# IMMUNOREGULATORY MECHANISMS DURING EARLY ONTOGENY

by

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I. **ABSTRACT**

Immunoregulatory Mechanisms During Early Ontogeny

The developing neonatal animal must have available an immune system sufficient to protect against invading pathogens and allo-aggressive maternal lymphocytes. Both *in vivo* and *in vitro* antigenic stimulation of the neonatal murine immune system results in a relatively weak immune response when compared with adult reactivity. However the present investigation shows that T lymphocytes purified from neonatal lymphoid organs can express substantially increased *in vitro* proliferative capacity toward mitogens as well as allo- and autoantigens. These findings imply the existence of naturally occurring immunoregulatory cells and inhibitory humoral factors. Further studies revealed that distinct populations of newborn regulatory cells can be observed, and identified as belonging to both T and B cell lineages. It was also determined that neonatal thymus contains a population of highly autoreactive T cells whose proliferative capacity could be effectively modulated by intrinsic levels of alpha-fetoprotein. Thus the newborn can be shown to possess humoral immunosuppressive factors as well as inhibitory cells with the potential to control possibly harmful allo- and auto-aggressive T lymphocytes.

**RESUME**

Les Méchanismes Immunorégulateurs tôt Lors de l'Ontogénèse

L'animal naissant, qui se développe doit être muni d'un système immunitaire suffisant afin de le protéger contre tous pathogènes envahisseurs ainsi que contre les lymphocytes maternels allo-agressifs. La stimulation, in vitro et in vivo, du système immunitaire de la souris nouvellement née entraîne dans les deux cas une faible réponse immunitaire lorsque comparée à celle d'un animal adulte. Toutefois, la présente investigation démontre que les lymphocytes T isolés des organes lymphoïdes peuvent exprimer une augmentation substantielle de leur capacité de prolifération lorsque mis en présence de mitogènes ainsi que d'antigènes auto- et allogéniques. Ces découvertes impliquent l'existence de cellules immunorégulatrices et de facteurs humoraux inhibiteurs qui sont naturels à l'animal. Des études plus poussées rélèvent par surcroît que des populations distinctes de cellules régulatrices peuvent être observées chez les nouveau-nés et identifiées comme appartenant aux lignées de cellules T et B. Il a également été déterminé que le thymus du nouveau-né renferme une population de cellules T hautement autoréactives dont la capacité de prolifération peut être bien contrôlée par des quantités intrinsèques d'alpha-fétoprotéine. Il peut être démontré que le nouveau-né possède des facteurs humoraux immunosuppresseurs ainsi que des cellules inhibitrices qui ont le potentiel de contrôler les lymphocytes allo- et auto-agressifs T qui pourraient s'avérer nuisibles.

II. **CONTENTS**

page no.

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IV. **PREFACE**

This thesis is the result of four and one-half years of investigation performed in the laboratory of Dr. Robert A. Murgita of the Department of Microbiology and Immunology, McGill University. A major contribution of this investigation to original knowledge is the description of two distinct classes of MLC-inhibitory cells in newborn mouse spleen. This finding is not only original, but also explains some of the contradictory data present in the literature concerning the exact characterization of the neonatal spleen cell(s) capable of suppressing MLRs. A second important pioneering development in this study is the demonstration that AFP has the capacity to directly inhibit the proliferative responses of particular populations of lymphocytes, especially those which respond to autologous stimulation. Other findings that should be considered as contibutory to original knowledge include:

1. Isolated newborn splenic T cells possess virtually adult levels of CON A and PHA reactivity.

2. T cells isolated from neonatal spleen proliferate in allogeneic MLCs with a magnitude higher than unselected newborn spleen cells whereas adult splenic T cells respond with approximately the same proliferation as whole adult spleen cells.

3. The cell surface antigenic phenotype of the newborn thymocytes responding in the Type I AMLR is Lyt $1^{+}23^{-}$. These cells respond to stimulation from genetically identical Ia-bearing adult Non-T cells in the absence of a requirement for xenogeneic proteins.

4. Both neonatal thymus responder and adult stimulator cells in the Type I AMLR are sensitive to *in vivo* anti-mu treatment.

5. In AMLCs, newborn thymocytes respond with greater proliferation than any lymphoid population in the adult.

6. Neonatal T and B spleen cells suppress autologous in addition to allogeneic MLCs.

7. Both T and B-like MLC-inhibitory cell populations show the potential for self-regulation.

8. Different inhibitory cells may be active in MLC versus PFC reactions.

9. AFP has stimulatory properties for certain lymphoid cell populations.

10. AFP preferentially inhibits mitogen induced proliferation of newborn and adult thymocytes versus peripheral lymphocytes.

## V. ABBREVIATIONS

| | |
|---|---|
| AFP: | Alpha-fetoprotein |
| AMLC: | Autologous mixed lymphocyte culture |
| AMLR: | Autologous mixed lymphocyte reaction |
| B Cell: | Bursa-equivalent derived cell |
| CML: | Cell mediated lympholysis |
| CON A: | Concanavalin A |
| CTL: | Cytotoxic T lymphocytes |
| FCS: | Fetal calf serum |
| Fe: | Carbonyl iron |
| GVH: | Graft versus host |
| HVG: | Host versus graft |
| Ig: | Immunoglobulin |
| LPS: | Lipopolysaccharide |
| MAF: | Mouse amniotic fluid |
| MHC: | Major histocompatibility complex |
| MICG: | Macromolecular Cold Insoluble Globulin |
| MLC: | Mixed lymphocyte culture |
| MLR: | Mixed lymphocyte reaction |
| NHS: | Normal human sera |
| NMS: | Normal mouse sera |
| Non-MHC: | Unrelated to the major histocompatibility complex |
| Non-T Cell: | Lymphoid cell that is not thymus derived |
| NRS: | Normal rabbit sera |
| PBL: | Peripheral blood lymphocytes |
| PBS: | Dulbecco's modified phosphate buffered saline |
| PFC: | Plaque forming cell |
| PHA: | Phytahemagglutinin |
| PNA: | Peanut agglutinin |
| $PNA^-$: | Not agglutinated by PNA |
| $PNA^+$: | Agglutinated by PNA |
| RC: | Rabbit complement |
| SBA: | Soybean agglutinin |
| $SBA^-$: | Not agglutinated by SBA |
| $SBA^+$: | Agglutinated by SBA |
| SRBC: | Sheep red blood cells |
| T Cell: | Thymus derived lymphocyte |
| TD: | T cell dependent |
| TI: | T cell independent |

## VI. INTRODUCTION

During ontogenesis the immune system acquires reactivity in a sequence of identifiable stages which is relatively constant for all members of a particular species [226,237]. Although certain species can mediate restricted immune functions during life *in utero*, the immunological responsiveness of the fetus and neonate can be seen to be markedly depressed in comparison with that of the mature animal [105,193]. The mouse in particular shows profound deficits in the ability to respond to immunological stimuli at birth, and does not develop adult-like reactivity for periods of time ranging from days to weeks afterwards [226]. Various explanations for the gradual postnatal development of the murine immune system have been put forward including: a relative deficiency of functionally mature lymphocytes and accessory cells, and/or the presence of serum regulatory factors [156,157,163,165,200,238, and reviewed in 171]. However, neonatal lymphocytes can be shown to elicit T-dependent antibody responses when immunized after adoptive transfer into lethally irradiated adult recipients [197]. This suggests that functional lymphocytes are in fact present during early life. Experimental manipulations of the newborn immune system have also demonstrated that mice within a few days of birth possess functional T and B lymphocytes [10], as well as antigen presenting cells, in numbers sufficient to allow a PFC response of considerable magnitude following immunization with T-dependent antigens [99,200]. Thus the immunological hyporeactivity of the mouse during the first week of life is not the result of a total lack of competent cellular components of the immune system and is therefore likely due, at least in part, to regulatory mechanisms active in early ontogeny. It is now apparent that the relatively poor performance of neonatal mouse lymphocytes in tests of immune function *in vivo* and *in vitro* is the result

of a combination of both low numbers as well as efficient down regulation of immunologically mature effector cells.

## i. Postnatal Development of Thymus and Spleen Cells Involved in the Immune Response.

### a) T Cells.

The lymphoid organs of the mouse are undergoing their most rapid increase in cell numbers during the perinatal period ranging from four days prior to and two days following birth [233]. Towards the end of gestation most fetal thymocytes possess virtually adult levels of Thy 1, Lyt 1, and Lyt 2 antigens [114]. Between one and four days after birth thymocytes attain Lyt 1 antigen density profiles comparable to those of adult thymocytes [91]. In fact, the lymphocytes migrating from the thymus of the newborn have been demonstrated to consist of 60-70% Lyt $1^+2^-$ and 30% Lyt $12^+$ T cells, which corresponds to the Lyt phenotypic composition of cells leaving the adult thymus [224]. Recently Hauptman has described a T cell antigen, Macromolecular Insoluble Cold Globulin (MICG), that is expressed on the surface of cells of the T lineage before the appearance of Thy 1 determinants [20]. Only cells previously expressing MICG were found to be capable of developing Thy 1 determinants [20]. All thymocytes remain MICG positive from birth through adulthood. During gestation thymocytes expressing cytoplasmic but not surface MICG determinants are demonstrable [20]. Hauptman and his co-workers have concluded that pre-T cells lacking Thy 1 and MICG determinants can be identified by the presence of cytoplasmic MICG [20]. At birth neonatal thymocytes have adult proportions of cells expressing binding sites for the lectin PNA [133]. The corticosteroid-sensitive thymocytes and minor fractions of peripheral T or null cells that bind PNA

are apparently less mature functionally than T cells that do not possess receptors for this lectin [133]. In addition to possessing comparable distributions of cells with equivalent antigenic profiles, neonatal and adult thymi apparently contain the same proportion of mature versus relatively immature T cells. However the neonatal thymus contains a significantly increased proportion of large blast-like cells in comparison with that of the adult, without having a parallel increase in the number of cells undergoing mitosis [172].

Although only minor phenotypic differences exist between adult and newborn thymocyte subpopulations, newborn spleen may contain a lower proportion of Thy 1 and Lyt 1 positive T cells than adult spleen. Estimates of the percentage of T cells in the spleen of the newborn at birth, based on the number of cells expressing Thy 1, range from nearly adult levels of over 20% [233] to as little as 1% [91,238]. Throughout the first week of life less than 1% of spleen cells bear Lyt 1 antigens [91]. Adult proportions of Thy 1, Lyt 1, and Lyt 2 positive cells have been demonstrated in spleen at approximately two weeks of age by Haaijman et. al. [91]. Their results indicate that at two weeks after birth there are approximately three Lyt $1^+$ T cells to every Lyt $2^+$ T cell [91]. This contrasts with earlier observations suggesting that most newborn splenic T cells are Lyt $123^+$ [39]. Thus during early ontogeny the spleen of the neonate, but not the thymus, appears to have relatively few T cells with adult levels of Thy 1 and/or Lyt antigens.

b) B Cells.

B lymphocytes are known to differentiate through a sequence of phenotypically distinct stages [2]. Pre-B cells with cytoplasmic but no surface IgM appear during mid-gestation [148,205] shortly before the appearance of B cells

with surface IgM [148,182]. At birth there are reported to be from 4% [75] to 20% [233] surface Ig-positive B cells in murine spleen. Jyonouchi and Kincade [112] have concluded that spleens of mice under one day of age contain approximately 27% lymphocytes of the B lineage of which nearly 70% do not bear surface immunoglobulin. Less than 20% of the Ig-negative B-derived cells were determined by these investigators to be classical pre-B cells containing cytoplasmic mu chain [112]. The remainder of the Ig-negative cells were shown to be B-derived on the basis of possessing a determinant detected by monoclonal 14.8 antibody [112]. However, a fraction of these Ig-negative cells could be T cells as 14.8 antibody binds to subpopulations of peripheral adult T as well as B cells [112]. From the day of birth to between seven and ten days of age the proportion of cells in spleen expressing Ig increases from approximately 30% to more than 85% of the total cells of B lineage [112]. Thus by one to two days of age approximately 25% of the spleen cells express surface Ig [75,233] and adult levels of 40% to 50% are achieved by two [75] to three [233] weeks of age. The B cells detected by the presence of membrane bound IgM during the first weeks of life are clearly phenotypically distinguishable from the majority of mature adult B cells by the absence of IgD [116,222,223,250], Ia antigens [94,116], Mls antigens [7], and C3 receptors [75]. From birth through ten days of age splenic B cells express intermediate to high amounts of surface IgM and essentially no IgD [223]. By three weeks of age the surface immunoglobulin pattern is becoming more like that of the adult with the appearance of IgD on approximately 60% of spleen B cells [116,223,250]. Ia antigens are also absent on splenic B cells at birth [94,116]. These antigens appear on the B cell membrane shortly after birth and by seven to nine days of age are present on an adult proportion of 95% of the IgM-positive

spleen cells [116,173]. Receptors for the C3 component of complement do not appear on splenic B cells until approximately two weeks after birth and are not present in significant numbers until three to four weeks of age [75]. Antigens encoded by the M locus are also absent from the B cells of the neonate and subsequently become expressed at approximately three weeks after birth [7].

c) Antigen Presenting Cells.

Macrophages possessing phagocytic activity and receptors for the Fc fragment of IgG first appear during mid-gestation [49]. During early postnatal life the spleen contains nearly adult proportions of macrophages as determined by adherence, phagocytosis, and the presence of Fc and C3 receptors [135,136]. However while adult splenic macrophages are 40 to 65% Ia-positive [107,134,136,173], virtually no one to three day old [134], and less than 10% of four to six day old [107,173] newborn splenic macrophages express these antigens. Adult proportions of Ia-positive macrophages are not detectable in spleen until three weeks after birth [107,134, 136]. On the other hand, the thymus of the newborn mouse contains significant numbers of Ia-positive macrophages with phagocytosis and adherence abilities equivalent to adult thymic macrophages [134]. While 35% of the macrophages in the adult thymus are Ia-positive, 11% of one to three day old thymic macrophages bear Ia antigens [134]. By six days after birth the neonatal thymus contains macrophages which are 27% Ia-positive and adult proportions of these cells are reached by twelve days of age [134]. It is noteworthy that 15% of both newborn and adult Ia-positive thymic macrophages have the properties of dendritic macrophages including the characteristic morphology, Fc receptors, and reduced phagocytic ability [22,134]. Thus during early ontogeny adult proportions of macrophages are demonstrable in both

spleen and thymus. While few macrophages in the spleens of neonates are Ia-positive, the fraction of newborn thymic macrophages expressing Ia antigens more closely approaches that of the adult.

## ii. The Ontogeny of Immunological Responsiveness In the Neonatal Mouse.

### a) T Cell Functions.

At birth neonatal thymocytes show a low (in comparison to adult cells) but detectable response to the T cell mitogen CON A [154,155,209,232,238]. Over the next several weeks of life the ability of thymocytes to respond to CON A gradually increases to achieve adult levels by approximately three weeks of age [155,209,232,238]. On the other hand, thymocytes from newborn mice have been demonstrated to be incapable of responding to the T cell mitogen PHA [4,35,38,105,155,232,238]. Fetal thymocytes may however possess a transient ability to respond to this mitogen [154,155]. In several studies the response of thymocytes to PHA was detectable approximately two weeks after birth and did not reach adult levels until six to twelve weeks of age [35,238]. A weak postnatal response of thymocytes to PHA which appeared to be constant from one week of age through adulthood was shown in another investigation [155]. Therefore during at least the first week of life thymocytes appear to have a reduced ability to respond to T cell mitogens.

In contrast with the relatively poor capacity of neonatal thymocytes to respond to mitogens these cells are efficient responders in both allogeneic [28,87,105,154] and autologous [19,27,28,29,101,102,103] mixed lymphocyte cultures. Two classes of murine AMLR have been described. The Type 1 AMLR consists of neonatal thymocytes responding to

genetically identical adult spleen cells [70,195]. In the Type 2 AMLR adult lymph node, splenic T cells, or $PNA^-$ thymocytes react against autochthonous spleen cells [70,195]. Both classes of AMLR are characterized by the proliferation of T cells apparently induced by self Ia antigens [19,26,70,83,84,85,127,194] on appropriate stimulator cells. However Ponzio, Finke and Battisto have presented evidence that two distinct murine differentiation antigens (MDAs 1 and 2) are responsible for stimulation in Type 1 and Type 2 AMLR [70,195]. While neonatal thymocytes can respond to both MDA 1 and MDA 2 adult lymph node cells react with only MDA 2 [70]. Interestingly, MDAs 1 and 2 are both apparently Ia antigens or closely related to these structures [70]. Initial characterizations of the stimulator cell in both Type 1 and 2 AMLRs indicated that these cells also express Ig as well as Ia antigens [19,27,103,195]. Further investigations in the Type 2 AMLR have indicated that Ia bearing B cells, macrophages, and dendritic cells can all induce this autologous reaction [19,30,83,127,177,194]. Since neonatal thymocytes can react to MDA 2, the stimulatory antigen in the Type 2 AMLR [70], it appears likely that Type 1 AMLR may be stimulated by Ia-positive macrophages and dendritic cells as well as B cells. The Lyt phenotype of the responder cells in the Type 2 AMLR has been determined to be Lyt $1^+23^-$ [19,30,85,127]. At present the Lyt phenotype of the neonatal thymocyte autologous responder has not been described. This cell is present during a restricted period of early postnatal ontogeny [28,102,103]. Peak activity of thymocytes in the Type 1 AMLR is seen within several days of birth [102,103]. The autologous reactivity of thymocytes begins to decline at approximately two days of age [102,103,104]. Initial studies indicated that adult thymocytes do not respond in the AMLR [28,29,44,103,104,183]. However, it has more recently been reported that isolated $PNA^-$ adult thymocytes can indeed

respond to autologous stimulation [30]. Direct comparison of the ability of neonatal versus adult thymocytes to respond in the AMLR indicates that the thymus contains an enlarged proportion of autoreactive cells during early ontogeny [103]. The AMLR can be interpreted as indicating self-cognitive processes that normally occur in vivo [83,104]. Thus the apparent decline with increasing age of the ability of thymocytes to respond in AMLR has been interpreted as indicative of the acquisition of self tolerance [104]. However criticism has been made of the suggestion that AMLRs measure anti-self proliferative reactions. It has been suggested that the heterologous serum additives used to supplement medium employed in numerous studies of AMLRs contribute to and may even be responsible for the reactions [106]. The objection that the proliferation in conventional AMLRs may be directed against foreign, or self plus foreign, antigenic determinants is becoming less important because of the increasing use of autologous serum supplements in AMLRs. However the possibility remains that AMLRs performed in medium with heterologous serum additives may not represent anti-self processes. Von Boehmer and Byrd have suggested that the Type 1 AMLR may not be an anti-self reaction because the stimulatory antigens present on adult splenocytes are not expressed on newborn, and therefore self, spleen cells [28]. Their conclusions are based on the fact that newborn spleen cells do not initiate proliferation in autochthonous thymocytes [27,28,103]. Ia determinants, which are presumed to be the stimulatory structures in AMLRs [19,26,70,83,84, 85,127,194] are initially expressed during gestation [57]. It has been reported that newborn spleen contains relatively low numbers of Ia bearing B cells [94,116], macrophages [107,134,135], and dendritic cells [235] when compared with adult spleen. Thus by virtue of low levels of Ia antigens and/or the presence of inhibitory cells neonatal spleen cells

may indeed be poor stimulators of AMLRs. However other potential autologous stimulator cells are present during early life. In fact newborn thymus has been demonstrated to contain significant populations of Ia positive macrophages and dendritic cells [134]. It would therefore appear that the Type 1 AMLR is an *in vitro* correlate of autologous interactions that may occur during early ontogeny *in vivo*.

In addition to proliferating in AMLRs, newborn thymocytes also show an enhanced [105] or equivalent [28,154] ability to respond in conventional allogeneic MLRs when compared with adult thymocytes. However newborn thymocytes appear incapable of mediating CML reactions until approximately one week of age [158]. On the other hand, thymocytes are capable of mounting a GVH reaction *in vivo* [87,105] and *in vitro* [45] shortly after birth. No difference has been noted in the ability of neonatal versus adult thymocytes to initiate GVH responses [45,87]. Analysis of the T cell helper function of newborn thymocytes in the production of an antibody response to T-dependent antigens has shown that at birth the cells are rapidly acquiring this ability, equalling that of adult thymocytes within 48 hours [47]. Day one newborn thymocytes are apparently limited to providing helper function for only direct PFC responses since they cannot mediate the shift to indirect PFCs in a primary response [92]. The ability to help B-cells in the transition from IgM to IgG and IgA antibody production has been found to be a separate maturational event occuring in the thymus between birth and two to four days of age [92]. Neonatal thymocytes therefore appear to be competent in some but not all tests of immune function. It is interesting to note that these cells respond in MLCs but do not generate cytotoxic lymphocytes. This perhaps indicates a deficiency in the function of the neonatal Lyt $1^{-}23^{+}$ thymocyte pre-CTL subpopulation. The ability of newborn thymocytes to mediate

GVH reactions may therefore possibly reside in the Lyt $1^{+}23^{-}$ cells as has been demonstrated for adult HVG reactive T cells [140].

While neonatal thymocytes show good responses in certain tests of immune function, spleen cells from mice of the same age respond poorly if at all in the same assays. Spleen cells at birth show little or no ability to respond to either CON A or PHA [105,232,238]. CON A reactivity is not detectable in spleen cells until one week after birth thereafter increasing to adult levels by three weeks of age [232,238]. On the other hand, the ability of spleen cells to respond to PHA appears to develop later in ontogeny [232,238]. A spleen cell response to PHA becomes detectable between one [238] and three weeks after birth [232] while adult levels of reactivity are not observed until six to eight weeks of age [232,238]. Early studies also indicated that spleen cells from mice under one to four weeks of age are incapable of responding to allogeneic stimulation in mixed lymphocyte cultures [6,105]. The age of onset of detectable reactivity appears to be related to the strains employed in the MLC [6]. Thus CBA/J splenocytes were seen to first obtain alloreactivity in MLCs one week after birth while C57BL/6 spleen cells did not respond until three weeks afterwards [6]. However both strains showed peak MLC reactivity in spleen cells between twelve and twenty weeks of age [6]. More recently, Wu et al have demonstrated that spleen cells from B10 mice at birth are capable of responding in allogeneic MLCs at a level of approximately 20% of the adult response [256]. In the same study, the ability of MLC activated B10 spleen cells to mediate CML was not detected until seven days after birth [256]. The appearance of CML activity in young spleen cells was found to be unrelated to any change in the magnitude of their MLR [256]. CTL precursors have been detected in the spleens of Balb/c mice

as young as two to three days old and significant cytotoxicity demonstrated one day later [192]. By twelve days of age the CTL response of Balb/c spleen cells has reached 23-32% of the response of 12-14 week old adult spleen cells [192]. On the other hand, CBA spleen cells acquired CML activity more slowly than Balb cells, with significant cytotoxicity not becoming apparent until nine days after birth [192]. Thus during ontogeny both MLC [6] and CML [192] abilities develop in the spleen at different rates in various mouse strains. Interestingly the Lyt phenotype of the cytotoxic precursors appears to be Lyt $123^+$ during the first two to three weeks of life becoming Lyt $1^-23^+$ between three and five weeks of age [34]. Different ages have also been reported for the onset of the ability of spleen cells to mediate GVH reactions in various strain combinations, ranging from one [244] to four days of age [87]. Spleen cells apparently develop mature GVH reactivity between four [244] and fourteen [87] days after birth. Dependent on the strain of mouse examined, it appears that adult levels of cell mediated immunity are not achieved until more than one week after birth.

The acquisition of the ability to produce antibody in response to T-D antigens parallels the slow development of cell mediated immunity in populations of spleen cells. The PFC response to T-D antigens is meager or absent for the first week or more of life and does not reach adult levels until four to eight weeks of age [69,156,204,232]. During ontogenetic development the ability of spleen cells to respond to T-independent antigens preceeds the ability to respond to T-dependent antigens [155,156,204,232]. In addition, substitution of neonatal splenic T cells by adult splenic T cells, or augmentation of their helper ability by exposure to mitogens or thymus extract, improves their PFC reactivity to T-D antigens [31,95,113,175,176,220,232]. Thus

the neonatal spleen cell deficit in T-dependent humoral responsiveness may be at least partly due to the functional incompetence of splenic T-helper cells in the newborn. Neonatal spleen T cells have in fact been shown to be unable to produce a T helper factor which is capable of replacing T helper function in an in vitro T-D antigen driven humoral system [31]. The capacity to produce this T helper factor is apparently lacking in the splenic T cells of mice less than one to two weeks of age [52]. Thus newborn T cells from spleen have profound deficits in the ability to proliferate in MLCs and provide poor helper activity in PFC assays stimulated by T-D antigens. In contrast, shortly after birth thymocytes are capable of responding in both autologous and allogeneic MLCs and also demonstrate significant, although restricted, helper activity in T-dependent humoral responses [19,27,28,29,47,86, 92,101,102,103,105,154]. In addition it has been shown that Lyt $1^{+}23^{-}$ cells migrate from the thymus during early ontogeny [224]. It is therefore possible that suppressor cells resident within the spleen of the neonate may prevent the activity of reactive T cells that have migrated from the thymus. Alternatively, there may be a selection process operative early in development which allows only immunoincompetent or inhibitory cells to leave the thymus.

b) B Cell Functions

While splenic T cells do not show significant mitogen reactivity until more than a week after birth, splenic B cells become mitogen reactive at birth [213]. Spleen B cells achieve adult responsiveness to LPS by two weeks of age [155]. B cells from seven day old spleen and bone marrow can be shown to synthesize antibodies to T-D antigens when immunized in the presence of adult thymocytes as a source of helper cells [72,86]. However newborn B cells produce

immunoglobulins which are restricted in heterogeneity of affinity in comparison with those elaborated by adult B cells [72,86]. During early ontogeny B cells appear to be particularly deficient in the capability of producing high affinity antibodies [72,86]. Interestingly, memory B cells for high affinity antibodies are elicited during primary immunization of one week old B cells since such antibodies are produced following secondary challenge with the antigen [72]. The heterogeneity of affinity of the immunoglobulins synthesized by young B cells becomes similar to that of adult B cells between two and four weeks of age [72,86]. Two classes of T-I antigens have been distinguished based on a differential ability to induce neonatal B cells to produce specific antibodies *in vitro* [159,160]. T-I type one antigens stimulate a humoral response by newborn B cells expressing IgM without IgD while T-I type two antigens do not [159,160]. Antibody production to T-I type two antigens occurs only after further maturation of the B cell population and is apparently related to the development of surface IgD immunoglobulins [159,160]. *In vivo* studies have indicated that the ability of the mouse to produce antibodies of the IgM class preceeds the capacity to produce IgG immunoglobulins during ontogeny [43,241]. The acquisition of Ia antigens on B cells may be related to a switch from production of IgM antibodies during early ontogeny to the subsequent synthesis of both IgM and IgG [94,131,171]. It is apparent that neonatal B cells have a limited but significant ability to synthesize immunoglobulins directed against both T-I and T-D antigens. The inability of newborn spleen to mediate a humoral response when immunized with T-D antigens [156,232] is therefore at least partly due to the undeveloped or down regulated neonatal splenic helper T cell population.

