anti-B10.S(7R)       | A.TL s k k k k k d            | 11,940-625      | 7,803-485                         |
| B10.S(7R)                 | s a a a a a a d              | 56,332-475      | 29,087-302                        |
| B10.S(7R)                 | s s s s s s s d              | 1,017-87        | 994-108                           |
| B10.S                     | s a a a a a a a             | 10,066-60       | 13,258-348                        |
| CBA-H-2*                  | CBA-H-2* k k k k k k         | 3,422-254       | 3,906-776                         |
| CBA/H                     | k k k k k k k k             | 41,440-546      | 37,156-213                        |
| B10.AKM anti-B10.BR       | B10.AKM k k k k k k q        | 4,864-1,031     | 5,772-120                         |
| B10.BR                    | k k k k k k k k             | 24,784-1,719    | 23,265-706                        |

* Blast cells isolated on 1 g velocity sedimentation gradients after peak primary MLC activation.
† Underlined letters designate MHC region(s) against whose gene products the responding T-cell population is sensitized in the primary MLC.
‡ AFP added at initiation of secondary MLC to a final concentration of 150 μg/ml. Control cultures received an equivalent concentration of normal mouse serum (NMS).

**Region Determinants of the MHC.** MHC recombinant strains possessing limited genetic differences permit the study of the effects of AFP on MLC reactions induced by individual region or subregions of the MHC. Results presented in Table III show the effects of AFP on selected primary and secondary MLC reactions directed specifically against I or SD region incompatibilities. AFP clearly suppresses the reaction directed towards I region differences, as defined by the combination A.TL-B10.S(7R), but has little or no effect on the proliferative responses directed towards SD region histoincompatibilities, as defined by the combinations B10.S(7R)-B10.S, CBA-H-2*-CBA/H, and B10.AKM-B10.BR. Although strains A.TL and B10.S(7R) differ genetically not only at the MHC I region but also at both the MHC S and G regions as well as in the background, the A.TL anti-B10.S(7R) blast cells can be shown in primed-lymphocyte typing analysis to be restimulated only by I region-associated gene products (A. B. Peck, unpublished data). This rules out the involvement of these additional non-I region genetic differences and suggests that AFP-induced suppression in MHC histoincompatible strain combinations must be due, at least in part, to suppression of cellular proliferation against I region alloantigens. It should also be noted that the addition of AFP to such secondary reactions of blast lymphocytes generally (but not absolutely) results in rapid suppression of the ongoing proliferation (e.g., the response of the A.TL anti-B10.S(7R) blast cells against syngeneic A.TL cells). It appears, therefore, that the residual reactivity of the activated blast cells against the I region gene products is also sensitive to inhibition by AFP. In contrast, primary and secondary proliferative responses directed towards isolated SD region alloantigens, as shown in Table III, are refractive to suppression by AFP. Two strain combinations, B10.S(7R) anti-B10.S and B10.AKM anti-B10.BR, which differ genetically at MHC D, exhibit specific responsiveness in primed-lymphocyte typing analysis towards the MHC D region-associated alloantigens defined by specificities H-2.12 and H-2.32, respectively (A. B. Peck, unpublished data).
Reactivity between strains CBA-H-2\textsuperscript{aa} and CBA/H is believed to result from a gain loss mutation associated with the MHC K region. Thus, the lymphocyte proliferation observed is most probably induced by the parent mutation strain-defined SD specificity H-2.60 (see reference 41).