Adult Ig bearing B cells have been implicated as

stimulatory cells in both autologous and allogeneic mixed lymphocyte reactions [27,29,70,103,104]. However spleen cells from mice under one week old do not possess a detectable capacity to stimulate allogeneic MLCs [5] or AMLCs [27,28,103]. Adult levels of stimulation are not seen in either system until at least four weeks after birth [5,27,103]. Thus during ontogeny there is apparently a parallel development of the ability to stimulate autologous and allogeneic MLCs. The poor stimulatory capacity of newborn spleen cells in MLRs may be related to the low numbers (relative to the adult) of B cells that express Ia antigens during early life. B cells from neonates can also be functionally distinguished from similar adult cells by the fact that they are more sensitive to suppression resulting from anti-immunoglobulin treatment [128,142,166]. In addition, newborn and adult B cells show differences in membrane properties which may indicate changes in membrane fluidity during development [122]. Therefore neonatal B cells, although capable of producing limited antibody responses, show numerous functional and phenotypic differences from adult B cells.

c) Antigen Presenting Cell Functions

In the expression of various types of immune reactivity macrophages are considered very important both in presenting antigen in an immunogenic form [151,245] and in their ability to bind [67,68,199,240] and transmit [198,203] regulatory factors. In addition $Ia^+$ dendritic macrophages have been implicated in the stimulation of allogeneic [236] and syngeneic [177] mixed lymphocyte reactivity. It is now apparent that significant numbers of functional macrophages and dendritic cells are resident within the thymus but not the spleen of the neonatal mouse [134]. Neonatal splenic macrophages show equivalent [135] or enhanced [107]

phagocytic activity as measured by latex bead ingestion when compared with adult splenic macrophages. However, splenic macrophages from newborn mice may not be as efficient in antigen presentation as adult macrophages. Neonatal in comparison with adult splenic macrophages show a decreased ability to induce a proliferative response in immune adult T cells [107,134,135]. The reduced antigen presenting ability of newborn splenic macrophages is apparent for up to three to four weeks after birth [135] and has been suggested to result from the scarcity of $Ia^+$ macrophages in the neonatal spleen [107,134,135]. Thymic adherent cells on the other hand develop the ability to present antigen in an immunogenic form in T cell proliferative assays by three days of age [134]. By six days after birth thymic macrophages are functionally equivalent to those of the adult, at least according to this index of activity [134]. Conflicting results have been presented concerning the ability of newborn splenic macrophages to co-operate with adult lymphocytes in the production of a humoral response [10,23,25,69,125,173,204]. Macrophages from the spleen of neonates under five days of age have been shown to fail to co-operate with competent adult lymphocytes in the development of a humoral response in vitro [125,173]. In contrast neonatal macrophages and adult lymphocytes were seen to synergize effectively to mediate antibody responses in vitro in other investigations [69,156]. The administration of adult macrophages with antigen has, in some cases, been demonstrated to overcome the poor humoral responsiveness of newborn mice in vivo [10,23,25]. This suggests that the neonatal macrophage may be responsible for at least part of the immune hyporeactivity during early life. Moreover, heat killed adult macrophages, while incapable of presenting antigen, enhance the ability of neonatal spleen cells to produce antibody in vitro and in vivo [200]. This implies that the spleen of the neonate does indeed contain

competent macrophages. It is therefore conceivable that the addition of adult macrophages to newborn spleen cells boosts their humoral reactivity by a function unrelated to antigen presentation. Thus the poor ability of neonates to respond in various tests of immune function in vitro and in vivo may not be the result of inadequate numbers of functional macrophages.

In addition to antigen presenting functions it is apparent that $Ia^+$ dendritic macrophages can stimulate both autologous and allogeneic MLCs [177,236]. The low numbers of Ia positive dendritic macrophages in newborn spleen [107,234,235] may contribute to the inability of neonatal spleen cells to stimulate syngeneic [27,28,103] as well as allogeneic MLCs [5]. However it is noteworthy that in addition to auto and alloreactive thymocytes [28,103] a significant proportion of Ia bearing dendritic macrophages is present within newborn thymus [134]. It would therefore appear that all of the cellular components necessary for the generation of an autologous reaction are in the thymus of the newborn.

### iii. Immunoregulation in the Neonate

#### a) Inhibitory Cells

Antigenic stimulation of a population of lymphocytes normally activates both helper and suppressor cells [42]. It is now generally believed that the magnitude of an immune response is the result of a delicate balance between, on one hand, helper and effector activities, and on the other, inhibitory activities [40]. Thus after immunization with a T-dependent antigen in the murine system, Lyt $1^+23^-$ T cells not only help B cells in the production of specific antibody but also induce a feedback inhibitory circuit through Lyt $123^+$ T cells. Activation of this regulatory pathway

culminates in the induction of Lyt $1^{-}23^{+}$ T-suppressor cells which can inhibit the activity of the T-helper cells [41,61,146]. Although the majority of suppressor cells have been identified as T cells, [76,77,78,150] inhibitory cells apparently of B cell [58,59,115,258] and macrophage [63,65, 117,120,132,196] lineages have also been described in various systems. It has been suggested that the ability of the neonate to respond to an immunogenic stimulus is depressed by inhibitory cells that naturally occur during early ontogeny [12,13,14,16,18,37,55,97,99,100,156,157, 161,163,165,184,191, 201,210,214,231].

The presence of immunoregulatory suppressor cells in neonatal mice was originally observed by Mosier and Johnson who showed that newborn spleen cells could inhibit the in vitro antibody responses of adult spleen cells to T-dependent and T-independent antigens [156]. The inhibitory cells described in their investigation were found to be non-adherent to nylon wool columns and therefore probably not B-cells or macrophages [156]. On the other hand the suppressor cells were not sensitive to conventional anti-Thy 1 plus RC treatment but only to negative selection procedures employing high concentrations of this antibody [156]. Thus the newborn splenic inhibitory cell appeared to be a T cell with low Thy 1 density. Mosier and his co-workers subsequently determined that neonatal thymus also contains a suppressor for antibody synthesis within the 5% of cortical thymocytes resistant to infection by mouse thymic virus [53,157]. This inhibitory population was characterized as large cells with high DNA content and a reduced sensitivity to treatment with anti-Thy 1 serum plus RC when compared with normal thymocytes [157]. The Lyt phenotype of the thymic inhibitory cell was determined by cytotoxic treatment with anti-Lyt 1 and anti-Lyt 2 sera to be Lyt $123^{+}$. Mosier et. al. concluded that migration of suppressor cells from thymus to spleen

is responsible for their observations of comparable inhibitory populations in both of these organs [157]. Debre has also detected the presence in newborn thymus and spleen of Lyt $1^+23^+$ cells capable of inhibiting both T-D and T-I humoral responses [55]. In addition, Luchenbach et. al. have described a suppressor cell population within the thymus of fetal and neonatal mice [137]. In agreement with the results of Mosier [157] these investigators also characterized the thymocyte suppressor cell as having low Thy 1 density [137]. However in contrast to the observations of Mosier and his co-workers that neonatal thymocytes suppress both T-D and T-I responses, Luchenbach et. al. concluded that these cells inhibit only T-dependent antibody responses [137].

While several investigators have detected inhibitory cells in neonatal thymus [53,137,157,161] Calkins et. al. were unable to demonstrate suppressor cells for T-D antibody synthesis in the thymus of one week old mice [37,211]. However they have shown that cells inhibitory for T-D PFC responses reside in the spleen at this stage of development [36,37,211]. Calkins and her collaborators characterized the splenic suppressor cell as being non-adherent to Sephadex G-10, glass beads, and nylon wool [36,37,211]. The inhibitory cells also appeared to be relatively insensitive to negative selection with anti-Thy 1 plus RC [36,37,211]. Thus a number of investigators have observed suppressor cells for T-D antibody responses in the thymus and spleen of neonates that do not appear to express the Thy 1 levels of a mature T cell. Due to partial sensitivity to anti-Thy 1 plus RC treatment, anatomical location in the thymus, and especially by virtue of being non-adherent to nylon wool it would appear that these regulatory cells are of the T cell lineage, or there are two suppressor cell populations; one consisting of T cells with low Thy 1, and the other of Non-T cells.

Murgita and his collaborators have described a newborn

spleen cell population which is an efficient inhibitor of T-D humoral responses and may also suppress, to a lesser extent, T-I antibody reactions [99,163,165]. These cells do not adhere to anti-Ig affinity columns, are sensitive to treatment with anti-Thy 1 plus RC, and are therefore T lymphocytes [163,165]. Negative selection studies indicate that this neonatal splenic T-inhibitory cell expresses I-J and Lyt 1 but not Lyt 2 antigens [99,165]. Murgita et. al. have identified the neonatal splenic inhibitor with a functionally and phenotypically identical cell that can be induced from adult spleen cells by incubation with AFP in vitro [99,165]. Thus it appears that the high levels of AFP prevalent during fetal and neonatal life may result in the in vivo induction of an Lyt $1^+23^-$ $I\text{-}J^+$ inhibitor of T-D antibody responses. In vivo treatment of newborn mice with anti-I-J serum significantly improves their ability to respond to the T-D antigen SRBC [165]. In addition the capacity of newborn splenic suppressor cells to suppress in vivo T-dependent humoral responses has also been shown. Adoptive transfer of newborn spleen cells into syngeneic adult mice inhibits the PFC response of the recipients to immunization with the T-dependent antigen SRBC in vivo [99]. Therefore the poor responsiveness of neonatal mice to T-D antigens is likely to be due in part to the activity of the $I\text{-}J^+$ inhibitory cells.

Conventional antigen stimulated suppressor cells possess Lyt 2 but not Lyt 1 antigens [42]. Thus both the neonatal inhibitory populations described by Mosier et. al. (Lyt $123^+$) and by Murgita et. al. (Lyt $1^+23^-$) have atypical Lyt phenotypes for suppressor cells. It is possible that the Lyt $1^+23^-$ newborn T cells do not directly inhibit T-D humoral reactions but instead act as suppressor inducers. Newborn T cells may preferentially activate inhibitory pathways in an immunological circuit [40] leading to the induction of

suppressor cells. Although a number of studies have concluded that T-D humoral reactions are more susceptible to inhibition by newborn splenic T cells [137,163,171] it is apparent that neonatal T cells can also suppress T-I antibody responses [56,97,152,156,163,171]. This implies that newborn T cells may have direct regulatory effects on B lymphocyte functions. On the other hand accumulating evidence suggests that the ultimate target of the inhibitory effects of newborn splenic Lyt $1^{+}23^{-}$ T cells in the T-D humoral response may be the T helper population [171]. These apparently distinct inhibitory activities may indicate the presence of more than one regulatory cell type within the T cell compartment of newborn spleen.

While both Mosier et. al. [156,157] and Calkins et. al. [36,37,211] have shown that the neonatal inhibitors of humoral responses are relatively resistant to cytotoxic treatment with anti-Thy 1 and complement they have concluded that these suppressors are T cells. In contrast other investigators that have confirmed that newborn PFC-inhibitory cells are resistant negative to selection with anti-Thy 1 have suggested that the inhibitory population is not a T cell [191,210]. Piguet et. al. showed that neonatal spleen cells capable of suppressing T-D antibody responses <u>in vitro</u> were not enriched by passage through nylon wool columns, which according to their data increased the proportion of T cells by 4 to 10 fold [191]. They therefore concluded that the neonatal splenic PFC-suppressor cells are not T cells [191]. On the other hand these investigators demonstrated that the inhibitory cell population is partially sensitive to treatment with silica particles which are toxic for macrophages [191]. They also observed that cultures of newborn spleen cells generate considerably higher numbers of macrophages than cultures of adult spleen cells [191]. Thus Piguet et. al. concluded that newborn spleen cells suppress

PFC reactions by virtue of an excess of macrophages and that the inhibitory cells are macrophages differentiating in vitro from monoblastic precursors [191]. It is noteworthy that other investigators have demonstrated that newborn splenic macrophages apparently possess adult levels of phagocytic and adherence activities [107,134,135]. However Piguet et. al. were unable to remove more than 10% of the inhibitory capacity of newborn spleen cells with silica treatment and did not show adherence of the suppressor cells to nylon wool [191]. It is therefore conceivable that these investigators may not be correct in their conclusions that newborn PFC-inhibitory cells are macrophages. Recently, Peck et. al. have produced evidence that macrophages treated in vitro with AFP may induce T cells inhibitory for T-D PFC responses [188]. Thusit is possible that Piguet et. al. obtained suppression of antibody reactions through in vitro induction of inhibitory T cells by neonatal macrophages which have been subjected to high concentrations of AFP in vivo. The report of Rodriguez et. al. suggested that newborn splenic suppressors are not T cells, primarily basing this conclusion on the results of experiments performed in MLCs [210]. Their observations therefore may not be relevant to the discussion of inhibitory cells active in humoral reactions and will be discussed below. Thus the majority of investigations have concluded that neonatal T cells can inhibit T-D and/or T-I PFC responses in vitro. However it is evident that there are distinct differences in the characterization of the inhibitory cell population within the various studies.

In addition to inhibiting humoral responses, newborn spleen cells can also suppress the in vivo generation and/or recruitment of $Ia^+$ macrophages [231]. Snyder et. al. showed that peritoneal exudates containing a high proportion (approximately 30 to 60 %) of $Ia^+$ macrophages are induced in adult mice by administration of Listeria-immune T cells and

heat killed Listeria or macrophage Ia-recruiting factor (MIRF) [231]. Adult mice that had received neonatal spleen cells were found to respond to these stimuli with peritoneal exudates containing significantly reduced numbers of $Ia^+$ macrophages [231]. The newborn spleen cell mediating this inhibitory effect was determined to be non-adherent to plastic plates and nylon wool yet resistant to treatment with anti-Thy 1 plus RC [231]. Comparable suppressor activity was found in adult bone marrow and peritoneal cells but not in neonatal thymocytes [231]. Snyder et. al. showed that the inhibition by newborn spleen cells of the appearance of $Ia^+$ macrophages in peritoneal exudates following Listeria-immune T cells plus heat killed Listeria or MIRF treatment is sensitive to indomethacin and aspirin administration in vivo [231]. Thus they concluded that the phagocytic system is involved in the suppression [231]. However further experiments are required in this system to determine the nature of the neonatal spleen cells that initiate this inhibitory mechanism.

Spleen cells from neonatal mice of numerous inbred strains also suppress MLRs [12,13,14,16,18,100,178,179,184, 210]. The inhibitory activity of newborn spleen cells in MLCs has in most cases been shown not to be genetically restricted. Thus, neonatal splenocytes can suppress MLC induced proliferation in various strain combinations involving syngeneic as well as allogeneic responders [178, 179]. Several investigations have reported different rates of decay for the inhibitory activity of newborn spleen cells with increasing age in MLCs [14,179,184]. Argyris has presented evidence that the apparent differences in the loss during maturation of the ability of spleen cells to suppress MLRs may be the result of different strains of mice being employed in the various investigations [14]. In four out of the five strains compared by Argyris, the ability of spleen

cells to suppress MLRs began to decrease approximately ten days after birth and disappeared between seven and eleven weeks afterwards [14]. However in a fifth strain, suppressor activity declined rapidly from ten to twelve days after birth and more gradually thereafter disappearing at approximately eight weeks of age [14]. Thus the rate of the loss with increasing age of the MLC-inhibitory capacity of neonatal spleen may be genetically determined. Interestingly, the loss in suppressor ability apparently parallels the development of the capacity of young spleen cells to stimulate in allogeneic MLCs [14,184]. This suggests that the MLC-stimulatory capacity of newborn spleen may be self-regulated by inhibitors within the spleen cell population.

The characterization of the newborn splenic MLC-suppressor has led to different conclusions as to the nature of the cell, comparable to those encountered in the definition of the neonatal cell type inhibiting humoral responses. Pavia and Stites showed that the MLC-inhibitors in newborn spleen are susceptible to anti-T cell serum [88] plus RC treatment [184]. They therefore concluded that the suppressors are T cells [184]. On the other hand, the results of Argyris indicate that the inhibitory activity of newborn spleen cells in MLCs is only partially sensitive to negative selection with anti-Thy 1 [12,13,16]. At the opposite extreme, Rodriguez et. al. demonstrated that this inhibitory population is insensitive to cytotoxic treatment employing anti-Thy 1 [210]. Rodriguez et. al. also showed that the MLC-inhibitors in newborn spleen are resistant to negative selection with anti-Ia serum [210]. In contrast, Argyris has presented evidence that these cells are partly susceptible to treatment with anti-I-J and anti-Lyt 1 but not anti-Lyt 2 plus RC [16]. However both groups have determined that the suppressor population consists of non-adherent spleen cells and that neonatal thymocytes do not have the

capacity to inhibit MLCs [12,13,210]. Rodriguez et. al. have further characterized the inhibitory cells as being adherent to Ig-anti-Ig affinity and nylon wool columns [210]. These investigators also demonstrated that the spleens of nude mice contain a similar suppressor cell [210]. While Argyris concluded that the neonatal splenic MLC-inhibitors are probably T cells [12,13,16], Rodriguez et. al. decided that they were neither T, B, nor macrophages [210]. These investigators termed the suppressors Non-T cells [210]. Removal of the Non-T population from newborn spleen by Ig-anti-Ig column passage was indeed shown by Rodriguez et. al. to improve the allogeneic MLC responsiveness of the remaining newborn T cells [210]. However Rodriguez and his collaborators were apparently unable to provide an explanation for the ability of the inhibitory cells to adhere to the affinity column. Thus there is no discernable agreement in the literature as to the nature of the population of cells in newborn spleen that is capable of inhibiting MLRs. While it is evident that during early ontogeny splenic T cells may have such activity, it is also possible that another, as yet undefined, class of neonatal spleen cell has the ability to suppress MLRs.

The ability of newborn spleen cells to inhibit the generation of effector cells in MLCs has also been examined. Okada and Strober have shown that neonatal spleen cells added to MLCs prevent the normal generation of antigen non-specific but not antigen specific suppressor cells in the cultures [178,179]. In addition they have demonstrated that newborn spleen cells inhibit the development of CTLs in MLC reactions involving various strain combinations [179]. Similar results were obtained by Argyris who also showed inhibition of CTL generation by neonatal spleen cells [14]. On the other hand, Pavia and Stites showed significant suppression of the proliferation but not CTL production in MLRs [187].

Rollwagen and Stutman have indicated that precultured, but not fresh, spleen cells from mice less than one week of age are capable of suppressing the allogeneic MLR but not the development of CTLs [211,212]. In contrast, Ross and Pilarski have determined that precultured but not fresh Thy 1 positive spleen cells from mice between 1 and 9 days of age inhibit the generation of CTLs in MLCs [214]. Both Rollwagen and Stutman [211,212] and Ross and Pilarski [214] detect differences in the activities of suppressor cells induced by preculture of adult versus neonatal spleen cells. However it is unknown why preculture is required in their systems, and not in others, to show the inhibitory capacity of newborn spleen cells. Factors responsible in the different studies for the opposing results concerning the ability of neonatal cells to suppress CTL induction are not readily apparent. Presumably these controversies may be consequential to differences in the strain combinations and/or culture systems employed by the various investigators.

The capacity of fetal and newborn spleen cells to inhibit reactions of cellular immunity in vivo has also been demonstrated [201,227]. Fetal and one day old neonatal spleen cells can suppress the ability of syngeneic adult cells to induce local graft versus host reactions in $F_1$ mice [227]. This inhibitory activity was found to be restricted to the spleen of fetal and newborn mice and was sensitive to treatment with anti-Thy 1 plus complement [227]. The splenic GVH suppressor appears to be short lived as it is not detectable five days after birth [227]. Newborn spleen cells have also been shown to inhibit the passive transfer of contact sensitivity to syngeneic recipients [201]. Thus it is evident that neonatal spleen cells can depress cell mediated immunity in vivo as well as in vitro.

The neonatal inhibitory cells discussed above suppress cellular and humoral reactions without known prior exposure

to the stimulatory or immunizing antigens. This implies that the suppressor cells are not activated by processes resulting from antigen administration. In addition, the activity of newborn regulatory cells does not appear to be restricted to particular antigenic determinants. Thus the neonatal suppressors are generally referred to as being naturally occurring, non antigen specific inhibitory cells. On the other hand, both antigen specific and non-specific suppressor cells can be induced from populations of resting newborn lymphocytes via conventional antigenic stimulation [37,179, 211]. There is now accumulating evidence that immunization of the neonate leads to preferential activation of inhibitory versus helper pathways within an immunological circuit [200]. This apparently results primarily in the induction of non-antigen specific suppressor cells [37,140, 214]. Thus in addition to having relatively high proportions of naturally occurring inhibitory cells, the neonatal immune system when stimulated is evidently predisposed to activating increased numbers of antigen non-specific suppressor cells.

b) Humoral Regulatory Factors

A number of investigations have demonstrated that normal serum from adult mice contains factors capable of regulating in vitro immunological activities [33,143,174,249]. At present it is unknown whether an overabundance, during early ontogeny, of one or more of these naturally occurring factors contributes to the depressed immune reactivity of the newborn. Neonatal mouse serum has been shown to inhibit in vitro T-dependent humoral responses [124,162], MLRs [124], and mitogen reactions [124]. Immunosuppressive effects of the fluid from the amniotic sac of fetal mice (MAF) are apparent both in vitro and in vivo [66,108,169,243]. MAF has been shown to inhibit antibody responses to T-dependent and

independent antigens as well as polyclonal antibody production induced by LPS [66,169]. The capacity of MAF to inhibit T-dependent humoral responses has been demonstrated to be due to AFP [169], a glycoprotein of approximately 70,000 molecular weight [217]. On the other hand, the ability to suppress T-independent antibody synthesis apparently resides in a MAF component of molecular weight less than 10,000 [171]. AFP, primarily synthesized by fetal liver [1,74,82], is a major component of amniotic fluid and serum during embryonic development [81,144] and is also present in high levels in newborn mouse serum [180]. After birth serum AFP concentrations decrease from milligram to low nanogram levels found in normal adult sera [180,216,217]. In adulthood AFP levels transiently rise during pregnancy and in association with certain disease processes [1,3]. Interestingly, some antigenic properties of AFP are retained among various mammalian species [109] and AFP appears to be structurally related to albumin [215,218]. AFP is normally present in a number of molecular subspecies [8,21,129,228, 247] partially due to differences in degree of sialylation [129,130].

Purified murine AFP has been demonstrated to have selective regulatory properties in various immunological reactions [reviewed in 170]. AFP significantly inhibits the in vitro PFC response of adult spleen to T-D [167,169,170] but not to T-I [169,170] antigens or polyclonal stimulation by LPS [169]. Dose response studies in vitro indicate that a hierarchy of sensitivity to inhibition by AFP of the various immunoglobulin classes exists, with IgA responses being more sensitive than those of the IgG class and IgM responses being the least sensitive [167]. It is now apparent that the differences in susceptibility of the immunoglobulin classes to AFP induced suppression result from their relative T cell dependency [50,138] and the preferential action of AFP on T

cell activities [171]. AFP has been shown to induce suppressor cells in vitro from unstimulated adult spleen cells and it is likely that the inhibition of T-dependent humoral responses by AFP is mediated through these cells [163,164,165]. The phenotype of the inhibitory cells induced from adult whole spleen or isolated splenic T cells by AFP is Thy $1^+$, Lyt $1^+23^-$, I-$J^+$ and identical to that of a naturally occurring suppressor T cell in the spleens of newborns which has the same immunoregulatory attributes [99,163,165]. Both newborn and adult AFP activated suppressor cells on adoptive transfer into adult mice inhibit the in vivo response of IgG and IgA immunoglobulin subclasses to SRBC [99]. Thus it is possible that AFP contributes to the depression of T-dependent humoral reactivity in the newborn mouse through induction of suppressor cells.

In addition to inducing inhibitory cells , AFP also appears to have direct anti-proliferative activity. AFP inhibits the reactions of adult spleen cells to the T-cell mitogens PHA and CON A as well as the B-cell mitogen LPS [168]. It has been suggested [171] that the targets for AFP mediated suppression of LPS mitogenic responses may be T-cells that regulate the reaction of B cells to this mitogen [176]. Earlier results with mitogen reactions indicated that T-cells responding to PHA may be more sensitive to inhibition by AFP than similar cells stimulated by CON A [169]. These findings suggest that AFP may have differential effects on subpopulations of T-cells [169].

Anti-proliferative effects of AFP are also observable in mixed lymphocyte reactions [168,186]. In addition AFP inhibits the generation of CTLs in the MLC [187]. The suppressive effects of AFP on both the proliferation and induction of CTLs in one-way MLRs resulting from combinations of lymphocytes from various strains of inbred mice have been extensively studied [186,187]. The results of these studies

indicate that both primary and secondary proliferative responses occurring solely as a result of I region incompatibilities between responder and stimulator cells are the most sensitive to AFP mediated inhibition. In contrast, proliferative responses induced by MHC K or D antigenic differences were determined to be largely unaffected by AFP. MLRs resulting from non-MHC Mls locus genetic differences also appear to be relatively susceptible to inhibition by AFP [186]. This is possibly a result of the requirement for concomitant recognition by the responder population in this class of MLR of self-Ia plus foreign Mls determinants on the stimulator cells [185]. In contrast to MLRs stimulated by Mls locus disparity, proliferation induced by other non-MHC differences was found to be either unaffected or strengthened by the addition of AFP [186]. Thus reactions directed against I region differences, mediated predominantly by Lyt $1^+23^-$ cells, are suppressed by AFP. In contrast responses to K or D antigens, consisting primarily of Lyt $1^-23^+$ cells, are not inhibited by AFP. On the other hand, both Lyt $1^+23^-$ and Lyt $1^-23^+$ cells react against non-MHC antigens in MLCs which are refractory to inhibition by AFP. It is therefore apparent that the susceptibility of a particular proliferative response is not related to the Lyt phenotype of the predominating responder population.

While AFP does not inhibit proliferation against K or D antigens, this protein has been shown to inhibit the generation of CTLs in MLCs induced by MHC differences at either I, K, or D loci [187]. Only MLRs against non-MHC antigens distinct from those encoded by the Mls locus were determined to be capable of generating CTLs in the presence of AFP [187]. The addition of AFP to the effector phase of the CTL assay instead of the proliferative phase was found to be without effect irrespective of the strain combinations employed in the MLR [187]. Therefore AFP does not have the

capacity to inhibit the function of activated CTLs while being able to prevent their development in certain classes of MLRs. While AFP appears to have direct inhibitory effects on T cells proliferating in MLRs, Peck et. al. [188] have concluded that AFP may not suppress CTL induction by interacting directly with pre-cytotoxic T cells. They have suggested that, alternatively, AFP may act through a monocyte enriched MLC-stimulating population which in turn activates T-suppressor cells capable of preventing the development of CTL effectors [188].

Evidently AFP has several selective immunoregulatory effects which can be exerted via direct anti-proliferative effects and through the activation of regulatory cells that can inhibit T-D humoral reactions and CTL generation. The presence of an elevated serum AFP concentration throughout early ontogeny suggests that inhibitory activities of this protein may contribute to the low immune reactivity apparent in the neonate. It is, of course, also likely that other factors elaborated by neonatal lymphoid cell populations in vitro may be active in vivo. For example, Argyris [15] has demonstrated that non-adherent Thy 1 negative newborn spleen cells produce a soluble suppressor factor when cultured for two to four days in 10% FCS. This factor inhibits MLC reactions of adult cells without noticeable antigenic specificity. Other investigators have also determined that neonatal spleen cells may suppress MLRs through the production of inhibitory factors [18,184]. However the most enticing evidence suggesting that immunoregulation in the newborn may occur at least in part through the action of immunosuppressive factors has been presented by Ptak et. al. [200]. They showed that heat killed macrophages are capable of binding but not transmitting suppressor factors [200]. Injected into neonatal mice, heat killed macrophages caused an increase in the ability of the animals to respond to T-

dependent antigens presumably by reducing the concentration of inhibitory factors [200].

iv. Overview.

There is little doubt that the acquisition of immunocompetence during ontogeny is related to the appearance of mature lymphocytes and macrophages. However, the presence of active cellular and humoral immunoregulatory mechanisms in the neonate alludes to the necessity of controlling some but not all immunological processes. Thus it seems likely that during fetal life limited expression of immune functions must develop to protect the animal from invading microorganisms. Within a few days of birth there are functional T cells especially in the thymus capable of responding in MLCs to stimulation from allogeneic as well as autologous cells. The newborn thymus also contains Ia-bearing dendritic cells which are presumed to be able to stimulate autologous reactions. It is therefore apparent that the neonate must be able to prevent potentially damaging autoreactive events. In addition it is evident that the developing animal has to defend itself from maternal lymphocytes sensitized against paternal and fetal antigens. Effective suppressor cells as well as inhibitory humoral factors such as AFP may prohibit such destructive processes and may be involved in the establishment of neonatal tolerance to self and other antigens. The results of this investigation will show that: a) isolated T lymphocyte populations from the newborn mouse possess nearly adult capacity to respond to mitogens, allogeneic and autologous stimulation at an age much earlier than previously thought; b) two distinct cell populations in neonatal spleen suppress MLC and AMLC induced proliferation; c) newborn spleen cells show the capability of self-regulation of proliferative responses; and d) AFP has

specialized direct inhibitory properties on specific populations of T cells particularly those responding to autologous stimulation.

## VII. MATERIALS AND METHODS

### i. Animals.

The inbred CBA/J and Balb/cJ mice employed in this investigation were either purchased from or bred from mice obtained from The Jackson Laboratory (Bar Harbor, Maine). Adult animals of both strains were used from six to thirty weeks of age. Whereas both male and female adult mice were used in mitogen and allogeneic cultures, only adult CBA/J females were employed in autologous cultures. Newborn CBA/J mice of both genders were used generally from the day of birth to five days of age in the analysis of inhibitory capacity and from the day of birth to three days of age as a source of responders in the autologous mixed lymphocyte cultures. All animals were bred and maintained in our animal facility at the Department of Microbiology and Immunology, McGill University. Newborn and adult C57Bl/6 x C3H $F_1$ mice were supplied by Dr. J. Gordon of the Division of Surgical Research, McGill University.

### ii. Cell Preparation.

Single cell suspensions were prepared from aseptically removed adult murine spleen, thymus, and lymph node as well as from newborn spleen and thymus by gentle mincing of the tissue through stainless steel mesh into PBS. Bone marrow cells were rinsed from excised adult femurs with PBS. All cell preparations were mixed with a pasteur pipette and placed in 15 ml polystyrene tubes (Fisher Scientific; Corning Cat. # 25311). Cell clumps were allowed to sediment for approximately 10 minutes before the cell suspensions were decanted off into new tubes. After a minimum of two washes in PBS by centrifugation at 1000 rpm in a Beckman TJ-6 centrifuge (Beckman Instruments Inc., Montreal, Quebec) the cells were counted and assayed for viability by their ability to exclude 0.1% trypan blue. Cell preparations found to

have less than 90% viability at this stage were discarded. Peripheral blood lymphocytes were separated from whole mouse blood collected by cardiac puncture and immediately diluted in Alsever's solution (1:1). The blood cells were pelleted by centifugation at 1500 rpm, washed twice in PBS, and then diluted in PBS and layered on a step gradient of 35% and 65% isotonic Percoll (Pharmacia Ltd., Dorval, Quebec) [123]. Following a 20 to 30 minute spin at 2000 rpm the isolated lymphocytes were removed from the 35/65% Percoll interphase with a pasteur pipette and washed twice in PBS prior to being counted in trypan blue. When desired, red blood cells were lysed by hypotonic shock using ice cold distilled $H_2O$.

iii. Antisera and Cytotoxic Treatments.

A variety of anti-sera specific for T cells were employed in this study including: monoclonal anti-Thy 1.2 purchased from NEN Canada (Lachine, Quebec; NEI-001, lot # FPA-171) and from Becton Dickinson (Mountain View, California; Cat. # 1330, lot # A0713); conventional AKR anti-C3H (anti-Thy 1.2; Bionetics, Kensington, Maryland; Cat. # 8301-02, lot # BJ107); heterologous rabbit anti-mouse brain associated T sera (anti-T) a gift of S. Kontiainen and also purchased from Cedarlane Laboratories (Hornby, Ontario; Cat. # CL2005, lots # 270, 280, 290); anti-macromolecular cold insoluble globulin (anti-MICG) generously provided by S. Hauptman and described in detail elsewhere [20]; monoclonal anti-Lyt 1.1 (NEN; Cat. # NEI-003, lot # FPA-172); and monoclonal anti-Lyt 2.1 (NEN; Cat. # NEI-004, lot # FPA-066). Other commercial anti-sera employed were: A.TH anti-A.TL anti-$Ia^k$ alloanti-serum (Cedarlane; Cat. # CL8701, lot # 4913); monoclonal anti-$Ia^k$ (Becton Dickinson; Cat. # 1300, lot # A0701); rabbit anti-mouse IgM (Daymar Laboratories, Toronto, Ontario; Cat. # R-317, lot # 19); and goat anti-mouse IgM (Meloy Laboratories, Springfield, Virginia; Cat. #

B107, lot # 51701). Anti-sera prepared in our laboratory by immunization of New Zealand albino rabbits and used in this investigation included rabbit anti-mouse poly IgG, rabbit anti-mouse AFP, and rabbit anti-mouse Ig. Two types of fluorescein conjugated anti-mouse IgM sera were employed in the enumeration of surface IgM bearing B cells: goat anti-mouse IgM (Meloy; Cat. # C-402, lot # 51765); and rabbit anti-mouse IgM (Cedarlane, Zymed Cat. # 61-6711, lot # 10602).

Cytotoxic depletion of cells sensitive to an anti-serum was carried out in a two step negative selection protocol. Approximately 10 to 30 x $10^6$ cells were treated with 0.3 to 0.5 ml of a previously determined optimal concentration of anti-serum for 30 to 45 minutes at 37°C in a waterbath. The cells were then pelleted by centrifugation at 1000 rpm for 10 minutes, the anti-serum removed, and the cells resuspended and incubated for a further 45 minutes at 37°C in 1.0 ml of a 1/8 to 1/10 dilution of rabbit complement (Cedarlane; Low Tox M, Cat. # CL3051, lots # 4045, 4072). Cells surviving this treatment were washed a minimum of three times prior to use. In experiments designed to assay the function of cell preparations specifically depleted of a subpopulation by anti-sera plus complement it is desirable that the numbers of cells added to treated versus control cultures reflects the loss of the specific population. This condition was fulfilled by performing the negative selection protocol as follows. Prior to treatment the starting cell preparation was aliquoted into a minimum of three identical fractions. Each fraction then received equivalent treatment, according to the above protocol for negative selection, with the exception that one group was treated with media instead of anti-sera anda second group received medium in the place of both anti-sera and complement. The third aliquot was treated with both anti-sera and complement. Following completion of the

cytolytic procedure and washes, all three aliquots were resuspended in medium to the same volume and counted in trypan blue. When a significant difference in the recovery of cells between medium and complement treated groups was detected, suggesting cytotoxicity due to the complement itself, the cells were discarded. In the majority of cases no such difference was noted. The dilution factor calculated to set the medium treated cells to the final working concentration was also used for the complement and anti-serum plus complement treated aliquots. Addition of equal volumes of diluted cells to the cultures resulted in the anti-serum plus complement treated cultures receiving only the fraction of resistant cells which is resident within the medium treated control cultures. Direct assays of cytotoxicity were performed using a similar two step protocol employing 1 x $10^6$ cells plus 0.1 ml of antiserum followed by 0.2 ml of complement in the wells of a microtitre plate.

iv. In Vivo Anti-IgM Treatment.

The newborn CBA/J mice to be used as donors of cells treated in vivo received 0.1 ml of undiluted NRS or rabbit anti-mouse IgM (Daymar) through intra-peritoneal injection on the day of birth and at 2 days of age. Lymphocytes were removed from these animals at 4 days of age and were prepared in the usual fashion. C57/BL6 x C3H $F_1$ mice were treated with anti-IgM from birth until their use at 6 to 7 months of age. These animals were supplied by Dr. J. Gordon and the protocol for their treatment is described in detail elsewhere [90].

v. Affinity Column Purification of T Cells.

T cells were isolated from the various lymphoid cell suspensions according to the protocol of Wigzell [254]. Cell preparations were adjusted to between 20 and 50 X $10^6$

cells/ml in PBS and then passed through columns containing glass beads consecutively coated with mouse immunoglobulin and then rabbit anti-mouse immunoglobulin. From a suspension of unselected spleen cells of either adult or newborn origin, the cells filtering through the Ig anti-Ig affinity column can be shown to consist of greater than 90% T cells by sensitivity to anti-T cell serum plus complement and of less than 1% Ig bearing B cells by direct immunofluorescence with anti-IgM. T cells prepared from spleen by this method consisted of approximately 18 to 30% of the starting cell population with a viability of at least 98%.

vi. Soybean Agglutinin Fractionation of Spleen Cells.

This technique is based on the ability of SBA to selectively agglutinate murine B-cells. SBA was purchased from Vector Laboratories (Cedarlane; Cat. # L1010-L) and the procedure employed was their adaptation of the technique of Reisner et. al. [208]. Briefly, equal amounts of 2 mg/ml SBA and 400 x $10^6$ spleen cells per ml in medium were mixed and then incubated for 15 minutes at room temperature. The mixture was then layered on 50% FCS (Gibco, Montreal, Quebec; Microbiological Associates, Bethesda, Maryland) and after 30 minutes at room temperature the bouyant T-cells and the pelleted agglutinated B-cells were recovered. After three washes in 0.2 M galactose, the competing sugar for SBA, and two washes in PBS the cells were ready for use. Typically, the resultant $SBA^+$ B-cell fraction contained over 80% IgM positive cells in adult preparations and approximately 50% IgM positive cells in newborn preparations as determined by immunofluorescence with FITC conjugated anti-IgM. In general, $SBA^-$ T-cell fractions from both age groups contained less than 5% IgM bearing cells. In our hands, functional analysis of the two cell fractions showed reactivity to the T cell mitogens CON A and PHA to be limited

to the $SBA^-$ population.

vii. Peanut Agglutinin Separation of Thymocytes.

PNA (Cedarlane; Vector Product # L-1070) was employed to divide thymocytes into mature ($PNA^-$) and immature ($PNA^+$) subpopulations. The technique was essentially that of Reisner et. al. [207]. Briefly, 400 x $10^6$/ml thymocytes were mixed with an equal volume of 1 mg/ml PNA in medium. Following incubation for 10 to 15 minutes at room temperature the mixture was layered on 20% FCS in PBS and allowed to sediment for 20 minutes. $PNA^-$ thymocytes floating on the FCS and pelleted $PNA^+$ were then separately removed using a pasteur pipette. The isolated cell preparations were washed once, then incubated for 20 minutes before being washed a second time in a solution of the competing sugar for PNA (D-galactose, 0.15 M in PBS). A final two washes in PBS were performed before use of the cells.

viii. Macrophage Depletion.

Three techniques were employed to remove macrophages from spleen cell suspensions including plate adherence, Sephadex G-10 column passage, and carbonyl iron plus magnet treatment. Plate adherence was performed by incubating 10 to 60 x $10^6$ cells in 5 mls of medium plus serum on 100 mm plastic petri plates (Fisher Scientific, Montreal, Quebec; Falcon # 1007) at 37°C for 1 hour [153]. Non-adherent cells were then siphoned off and the procedure repeated one to two times on this fraction before their use. Adherent cells were recovered from the first plates, after they had been thoroughly rinsed with PBS, by mechanical means using a rubber policeman. The depletion of adherent cells by passage through a Sephadex G-10 (Pharmacia) column was based on the technique of Ly and Mishell [139]. Up to 200 x $10^6$ cells at 20 x $10^6$/ml in medium plus sera were run through 10

cm by 1.5 cm diameter columns of Sephadex G-10 pre-warmed to 37°C. Carbonyl iron (A.D. Mackay Rare Metals and Chemicals, Darien, Connecticut) was used to remove phagocytic cells as follows. 10 mg/ml Fe was mixed with spleen cells at a density of $10 \times 10^6$/ml in medium plus serum for 30 minutes at room temperature. Free and phagocytosed iron was then pelleted by magnetism and the cell suspension was passed through a pasteur pipette over a magnet twice. Following these procedures the cells were washed at least twice.

ix. Rosetting Through Fc and C3b Receptors.

Tests for the presence of Fc or C3b receptors on newborn spleen suppressor cells was analysed using a modification of the rosetting technique of Gelfand et. al. [75]. For Fc receptor rosettes, SRBC (5%, v/v) sensitized for 30 minutes at 37°C with a 1/1000 dilution of hyperimmune mouse anti-SRBC serum (primarily non IgM based on insensitivity to 2-mercaptoethanol) were used. SRBC similarly sensitized with a 1/100 dilution of mouse anti-SRBC serum from day 5 of a primary immunization (primarily IgM on the basis of sensitivity to 2-mercaptoethanol) followed by a 1/10 dilution of fresh CBA/J serum were employed for C3b receptor rosettes. In both cases spleen cells were rosetted with sensitized SRBC at a ratio of 1:20 for 30 minutes at 37°C and then layered on a step density gradient of 30% and 60% Percoll. The gradient was centrifuged at 2000 rpm for 20 minutes which pelleted the rosetted cells through the Percoll while leaving the non-rosetting lymphocytes floating on the 60% fraction. SRBC were removed from the lymphocytes by lysis through hypotonic shock with $H_2O$. Approximately 30% of adult spleen cells were found in the C3b rosetted fraction in contrast with less than 3% found in the equivalent newborn preparation. The relationship between adult and newborn spleen cells for Fc receptor rosetting cells was determined to be approximately

the same.

x. Isolation of Surface Ig-positive Cells.

Two techniques were employed to separate cells on the basis of membrane Ig expression. The first procedure involved pretreating cells with anti-Ig followed by rosetting the cells with protein-A coated SRBC using the protocol of Johnson [111]. 0.2 ml of packed SRBC were incubated with 0.2 ml of protein-A (Pharmacia) plus 0.2 ml of 1 mg/ml $CrCl_3$ for 4 minutes and then washed thoroughly. Non-T lymphocytes prepared by SBA agglutination as described above were treated with a 1/6 dilution of rabbit anti-mouse Ig for 1 hour in an icebath, washed, and then mixed with the protein-A coated SRBC at a ratio of 1:50. Following a 10 minute centrifugation at 100 g and 4°C the cells were allowed to rosette for 1 hour in the icebath. Rosetted cells were then isolated using a 60%/30% Percoll step gradient as described in the preceeding section.

Ig bearing cells were also separated using the specific anti-Ig plate adherence technique of Wysocki and Sato [257]. 100 mm plastic petri plates (Fisher Scientific; Cat. # 8-757-12) were coated for approximately 1 hour at room temperature with 8 ml of the twice 18% $Na_2SO_4$ precipitated gamma globulins of rabbit anti-mouse Ig serum at a final concentration of 500 ug/ml in Tris buffer (0.8 M, pH 9.5). Following thorough washing of the plates, SBA separated Non-T lymphocytes in 4 mls of PBS at 5 x $10^6$/ml were added and incubated for 70 minutes at room temperature with the cells being gently mixed at 40 minutes. Ig-negative non-adherent cells were then aspirated off and the plates washed 3 times with PBS before the Ig-positive cells adhering to the plate were removed by vigorous pipetting. All cells were washed at least once in PBS prior to use.

xi. Purification of Alpha-fetoprotein.

Mouse AFP was purified from amniotic fluid by passage through an anti-NMS affinity column as previously described [167]. AFP isolated using this procedure can be demonstrated to be pure on the basis of specific immunoprecipitation in gel and by polyacrylimide gel analysis. Purified AFP was quantified by absorbance at 280 nm with a calculated extinction coefficient of 0.443 absorbance units per mg. Quantitation of AFP was also performed by rocket immunoelectrophoresis using monospecific rabbit anti-mouse AFP and following the technique of Weeke [17,252]. Rat AFP was purified from rat amniotic fluid by passage over an anti-NMS column while human AFP was isolated from the ascitic fluid of patients with hepatoma according to the protocol of O'Neill et. al. [181].

xii. Microtiter Cell Culture and Assay of Proliferation.

Cultures were performed in 96 well round bottom microtitre plates (Flow Laboratories, Mississauga, Ontario; Linbro Cat. # 76-013-05) at 37°C in an atmosphere of 5% $CO_2$, 95% air, and 98% humidity. The medium employed was $RPMI_{1640}$ (Flow Labs; Cat. # 12-602-54; Johns Scientific, Toronto, Ontario; M.A. Bioproducts # 12-702Y) supplemented with 20 mM HEPES (Gibco; Cat. # 380-5630; Johns Scientific; M.A. Bioproducts # 17-737A), 5 x $10^{-5}$ M 2-mercaptoethanol (Eastman, Rochester, New York), 4 mM l-glutamine (Gibco; Cat. # 320-5030; Johns Scientific; M.A. Bioproducts # 17-605C), 100 U/ml penicillin "G" (Gibco; Cat. # 860-1840), and 100 ug/ml streptomycin sulfate (Gibco; Cat. # 860-1860). Cultures were further supplemented with 1 % FCS or 0.5 % fresh CBA/J NMS. In the different experiments either 200 or 220 ul of medium were used per microtiter well. At desired intervals after initiation, cultures were pulsed with 1 uCi per well of $^{3}H$-thymidine of specific activity from 50 to 85

Ci/mmole (NEN; Cat. # NET-027Z) in 20 ul of media. 5 to 6 hours afterwards the cultures were harvested onto glass fibre mats (Flow Laboratories; Cat. # 78-105-05) using a Titertek multiple sample harvester (Flow Laboratories) and the insoluble radioactivity was counted using standard liquid scintillation procedures in a Beckman LS8000 or an LKB 1216 Rackbeta (Fisher).

xiii. Mitogen Induced Proliferation.

Three mitogens were employed in this study, the T-cell mitogens PHA and CON A as well as the B-cell mitogen LPS. In all mitogen experiments 3 or 4 replicate cultures containing 250,000 cells per microtitre well and a previously determined optimal concentration of mitogen were set up as described above. PHA was obtained from Wellcome Reagents Ltd. (Beckenham, Great Britain; Purified Phytahaemagglutinin, Cat. # HA 17, lot #s K4515 and K4568). Dilution analyses showed that these lots of PHA induced maximal proliferation, assayed by $^{3}$H-thymidine incorporation, in all adult and newborn T-cell populations tested when used at concentrations from 1 to 2 ug/ml. CON A (Pharmacia; lot # DB 5333) showed maximal mitogenic activity with concentrations from 0.5 to 1 ug/ml in similar analysis while LPS (Sigma Chemical Company, St. Louis, Missouri; E. coli serotype 055:B5, Cat. # L-2880, lot # 89C-0529) induced optimum proliferation in spleen cells from both adult and newborn CBA/J mice at a concentration of 25 ug/ml. An investigation into the kinetics of mitogen induced proliferation in our culture system indicated that the three mitogens employed caused maximal $^{3}$H-thymidine incorporation within an interval of 36 to 48 hours of culture in all cell populations tested. In subsequent experiments with mitogens, cultures were routinely pulsed 5 to 6 hours prior to their harvest at approximately 48 hours after initiation.

xiv. Allogeneic Mixed Lymphocyte Culture.

Responder populations in the majority of experiments reported here consisted of 250,000 cells from various lymphoid organs of adult as well as from spleen and thymus of newborn CBA/J mice. Stimulator cells were either 250,000 or 500,000 spleen cells from adult Balb/c mice, a strain differing from the CBA/J strain at a number of genetic loci including H-2 and Mls encoding regions. Balb/c spleen stimulator cells were prevented from proliferating by pretreatment for 30 minutes at 37°C with 25 to 50 ug/ml of mitomycin C (Sigma; Cat.# M0503). In allogeneic experiments designed to assay newborn spleen suppressor activity 250,000 adult CBA/J spleen cells were used as responders in the control cultures. Up to 250,000 newborn CBA/J spleen derived cells were added to control cultures. In the experiments reported here the addition of 250,000 adult CBA/J spleen cells to control cultures did not significantly reduce the peak $^{3}$H-thymidine incorporation of the cultures, and is often omitted in the interest of simplification. Allogeneic cultures were performed in triplicate or quadruplicate.

xv. Autologous Mixed Lymphocyte Culture.

Autologous cultures generally consisted of 250,000 CBA/J from various lymphoid sources as responders plus an equal number of whole or T-depleted spleen cells from adult CBA/J spleen as stimulators. In studies on newborn suppressor cells the control AMLC was composed of 250,000 of each of newborn CBA/J thymocytes and syngeneic adult spleen cells. 250,000 or less newborn CBA/J spleen derived cells were added to the control cultures. Addition of 250,000 adult spleen cells to the control cultures did not significantly decrease the peak $^{3}$H-thymidine incorporation of the cultures. This control was routinely performed but is generally not

reported. Autologous cultures were also set up in replicates of either three or four.

xvi. Backgrounds.

In conjunction with the MLCs, the various cellular components of the reaction mixtures were cultured separately to determine their unstimulated $^{3}H$-thymidine incorporation. The backgrounds reported are usually the sum of the results obtained for the proliferation of separate cultures of responder and stimulator cell populations. The backgrounds of mixed cultures containing newborn spleen regulator cells were determined by the incorporation of cultures of the newborn spleen cells plus the 250,000 adult CBA/J spleen cells used as responders in the control allogeneic MLC and as stimulators in the control autologous MLC. In the backgrounds of these latter MLCs the contribution of inactivated Balb/c stimulators and newborn CBA/J thymocyte responders respectively were negligible and are reported separately in order to simplify the presentation of the data. The background cultures were performed either in triplicate or quadruplicate.

xvii. PFC Culture and Assay.

Both Marbrook and Mishell-Dutton culture systems were used to analyze the regulation of the primary *in vitro* TD antibody response to SRBC by the addition of newborn spleen cells. The medium employed was identical to that used in the microtiter cultures with the exception that a serum supplement of 10% FCS was used. Marbrook assay culture flasks contained $20 \times 10^6$ adult CBA/J spleen cells plus $3 \times 10^6$ SRBC in a total volume of 2.1 ml in the inner chamber with 8 ml of medium in the outer chamber. Mishell-Dutton assay cultures consisted of $10 \times 10^6$ adult CBA/J spleen cells plus $3 \times 10^6$ SRBC in a volume of 2.1 ml in the 16 mm wells of

a 24 well plate (Costar, Cambridge, MA; Cat. # 3524). On day 2 of incubation of the Mishell-Dutton cultures 0.5 ml of medium was removed from each well and replaced with fresh supplemented medium. $1 \times 10^6$, or less, Ig-anti-Ig affinity column purified neonatal CBA/J spleen cells were added to the assay cultures. These regulator cells, when pretreated, were adjusted so that the cultures received only the fraction of the $1 \times 10^6$ cells resistant to the treatment. The numbers of specific anti-SRBC PFCs per culture were assayed on day 4 of incubation using a hemolytic plaque assay described in detail elsewhere [165]. The results are expressed as the mean PFCs $\pm$ S.E.M. of triplicate cultures.

xviii. Data Manipulation.

The results from assays of $^3$H-thymidine incorporation are presented as the mean cpm $\pm$ S.E.M. of either three or four replicate cultures.

The relative response was calculated as 100 x

$$\left[ \frac{\text{(mean cpm of test MLC - mean cpm test background)}}{\text{(mean cpm of control MLC - mean cpm control background)}} \right].$$

The stimulation index was determined from the formula:

$$\text{S.I.} = \frac{\text{mean cpm of MLC}}{\text{mean cpm of background}}.$$

The percent suppression was obtained by:

$$\% \text{ suppression} = \left[ 1 - \frac{\text{mean cpm of test MLC}}{\text{mean cpm of control MLC}} \right] \times 100.$$

Statistical significance between the means of different culture replicates was tested using the t-statistic evaluation and Student's t-distribution [73].

## VIII. RESULTS

### i. Phenotypic and Functional Characterization of Newborn Lymphoid Cell Populations from Spleen and Thymus.

In preparation for a comprehensive analysis of the cellular immunoregulatory mechanisms operative during early neonatal life, it was considered important to carefully determine the efficacy of reagents to be used to identify and isolate various populations of lymphocytes from newborn mice. While it has been reported that newborn spleen cells have little or no capacity to respond to CON A and PHA [105,233,238], we were able to obtain relatively weak but significant reactivity of splenocytes from mice of under 5 days of age to these mitogens as well as to LPS (see fig. 4). We could therefore test the specificity of various anti-T cell sera plus RC on the basis of their ability to remove the reactivity to CON A and PHA versus LPS. The results of this analysis are shown in Figure 1. Negative selection with anti-T (rabbit anti-mouse brain), anti-Thy 1, or anti-MICG plus RC removes both adult and newborn spleen cells reactive to CON A and PHA without significantly affecting those responsive to LPS. We next sought to determine whether T cell populations isolated from adult versus newborn spleen have comparable sensitivity to cytotoxic treatment with various anti-T cell reagents plus RC. Figure 2a shows the results of a representative experiment comparing the susceptibility of newborn and adult Ig-anti-Ig affinity purified splenic T cells to treatment with varying dilutions of anti-T, anti-Thy 1, and anti-MICG sera plus RC. Through a wide range of concentrations the anti-T reagents were capable of lysing over 90% of adult splenic T cells in the presence of complement. However all three antisera plus RC are less effective on newborn splenic T cells. Only 10 to 20% of the neonatal T cells are sensitive to treatment with anti-Thy 1

## SPECIFICITY OF ANTI-T CELL REAGENTS

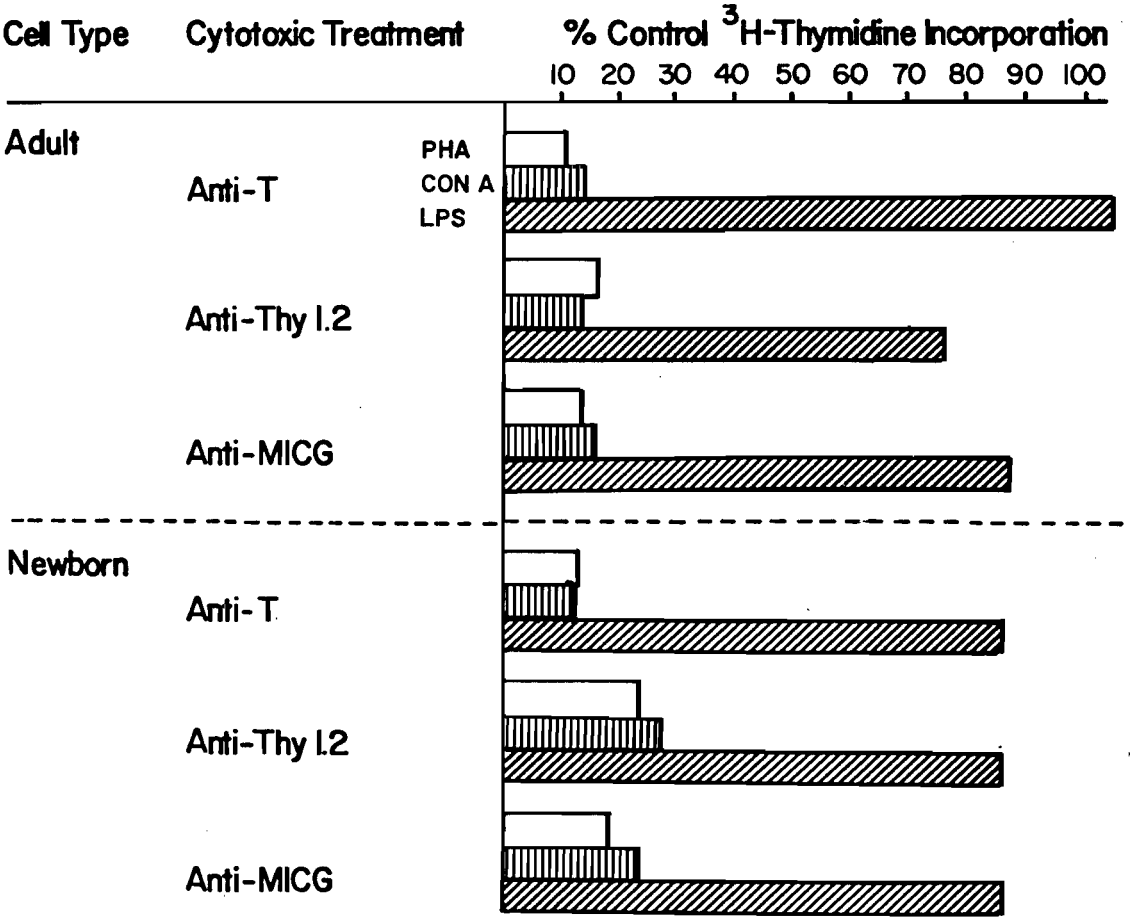



Figure 1. Functional specificity of anti-T cell reagents. Spleen cells from adult and newborn CBA/J mice were treated with the indicated antisera plus RC. The cells resistant to the treatment, out of a possible 250,000, were cultured with CON A, PHA, or LPS for 48 hours at which time the resulting proliferation was assayed. The protocols employed for the preparation of the cells, their culture and mitogen stimulation, as well as the proliferative assay are detailed in Materials and Methods.