This selective inhibitory action of AFP on anti-MHC I region responses is again very clearly exemplified in Fig. 2. The two strain combinations examined here are B10.S(7R) responding against B10.HTT or B10.G responding against B10.T(6R). The major genetic difference between B10.S(7R) and B10.HTT important for MLC activation is the presence of I region antigen Ia.7 expressed in strain B10.HTT (42, 46). On the other hand, B10.G and B10.T(6R) share MHC region identity except at D, and the reaction of B10.G against B10.T(6R) appears to be directed against a portion of the SD molecule defined by, but distinct from, SD specificity H-2.4 (ABP, unpublished data). Both the anti-Ia.7 and the anti-SD reactions exhibit typical secondary-like profiles with peak \([^{3}H]\text{TdR}\) incorporation occurring between 24 and 48 h of culture. Again, AFP can be seen to exert a highly selective activity: the restimulation of B10.S(7R) anti-B10.HTT blasts by Ia.7 is strongly suppressed, whereas the restimulation of B10.G anti-B10.T(6R) blasts by the D region molecule(s) is unaffected.

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**Fig. 2.** Differential effects of AFP on the secondary MLC activation of isolated, alloantigen-activated T lymphocytes sensitized to either MHC I or MHC SD region determinants.

A. The kinetics of secondary MLC activation for B10.S(7R) anti-B10.HTT blast cells with spleen cells from B10.HTT in the absence (●—●) or presence (○—○) of AFP. B. The kinetics of secondary MLC activation for B10.G anti-B10.T(6R) blast cells with X-irradiated B10.T(6R) spleen cells in the absence (●—●) or presence (○—○) of AFP. 0.8 μCi \([^{3}H]\text{TdR}\) was added to each culture 4 h before harvesting the cells at the times indicated.
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Effects of AFP on Primary and Secondary MLC Responses against Non-MHC-Associated Alloantigens. To test the effects of AFP on the proliferative responses towards non-MHC alloantigens, the kinetics of primary MLC activation for whole splenic leukocytes and purified splenic T lymphocytes from B10.BR mice stimulated with spleen cells from C3H/HeJ mice were studied. Both B10.BR and C3H/HeJ carry the MHC k haplotype but differ genetically at numerous non-MHC loci, including the MLC-stimulating Mls locus (43, 44). Results, shown in Fig. 3, indicate that AFP effectively suppresses this anti-non-MHC response elicited by both responding cell populations.

Examples of secondary MLC responses against non-MHC alloantigens in the presence and absence of AFP are shown in group A, Table IV. Results with the three reactions of B10.BR anti-C3H/Tif, B10.M anti-A.CA, and B10.S(7R) anti-A.TH indicate that AFP can also exert strong suppressive effects on secondary MLC activation induced by non-MHC genetic differences. The major stimulating determinant(s) in each of these three reactions are considered to be the Mls locus product.

A limited number of strain combinations possessing non-MHC genetic differ-
TABLE IV

Differential Effects of AFP on the In Vitro Activation of Splenic T Lymphocytes by Non-MHC Alloantigens

| Group | Responding T lymphocytes* | Stimulating cells | Genetic differences |[^3H]Tdr Incorporation (cpm ± SD) |
|-------|---------------------------|-------------------|--------------------|-----------------------------|
|       |                           |                   | in response to     | NMS Control§ | + AFP§ |
| A     | B10.BR anti-C3H/IF       | B10.BR            | None               | 9,159 ± 631 | 10,357 ± 880 |
|       |                           | C3H/IF            | Non-MHC, Mls locus | 187,767 ± 3,256 | 78,330 ± 3,503 |
|       |                           | C3H/BL/6          | Non-MHC cross-reactivity | 80,455 ± 1,368 | 40,830 ± 2,115 |
|       | B10.M anti-A.CA          | B10.M            | None               | 21,360 ± 100 | 19,214 ± 1,378 |
|       |                           | A.CA              | Mls locus          | 46,874 ± 3,369 | 18,900 ± 2,727 |
|       | B10.B(7R) anti-A.TH      | B10.B(7R)        | None               | 16,997 ± 765 | 20,183 ± 2,747 |
|       |                           | A.TH              | Mls locus          | 10,546 ± 752 | 15,996 ± 675 |
|       | B10.D2/n                 | B10.D2/n         | None               | 1,382 ± 25 | 2,017 ± 134 |
|       |                           | DBA/2             | Non-MHC, Mls locus | 2,457 ± 334 | 7,446 ± 1,190 |
|       | CBA/J                    | CBA/J            | None               | 1,012 ± 100 | 676 ± 149 |
|       |                           | DBA/2             | MHC, Non-MHC, Mls locus | 59,855 ± 2,231 | 93,385 ± 4,181 |
|       | B10.BR anti-CBA/J        | B10.BR           | None               | 976 ± 210 | 1,887 ± 188 |
|       |                           | CBA/J            | Non-MHC, Mls locus | 34,891 ± 3,184 | 34,233 ± 1,161 |
|       |                           | DBA/2             | Non-MHC cross-reactivity | 21,299 ± 4,840 | 19,045 ± 503 |