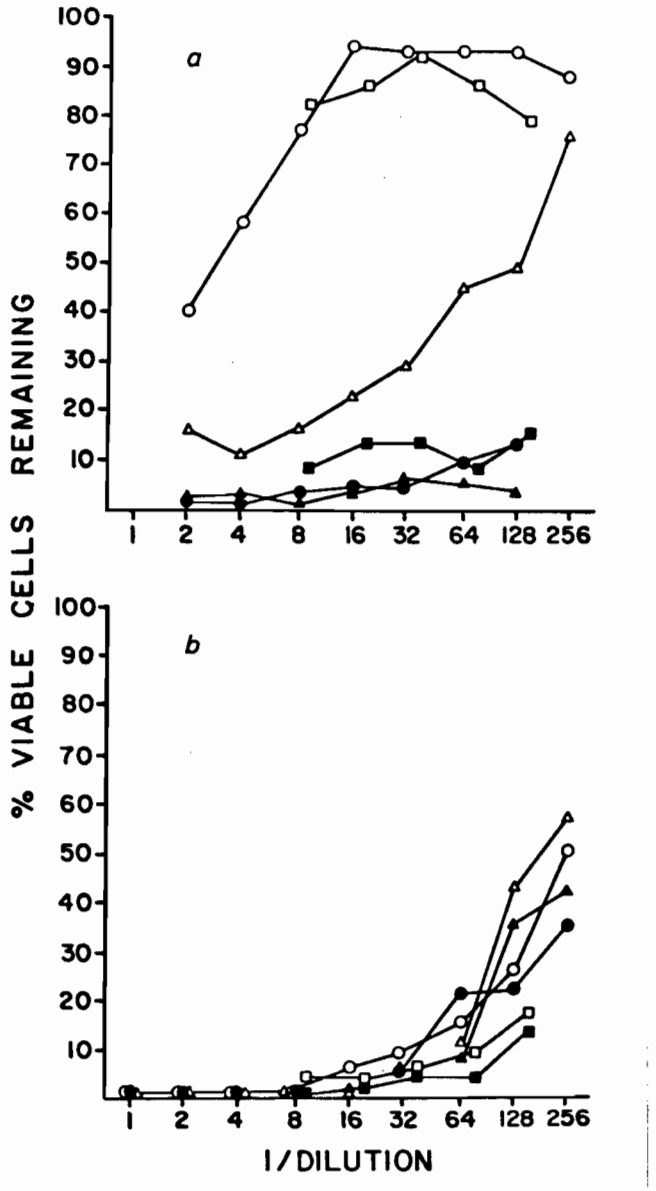



Figure 2. The sensitivity of CBA/J adult versus newborn T cells from spleen (panel a) and thymus (panel b) to direct cytotoxicity with various anti-T cell sera plus RC. 1 x $10^6$ adult (closed symbols) and 4 day old newborn (open symbols) thymocytes and Ig-anti-Ig affinity purified splenic T cells were treated as described in Materials and Methods with varying dilutions of anti-T (▲,△), anti-MICG (●,○), and anti-Thy 1.2 (■,□) plus RC. The viable cells remaining after treatment were scored by the ability to exclude trypan blue and are represented as a percentage of cells surviving treatment with RC alone.

plus RC. While a large part of the newborn splenic T cells can be lysed by anti-MICG plus RC, dilutions of less than the 1/10 normally employed for adult cells are necessary to kill over 10% of the neonatal T cells. On the other hand, the newborn T cells from spleen are more sensitive to cytotoxic treatment with anti-T serum plus RC with approximately 80% being removed with the use of a 1/10 dilution of this specific type of antiserum. Figure 2b shows the results of a similar comparative analysis of the susceptibility of newborn versus adult thymocytes to negative selection with various concentrations of the anti-T cell sera plus RC. In contrast to the differences in sensitivity between adult and newborn splenic T cells, thymocytes from adult and neonatal mice are not significantly different in their susceptibility to cytotoxic treatment with anti-Thy 1, anti-MICG, or anti-T sera plus RC. Only anti-T and high concentrations of anti-MICG, but not anti-Thy 1, plus RC are able to kill the majority of newborn splenic T cells. However, neonatal thymocytes and CON A and PHA reactive spleen cells as well as adult thymocytes and splenocytes are effectively lysed by treatment with conventional dilutions of anti-T, anti-MICG, and anti-Thy 1 plus RC.

The data in Table 1 indicates that most newborn splenic T cells, in contrast to adult splenic T cells, are also resistant to negative selection with anti-Lyt 1 plus RC. On the other hand as demonstrated in Figure 3, thymocytes from mice as young as one day of age show nearly adult profiles of sensitivity to cytotoxic treatment with various concentrations of anti-Lyt 1 and anti-Lyt 2 plus RC. With the exception of a higher percentage of cells bearing low levels of both Lyt 1 and 2 antigens, newborn thymocytes as previously reported [114] appear to have adult proportions of Lyt $123^+$, Lyt $1^+23^-$, and Lyt $1^-23^+$ cells.

Previous investigations into the ontogenetic development

Table 1. Lyt phenotype of adult and newborn splenic T cells.

| T-CELL SOURCE[a] | PERCENT VIABLE CELLS REMAINING AFTER NEGATIVE SELECTION WITH: | | |
|---|---|---|---|
| | ANTI-LYT 1 | ANTI-LYT 2 | ANTI-LYT 1 + 2 |
| ADULT SPLEEN | 14 | 65 | 10 |
| NEWBORN SPLEEN | 86 | 94 | 86 |

[a]T cells were purified from adult or newborn CBA/J spleen by passage through Ig-anti-Ig affinity columns. Purified T cells were treated, as described in Materials and Methods, with monoclonal anti-Lyt 1.1, anti-Lyt 2.1, or a mixture of anti-Lyt 1.1 + anti-Lyt 2.1 plus RC. Viable cells were scored on the basis of exclusion of trypan blue. The results are presented as a percentage of the viable cells remaining in aliquots of cells treated with anti-serum plus RC versus those treated with RC alone, and are the average of values obtained in a number of experiments.

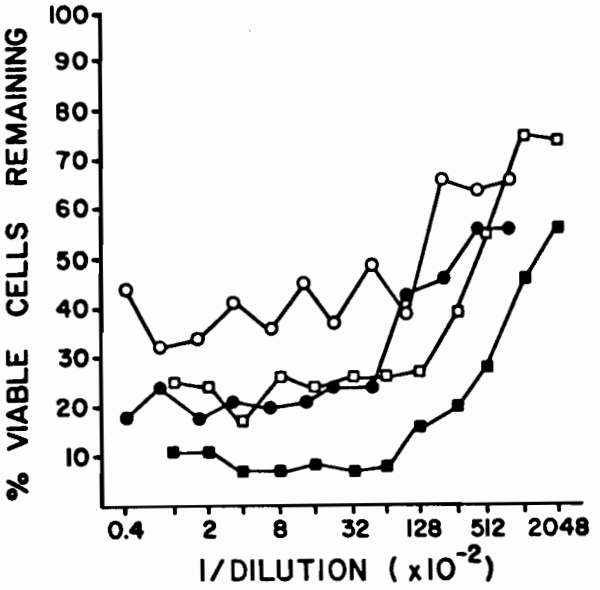
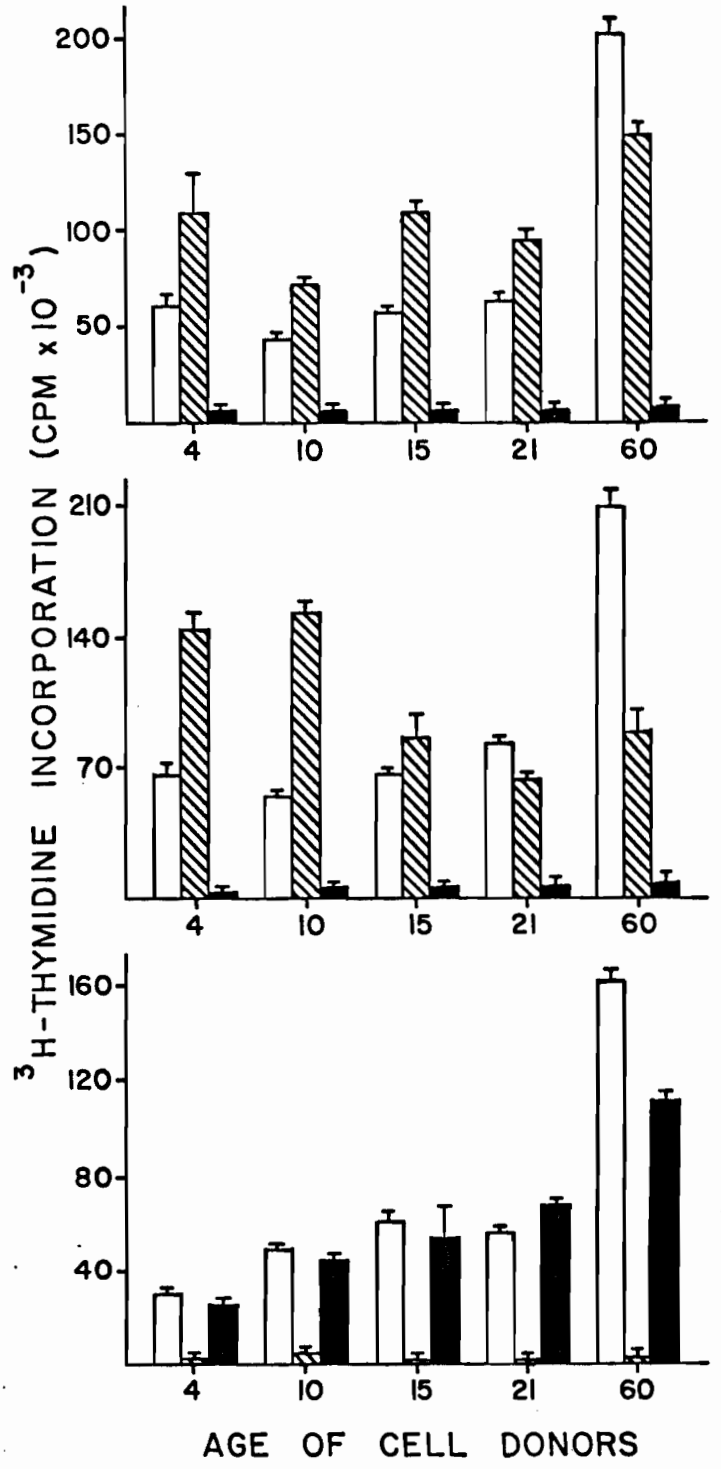



Figure 3. The sensitivity of newborn (open symbols) versus adult CBA/J (closed symbols) thymocytes to direct cytotoxicity with anti-Lyt sera plus RC. 1 x $10^6$ cells from adult and 4 day old newborn thymus were treated as described in Materials and Methods with varying concentrations of anti-Lyt 1.1 (■,□) or anti-Lyt 2.1 (●,O) plus RC. At antiserum dilutions of less than 1/12,800 the percentages of newborn thymocytes left viable after cytotoxic treatment with mixtures of anti-Lyt 1.1 and anti-Lyt 2.1 plus RC were not significantly different from the cells remaining after treatment with anti-Lyt 1 plus RC and are not shown. Similar treatment with mixtures of anti-Lyt 1 and anti-Lyt 2 plus RC killed approximately 98% of the adult thymocytes. The results were determined and are expressed as outlined in the legend to figure 1.

of the ability of splenocytes to respond to mitogens have primarily examined the reactivity of unseparated spleen cell populations [105,155,213,233,238]. Numerous studies have demonstrated the presence in neonatal spleen of regulatory cells (see Section IV. iii. a)) which we felt could possibly inhibit the mitogenesis of subpopulations of splenocytes. Consequently we reasoned that it would be valuable to examine the mitogen reactivity of isolated T and B as well as unseparated cells from the spleens of mice of different ages. Figure 4 shows the results of a representative experiment of this type. Unselected spleen cells from animals of up to several weeks of age are deficient, when compared with adult spleen cells, in their ability to respond to CON A, PHA, and LPS. In contrast, affinity purified splenic T cells from mice of all the ages tested show a similar capacity to proliferate in response to the T cell mitogens. On the other hand, whole spleen cells as well as Non-T cells prepared by treating spleen with anti-T serum plus RC demonstrate an ability to respond to LPS which increases with age. During the first three to five days after birth the newborn splenic T cell population possesses mitogen reactivity approaching that of the adult while T-depleted spleen cells have less than one third of the adult ability to respond to LPS. It is therefore apparent that during early life the T cells within spleen may be prevented from expressing optimal reactivity to mitogens because of the regulatory influences of cells within the Non-T fraction.

While newborn spleen cells show little or no ability to respond in assays of mitogenesis [105,213,233,238] and in MLCs [6,105,256], neonatal thymocytes can respond significantly in MLCs to stimulation from autologous as well as allogeneic cells [19,27,28,29,87,101,102,103,105,154]. Since adult thymocytes do not appear to have comparable autoreactivity [27,28,103], the capacity of neonatal

Figure 4. A comparison of the reactivity of whole, T, and Non-T spleen cells from CBA/J mice of different ages to PHA (panel a), CON A (panel b), and LPS (panel c). Unselected cells (open bars), Ig-anti-Ig affinity isolated T cells (hatched bars), or Non-T cells depleted of T cells by anti-T serum plus RC treatment (filled bars) were cultured in triplicate at a cell density of 200,000 cells per microtitre well for 48 hours. All the techniques employed are detailed in Materials and Methods.


200
150
100
50
4
10
15
21
60
210
140
70
160
120
80
40
$^3$H-THYMIDINE INCORPORATION (CPM x $10^{-3}$)
AGE OF CELL DONORS

thymocytes to proliferate in response to genetically identical adult splenic stimulator cells in Type I AMLRs is particularly interesting. Initial studies revealed that the culture conditions employed in the generation of AMLRs are very important in determining the magnitude of the response. Table 2 shows the results of an experiment comparing different serum supplements in a Type I AMLC. Media without serum additives supports a weak but significant autologous reaction. Addition of conventional serum supplements such as NMS, FCS, or NHS in appropriate amounts can support an increased AMLR. The use of FCS, at a concentration found to be optimal for allogeneic reactions in our culture system (1%), resulted in a proliferative response which was somewhat weaker than that obtained in cultures supplemented with either NHS or NMS. Although not shown here only select batches of FCS appeared to be capable of supporting the AMLR. On the other hand NHS (1%) added to the cultures increased both the incorporation of thymidine as well as the stimulation index but shifted the kinetics of the AMLR such that the peak response occurred twenty-four hours later than the peak seen with other serum supplements or without added serum. Autologous NMS (0.5%) supported a strong AMLR as determined by thymidine incorporation and stimulation index and did not appear to modify the kinetics of the reaction. Thus significant autoreactivity of neonatal thymocytes can be demonstrated in AMLCs in the absence of heterologous serum additives. Subsequent AMLCs were supplemented with 0.5% NMS.

The Type II AMLR has been characterized as the proliferation of adult Lyt $1^{+}23^{-}$ T cells in response to stimulation from Ia-positive B and/or dendritic cells [19,30,83,127,177,194]. We therefore sought to determine whether the same classes of cells are interacting in the Type I AMLR. The cell surface antigen phenotype of the responders was examined by negative selection of newborn

Table 2. Serum dependency of the neonatal AMLR.

| SERUM SUPPLEMENT[a] | BACKGROUND | AUTOLOGOUS MLC | STIMULATION INDEX |
|---|---|---|---|
| | $^3$H-THYMIDINE INCORPORATION cpm ± S.E. | | |
| NONE | 599 ± 93 | 1,491 ± 292 | 2.5 |
| NMS 0.5% | 3,962 ± 1,50 | 29,260 ± 1,296 | 7.4 |
| FCS 1.0% | 3,641 ± 225 | 14,746 ± 1,574 | 4.0 |
| NHS 1.0% | 5,082 ± 1,380 | 44,515 ± 5,687 | 8.8 |

[a]AMLCs consisted of a responder population of 250,000 newborn CBA/J thymocytes co-cultured with a stimulator population of an equal number of syngeneic adult spleen cells. All cultures showed maximal incorporation at 96 hours of culture except for those supplemented with NHS which showed peak activity 24 hours later.

thymocytes using various antisera plus RC prior to culturing the residual cells with T-depleted adult spleen stimulator cells in AMLCs. As shown in Figure 5, the AMLR of thymocytes depleted of Lyt $2^+$ cells does not differ significantly from the AMLR of untreated thymocytes. However, selective removal of Lyt $1^+$ cells from the thymocyte population abrogates the autologous response. Table 3 details the results of several experiments designed to determine the phenotype of the neonatal thymocytes responding in the type I AMLR. These data indicate that the AMLR responsive thymocyte is sensitive to treatment with anti-T anti-MICG or anti-Lyt 1 plus RC. Elimination of cells expressing Lyt 1 and/or Lyt 2 by negative selection using a mixture of anti-Lyt 1.1 and anti-Lyt 2.1 plus complement also completely removed the AMLR responder. On the other hand the AMLR reactive thymocytes are only partially susceptible to negative selection with anti-Thy 1 plus RC. Pretreatment of this population with anti-$Ia^k$ plus complement had no significant effect on the AMLR. Thus the newborn thymocyte population that responds in the Type I AMLR is $MICG^+$, Thy $1^+$ (but with relatively low numbers of these determinants), and $Ia^-$. The AMLR responsive neonatal thymocytes resemble adult T cells reactive in Type II AMLRs in that both populations are Lyt $1^+23^-$.

Previous studies have indicated that it is possible to separate functionally mature and immature thymocytes by differential agglutination with PNA [133,207]. Thymocytes that appear to be functionally less mature are preferentially agglutinated by PNA [133]. It has recently been shown that $PNA^-$ adult thymocytes can proliferate in response to stimulation from $Ia^+$ autochthonous Non-T thymic cells [30]. The data presented in Table 4 indicates that the newborn thymocytes that respond in both autologous and allogeneic MLCs are also $PNA^-$. Autologous and allogeneic MLC-induced proliferation of isolated $PNA^-$ thymocytes was two to three

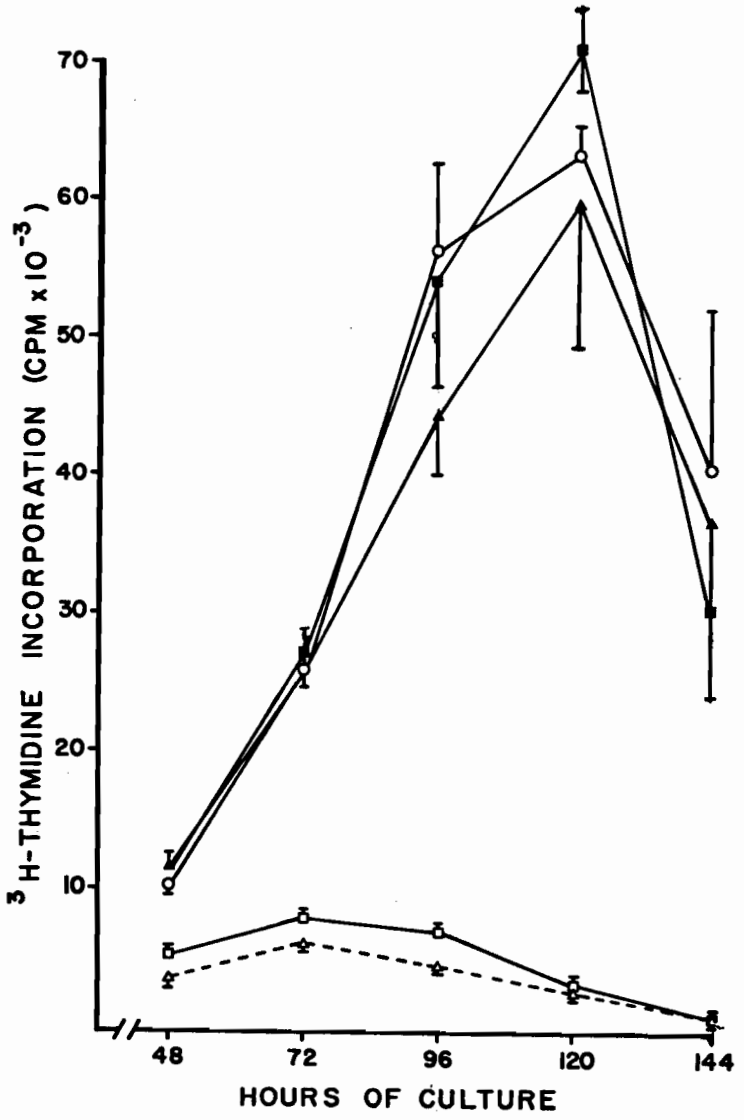



Figure 5. Lyt phenotype of the neonatal thymocyte Type I AMLR responder. Thymocytes from 2 day old CBA/J mice were were either left untreated (▲), treated with RC alone (■), or treated with monoclonal anti-Lyt 1.1 (□) or anti-Lyt 2.1 (○) plus RC. The thymocytes were manipulated such that 250,000 untreated thymocytes or the fraction resistant to the cytotoxic pretreatment were subsequently added to MLC with 250,000 adult CBA/J spleen cells depleted of T cells by treatment with anti-T plus RC. The background of the 250,000 adult CBA/J stimulators plus that of 250,000 untreated newborn thymocytes cultured separately (△) did not significantly differ from the backgrounds of the treated thymocytes plus stimulator cells which are not shown. All the techniques employed are described in Materials and Methods.

Table 3. The neonatal thymocytes responding in the Type I AMLR are T cells expressing MICG, THY 1, and Lyt 1 but not Lyt 2 or Ia determinants.

| PRETREATMENT OF NEWBORN THYMOCYTE AMLR RESPONDERS[a] | BACKGROUND | AUTOLOGOUS MLR | |
|---|---|---|---|
| | $^3$H-THYMIDINE INCORPORATION cpm ± S.E. | [Relative Response] | |
| EXPERIMENT 1. | | | |
| NONE | 14,945 ± 1,329 | 101,172 ± 8,627 | [100] |
| RC | 14,548 ± 1,333 | 92,446 ± 626 | [90] |
| ANTI-T + RC | 13,973 ± 1,185 | 12,713 ± 894 | [0]* |
| ANTI-Ia$^k$ + RC | 15,533 ± 1,350 | 102,140 ± 11,485 | [100] |
| EXPERIMENT 2. | | | |
| NONE | 2,765 ± 426 | 67,930 ± 9,713 | [100] |
| RC | 3,259 ± 450 | 70,829 ± 2,950 | [104] |
| ANTI-THY 1.2 + RC | 2,362 ± 307 | 30,791 ± 4,793 | [44]* |
| ANTI-MICG + RC | 2,091 ± 363 | 4,031 ± 385 | [3]* |
| ANTI-LYT 1.1 + RC | 2,169 ± 391 | 3,254 ± 378 | [2]* |
| ANTI-LYT 2.1 + RC | 2,665 ± 314 | 63,376 ± 1,845 | [93] |
| ANTI-LYT 1.1 + ANTI-LYT 2.1 + RC | 3,513 ± 848 | 2,535 ± 227 | [0]* |

[a]Thymocytes from 3 day old CBA/J mice were treated as indicated and added to 250,000 adult CBA/J spleen cells which had been depleted of T cells by treatment with anti-T serum plus complement. Newborn thymocytes were manipulated, as described in Materials and Methods, such that only the cells resistant to treatment, out of a possible 250,000 cells, were added to cultures. The peak proliferative responses are shown. These occurred at 96 hours in experiment 1 and at 120 hours of culture in experiment 2.

*The mean response of cultures treated with antiserum plus RC is significantly less than that of cultures treated with RC alone; $p \leq 0.001$.

Table 4. The stimulatory population in neonatal Type I AMLRs consists of Ia-positive Non-T cells.

| PRETREATMENT OF ADULT SPLEEN CELL AMLR STIMULATORS | BACKGROUND | AUTOLOGOUS MLR | |
|---|---|---|---|
| | $^3$H-THYMIDINE INCORPORATION cpm ± S.E. [Relative Response] | | |
| EXPERIMENT 1. | | | |
| NONE | 14,447 ± 1,588 | 119,401 ± 9,862 | [100] |
| RC | 13,140 ± 402 | 98,276 ± 6,113 | [81] |
| ANTI-T + RC | 7,463 ± 602 | 98,326 ± 5,402 | [87] |
| EXPERIMENT 2. | | | |
| NONE | 3,909 ± 329 | 92,644 ± 13,180 | [100] |
| RC | 4,218 ± 340 | 81,957 ± 5,767 | [88] |
| ANTI-Ia$^k$ + RC | 1,653 ± 172 | 7,737 ± 1,407 | [7]* |

[a]AMLCs consisted of a responder population of 250,000 newborn CBA/J thymocytes co-cultured with a stimulator population prepared from syngeneic adult spleen cells. Stimulator cells were treated, as described in Materials and Methods, such that 250,000 cells minus those susceptible to the particular treatment were added to the cultures. The peak proliferative response of experiment 1 occured at 96 hours of culture and is shown here. In experiment 2 the peak proliferation of the different cultures ranged from 96 to 120 hours of culture and the mean ± S.E. of cultures at both of these time points are shown.

*The mean response of cultures treated with antiserum plus RC is significantly less than that of cultures treated with RC alone; $p \leq 0.001$.

times higher than that of an equal number of unselected cells from the same initial preparation. In contrast, the same number of purified $PNA^+$ thymocytes showed no significant response in either class of MLC. Thus autologous and allogeneic MLC reactive newborn thymocytes are $PNA^-$.

It has previously been shown that the stimulator population in Type II AMLRs consists of $Ia^+$ Non-T cells [19,30,83,127,177,194]. As demonstrated in Table 5 $Ia^+$ Non-T cells also trigger the Type I AMLR. Removal of T cells from the adult spleen cell stimulator population does not significantly reduce the AMLR. However, depletion of $Ia^+$ cells virtually eliminates the reaction. Therefore in Type I AMLRs Lyt $1^+23^-$ $Ia^-$ neonatal thymocytes proliferate in response to stimulation from $Ia^+$ Non-T cells.

While prior investigations have concluded that AMLRs are stimulated by $Ia^+$ Non-T cells, this population has been further characterized as either Ig-bearing B cells [19,27, 103,195] or dendritic macrophages [177]. If B cells or their products are involved in the initiation of Type I AMLRs, we considered that spleen cells from adult mice deprived of B cells and immunoglobulin from birth by *in vivo* anti-mu treatment [90,128,142,166] should be incapable of stimulating this reaction. Thus adult spleen cells as well as neonatal thymocytes from anti-mu treated mice were assayed for their respective abilities to stimulate and respond in the AMLR (Table 6). Thymocytes from 4 day old mice treated *in vivo* with rabbit anti-mouse IgM within 24 hours of birth and at 2 days of age were unable to respond in AMLR while thymocytes from untreated and normal rabbit serum treated littermates showed significant autologous reactivity. Thymocytes from C57/Bl6 x C3H F1 neonates proliferated when cultured in AMLCs with unmanipulated syngeneic adult spleen cells but did not react with spleen cells from identical mice treated from birth with anti-mu. Thus it appears that *in vivo* anti-mu

Table 5. The newborn thymocyte responders in autologous and allogeneic MLCs are PNA-negative.

| NEWBORN THYMOCYTE[a] MLR RESPONDERS | BACKGROUND[b] | AUTOLOGOUS MLR | ALLOGENEIC MLR |
|---|---|---|---|
| | $^3$H-THYMIDINE INCORPORATION | | |
| | cpm ± S.E. | [Relative Response] (Stimulation Index) | |
| UNSELECTED | 422 ± 97 | 31,682 ± 16,337 [100] (15) | |
| | 766 ± 202 | | 44,645 ± 5,104 [100] (49) |
| PNA-NEGATIVE | 497 ± 151 | 59,674 ± 6,545 [200] (28) | |
| | 1,453 ± 276 | | 104,240 ± 6,319 [235] (65) |
| PNA-POSITIVE | 141 ± 28 | 3,503 ± 737 [11] (2) | |
| | 276 ± 101 | | 1,139 ± 493 [2] (3) |

[a]250,000 unselected or PNA fractionated thymocytes from 2 to 3 (average 2.5) day old newborn CBA/J mice were cultured with an equal number of syngeneic adult spleen cells in AMLCs and with an equal number of mitomycin C inactivated Balb/c spleen cells in allogeneic MLCs. The data shown was obtained at the peak of culture proliferation, 96 hours of culture for the autologous reaction and 24 hours later for the allogeneic MLR. Cell separation, culture, and proliferative assay are described in Materials and Methods.

[b]The background is the $^3$H-thymidine incorporation of cultures of 250,000 of the various thymocyte subpopulations cultured alone. The adult CBA/J spleen stimulators in the AMLCs and the Balb/cJ stimulators in the allogeneic MLCs showed 1,648 ± 230 and 139 ± 61 mean cpm ± S.E.M. incorporation respectively when cultured separately.

Table 6. Both the responder and stimulator populations in the Type I AMLR are removed by *in vivo* anti-mu treatment.

| AMLR CELL POPULATION[a] | IN VIVO PRETREATMENT | BACKGROUND | AUTOLOGOUS MLR | |
|---|---|---|---|---|
| | (# animals) | $^3$H-THYMIDINE INCORPORATION cpm ± S.E. [Relative Response] | | |
| EXPERIMENT 1. | | | | |
| NEWBORN THYMOCYTE RESPONDERS | NONE (15) | 12,249 ± 547 | 52,047 ± 3,785 | [100] |
| | NORMAL RABBIT SERUM (11) | 12,252 ± 557 | 44,295 ± 3,818 | [81] |
| | RABBIT ANTI-MOUSE IgM (10) | 12,189 ± 553 | 15,317 ± 990 | [8]* |
| EXPERIMENT 2. | | | | |
| ADULT SPLEEN CELL STIMULATORS | NONE (3) | 4,876 ± 445 | 97,148 ± 14,085 | [100] |
| | RABBIT ANTI-MOUSE IgM (3) | 3,819 ± 352 | 5,364 ± 1,633 | [2]* |
| EXPERIMENT 3. | | | | |
| ADULT SPLEEN CELL STIMULATORS | NONE (5) | 6,158 ± 2,402 | 33,408 ± 3,480 | [100] |
| | RABBIT ANTI-MOUSE IgM (2) | 4,209 ± 448 | 2,614 ± 284 | [0]* |

[a]AMLCs consisted of 250,000 newborn thymocytes co-cultured with an equal number of syngeneic adult spleen cells. In experiment 1 the mice employed were of the CBA/J strain while in experiments 2 and 3 C57Bl/6 x C3H F1 mice supplied by Dr. J. Gordon were used. *In vivo* treatments with anti-mu and NRS were performed as described in Materials and Methods. In all experiments the peak MLC response is shown, 120 hours of culture for experiment 1 and 144 hours of culture for experiments 2 and 3.

*The mean response of cultures containing *in vivo* anti-IgM treated cells is significantly different from that of cultures containing the relevant untreated or NRS treated control populations; $p \leq 0.001$.

treatment not only abrogates the ability of spleen cells to stimulate the AMLR, but also results in the disruption of the responder population.

The results of the preceeding experiments indicate that newborn thymocytes respond in Type I AMLRs to stimulation from Ia bearing Non-T cells that are sensitive to in vivo anti-mu treatment. This finding is particularly interesting in the light of the observations of Hamano et. al. who have presented evidence that anti-mu treatment can reduce the levels of Ia antigen expressed on the cell surface [93]. Titration experiments, shown in Figure 6, demonstrate that the AMLR induced proliferative response can be blocked by monoclonal anti-Ia. These findings add weight to the suggestion that Ia antigenic determinants may be directly involved in the stimulation of the Type I AMLR.

To determine whether the newborn thymus contains an equivalent capacity to respond to autologous versus allogeneic stimulation we compared the proliferation of newborn CBA/J thymocytes cultured in MLCs with adult syngeneic versus allogeneic Balb/cJ spleen cells (Figure 7). Throughout the culture period the mean thymidine incorporation of newborn thymocytes in AMLCs was greater than that of the same cell population in allogeneic MLCs. Thus the neonatal thymus may have more autoreactive cells than cells capable of responding to a particular allogeneic haplotype.

We next sought to compare newborn CBA/J thymocytes with various adult CBA/J lymphoid cell populations for the capacity to respond in AMLCs. As shown in Figure 8a Ig-anti-Ig affinity column purified T cells from adult spleen and lymph node both proliferated significantly in AMLCs while unselected adult thymocytes did not respond during the culture period. The same number of newborn thymocytes, however, showed an autologous reaction approximately ten fold

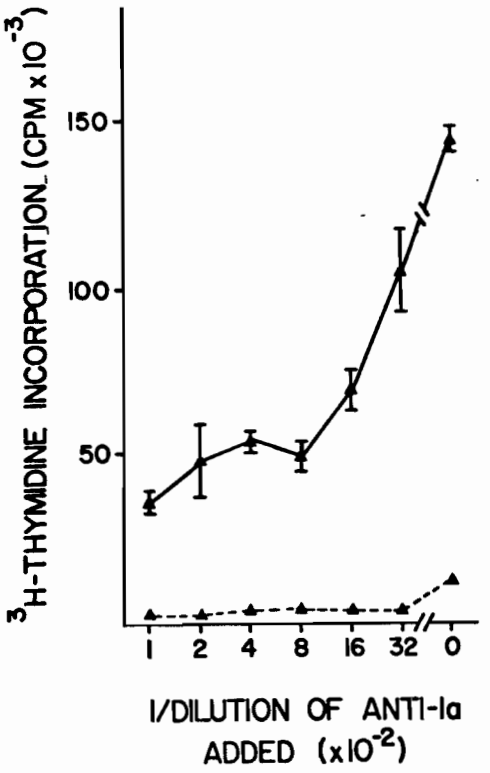



Figure 6. Titration of anti-$Ia^k$ into the neonatal CBA/J AMLC. Varying dilutions of monoclonal murine anti-$Ia^k$ were added to AMLCs consisting of 250,000 thymocytes from newborn CBA/J mice responding to an equal number of syngeneic adult spleen cells. The peak proliferative response, at 120 hours of culture, was assayed as described in Materials and Methods and is expressed by (▲——▲). The added background proliferation of responder and stimulator populations cultured separately is represented by (▲- - -▲).

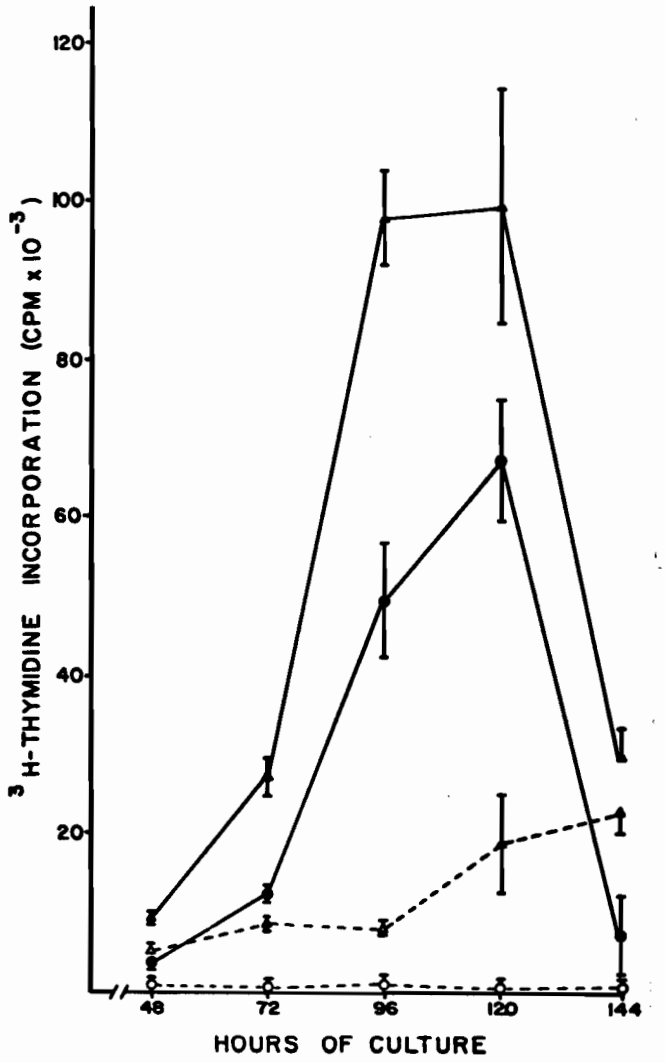
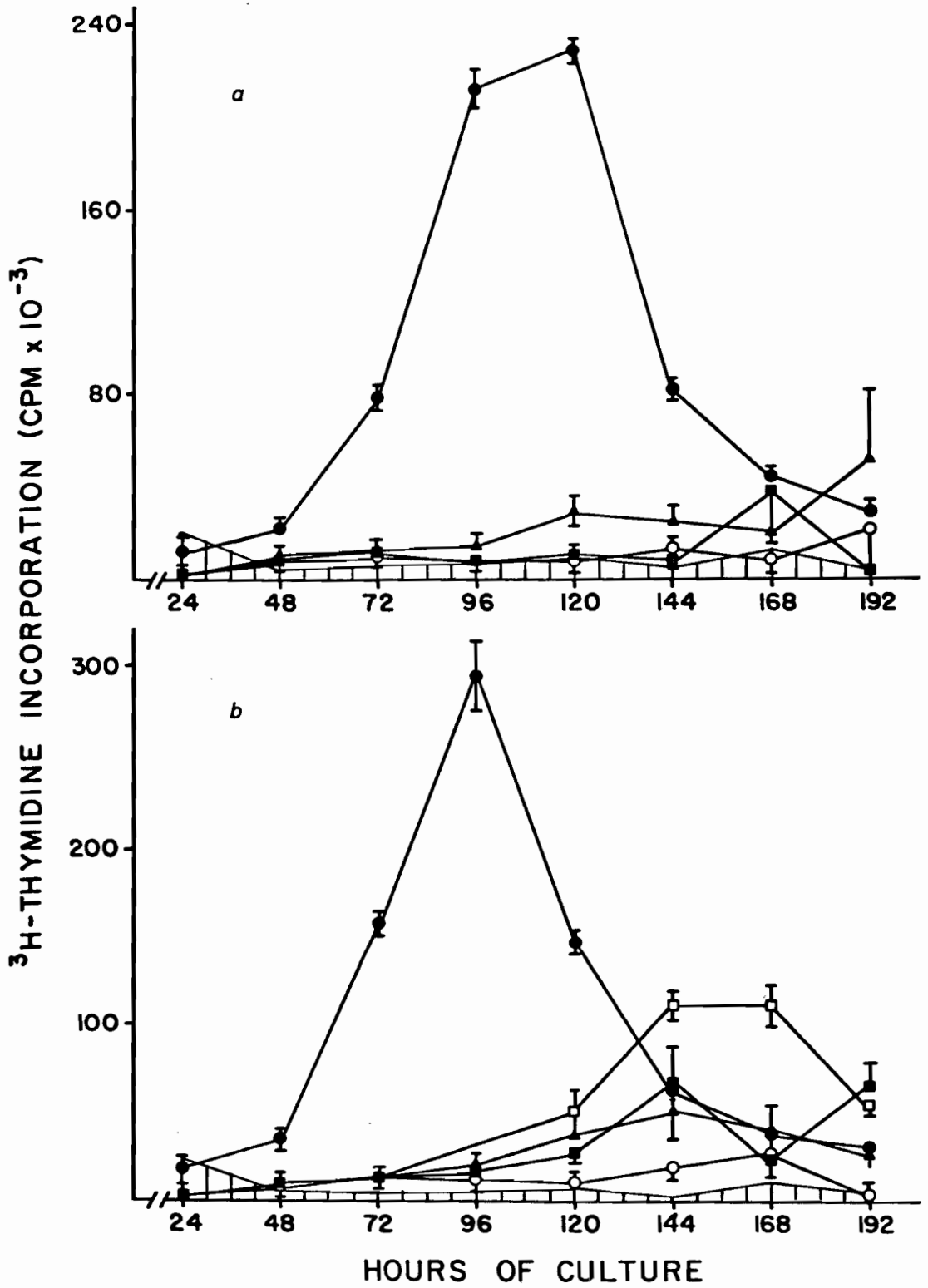



Figure 7. Newborn CBA/J thymocytes responding in autologous and allogeneic MLCs are contrasted. 250,000 thymocytes from 1 to 3 day old CBA/J mice were cultured with equal numbers of syngeneic adult spleen cells (▲) or with equal numbers of mitomycin C inactivated Balb/c spleen cells (●). The backgrounds of the AMLCs (△) and allo-MLCs (○) are also shown. Results were determined and are expressed as described in Materials and Methods.

Figure 8. A comparison of the ability of various CBA/J newborn and adult T cell (panel a) or isolated Lyt $1^{+}23^{-}$ T cell (panel b) populations to respond in AMLCs. The tissue sources of the various responder cells included thymus from 1 to 3 day old neonates (●), and thymus (○), spleen (▲), lymph node (■), and peripheral blood (□) from 30 week old adult female mice. Responder T cells were either prepared by Ig-anti-Ig affinity column passage of whole cell suspensions or, in the case of thymocytes, left as unselected cell populations. Lyt $1^{+}23^{-}$ cells were separated from the T cell preparations by treatment with anti-Lyt 2.1 serum plus RC. Responder cells (250,000) were cultured with an equal number of adult splenic stimulator cells depleted of T cells by treatment with anti-Thy 1.2 plus RC. The backgrounds were all within the hatched area. Cell preparation, culture, and assay of proliferation were performed as detailed in Materials and Methods.


240
160
80
a
24
48
72
96
120
144
168
192
300
200
100
b
$^3$H-THYMIDINE INCORPORATION (CPM x $10^{-3}$)
HOURS OF CULTURE

stronger than the adult T cell response. On the other hand, Lyt $1^+$ $23^-$ T lymphocytes isolated from newborn thymus and adult thymus, spleen, lymph node, and peripheral blood all showed significant proliferation in AMLCs (Figure 8b). Neonatal Lyt $1^+$ $23^-$ thymocytes were more reactive in AMLRs than comparable Lyt $1^+23^-$ adult cells with a magnitude of about three times that of the most responsive adult cell population. In addition, newborn thymocytes showed peak activity in AMLCs at 96 hours of culture which was at least 48 hours prior to that seen in AMLCs with adult responder cells. It is therefore evident that in comparison with adult lymphoid organs neonatal thymus harbors a relatively large proportion of autoreactive cells.

### ii. Regulation of MLC Induced Proliferation by Newborn CBA/J Inhibitory Cells.

#### a) Evidence for Two Distinct Lymphoid MLC-Inhibitory Populations in Newborn Spleen.

Early investigations suggested that neonatal spleen cells could not respond in allogeneic MLCs [6,104]. It has since been established that while capable of reacting in such MLCs newborn spleen cells do not respond with the same proliferative magnitude as similar numbers of adult spleen cells [210,256]. Figure 9 shows a comparison of the allogeneic reactivity of newborn versus adult spleen cells and purified splenic T cells. Newborn spleen cells showed approximately one-fourth of the proliferative response of an equal number of adult spleen cells. However T cells isolated from neonatal spleen showed an MLR that was nearly double that of unselected newborn spleen cells. Purified adult splenic T cells did not demonstrate a significantly better reaction than whole adult spleen cells. The improvement in

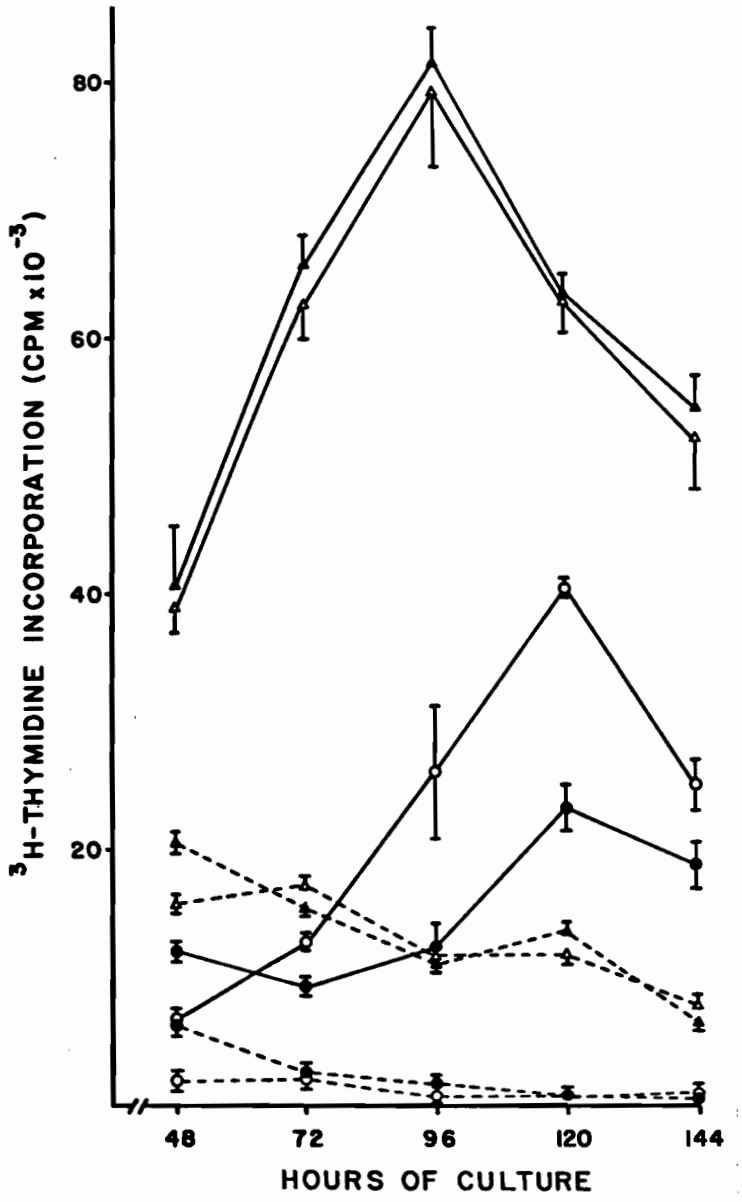



Figure 9. Comparison of the response of adult versus newborn unselected spleen and isolated splenic T cells in the allogeneic MLC. CBA/J spleen or Ig-anti-Ig affinity purified T cells (250,000) were co-cultured with 500,000 mitomycin C inactivated adult Balb/c spleen cells. The MLRs of newborn whole spleen (●) and splenic T cells (○) as well as adult whole spleen (▲) and splenic T cells (△) are shown. Backgrounds are denoted by the broken lines. All the techniques employed are described in Materials and Methods.

the ability of neonatal splenic T cells to respond in allogeneic MLCs may be the result of removal of Non-T inhibitory cells. This suggests that during early ontogeny Non-T cells within spleen may be inhibiting the expression of alloreactivity by splenic T cells. Although possessing an appreciably better response than whole newborn spleen cells, purified splenic T cells from neonatal mice demonstrated only one-half of the allogeneic MLR induced proliferation of an equal number of adult splenic T cells (Figure 9). Therefore newborn T cells from spleen do not possess adult levels of alloreactivity and/or there is an inhibitory T cell population within neonatal spleen. Thus a comparison of the MLRs of whole and isolated T cells from newborn versus adult spleen suggests that there may be both T and Non-T suppressor cells resident in the spleen during early postnatal life.

It is evident from section iii a) of the Introduction that considerable disaggreement exists in the literature as to the nature of the neonatal spleen cell population(s) capable of inhibiting conventional allogeneic MLCs. We considered that our observations suggesting that there may be more than one class of suppressor cell in newborn spleen could possibly provide an explanation for this earlier controversy. In addition we sought to determine whether newborn splenic suppressor cells could also regulate autoreactive processes. Thus we employed a number of fractionation techniques on newborn CBA/J spleen and assayed the resultant cells for suppressor activity in conventional one way CBA/J anti-Balb/cJ allogeneic MLCs and in neonatal CBA/J Type I AMLCs. The initial approach was to isolate T cells from the spleens of one to five day old CBA/J mice using Ig-anti-Ig affinity columns and to add the T cells as well as whole spleen cells to the autologous and allogeneic assay cultures. The results from both the autologous (Figure 10) and allogeneic systems (Figure 11) indicate that whole

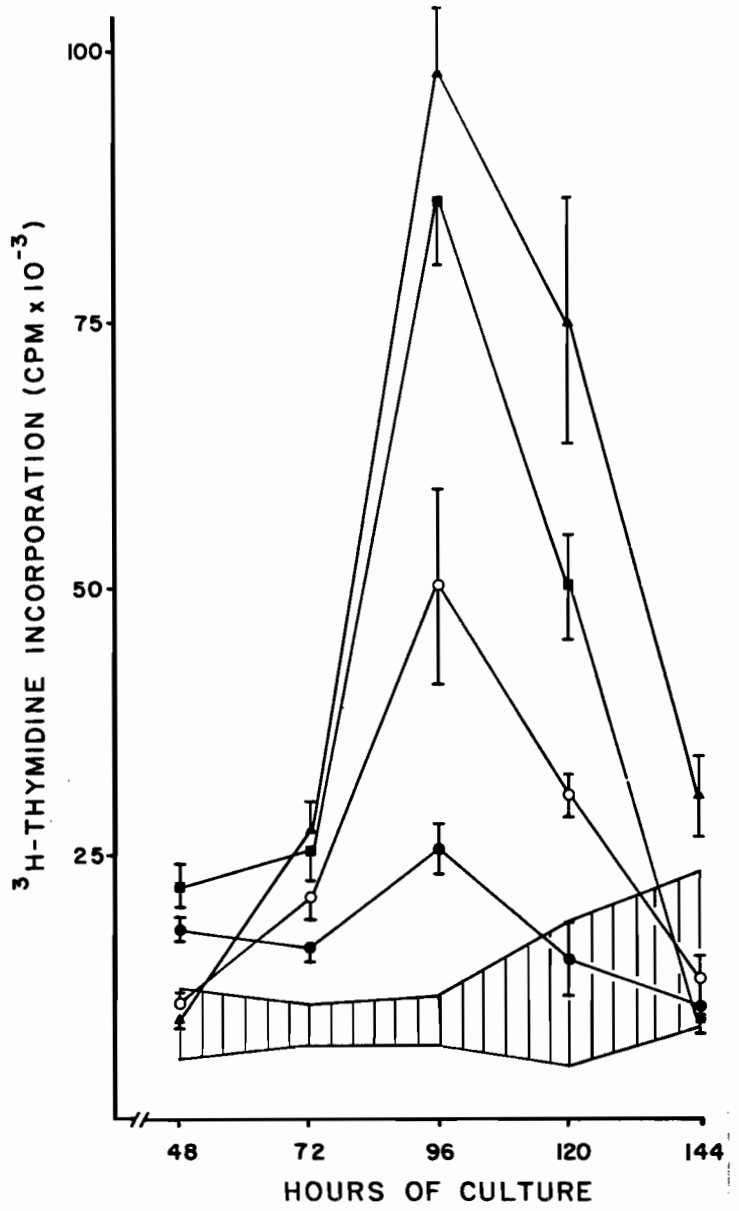



Figure 10. Inhibition of the autologous MLR by the addition of newborn spleen and spleen derived T cells. Control AMLCs (▲) consisted of 250,000 newborn CBA/J thymocytes plus an equal number of syngeneic adult spleen cells. Newborn CBA/J whole spleen cells (●) as well as T cells (O) Ig-anti-Ig affinity purified from the newborn spleen were added to the control cultures in a 1:1 ratio with the adult spleen cell stimulators. Also added to the control cultures were an additional 250,000 adult CBA/J spleen cells (■). The background were within the hatched area. Details of the techniques employed are in Materials and Methods.

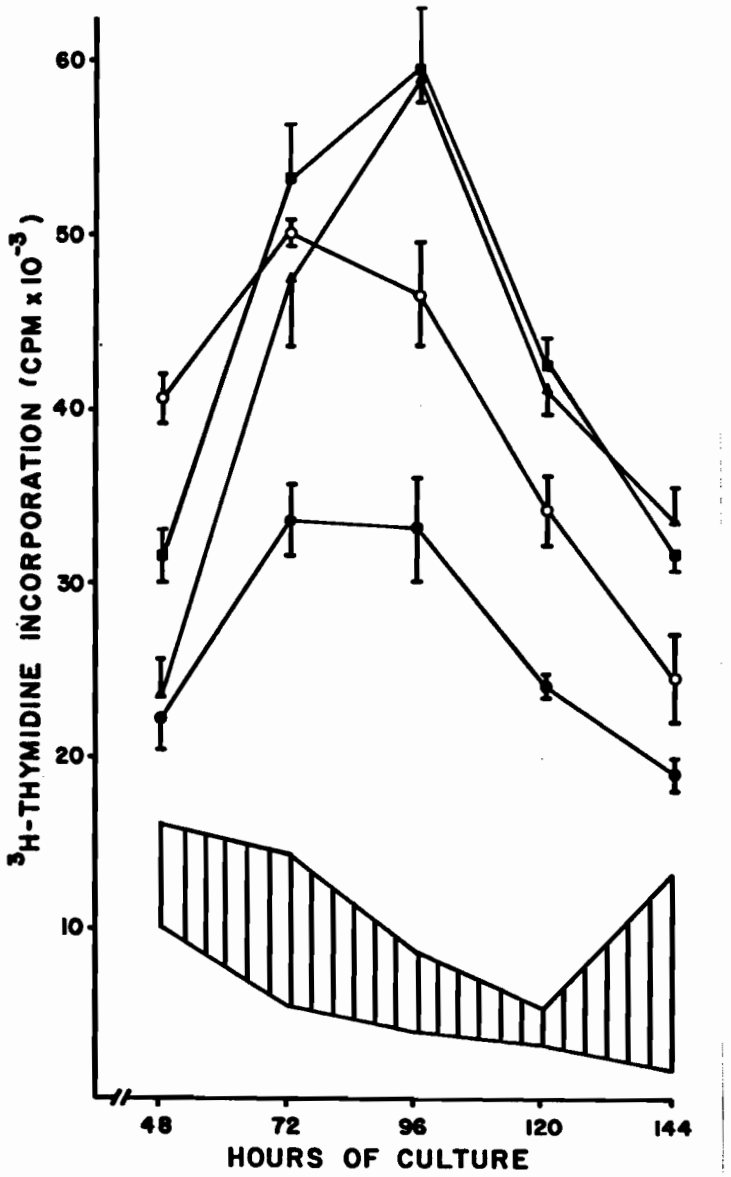



Figure 11. Suppression of the adult allogeneic MLR by the addition of newborn whole spleen and spleen derived T cells. Newborn CBA/J spleen cells (●) as well as T cells purified from the newborn spleen by Ig-anti-Ig affinity column passage (O) were added to control MLCs (▲) consisting of 250,000 adult CBA/J spleen cells responding to 500,000 mitomycin C inactivated Balb/c spleen cells. Adult CBA/J spleen cells (■) were also added to control cultures. All cell additions were ina 1:1 ratio with the adult spleen responder population. The backgrounds were within the hatched area. The techniques employed are described in Materials and Methods.

newborn spleen and also the purified splenic T cells are suppressive. However, newborn splenic T cells were less inhibitory than an equal number of unselected spleen cells. This suggests that neonatal spleen may harbor a second population of inhibitory cell which is removed by the affinity column. As indicated in Figures 10 and 11, addition of adult CBA/J spleen cells to the assay cultures in numbers equivalent to the neonatal additions did not significantly effect the MLRs. Thus the suppressive effects of the added newborn cells was not due to the trivial explanation of overcrowding. Control adult cell additions were routinely performed in all experiments, but in most cases are not presented in the interests of simplifying the following figures and tables.