* T lymphocytes purified by passage through Ig-anti-Ig columns. Blast cells isolated on 1 g velocity sedimentation gradients after peak primary MLC activation.
† Genetic difference considered responsible for activation of T cells in MLC.
§ AFP added at initiation of primary or secondary MLC to a final concentration of 150 μg/ml. Control cultures received an equivalent concentration of normal mouse serum (NMS).

ences have been found whose proliferative responses were either not significantly inhibited by AFP or were actually enhanced. Such combinations, as a rule, contained either DBA/2 or CBA/J as the stimulating cell type. One example, shown in Fig. 4, is the kinetic responses of whole splenic leukocytes and purified splenic T lymphocytes from B10.G responding against DBA/2 cells, a strain combination possessing both MHC and non-MHC genetic differences. In addition, the MHC activations of other strain combinations, including B10.D2/n anti-DBA/2, CBA/J anti-DBA/2, and B10.BR anti-CBA/J, are also unaffected or slightly enhanced (group B, Table IV). It appears, therefore, that AFP suppresses proliferative responses against Mls locus incompatibility, but enhances the proliferative responses between a few strain combinations possessing additional MLC-activating, non-MHC differences.

The different effects of AFP in the various strain combinations is a reproducible phenomenon, thus it can be concluded from this series of experiments that the suppressive activity of AFP on primary and secondary proliferative responses depends to a great extent on the genetic relationship between responding and stimulating strains.

Effects of AFP on Different Ly Subclasses of Responding T Cells. Data obtained in the previous sections indicate that AFP affects primarily MLC reactions directed against I region gene products, with little or no activity on responses against the SD region products. Several recent studies (40, 49, 50) have suggested that the predominant class of T cells responding against the MHC K and D region gene products carry the Ly antigen phenotype Ly 1-, 2+, 3+ (Ly 2,3 cells), whereas the T cells which proliferate in response to I
region incompatibilities are mainly of the phenotype Ly 1+, 2−, 3− (Ly 1 cells). The following experiments were performed to determine whether AFP acts principally on Ly 1 cells as might be predicted on the basis of the previous results.

The activated blast cell population isolated from primary mixed cultures of B10.S(7R) responding against B10.HTT (anti-Ia.7 reaction) has been shown previously (46) to consist of T cells which are 60−70% sensitive to treatment with anti-Ly-2.1 antiserum plus complement. Thus, B10.S(7R) anti-B10.HTT blasts were treated with anti-Ly 2.2 antiserum plus complement to obtain a responding population highly enriched for Ly 1 cells (usually about 90% pure). As shown in Table V, this cell population is nearly twice as reactive in secondary MLC as the control population treated with normal mouse serum plus complement (compared on a culture to culture basis). AFP suppresses the secondary MLC activation of both the control and the Ly 2,3 cell-depleted populations, thereby proving that AFF can effectively inhibit proliferation of Ly 1 T cells.

In contrast, the T-blast cell populations obtained from the reaction of B10.G
The Effects of AFP on the In Vitro Restimulation of Ly Subclasses of Splenic T Lymphocytes Sensitized against MHC I or MHC SD Region Alloantigens

| Responding T lymphocytes* | Antiserum treatment | Secondary MLC stimulating cells | Genetic difference | [H]TdR Incorporation NMS Control (cpm ± SD) + AFP* |
|---------------------------|---------------------|---------------------------------|--------------------|-----------------------------------------------|
| B10.S(7R) anti-B10.HTT    | NMS + C’§           | B10.S(7R) None                  | 11,012 ± 1,365     | 5,013 ± 2,638                                |
|                           |                     | B10.HTT I-C (la.7)              | 24,341 ± 668       | 12,854 ± 2,961                               |
|                           | anti-Ly 2.2 + C’    | B10.S(7R) None                  | 11,700 ± 815       | 5,444 ± 149                                  |
|                           |                     | B10.HTT I-C (la.7)              | 40,276 ± 2,081     | 20,076 ± 2,121                               |
| B10.G anti-B10.T(6R)      | None               | B10.G None                      | 22,586 ± 2,509     | 15,285 ± 2,136                               |
|                           |                     | B10.T(6R) MHC D                 | 74,241 ± 461       | 68,099 ± 1,127                               |

* Blast cells isolated on 1 g velocity sedimentation gradients after peak primary MLC activation.
§ Complement-dependent cytotoxicity performed according to the protocol described by Shiku et al. (40).

| Table VI |
|----------|
| Effect of AFP on the Secondary Responses of Different Ly Subclasses for the Reaction C57BL/6 Anti-DBA/2 |

| Responding T lymphocytes* | Antiserum treatment | Secondary MLC stimulating cells | Genetic difference | [H]TdR Incorporation NMS Control (cpm ± SD) + AFP* |
|---------------------------|---------------------|---------------------------------|--------------------|-----------------------------------------------|
| C57BL/6 anti-DBA/2        | NMS + C’§           | C57BL/6 None                    | 898 ± 168          | 2,404 ± 202                                   |
|                           |                     | DBA/2 MHC, Non-MHC              | 26,462 ± 2,008     | 33,288 ± 6,000                               |
|                           | Anti-Ly 2.1 + C’    | C57BL/6 None                    | 878 ± 423          | 2,040 ± 910                                  |
|                           |                     | DBA/2 MHC, Non-MHC              | 49,193 ± 3,239     | 41,450 ± 4,020                               |
|                           | Anti-Ly 2.2 + C’    | C57BL/6 None                    | 3,540 ± 618        | 6,200 ± 1,038                                 |
|                           |                     | DBA/2 MHC, Non-MHC              | 40,200 ± 122       | 46,760 ± 5,215                               |

* Blast cells isolated on 1 g velocity sedimentation gradients after peak primary MLC activation.
§ Complement-dependent cytotoxicity performed according to the protocol described by Shiku et al. (40).

Responding against B10.T(6R), a pure anti-SD reaction, were found to carry Ly antigen Ly 2.2 on close to 100% of the responding cells. As shown in both Table V and previously in Fig. 2, AFP does not affect this cell population.

It was earlier noted that primary MLC reactivity against strain DBA/2, irrespective of whether the responding cells differed genetically at the MHC or non-MHC loci (e.g., CBA/J, B10.G, or B10.D2/n responding against DBA/2 as shown in Fig. 4 and Table IV), is either enhanced or unaffected by the presence of AFP. This feature is preserved in the secondary MLC responses of activated blasts against DBA/2, as evidenced by the reaction of C57BL/6 anti-DBA/2 cells presented in Table VI. If the C57BL/6 anti-DBA/2 cells are treated with either anti-Ly 2.1 or anti-Ly 2.2 antisera plus complement, cell populations result which are considerably more responsive than the control cultures (Table VI). However, like the control cultures, the proliferative responses of the Ly 1 or Ly 2,3 cell-depleted populations fail to be altered by the presence of AFP. Thus, the nature of the stimulating alloantigenic system, rather than the actual Ly phenotype class of the responding T-cell population may be the definitive factor determining susceptibility to the suppressive activity of AFP.
Discussion