If both T and Non-T MLC-suppressor cells exist in newborn spleen it might be expected that removal of T cells by negative selection would reduce but not eliminate their inhibitory activity. Table 7 details the results of experiments on the allo-MLR and AMLR in which the inhibition of whole newborn spleen was compared with that of spleen depleted of T cells by anti-T plus RC treatment. In both systems a significant decrease in the suppression was seen on removal of T cells with antisera plus RC while RC treatment alone had no significant effect. The cells remaining after selective T cell depletion, approximately 80% of the whole spleen cells, retained significant inhibitory capacity on both types of MLR. These results support the hypothesis that two distinct MLC-suppressor cell populations reside in newborn spleen.

Piguet et. al. have presented evidence that neonatal macrophages may inhibit T-D PFC responses [191]. We therefore decided to examine whether macrophages could be responsible for part of the inhibition of MLRs. Table 8 shows the results of a comparison of the effects of

Table 7. Depletion of T lymphocytes by negative selection reduces but does not remove the capacity of newborn spleen cells to inhibit autologous and allogeneic MLRs.

| NEWBORN CELLS ADDED TO CONTROL CULTURES[a] | PRETREATMENT OF ADDED CELLS | BACKGROUND[b] | AUTOLOGOUS MLR | ALLOGENEIC MLR |
|---|---|---|---|---|
| | | $^3$H-THYMIDINE INCORPORATION cpm ± S.E. [Relative Response] (% Suppression) | | |
| NONE | ——— | 4,964 ± 778 | 144,087 ± 4,592 [100] | |
| | | 10,103 ± 776 | | 67,291 ± 974 [100] |
| WHOLE SPLEEN | ——— | | 23,414 ± 4,580 [13] (84)* | |
| | | 9,044 ± 1,124 | | 43,152 ± 739 [60] (36)* |
| | RC | | 24,469 ± 3,545 [14] (83)* | |
| | | 12,214 ± 1,449 | | 47,807 ± 1,051 [62] (29)* |
| | anti-T + RC | 6,452 ± 1,427 | 44,663 ± 5,674 [27] (60)* † | |
| | | 9,866 ± 1,028 | | 57,268 ± 3,246 [83] (15)§ † |

[a]Newborn CBA/J spleen cells were added to control autologous cultures consisting of 250,000 newborn CBA/J thymocytes plus an equal number of syngeneic adult spleen cells and to control allogeneic cultures of 250,000 adult CBA/J spleen cells plus 500,000 mitomycin C inactivated Balb/c adult spleen cells. 250,000 untreated newborn spleen cells were added to the cultures while newborn spleen cells, treated as indicated, were added such that the cultures received 250,000 cells minus those sensitive to treatment, as described in Materials and Methods. The peak responses of the cultures are shown; 120 hours of culture for the autologous reaction and 96 hours of culture for the allogeneic MLR.

[b]The background is the $^{3}$H-thymidine incorporation of 250,000 adult CBA/J spleen cells plus the specified cell additions cultured in the absence of newborn thymocytes or Balb/c spleen cells. The latter two cell populations each incorporated less than 1400 cpm when cultured alone.

[*]The inhibition of control cultures by the added cells is significant; $p \leq 0.001$.

[§]The inhibition of control cultures by the added cells is significant; $p \leq 0.050$.

[†]The difference in the response of cultures with added complement treated newborn CBA/J spleen cells and cultures with added anti-T plus complement treated newborn spleen cells is significant; $p \leq 0.050$.

Table 8. The effects of titration of adherent versus non-adherent adult or newborn spleen cells into MLRs.

| SPLEEN CELLS ADDED[a] TO CONTROL CULTURES | RATIO OF ADDED CELLS TO ADULT CBA/J SPLEEN | BACKGROUND[b] | AUTOLOGOUS MLR | ALLOGENEIC MLR |
|---|---|---|---|---|
| | | $^3$H-THYMIDINE INCORPORATION cpm ± S.E. [Relative Response] (% Suppression) | | |
| NONE | ——— | 15,921 ± 784 | 127,907 ± 7,335 [100] | 107,994 ± 3,018 [100] |
| ADULT NON-ADHERENT | 1:1 | 43,703 ± 3,866 | 144,539 ± 10,405 [90] (-) | 136,014 ± 6,425 [100] (-) |
| | 1:2 | 25,355 ± 2,327 | 105,377 ± 11,257 [71] (18) | 117,544 ± 6,262 [100] (-) |
| | 1:4 | 22,451 ± 1,852 | 131,153 ± 6,820 [97] (-) | 103,566 ± 11,513 [88] (4) |
| | 1:8 | 20,480 ± 1,751 | 107,284 ± 2,742 [78] (16) | 114,046 ± 7,020 [102] (-) |
| NEWBORN NON-ADHERENT | 1:1 | 22,825 ± 2,853 | 29,068 ± 3,124 [6] (77)* | 59,064 ± 2,027 [39] (45)* |
| | 1:2 | 26,614 ± 3,597 | 39,924 ± 5,990 [12] (69)* | 64,710 ± 6,077 [41] (40)* |
| | 1:4 | 22,326 ± 3,049 | 77,841 ± 9,134 [50] (39)† | 71,840 ± 5,981 [54] (33)* |
| | 1:8 | 19,752 ± 3,119 | 95,606 ± 7,159 [68] (25) | 83,755 ± 4,471 [70] (22)¶ |
| ADULT ADHERENT | 1:1 | 38,656 ± 3,383 | 94,275 ± 5,531 [50] (26) | 94,503 ± 5,653 [61] (12) |
| | 1:2 | 27,365 ± 1,233 | 92,108 ± 2,642 [58] (28)† | 88,534 ± 9,521 [66] (18)§ |
| | 1:4 | 17,008 ± 563 | 97,257 ± 11,397 [72] (24) | 90,840 ± 4,126 [80] (16)§ |
| | 1:8 | 15,738 ± 361 | 99,937 ± 4,112 [75] (22) | 84,049 ± 2,593 [74] (22)¶ |
| NEWBORN ADHERENT | 1:1 | 9,809 ± 436 | 39,713 ± 8,013 [27] (69)* | 56,027 ± 2,458 [50] (48)* |
| | 1:2 | 13,362 ± 2,196 | 93,866 ± 5,319 [72] (27) | 60,692 ± 3,936 [51] (44)* |
| | 1:4 | 11,698 ± 964 | 106,500 ± 4,134 [85] (17) | 77,184 ± 6,654 [71] (29)* |
| | 1:8 | 15,626 ± 1,429 | 96,102 ± 10,550 [72] (25) | 90,352 ± 6,647 [81] (16)§ |

[a]Spleen cells from newborn and adult CBA/J mice were fractionated by adherence/non-adherence to plastic petri plates as described in Materials and Methods and then titrated into control MLCs. Control reaction mixtures consisted of 250,000 adult CBA/J spleen cells plus an equal number of syngeneic newborn thymocytes in AMLRs or an equal number of mitomycin C inactivated adult Balb/c spleen cells in allogeneic MLCs. CBA/J spleen cells were titrated into control MLCs in the indicated ratios with the 250,000 adult CBA/J spleen cells present in both autologous and allogeneic control MLCs. The proliferation reported is that of peak culture activity which occurred at 96 hours of culture.

[b]The background is the $^{3}$H-thymidine incorporation of cultures containing 250,000 adult CBA/J spleen cells plus the cells added to the control MLCs, where indicated, but without newborn thymocytes or Balb/c spleen cells. Newborn CBA/J thymocytes and Balb/c mitomycin inactivated spleen cells showed $^{3}$H-thymidine uptake of 852 $\pm$ 288 and 758 $\pm$ 225 cpm $\pm$ S.E. respectively when cultured alone.

*The inhibition is significant; $p \leq 0.001$.

¶The inhibition is significant; $p \leq 0.005$.

†The inhibition is significant; $p \leq 0.010$.

§The inhibition is significant; $p \leq 0.050$.

titration of adult and newborn plate adherent versus non-adherent spleen cells into allogeneic MLCs and AMLCs. Non-adherent adult spleen cells added to the cultures had no inhibitory effect on the MLRs. In contrast the addition of adherent adult spleen cells suppressed both allogeneic and autologous reactions. The MLRs were also significantly inhibited by the addition of either non-adherent or adherent newborn spleen cells. Thus non-adherent newborn spleen cells possess an ability to inhibit MLRs which is lacking in non-adherent adult spleen cells. On the other hand the addition of either neonatal or adult plate-adherent spleen cells can suppress MLRs. Newborn splenic adherent cells appear to inhibit MLRs to a greater extent than similar numbers of adult adherent spleen cells. However it is possible that this enhanced inhibitory capacity is the result of contamination of the newborn adherent cell population with non-adherent suppressor cells. Therefore it became necessary to determine whether removal of the macrophage population from T-depleted newborn spleen would also affect the Non-T suppressor activity. Three techniques were employed in the depletion of phagocytic and adherent populations from neonatal spleen; carbonyl iron plus magnet treatment, Sephadex G-10 column passage, and plate adherence. As detailed in Table 9, application of these techniques did not reduce the regulatory activity of newborn T-depleted spleen cells as assayed on both allogeneic and autologous MLRs. The addition of macrophage-depleted Non-T newborn spleen cells to MLCs resulted in highly significant inhibition which was in most cases greater than that caused by the addition of untreated Non-T cells. Thus we conclude that: 1) the MLR-inhibitory capacity of newborn spleen resides for the most part within the non-adherent cell population; and 2) during early ontogeny macrophages offer little or no contribution to the suppression of MLRs by spleen cells.

Table 9. The MLC-inhibitory capacity of newborn spleen cells is not effected by the removal of macrophages.

| NEWBORN CELLS ADDED TO CONTROL CULTURES[a] | MACROPHAGE REMOVAL BY | BACKGROUND[b] | AUTOLOGOUS MLR | ALLOGENEIC MLR |
|---|---|---|---|---|
| | | $^3$H-THYMIDINE INCORPORATION | | |
| | | cpm ± S.E. [Relative Response] (% Suppression) | | |
| NONE | ——— | 10,866 ± 1,508 | 131,431 ± 10,136 [100] | 164,066 ± 3,168 [100] |
| T-DEPLETED SPLEEN | ——— | 8,044 ± 537 | 45,990 ± 187 [31] (65)* | 64,099 ± 3,008 [37] (61)* |
| | CARBONYL IRON + MAGNET TREATMENT | 9,550 ± 3,192 | 42,850 ± 843 [28] (67)* | 51,394 ± 2,360 [27] (69)* |
| | SEPHADEX G-10 COLUMN PASSAGE | 7,348 ± 722 | 37,668 ± 2,233 [25] (71)* | 55,748 ± 3,564 [32] (66)* |
| | 2X PLATE ADHERENCE | 5,708 ± 323 | 27,125 ± 2,463 [18] (79)* | 41,211 ± 984 [23] (75)* |

[a]Newborn CBA/J spleen cells were depleted of T cells by treatment with anti-T cell serum plus complement and then subjected to macrophage removal by the various techniques as described in Materials and Methods. 250,000 of the treated newborn spleen cells were then added to control AMLCs consisting of 250,000 newborn CBA/J thymocytes plus an equal number of syngeneic adult spleen cells, and to control allogeneic MLCs consisting of the same number of adult CBA/J spleen cells plus 250,000 mitomycin C inactivated Balb/c spleen cells. The peak proliferative activity of the cultures is reported and occurred at 96 hours of culture.

[b]The background activity is the $^{3}$H-thymidine incorporation of cultures of CBA/J adult spleen cells plus, where indicated, newborn spleen cells cultured in the same numbers as in the MLCs but without newborn CBA/J thymocytes or adult Balb/c spleen cells. The latter two cell populations cultured alone each incorporated less than 150 mean cpm of $^{3}$H-thymidine.

*The inhibition of the control reaction is statistically significant; $p \leq 0.001$.

We have demonstrated that T as well as Non-T cells from newborn spleen can both inhibit AMLRs and allogeneic MLRs. An important step in conclusively demonstrating two classes of MLC-inhibitory cells is the ability to separate and recover them from the same spleen cell preparation using a single technique. This would subject each cell fraction to similar manipulations and henceforth promote a better comparison of the activity of the two cell types. The technique employed in this study to isolate two distinct classes of MLC-inhibitory cell from newborn spleen is based on the ability of SBA to preferentially agglutinate murine B cells from spleen while not binding the splenic T cells [208]. Prior to the use of the SBA protocol in the analysis of MLC-suppressor cells a series of experiments were performed to show that SBA demonstrates a specificity for B cells in neonatal spleen as it is known to do in adult spleen. Through the use of T and B-cell mitogens, immunofluorescence with flourescein conjugated anti-IgM, and negative selection with anti-T serum plus complement, SBA was found to specifically agglutinate neonatal B cells. This allowed us to separate both a T-enriched and a B-enriched fraction from a single preparation of newborn spleen. The results of a typical experiment on the inhibitory capacity in the AMLR of unselected as well as SBA fractionated T and B cells from newborn spleen are shown in Figure 12. Both T and B cell fractions significantly inhibited the control AMLR. While addition of equal numbers of whole newborn spleen or $SBA^+$ B cells to assay cultures reduced the control thymidine incorporation to virtually background levels, addition of similar numbers of $SBA^-$ T cells inhibited the peak control reaction by approximately 50%. Thus $SBA^-$ neonatal splenic T cells have a significantly lower inhibitory effect on the MLR than unselected spleen cells. This is in agreement with the results of experiments employing affinity purification of

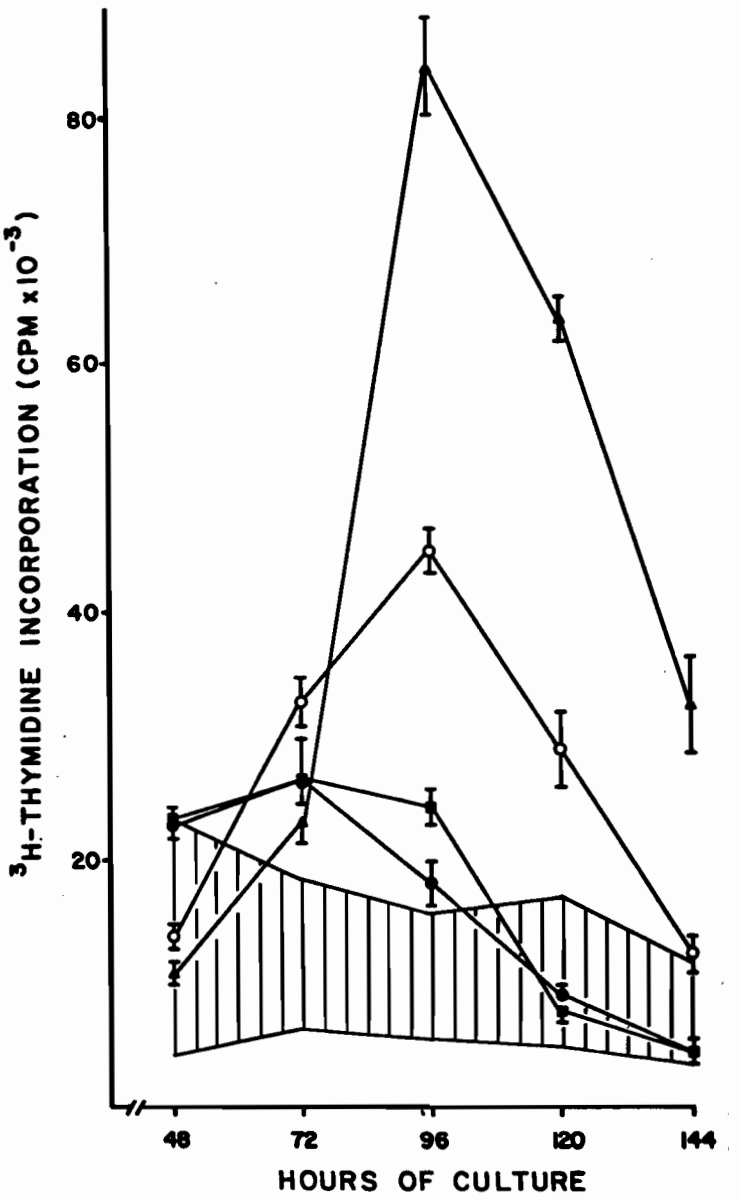



Figure 12. Evidence for two distinct populations of newborn spleen cells capable of inhibiting the AMLR. Unselected newborn CBA/J spleen cells (●), as well as cells fractionated by Soybean agglutinin into $SBA^-$ (O) and $SBA^+$ (■) populations, were added to control AMLCs (▲) consisting of 250,000 of each of newborn CBA/J thymocytes and adult CBA/J spleen cells. Newborn spleen derived cells were added to the control cultures in a 1:1 ratio with the adult CBA/J spleen cells. The backgrounds were within the hatched area. All techniques employed are detailed in Materials and Methods.

Table 10. Newborn spleen cells depleted of macrophages are separable into two AMLR-suppressor cell populations on the basis of agglutination with SBA.

| NEWBORN SPLEEN CELLS[a] ADDED TO CONTROL CULTURES | BACKGROUND[b] | AUTOLOGOUS MLR |
|---|---|---|
| | $^3$H-THYMIDINE INCORPORATION | |
| | cpm ± S.E. | [Relative Response] (% Suppression) |
| NONE | 3,385 ± 339 | 37,098 ± 4,546 [100] |
| WHOLE SPLEEN | 5,922 ± 403 | 10,159 ± 319 [13] (73)* |
| FE + MAGNET TREATED | 10,226 ± 956 | 8,248 ± 1,578 [0] (78)* |
| FE + MAGNET TREATED SBA$^-$ | 25,958 ± 400 | 23,969 ± 2,002 [0] (35)* |
| FE + MAGNET TREATED SBA$^+$ | 11,365 ± 671 | 8,114 ± 526 [0] (78)* |

[a]250,000 newborn CBA/J spleen cells were added to control autologous MLCs consisting of 250,000 newborn CBA/J thymocytes plus an equal number of syngeneic adult spleen cells. Prior to fractionation on the basis of agglutination with SBA, newborn spleen cells were treated with carbonyl iron and phagocytic cells removed by magnetism as described in Materials and Methods. The 96 hour peak proliferative activity of the cultures is reported.

[b]The background is the $^3$H-thymidine incorporation of cultures of newborn and adult spleen cells identical to the MLCs with the exception that newborn thymocytes were lacking. Cultured alone, 250,000 newborn CBA/J thymocytes incorporated 138 ± 18 cpm.

*The inhibition of the control cultures by the addition of cells is significant; $p \leq 0.001$.

T cells from newborn spleen where the T cells were also found to be less suppressive on MLRs than whole spleen cells. On the other hand, $SBA^+$ neonatal spleen B cells demonstrated essentially the same ability to suppress the AMLR as whole spleen cells. As shown in Table 10, newborn spleen cells depleted of macrophages by carbonyl iron plus magnet treatment can also be separated into $SBA^-$ and $SBA^+$ AMLC-suppressor cell populations. The $SBA^+$ cells showed an inhibitory capacity in the AMLC which was similar to that of equivalent numbers of unselected spleen cells while the same proportion of $SBA^-$ cells was relatively less efficient. Thus the removal of potential macrophage contamination has no apparent effect on the ability of either $SBA^-$ or $SBA^+$ neonatal spleen cells to inhibit the AMLR. The inhibitory effects of newborn SBA separated macrophage-depleted spleen cells on the allogeneic MLR were also examined (Figure 13). Addition of the $SBA^+$ fraction to the MLCs suppressed the allogeneic reaction by approximately 75%, only slightly less than the inhibition resulting from addition of similar numbers of unfractionated neonatal spleen cells. On the other hand, the same number of $SBA^-$ newborn cells added to the cultures resulted in 50% inhibition, again significantly less suppression than that shown by the whole or $SBA^+$ cell additions. Therefore newborn spleen cells can be separated by differential agglutination with SBA into two cell populations each with the ability to inhibit both autologous and allogeneic MLRs.

Experiments outlined above have shown that the inhibitory cells in newborn spleen as measured on MLRs are partially sensitive to treatment with anti-T serum plus RC (see Table 7). To test the preliminary conclusions that separation with SBA results in suppressor populations of apparent T cell lineage ($SBA^-$) as well as of Non-T derivation ($SBA^+$), newborn spleen cells fractionated with SBA were

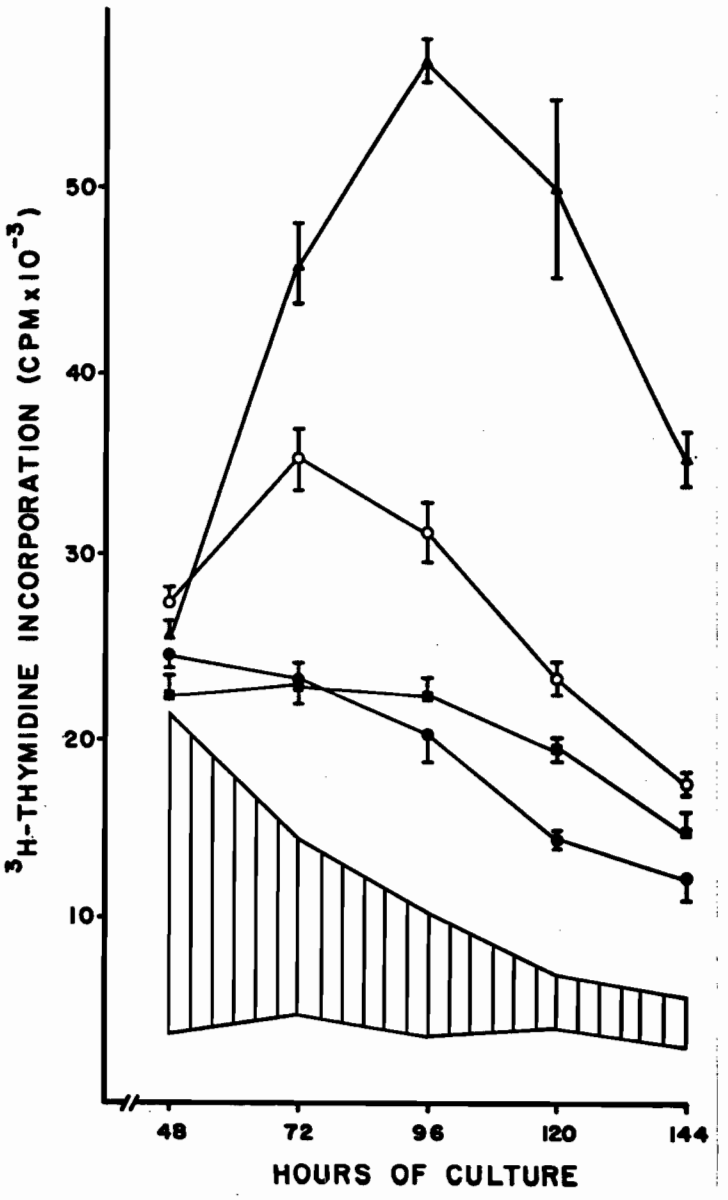



Figure 13. Evidence for two distinct newborn spleen cell populations capable of inhibiting the allogeneic MLC. The control MLC (▲) consisted of 250,000 adult CBA/J spleen cells plus an equal number of mitomycin C inactivated Balb/c spleen cells. Whole spleen cells (●) as well as isolated $SBA^-$ (O) and $SBA^+$ (■) from newborn spleen were added to the control MLCs in a 1:1 ratio with the adult CBA/J spleen responder cells. Newborn spleen cells were depleted of macrophages by carbonyl iron plus magnet treatment prior to fractionation with SBA. Backgrounds were within the hatched area. All techniques employed are described in Materials and Methods.

treated with anti-T serum plus RC prior to their addition to control MLCs (Table 11). The results from both autologous and allogeneic culture systems indicate that the $SBA^-$ inhibitory cell population is sensitive to negative selection with anti-T serum plus RC. In both MLRs a significant reduction in the inhibitory capacity of $SBA^-$ cells was apparent after treatment with anti-T serum plus RC. $SBA^-$ neonatal spleen cells treated with anti-T serum plus RC before addition to assay cultures did not inhibit the AMLR while the addition of untreated or RC treated $SBA^-$ cells resulted in a minimum of 42% suppression. On the other hand pretreatment of the $SBA^+$ newborn spleen cells with anti-T plus RC had no noticeable effect on their ability to inhibit the AMLR. Essentially the same results were seen in the allogeneic MLR although a residual 18% inhibition remained in cultures that had received anti-T plus RC treated $SBA^-$ cells. The data from these experiments indicates that two distinct non-phagocytic neonatal spleen cell populations, separated by differential SBA agglutination, are capable of suppressing both autologous and allogeneic MLCs. The $SBA^-$ and $SBA^+$ inhibitory populations can also be distinguished on the basis of sensitivity to anti-T serum plus complement treatment.

The results of the experiments presented above indicate that when newborn spleen derived cells are added at a 1:1 ratio with the adult CBA/J spleen cells in autologous or allogeneic cultures the T cells are less efficient inhibitors than the Non-T cells. To further test this possibility, spleen cells from newborn CBA/J mice were separated using SBA and the resultant T and Non-T fractions were then titrated into allogeneic MLCs as well as AMLCs. The results of this investigation are shown in Figure 14. When added to cultures at a 1:1 ratio with the adult spleen stimulators both T and Non-T cells significantly suppress the AMLR (Figure 14a).

Table 11. The newborn splenic SBA-separated MLC-inhibitory cell populations are distinguishable by differential sensitivity to treatment with anti-T-cell serum plus complement.