The immune system can be viewed as functioning through a complicated series of intricate collaborations which involve distinct sets of lymphoid and nonlymphoid cells as well as soluble factors interacting in a stimulatory or suppressive manner to create and regulate the immune response. One soluble factor capable of exerting significant immunosuppressive effects on T cells in vitro at physiological levels is AFP (7-14). Since AFP is a major alphaglobulin component of fetal and newborn sera, and since the serum levels of AFP in pregnant females is elevated, it must be considered a possible contributing regulatory factor in vivo protecting the fetus from maternal-immune attack. However, after a number of recent investigations which failed to find a uniform inhibitory activity for AFP (25-30), the possible importance of AFP as an immunoregulatory substance has been questioned. We believe that the present observations not only help to resolve these discrepancies by pointing out that the differential effects which AFP can exert on T-cell proliferation depend on the nature of the stimulating alloantigen(s), but also by demonstrating how consistent the previous observations are in reality (9, 10, 23-30).

The primary aim of this study has been to determine the effects of AFP on the recognition and subsequent proliferation of T lymphocytes reacting in MLC against histocompatibility-associated alloantigens. In the mouse, at least four distinct genetic systems are known to induce strong MLC activation (41-46): the Ia antigens encoded by genes of the MHC I region, the SD products of the MHC K or D regions, the Mls locus products, and the products of an as yet undefined non-MHC system (listed in Table I). Analysis of the impact exerted by AFP on T-cell proliferative responses towards alloantigens determined by these four respective genetic systems reveals drastic differences. T-cell reactivity involving MHC I region differences were markedly suppressed. This included reactions against isolated I region incompatibilities, reactions against I plus SD region differences, as well as reactions against whole MHC haplotype histoincompatibilities. In contrast, no detectable inhibition of T-cell proliferation against isolated SD alloantigens were observed (responses compared in Fig. 2). Furthermore, the partial suppression noted when measuring MLC reactions against a combination of I and SD differences no doubt results from this selective interference with the proliferation against only the I region structures.

The effects of AFP on T-cell responses against non-MHC alloantigenic structures further reveal a selective suppression. Nearly total inhibition by AFP of stimulation attributable primarily to Mls locus genetic differences was noted, whereas stimulation by the second non-MHC locus structures proved refractory to suppression. This is quite logical because a successful response against Mls locus structures requires a concomitant recognition of I region gene products on the stimulating cells (46). No similar I region requirements exist for stimulation by this second non-MHC locus structure (46). Thus it is clear that AFP would exert its suppressive activity in MLCs via selective interference with the I region triggering system(s), while failing to inhibit systems where I region association is not required.

One might speculate at this point that stimulation by any antigen whose
recognition requires concomitant recognition of \( I \) region gene products would be suppressed in the presence of AFP. In addition, the proliferative responses against chemically-modified (61) or virus-induced (52) determinants, which at least functionally occur in association with SD antigens, would remain refractive to suppression.

It is of interest to note that lymphocyte proliferation was at times enhanced by AFP in particular strain combinations, most notably in reactions against DBA/2 (see Table IV). Because the AFP preparations used in this study fulfilled strict criteria for protein purity, the stimulatory effect observed in the presence of AFP is most likely due to an intrinsic effect of the added glycoprotein per se rather than of a contaminating substance. Immunostimulatory effects of AFP in vitro have also been observed in the human system (53, 54). The idea that AFP may promote growth of distinct cell types in vitro is consistent with its frequent association in vivo with rapidly dividing cell populations in normal, restorative, and malignant conditions. Moreover, albumin, which shares many physicochemical characteristics with AFP (55), is actually reported to be essential for growth of mammalian lymphocytes in vitro (56). We consider it entirely possible, therefore, that AFP possesses both lymphocyte-suppressive and lymphocyte-stimulating properties. On the basis of the data presented within this report, these two opposing regulatory effects may be directed towards functionally distinct lymphocyte targets.