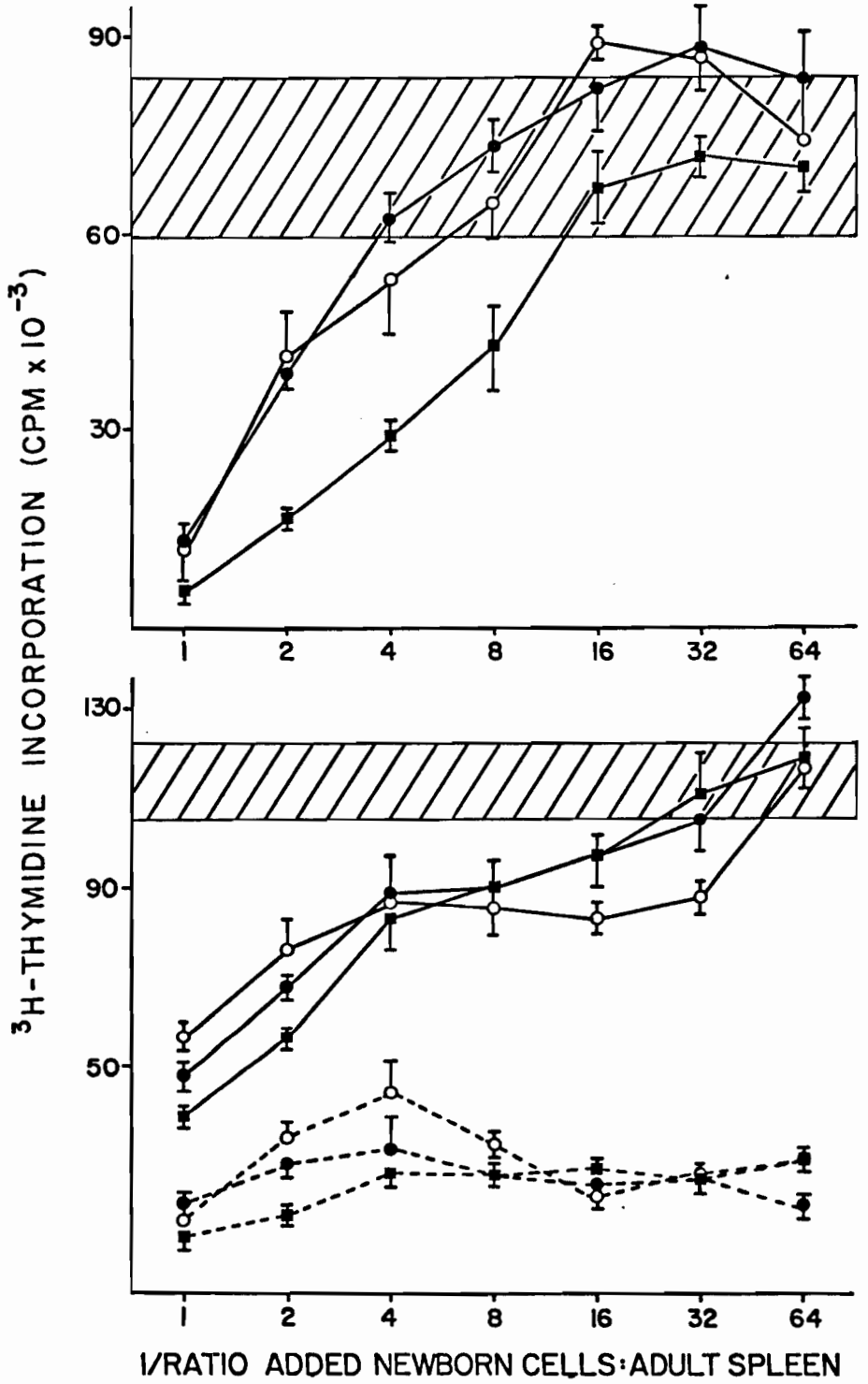
| NEWBORN CELLS ADDED TO CONTROL CULTURES[a] | PRETREATMENT OF ADDED CELLS | BACKGROUND[b] | AUTOLOGOUS MLR | ALLOGENEIC MLR |
|---|---|---|---|---|
| | | $^3$H-THYMIDINE INCORPORATION | | |
| | | cpm ± S.E. [Relative Response] (% Suppression) | | |
| NONE | ——— | 26,571 ± 2,902 | 93,209 ± 13,754 [100] | 178,859 ± 4,830 [100] |
| WHOLE SPLEEN | ——— | 25,751 ± 5,225 | 23,795 ± 4,712 [0] (74) | 74,075 ± 8,127 [32] (57) |
| SBA$^-$ SPLEEN T | ——— | 69,108 ± 9,291 | 53,784 ± 6,057 [0] (42) | 84,410 ± 6,133 [10] (51) |
| | RC | 47,860 ± 6,596 | 41,292 ± 5,795 [0] (56) | 71,617 ± 5,918 [16] (59) |
| | anti-T + RC | 21,682 ± 1,451 | 116,147 ± 27,025 [142] (0)* | |
| | | 19,040 ± 2,897 | | 141,924 ± 4,362 [81] (18)* |
| SBA$^+$ SPLEEN NON-T | ——— | 7,965 ± 1,639 | 5,592 ± 930 [0] (94) | 60,169 ± 2,715 [34] (65) |
| | RC | 7,821 ± 725 | 6,764 ± 1,332 [0] (93) | 50,671 ± 5,823 [33] (72) |
| | anti-T + RC | 4,426 ± 368 | 3,055 ± 221 [0] (97) | 52,736 ± 1,004 [32] (70) |

[a]Newborn CBA/J spleen cells were added to control cultures consisting of 250,000 adult CBA/J spleen cells plus an equal number of CBA/J newborn thymocytes in the autologous MLC and the same number of mitomycin C inactivated Balb/c spleen cells in the allogeneic MLC. Newborn spleen cells were separated on the basis of SBA agglutination prior to antiserum plus complement treatment and 250,000 cells minus those cells sensitive to antiserum plus complement treatment were added to control cultures as described in Materials and Methods. Proliferation was assayed at 120 hours of culture.

[b]The background proliferation is that of 250,000 CBA/J adult spleen cells without and with newborn spleen cells treated as indicated and added in the same numbers as in the MLCs. The proliferation of CBA/J newborn thymocytes and mitomycin inactivated Balb/c spleen cells cultured alone were both under 1000 cpm.

*The difference in mean response between cultures with added RC treated newborn spleen cells and cultures with added anti-T plus RC treated newborn spleen cells is significant; $p \leq 0.001$.

Figure 14. Comparison of the suppression of autologous (panel a) versus allogeneic (panel b) MLCs by whole and SBA separated newborn spleen cells. Neonatal CBA/J unselected (●), $SBA^-$ (○), and $SBA^+$ (■) spleen cells were titrated into control autologous MLCs consisting of 250,000 newborn CBA/J thymocytes plus an equal number of syngeneic adult spleen cells and into control allogeneic MLCs consisting of 250,000 adult CBA/J spleen cells plus an equal number of mitomycin C inactivated adult Balb/c spleen cells. Proliferation of the cultures was assayed at 96 hours of culture. The backgrounds (broken lines) of CBA/J adult plus the indicated newborn spleen cell populations but without CBA/J newborn thymocytes or Balb/c spleen cells are shown. CBA/J newborn thymocytes and mitomycin inactivated Balb/c spleen cells cultured alone incorporated 1,213 $\pm$ 483 and 394 $\pm$ 62 cpm $\pm$ S.E. respectively. The hatched area represents the 0.95 confidence interval for the mean $^3$H-thymidine incorporation of cultures without added newborn cells. All techniques are detailed in Materials and Methods.

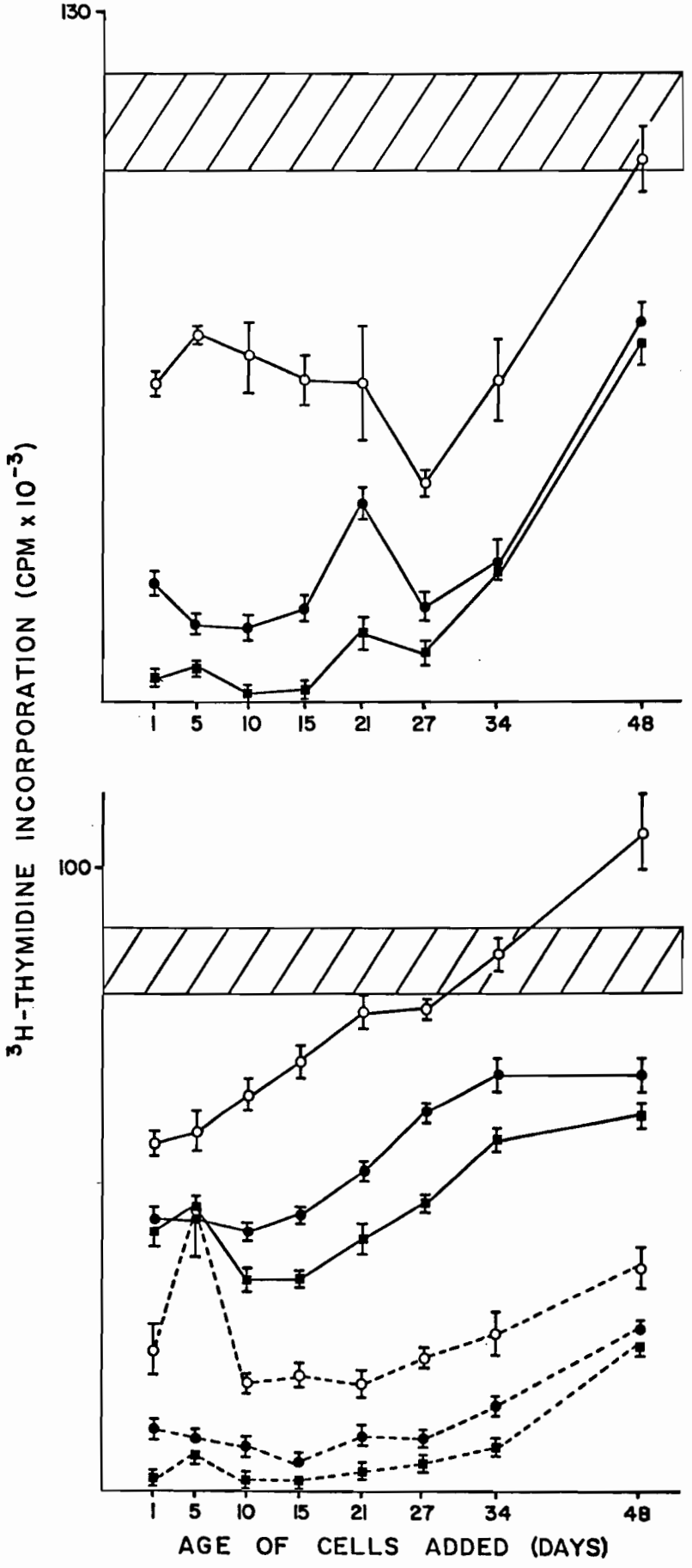

90
60
30
130
90
50
1
2
4
8
16
32
64
$^3$H-THYMIDINE INCORPORATION (CPM x $10^{-3}$)
1/RATIO ADDED NEWBORN CELLS:ADULT SPLEEN

The inhibitory effects of the T cell preparation were less than those of equal numbers of Non-T cells. T cells added in a 1:4 ratio did not cause significant inhibition of the autologous response. On the other hand, neonatal Non-T cells added in ratios as low as 1:8 significantly suppressed the reaction. In the allogeneic MLC (Figure 14b) the addition of Non-T cells caused greater inhibition than equivalent numbers of T cells added at ratios of 1:1 and 1:2. However at dilutions over 1:4, T cells effected more inhibition than Non-T cells. The loss of significant inhibitory effects in the allogeneic MLC was seen when Non-T cells were added at 1:16 and T cells were added at 1:64. Although the AMLR was suppressed to a greater extent than the allogeneic reaction by the presence of either inhibitory cell type in a ratio of 1:1, the T cell population showed more pronounced inhibition in the allogeneic reaction above 1:4. Thus small numbers of newborn splenic T cells are apparently capable of suppressing allogeneic MLRs but not AMLRs. Evidence has previously been presented that immunization of the neonate leads preferentially to activation of suppressor versus helper pathways in an immunological circuit [200]. It is possible that newborn inhibitory T cells may be triggered in the MLC to induce suppressor cells from other cell populations within the culture. As a result small numbers of neonatal splenic T cells may be capable of causing significant inhibitory effects by acting as suppressor inducers. Conceivably allogeneic MLRs may be more efficient than AMLRs in the activation of newborn T cells involved in inhibitory pathways. This may result in noticeable suppression by the addition of low numbers of neonatal T cells to allogeneic but not autologous MLCs.

The physical and functional distinction between the two types of newborn splenic MLC-inhibitory cells raises the possibility that the two classes of cell may be active during

different stages of postnatal ontogeny. To test for this, spleen cells from CBA/J mice at different ages were separated using SBA and then added to control autologous (Figure 15a) and allogeneic MLCs (Figure 15b). The addition to autologous cultures of unselected or isolated T or Non-T cells from the spleens of one day to four week old mice caused relatively consistent inhibition of the AMLR. While Non-T spleen cells throughout this period of ontogeny virtually ablated the autologous reaction, equivalent numbers of T cells inhibited the AMLR by approximately 50%. Unseparated spleen cells from animals aged from one day to four weeks suppressed the autologous response to a slightly lesser extent than equal numbers of Non-T cells. During the period of development from four to seven weeks of age, unseparated as well as T and Non-T spleen cells gradually lose in parallel their ability to inhibit the autologous reaction. However the Non-T fraction may begin a more subtle loss in suppressor ability as early as at the end of two weeks of life. Thus at seven weeks of age whole and Non-T spleen cells still retain significant inhibitory effects on the AMLR while the T cell population has lost the ability to suppress this reaction. On the other hand, a different pattern is seen on the addition of the various spleen cell fractions to allogeneic MLCs. Unfractionated cells, T cells, and Non-T cells from the spleens of one day old animals suppressed the reaction by approximately 60, 40, and 60% respectively. Inhibition mediated by unfractionated spleen cells from mice of different ages in the MLCs was constant for the first two weeks of life. However from five to ten days of age the inhibitory activity of the Non-T cells appeared to increase, thereafter remaining constant until two weeks following birth. The suppressive capacity of both whole and Non-T spleen cells then decreased in a linear fashion until five weeks of age. Significant inhibition by the addition of

Figure 15. Age versus MLR inhibitory capacity of newborn whole (●), T (○) and Non-T (■) spleen cells. AMLCs (panel a) consisted of 250,000 thymocytes from newborn CBA/J mice responding to an equal number of syngeneic adult spleen cells. Allogeneic MLCs (panel b) were composed of 250,000 adult CBA/J spleen cells plus the same number of mitomycin C inactivated Balb/c spleen cells. 250,000 of each of the newborn spleen cell populations were added at the initiation of the cultures. Proliferation was assayed at 120 hours of culture. The hatched area represents the 0.95 confidence interval for the mean $^{3}$H-thymidine incorporation of cultures without added newborn cells. Cell manipulation, culture, and assay of proliferation were carried out as described in Materials and Methods.


130
100
$^3$H-THYMIDINE INCORPORATION (CPM x $10^{-3}$)
1
5
10
15
21
27
34
48
AGE OF CELLS ADDED (DAYS)

Non-T cells was observed up to seven weeks after birth. In contrast, splenic T cells showed a linear decrease in inhibitory capacity with increasing age from birth until three weeks of life at which time no suppressive ability remained. These findings indicate that during early postnatal development the inhibitory capacity of splenic T and Non-T cell populations decays at different rates in the autologous and allogeneic MLCs. The T and Non-T cells both showed relatively consistent inhibition in the AMLR throughout the first seven weeks of life before a rapid loss in this activity became apparent. When assayed in the allogeneic MLC, splenic T inhibitors showed a constant loss in inhibitory potential beginning shortly after birth. The Non-T population, on the other hand, became more suppressive from days one and five to days ten and fifteen of life, with decreasing inhibitory activity becoming apparent afterwards. The differences in the changes in splenic T versus Non-T MLC suppressor capacity during early ontogeny, particularly noticeable when assayed in the allogeneic MLC, perhaps indicate a further distinction between the two inhibitory cell populations.

b) Phenotypic Characterization of Newborn Splenic MLC-Inhibitory T Cells.

Evidence presented above indicates that one population of newborn splenic MLC suppressor cells is apparently of T cell lineage. These inhibitors are not retained by Ig-anti-Ig affinity columns, are not agglutinated by SBA, and are sensitive to cytotoxic treatment with anti-T serum plus complement. To further analyze the surface antigenic phenotype of this class of suppressor cell, T cells were affinity column purified from the spleens of 1 to 4 day old CBA/J mice and then treated with a variety of antisera plus RC. Treated cells were added to MLCs such that assay

cultures received only the fraction of cells resistant to a particular negative selection procedure. The results of a representative experiment using this approach are tabulated in Table 12. The addition of untreated newborn splenic T cells to autologous and to allogeneic MLCs resulted in approximately 50% inhibition of each control response as measured by thymidine uptake. Pretreatment of the added cells with anti-T serum plus RC virtually removed their ability to inhibit the AMLR while reducing their suppressive effects on the allogeneic reaction by over 50%. AMLCs and allogeneic MLCs with added neonatal cells pretreated with anti-MICG plus RC showed significantly greater proliferative responses than assay cultures with added control treated regulator cells. Anti-$Ia^k$ plus RC treatment of the inhibitory cells also resulted in a slight decrease in their suppressive capacity in both classes of MLR, but this was marginally significant in only the allogeneic reaction. Negative selection with RC plus either anti-THY 1.2 or anti-Lyt 2.1 did not appear to alter the ability of the newborn cells to inhibit either the AMLR or the allogeneic MLR. Neonatal splenic T cells pretreated with anti-Lyt 1 plus RC showed a reduced capacity to inhibit the AMLR but no significant decrease in the ability to suppress the allogeneic reaction. These results indicate that the majority of neonatal MLC regulatory spleen T cells express antigenic determinants detected in cytotoxic assays by anti-T serum. A large percentage of these cells also seem to possess surface MICG determinants. On the other hand, most of these cells are either Thy 1, Lyt, and Ia negative or do not appear to have sufficient surface density of these antigens to support their destruction through the use of the relevant antisera plus complement.

Neonatal spleen has previously been shown to contain an efficient T cell inhibitor of the T-dependent humoral

Table 12. The cell surface antigenic phenotype of newborn splenic T MLC-inhibitory cells determined by negative selection.

| NEWBORN CELLS ADDED TO CONTROL CULTURES[a] | PRETREATMENT OF ADDED CELLS | BACKGROUND[b] | AUTOLOGOUS MLR | ALLOGENEIC MLR |
|---|---|---|---|---|
| | | $^3$H-THYMIDINE INCORPORATION | | |
| | | cpm ± S.E. | [Relative Response] (% Suppression) | |
| NONE | ——— | 26,663 ± 2,208 | 103,893 ± 5,627 [100] | 126,521 ± 5,999 [100] |
| WHOLE SPLEEN | ——— | 29,099 ± 688 | 28,120 ± 187 [0] (73) | 51,848 ± 4,353 [23] (59) |
| SPLENIC T | ——— | 47,341 ± 3,548 | 46,829 ± 2,544 [0] (55) | 61,757 ± 1,278 [14] (51) |
| | RC' | 47,426 ± 3,662 | 50,105 ± 1,936 [3] (52) | 62,822 ± 7,667 [15] (50) |
| | anti-T + RC' | 34,527 ± 2,013 | 100,040 ± 8,885 [85] (4)* | 96,176 ± 4,301 [62] (24)¶ |
| | anti-MICG + RC' | 35,467 ± 2,989 | 87,361 ± 3,128 [67] (16)* | 86,043 ± 7,472 [51] (32)§ |
| | anti-THY 1.2 + RC' | 29,280 ± 1,556 | 50,636 ± 2,299 [28] (51) | 61,078 ± 7,702 [32] (52) |
| | anti-Lyt 1.1 + RC' | 35,757 ± 386 | 65,184 ± 7,030 [38] (37)§ | 68,671 ± 5,823 [33] (46) |
| | anti-Lyt 2.1 + RC' | 37,613 ± 2,562 | 55,228 ± 1,974 [23] (47) | 66,793 ± 4,843 [29] (47) |
| | anti-Ia$^k$ + RC' | 49,156 ± 7,315 | 60,496 ± 7,315 [15] (42) | 81,845 ± 4,876 [33] (35)§ |

[a]250,000 newborn CBA/J spleen cells or splenic T cells isolated by Ig-anti-Ig column passage minus those cells removed by cytotoxic pretreatment were added to autologous cultures consisting of 250,000 newborn CBA/J thymocytes plus an equal number of syngeneic adult spleen cells, and to allogeneic cultures of the same number of adult CBA/J spleen cells plus 250,000 mitomycin inactivated Balb/c spleen cells. Newborn splenic T cells were treated as described in materials and methods with anti-T (rabbit anti-mouse brain, S. Kontainen) 1/15; anti-MICG (S. Hauptman) 1/5; anti-THY 1.2 (NEN) 1/10; anti-Lyt 1.1 (NEN) 1/100; anti-Lyt 2.1 (NEN) 1/100; and anti-$Ia^k$ (Cedarlane) 1/5. The proliferation shown was determined at the 96 hour peak activity of control cultures.

[b]The background is the proliferation of 250,000 adult CBA/J spleen cells with, where indicated, the newborn CBA/J spleen derived cells addition co-cultured in the absence of newborn thymocytes or Balb/c spleen cells. The newborn thymocytes and Balb/c mitomycin inactivated spleen cells incorporated 473 $\pm$ 128 and 367 $\pm$ 19 cpm respectively.

[*]The difference between the response of cultures with added RC' treated newborn T cells and that of cultures with added antisera plus complement treated cells is statistically significant; $p \leq 0.001$.

[¶]The difference between the response of cultures with added RC' treated newborn T cells and that of cultures with added antisera plus complement treated cells is statistically significant; $p \leq 0.005$.

[§]The difference between the response of cultures with added RC' treated newborn T cells and that of cultures with added antisera plus complement treated cells is statistically significant; $p \leq 0.050$.

response [99,163,165,170]. Comparison of the cell surface antigenic phenotypes of the newborn T cells capable of suppressing MLC and TD PFC responses offers a clear distinction between these two classes of regulatory lymphocytes. Table 13 details the results of an analysis of the phenotype of the newborn TD PFC inhibitory T cells, affinity purified from spleen. Newborn T cells were treated with various cytotoxic antisera plus complement in an analogous fashion to the experiments reported in Table 12. Cells were added such that the assay cultures received only the fraction of cells resisting negative selection. Control suppression of the primary in vitro anti-SRBC PFC response resulting from the addition to assay cultures of untreated newborn splenic T cells ranged from 61% to 81% in the different culture systems. Treatment of the neonatal T-cells with anti-THY 1.2, anti-Lyt 1.1, or anti-$Ia^k$ plus RC effectively removed their ability to inhibit the TD PFC response while similar treatment with anti-Lyt 2.1 plus RC had no noticeable effect. These results suggest that the newborn splenic T cell inhibitors of the TD PFC response possess Thy 1, Lyt 1, and Ia antigens in sufficient density to be detected in a cytotoxic assay. This contrasts with the apparent lack of these antigens on the MLC regulatory T cell as determined using a similar approach. Therefore T-D PFC assays and MLCs are suppressed by phenotypically distinct populations of newborn splenic T cells.

c) Further Characterization of the Newborn Splenic Non-T MLC-Inhibitory Cell Population.

Data presented earlier in this investigation indicates that the spleen of neonatal CBA/J mice contains a population of cells capable of suppressing MLCs which is not thymus derived. This class of regulatory cell is resistant to the cytotoxic effects of anti-T serum plus complement and is

Table 13. Effect of cytotoxic pretreatment with anti-Ia and anti-T-cell anti-sera on the ability of newborn T-cells to inhibit primary antibody responses to SRBC.

| SOURCE OF T-CELLS[a] | PRETREATMENT[b] | ASSAY CULTURE[c] IgM SRBC-PFC/culture ± S.E.M. (% suppression) | |
|---|---|---|---|
| | | Exp. 1 | Exp. 2 |
| - | - | 955 ± 53 | 2,246 ± 148 |
| ADULT | - | 863 ± 58 (10) | 2,721 ± 94 (0) |
| NEWBORN | - | 370 ± 28 (61) | 360 ± 51 (84) |
| | RC | 340 ± 20 (64) | 295 ± 91 (81) |
| | ANTI-THY 1 + RC | 1,100 ± 60 (0) | 2,059 ± 74 (8) |
| | ANTI-LYT 1 + RC | 911 ± 72 (5) | 2,324 ± 151 (0) |
| | ANTI-LYT 2 + RC | 365 ± 45 (62) | 495 ± 42 (78) |
| | ANTI-$Ia^k$ + RC | 846 ± 28 (11) | 2,055 ± 119 (9) |

[a]T-cells were purified from the spleens of adult and newborn CBA/J mice by Ig-anti-Ig affinity column passage.

[b]Aliquots of 3 x $10^6$ T-cells were treated with antiserum plus complement, complement alone, or medium and adjusted such that assay cultures received $10^6$ cells minus those sensitive to the cytotoxic treatment.

[c]The assay cultures in Experiment 1 consisted of 20 x $10^6$ adult CBA/J spleen cells plus 3 x $10^6$ SRBC while those in Experiment 2 consisted of the same number of SRBC plus 10 x $10^6$ adult CBA/J spleen cells. PFCs were assayed after 4 days of culture. The cell preparation, culture, and PFC assay are described in Materials and Methods.

agglutinated by the lectin SBA which has an affinity, in murine spleen, for B-cells. In addition, these inhibitory cells are retained by Ig-anti-Ig affinity columns although they are not adherent to plastic plates or Sephadex G-10. This data suggests that the Non-T suppressors of MLRs may be Ig and/or Fc receptor bearing B lymphocytes. As a result, an investigation was conducted to determine whether or not the Non-T regulatory cells possess any other markers common to mature B-cells. Table 14 details the results of experiments investigating the sensitivity of newborn Non-T MLC inhibitor cells to treatment with RC plus antiserum specific for IgM, Ig, or $Ia^k$. In autologous and allogeneic culture systems, the regulatory ability of T-depleted neonatal spleen cells was unaffected by treatment with RC alone or anti-$Ia^k$ plus RC. On the other hand, pretreatment of the neonatal Non-T cells with anti-IgM or anti-Ig plus RC caused a decrease in their inhibitory capacity, assayed in both classes of MLC, of approximately 10 to 20%. Thus the Non-T MLC suppressor cell is partially sensitive to cytotoxic treatment with antisera directed at surface Ig. However we had observed by immunofluoresence that more than 50% of isolated Non-T newborn spleen cells express surface IgM (not shown). We therefore concluded that negative selection directed at Ig determinants was not capable in our hands of killing the majority of Ig-bearing neonatal B cells.

Since negative selection of newborn B cells seemed largely ineffective, we employed two different experimental approaches in an attempt to physically separate surface Ig-positive from surface Ig-negative Non-T spleen cells. The techniques involved specific adherence of Ig-positive cells to anti-Ig coated plates by panning [141,257], and formation of rosettes between anti-Ig pretreated Ig-positive cells and protein-A coated SRBC [80,111]. Both procedures fractionated newborn Non-T spleen cells prepared by agglutination with SBA

Table 14. The effect of treatment of Non-T MLC-inhibitory cells with anti-Ig or anti-Ia plus complement.

| NEWBORN CELLS ADDED TO CONTROL CULTURES[a] | PRETREATMENT OF ADDED CELLS[b] | BACKGROUND[c] | AUTOLOGOUS MLR | ALLOGENEIC MLR |
|---|---|---|---|---|
| | | $^3$H-THYMIDINE INCORPORATION | | |
| | | cpm ± S.E. | [Relative Response] (% Suppression) | |
| NONE | ——— | 6,953 ± 997 | 90,732 ± 7,331 [100] | |
| | | 1,279 ± 56 | | 114,140 ± 7,614 [100] |
| WHOLE SPLEEN | ——— | 8,733 ± 1,526 | 27,823 ± 1,578 [23] (69) | |
| | | 4,449 ± 1,018 | | 29,542 ± 2,602 [22] (74) |
| NON-T SPLEEN | ——— | 7,542 ± 922 | 26,998 ± 1,886 [23] (70) | |
| | | 1,376 ± 121 | | 18,790 ± 122 [15] (84) |
| | RC | 5,572 ± 475 | 20,442 ± 5,825 [18] (77) | |
| | | 1,650 ± 365 | | 18,056 ± 1,221 [15] (84) |
| | NRS | 5,302 ± 489 | 18,653 ± 1,452 [16] (79) | |
| | anti-IgM + RC | 5,801 ± 235 | 33,969 ± 7,065 [34] (63) | |
| | | 2,336 ± 358 | | 41,798 ± 5,455 [35] (64)* |
| | anti-Ig + RC | 8,718 ± 857 | 36,426 ± 4,093 [33] (60)§ | |
| | | 5,694 ± 867 | | 29,692 ± 1,974 [21] (74)¶ |
| | anti-Ia$^k$ + RC | 4,996 ± 234 | 16,297 ± 2,828 [13] (82) | |
| | | 2,297 ± 64 | | 14,009 ± 768 [10] (88 |

[a]250,000 newborn CBA/J spleen cells or non-T cells, minus those cells susceptible to cytotoxic pretreatment, were added to control cultures. Newborn Non-T cells were prepared from spleen by removal of phagocytic cells using carbonyl iron and magnetism followed by cytotoxic depletion of T cells using anti-T serum plus complement. Control autologous cultures were composed of 250,000 CBA/J newborn thymocytes plus an equal number of syngeneic adult spleen cells. The control allogeneic cultures consisted of 250,000 adult CBA/J spleen cells plus an equal number of mitomycin C inactivated Balb/c spleen cells. Newborn mice employed in the autologous experiment ranged from 1 to 5 days of age (average 2.8 days) while in the allogeneic experiment the newborn mice were used from the day of birth to 5 days of age (average 2.3 days). In both experiments the peak proliferative activity of the cultures is shown; 96 hours of culture for the autologous MLC and 120 hours of culture for the allogeneic MLC.