The possibility that AFP exerts a suppressive activity on distinct subsets of T lymphocytes as defined by Ly antigen phenotypes was also explored in view of successful delineation in other systems using this classification of functional subsets of T cells (40, 49, 50). From the present data (Tables V and VI) it is clear that AFP will be a successful inhibitor of Ly 1+ T-blast cells reacting against \( I \) region structures including reactions against Mls locus products, while failing to inhibit Ly 2+ cells being stimulated by SD antigens. However, when using the non-MHC locus alloantigen(s) as stimulating agents, both Ly 1+ as well as Ly 2+ blast cells fail to become inhibited by AFP. We would have to conclude, therefore, that the mere fact that a T-blast cell expresses the Ly antigen phenotype Ly 1+,2− does not automatically indicate sensitivity to suppression by AFP. Rather, the more decisive factor determining whether AFP will function as an efficient inhibitor or not lies at the level of the triggering structures. It should be realized, in any case, that AFP may have a dual requirement, first for a particular subset of T cells, and second for a distinct stimulating unit.

Although the precise mechanism of AFP suppression remains obscure, the present data add further weight to the earlier findings that AFP does exert a selective inhibitory capacity on T-dependent immune responses (7-10, 23). This inhibition depends, at least in part, on the ability of AFP to induce highly efficient suppressor T cells (24). The potential importance of the production of suppressor cells for inhibition of the proliferative MLC responses noted here still must be explored. Furthermore, the present data leave open the question of whether AFP exerts a direct anti-proliferative effect on specific MLC reactive T cells or suppresses through interference by binding to relevant recognition structures on target cell surfaces. Nevertheless, AFP appears to regulate T-
cell-dependent-immune responses in vitro in a highly selective manner which we suggest is indicative of a potentially important immunoregulatory role in vivo. Further discussion of this topic is presented in the second paper of this series in which AFP is shown to inhibit development of cytotoxic T cells against SD region antigens despite the fact that the proliferative responses are quantitatively unaltered.

In conclusion, these data indicate that the normal fetal serum protein AFP is endowed with selective ability to abrogate in vitro T-cell proliferation to I region determinants. Although not resolving the question as to the in vivo relevance of AFP as an immunosuppressive agent, these results provide an explanation why earlier reports which failed to investigate the underlying genetic requirements yielded contradictory results as to the in vitro immunosuppressive potential of AFP. Further analysis of how AFP exerts this selective activity with respect to anti-I region reactions may provide a new approach in the search for the actual functions of I region gene products.

Summary

Alpha-fetoprotein (AFP), a major alpha-globulin component of fetal and newborn sera, has earlier been shown to exert significant immunosuppressive activity in vitro on T-dependent-immune responses. In the present investigation we have examined the effects of AFP on the recognition and proliferation of T lymphocytes responding in mixed leukocyte culture against histocompatibility-associated alloantigens. Fetal-derived AFP could be shown to exert differential effects on both primary and secondary responses ranging from strong inhibition to occasional enhancement, depending on the stimulating antigens. Proliferative responses against major histocompatibility complex (MHC) I region determinants, mediated predominantly by Ly 1+ cells, were markedly suppressed. Suppression was also observed in responses against Mls locus products, an antigenic system whose recognition requires concomitant recognition of I region gene products on the stimulating cells. In contrast, responses against MHC K or D region determinants, mediated predominantly by Ly 2+ cells, were generally unaffected by AFP. Similarly, non-MHC loci alloantigens distinct from Mls locus products also induced T-cell proliferation which was refractive to suppression by AFP. Because neither Ly 1+ nor Ly 2+ cells responding in this latter situation could be inhibited by AFP, we conclude that the mere fact that a T cell expresses a particular Ly phenotype does not predetermine sensitivity to AFP-induced suppression. In any case, AFP exerts a highly selective suppressive activity on I region-associated immune responses. These data may help to resolve the present controversy over the possibility that AFP has an in vivo relevance as an immunosuppressive agent by pointing out the importance of selecting proper genetic situations for study.

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