[b]Newborn CBA/J Non-T cells were treated, as described in Materials and Methods, with anti-sera at the following dilutions: anti-IgM 1/10 in the autologous experiment and 1/5 in the allogeneic experiment; anti-Ig 1/5; anti-$Ia^k$ 1/10; and NRS 1/5.

[c]The background is the $^3$H-thymidine incorporation of cultures of newborn CBA/J spleen derived cells plus adult CBA/J spleen cells equivalent to the MLCs except the lack of newborn thymocytes in the autologous cultures and Balb/c spleen cells in the allogeneic cultures. 250,000 newborn CBA/J thymocytes cultured alone and the mitomycin inactivated Balb/c splenocytes cultured alone incorporated $149 \pm 4$ and $588 \pm 408$ mean cpm $\pm$ S.E. respectively.

*The difference between the response of cultures with added antiserum plus RC' treated newborn Non-T spleen cells and those with added cells treated with RC'alone is statistically significant; $p \leq .001$.

¶The difference between the response of cultures with added antiserum plus RC' treated newborn Non-T spleen cells and those with added cells treated with RC'alone is statistically significant; $p \leq .005$.

§The difference between the response of cultures with added antiserum plus RC' treated newborn Non-T spleen cells and those with added cells treated with RC'alone is statistically significant; $p \leq .05$.

into two populations of roughly equal proportions. As can be seen in Table 15, the addition of Ig-positive or Ig-negative $SBA^+$ neonatal spleen cells to assay cultures suppressed the autologous and allogeneic reactions to a similar extent. It is evident that separation of Non-T cells on the basis of surface Ig expression using these techniques does not result in any significant separation of inhibitory and non-inhibitory cells. Since a large segment of the Non-T MLC-suppressor cell population consists of cells expressing surface Ig we concluded that these inhibitory cells may in fact be B cells.

Although it has been reported that newborn murine spleen, in comparison with that of the adult, contains few cells that bear Fc or C3b receptors [75], experiments were conducted to test for the presence of these markers on the newborn MLC-regulatory cells. The results of this investigation are shown in Table 16. Newborn spleen cells were fractionated on the basis of formation of rosettes with either C3b or Ig coated SRBC [75]. Less than 10% of the whole spleen cells were recovered in either the Fc or C3b receptor rosetting fractions. The cells collected from the C3b rosettes showed no significant ability to inhibit the autologous MLR while significantly suppressing the allogeneic reaction. The few cells found in the FcR rosetting fraction significantly suppressed both the AMLR and allogeneic MLR. In all cases the proportion of receptor positive cells isolated by rosette formation did not inhibit the reactions to as great an extent as the proportion of cells which did not rosette. In addition, the receptor-negative fractions showed improved inhibition over that of an equal number of unselected spleen cells. It would thus appear that newborn splenic MLC-regulatory cells, for the most part , do not possess Fc and/or C3b receptors.

From the results presented in the preceding sections we

Table 15. The inhibitory effects on autologous and allogeneic MLRs of newborn SBA-positive spleen cells further fractionated on the basis of surface Ig expression.

| NEWBORN SPLEEN CELLS ADDED TO CONTROL CULTURES[a] | BACKGROUND[b] | AUTOLOGOUS MLR | | | ALLOGENEIC MLR | | |
|---|---|---|---|---|---|---|---|
| | $^3$H-THYMIDINE INCORPORATION | | | | | | |
| EXPERIMENT I. | cpm ± S.E. | [Relative Response] | (% Suppression) | | [Relative Response] | (% Suppression) | |
| NONE | 19,155 ± 1,530 | 55,610 ± 5,247 | [100] | | 69,618 ± 3,524 | [100] | |
| WHOLE SPLEEN | 10,496 ± 1,804 | 7,347 ± 693 | [0] | (87)* | 41,207 ± 2,240 | [61] | (41)* |
| SBA$^+$ SPLEEN | 3,429 ± 795 | 2,710 ± 416 | [0] | (95)* | 25,292 ± 2,019 | [43] | (64)* |
| ANTI-IG PLATE NON-ADHERENT SBA$^+$ | 5,537 ± 656 | 3,997 ± 414 | [0] | (93)* | 30,705 ± 2,676 | [50] | (56)* |
| ANTI-IG PLATE ADHERENT SBA$^+$ | 928 ± 125 | 666 ± 51 | [0] | (99)* | 35,921 ± 1,520 | [69] | (48)* |
| EXPERIMENT II. | | | | | | | |
| NONE | 2,837 ± 793 | 89,377 ± 9,874 | [100] | | | | |
| WHOLE SPLEEN | 7,298 ± 1,411 | 17,120 ± 3,400 | [11] | (81)* | | | |
| SBA$^+$ SPLEEN | 4,386 ± 557 | 5,442 ± 423 | [1] | (94)* | | | |
| PROTEIN-A ROSETTE NEGATIVE SBA$^+$ | 4,910 ± 730 | 5,328 ± 530 | [0] | (94)* | | | |
| PROTEIN-A ROSETTE POSITIVE SBA$^+$ | 3,491 ± 285 | 6,818 ± 368 | [4] | (92)* | | | |

[a]250,000 newborn CBA/J spleen cells were added to control cultures consisting of 250,000 adult CBA/J spleen cells co-cultured with an equal number of syngeneic newborn thymocytes in autologous MLCs and with an equal number of mitomycin C inactivated adult Balb/c spleen cells in allogeneic MLCs. In experiment I newborn spleen cells were first depleted of macrophages by carbonyl iron plus magnet treatment and then separated by SBA agglutination prior to fractionation on the basis of surface immunoglobulin by adherence/non-adherence to rabbit anti-mouse Ig coated plates. In experiment II CBA/J newborn spleen cells were agglutinated by SBA, treated with rabbit anti-mouse Ig, and then separated by rosetting with protein A coated SRBC. All techniques are described in Materials and Methods. The peak activity of the reactions is reported and occurred at approximately 120 hours of culture in both experiments.

[b]The background is the proliferative activity of 250,000 CBA/J adult spleen cells with and without the addition of the specified newborn spleen derived cells cultured in the absence of newborn thymocytes or Balb/c spleen cells. In both experiments the mean thymidine incorporation of the newborn thymocytes or mitomycin inactivated Balb/c spleen cells cultured alone was less than 750 cpm.

*The inhibition of control cultures by the addition of newborn spleen derived cells is significant; $p \leq 0.001$.

Table 16. The inhibitory effects on autologous and allogeneic MLRs of newborn spleen cells separated on the basis of expression of Fc or C3b receptors.

| NEWBORN SPLEEN CELLS ADDED TO CONTROL CULTURES[a] | BACKGROUND[b] | AUTOLOGOUS MLR | ALLOGENEIC MLR |
|---|---|---|---|
| | $^3$H-THYMIDINE INCORPORATION | | |
| | cpm ± S.E. | [Relative Response] (% Suppression) | |
| NONE | 3,709 ± 481 | 83,055 ± 8,122 [100] | 136,815 ± 839 [100] |
| WHOLE SPLEEN | 7,299 ± 1,071 | 21,649 ± 2,327 [18] (74)* | 46,173 ± 2,788 [29] (66)* |
| C3b ROSETTE NEGATIVE | 10,654 ± 1,621 | 12,471 ± 1,349 [2] (85)* | 33,673 ± 3,452 [17] (75)* |
| C3b ROSETTE POSITIVE | 15,413 ± 4,297 | 71,985 ± 5,741 [71] (13) | 79,299 ± 3,798 [48] (42)* |
| IG ROSETTE NEGATIVE | 10,134 ± 1,018 | 10,577 ± 694 [1] (84)* | 31,308 ± 1,016 [16] (77)* |
| IG ROSETTE POSITIVE | 15,774 ± 2,341 | 56,691 ± 3,284 [52] (32)§ | 58,883 ± 5,855 [32] (57)* |

[a]Newborn CBA/J spleen derived cells were added to autologous MLCs consisting of 250,000 of each of syngeneic newborn thymocytes and adult spleen cells, and to allogeneic reaction mixtures of 250,000 adult CBA/J spleen cells and an equal number of mitomycin C inactivated adult Balb/c spleen cells. Newborn spleen cells were separated on the basis of rosette formation through Fc and C3b receptors as described in Materials and Methods, 250,000 of the receptor negative cells being added to the cultures while 10,000 C3b receptor positive and 20,000 Fc receptor positive cells were added to the cultures in direct proportion to the percentages of these cells recovered. The 96 hour peak proliferative activity of each MLR is shown.

[b]The background is the incorporation of cultures of adult CBA/J spleen cells with the specified cell additions resulting in the cultures having the same cell numbers as the MLCs but without newborn CBA/J thymocytes or adult Balb/c spleen cells. In all experiments the latter two populations each demonstrated a mean incorporation of less than 1600 cpm.

*The inhibition is statistically significant; $p \leq 0.001$.

§The inhibition is statistically significant; $p \leq 0.050$.

infer that the neonatal Non-T inhibitory cell population, detected in the MLC, consists of B cells that express variable levels of surface immunoglobulin. These cells are relatively immature with respect to adult B cells in that they do not possess significant levels of C3 or Fc receptors, or Ia antigens.

d) Internal Regulation of Allogeneic MLC Reactivity by Neonatal Spleen Cells.

As demonstrated in the earlier sections of this paper, both T and Non-T newborn CBA/J spleen cells are capable of suppressing MLRs. Allogeneic reactions resulting from the co-culture of adult lymphoid populations (see Figure 13) as well as Type I AMLCs composed of neonatal thymocytes responding to syngeneic adult stimulator cells (see Figure 12) are both susceptible to these inhibitory effects. Since both the responders and regulatory cells in the AMLCs were obtained from the same pool of newborn mice, this latter finding demonstrates the capacity of the neonate for self-regulation of autoreactive thymocytes by both T and B splenic inhibitory cells. Thus during early ontogeny the activity of autoreactive T cells migrating from the thymus may be regulated by peripheral inhibitory cells including those within the spleen.

As shown by Rodriguez et. al. [210] and in Figure 9 of this paper purification of newborn T cells from spleen improves their ability to respond in allogeneic MLCs. This has been taken as presumptive evidence that neonatal Non-T spleen cells suppress the MLC reactivity of splenic T cells [210]. Figure 16 details the results of a typical experiment demonstrating that the allogeneic MLR of T cells isolated from a population of neonatal spleen cells can indeed be inhibited by the addition of Non-T cells obtained from the same newborn spleen cell population. The proliferative

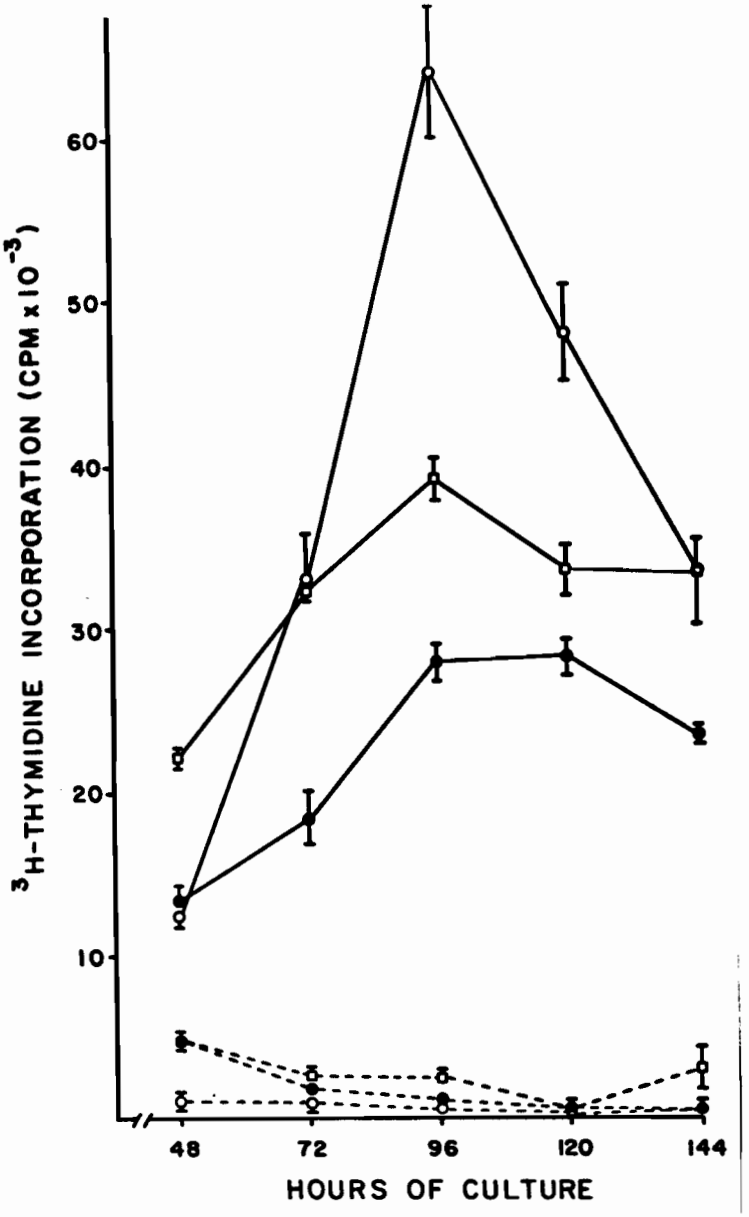



Figure 16. The regulation of newborn splenic T cell allogeneic MLC reactivity by newborn spleen Non-T cells. The control MLC (O) consisted of 250,000 T cells, prepared by Ig-anti-Ig affinity column passage of $SBA^-$ newborn CBA/J spleen cells, plus 500,000 mitomycin C inactivated Balb/c spleen cells. 250,000 Non-T cells isolated from whole newborn spleen by agglutination with SBA were added to the control cultures (□). Also shown is the MLR of 250,000 unselected newborn CBA/J spleen cells (●). The backgrounds are represented by the broken lines. Details of the techniques employed are in Materials and Methods.

response of newborn T cells in the allogeneic MLR was at least twice that of an equal number of whole spleen cells. Addition of $SBA^+$ cells to MLCs containing isolated T responders suppressed their reactivity to approximately the level of the MLR of unseparated spleen cells. Thus Non-T cells from neonatal murine spleen can regulate the allogeneic MLC reactivity of the co-resident T cells.

Although purification of T cells from newborn spleen significantly improves their capacity to respond in allogeneic MLCs the isolated T cells do not possess adult splenic T cell levels of allogeneic reactivity (see Figure 9). The ability of newborn splenic T cells to inhibit the proliferation of adult cells in allogeneic MLRs (see Figure 11) suggests that internal regulation by suppressor T cells may be partly responsible for the relatively poor alloreactivity of neonatal spleen T cells. We therefore sought to examine whether this could in fact be the case. Newborn thymocytes were employed as the allo-responders in these experiments because these T cells proliferate vigorously in response to allogeneic stimulation and appear to have little or no ability to inhibit MLRs (not shown). Table 17 shows the results of a representative experiment. They indicate that affinity purified splenic T cells are capable of suppressing the allogeneic MLR of thymocytes from the same newborn mice. However the T cells inhibited the MLR to a lesser extent than an equivalent number of whole newborn spleen cells. This is precisely the same relationship seen in the analysis of the suppression by newborn T versus whole spleen cells of allogeneic MLRs with adult responders (see Figure 11). Thus neonatal splenic T suppressor cells seem to have comparable effects on alloreactive adult versus newborn cell populations.

It is evident from the findings described above that newborn spleen has a considerable capacity for self-

Table 17. Internal Regulation of neonatal allogeneic MLC reactivity by newborn splenic T cells.

| NEWBORN SPLEEN CELL ADDITION TO CONTROL CULTURES[a] | BACKGROUND | ALLOGENEIC MLR |
|---|---|---|
| | $^3$H-THYMIDINE INCORPORATION | |
| | cpm ± S.E. | [Relative Response] (% Suppression) |
| NONE | 553 ± 137 | 58,068 ± 6,246 [100] |
| WHOLE SPLEEN | 1,358 ± 163 | 16,395 ± 1,716 [26] (72)* |
| SPLENIC T | 485 ± 68 | 35,408 ± 6,328 [61] (39)¶ |

[a]250,000 newborn CBA/J whole spleen cells or T cells isolated from spleen by Ig-anti-Ig affinity column passage were added to control allogeneic MLCs consisting of 250,000 newborn CBA/J thymocytes responding to an equal number of mitomycin inactivated adult Balb/c spleen cells. The responses shown are the means ± S.E. of 8 cultures, 4 from each of the two time points (96 and 120 hours) that the different cultures showed peak incorporation.

*The inhibition of the MLR is significant; $p \leq 0.001$.

¶The inhibition of the MLR is significant; $p \leq 0.005$.

regulation of MLC-induced proliferative responses in both the T and Non-T cell populations. Therefore a large part of the relatively poor ability of neonatal spleen to respond in as well as stimulate allogeneic and autologous MLRs [5,6,27,28,102 105,256] is conceivably due to the activity of these suppressor cells.

iii. The Effects of AFP on Unstimulated Cell Populations.

Lymphocytes prepared from various organs of adult CBA/J mice were incubated for up to 72 hours in the presence of different concentrations of AFP. At different intervals the cultures were assayed for the number of viable cells remaining using trypan blue. As shown in Figure 17, the presence of AFP in a concentration of 200 ug/ml had little effect on the number of viable cells recovered from the various cultures. These findings, as well as the results of a similar analysis using newborn CBA/J lymphocytes from various sources (not shown), indicate that affinity purified AFP has no direct cytotoxic properties on any murine lymphoid cell population.

Although AFP has been shown to have direct anti-proliferative effects particularly in MLRs [186,187], it is evident that this protein also has specific stimulatory properties. Thus AFP induces a suppressor T cell from cultures of adult spleen cells that can inhibit T-D PFC reactions [99,163,164]. We have observed that AFP can initiate *in vitro* proliferative responses in certain populations of lymphoid cells, particularly adult CBA/J bone marrow cells and splenic T cells. Figure 18 shows a time course of the stimulatory effects of AFP versus NMS on bone marrow cells and isolated splenic T cells from adult CBA/J mice. Essentially similar patterns of proliferation were seen in serum-free cultures (Fig. 18a) and those

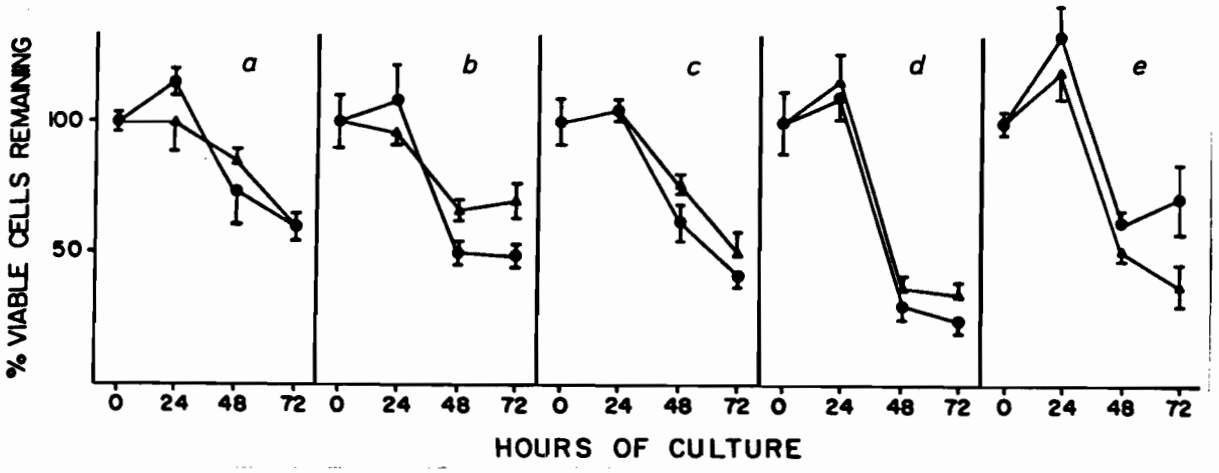



Figure 17. The absence of an adverse effect of AFP on the viability of cells in culture. 250,000 cells from adult CBA/J spleen (panel a), peripheral blood (panel b), lymph node (panel c), thymus (panel d), and bone marrow (panel e) were incubated in medium supplemented with 0.5% NMS in the presence (●) and absence (▲) of 200 ug/ml of AFP. After the periods of time indicated, the number of viable cells remaining per culture were determined by trypan blue exclusion. A minimum of 100 cells per field in each of two counts per duplicate culture were examined. The results for the mean of the viable cells remaining at the different culture intervals are expressed as a percent of the mean number of viable cells present at the initiation of the cultures $\pm$ S.E.M. also expressed as a percentage.

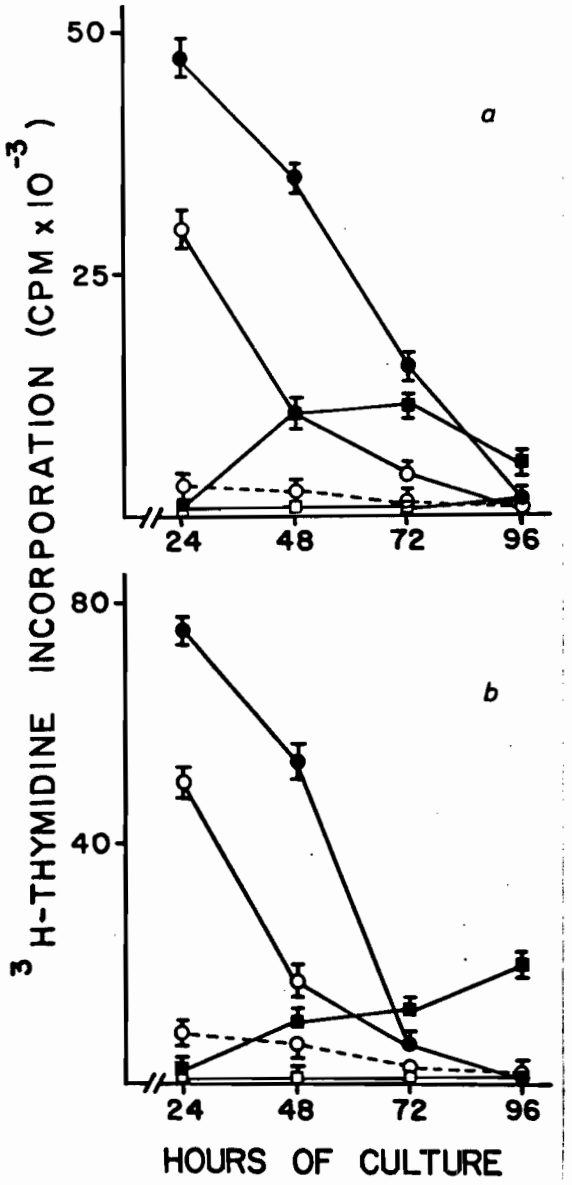



Figure 18. A kinetic analysis of the stimulatory effects of AFP on adult CBA/J bone marrow and splenic T cells. 250,000 unselected cells from bone marrow (○, ●) or the same number of Ig-anti-Ig affinity column purified T-lymphocytes from spleen (□, ■) were cultured in the absence of added protein (open symbols) or in the presence of 200 ug/ml of AFP (filled symbols). Cultures were performed in serum-free medium (panel a) or in medium supplemented with 0.5% NMS (panel b). At the time points indicated the proliferation in the cultures was determined by $^3$H-thymidine uptake. The protocols employed for cell preparation, culture, and proliferative assay are described in Materials and Methods.

supplemented with 0.5% NMS (Fig. 18b). The bone marrow cells responded with peak proliferation to both AFP and NMS at 24 hours after initiation of culture and maintained a significant response to AFP for at least 24 hours afterwards. The splenic T cells, on the other hand, showed a significant proliferative reaction to AFP between 48 and 72 hours of culture but did not respond to NMS. Since both cell types showed a significant response to AFP 48 hours after the commencement of culture, further experiments concerning induction of proliferation by AFP were examined at the 48 hour time point.

The effects of AFP on the proliferation of unfractionated and anti-Ig affinity purified cells from different lymphoid organs are shown in Tables 18 and 19 respectively. Whereas the resting thymidine incorporation of unseparated cells from most sources was relatively unaffected by the addition of AFP, adult bone marrow cells showed a highly significant response. On the other hand, a number of experiments (not shown and Figures 20 and 21) have suggested that adult and newborn spleen cells can occasionally respond in a significant manner to AFP. Cultures of Ig-anti-Ig affinity column passed adult spleen, bone marrow, and to a lesser extent peripheral blood lymphocytes all showed increased proliferation in the presence of AFP versus an equivalent concentration of NMS or without added protein. Similarly prepared cells from newborn spleen also showed the AFP induced proliferative effects.

It is evident that 200 ug/ml of AFP can stimulate proliferation in select populations of cells. However we decided that more comprehensive experiments were necessary to ascertain whether these responses are specific for AFP or, alternatively, are related to the non-specific addition of any protein source. Thus AFP and NMS were titrated into parallel cultures of cells from CBA/J adult bone marrow

Table 18. AFP induced proliferation in various lymphoid cell populations.

| CELL SOURCE[a] | PROTEIN ADDED | | |
|---|---|---|---|
| | NONE | AFP 200 ug/ml | NMS 200 ug/ml |
| | $^3$H-THYMIDINE INCORPORATION cpm ± S.E. | | |
| ADULT SPLEEN | 2,865 ± 1,561 | 1,150 ± 264 | 2,753 ± 2,054 |
| ADULT LYMPH NODE | 523 ± 65 | 674 ± 140 | 394 ± 27 |
| ADULT PBL | 2,506 ± 124 | 1,021 ± 80 | 1,337 ± 101 |
| ADULT PEC | 2,290 ± 156 | 565 ± 77 | 2,680 ± 140 |
| ADULT THYMUS | 478 ± 75 | 411 ± 84 | 323 ± 82 |
| ADULT BONE MARROW | 4,846 ± 349 | 46,626 ± 1,826 | 3,089 ± 39 |
| NEWBORN SPLEEN | 27,139 ± 919 | 33,059 ± 804 | 20,819 ± 343 |
| NEWBORN THYMUS | 158 ± 33 | 143 ± 33 | 193 ± 65 |

[a]250,000 cells from CBA/J mice were incubated for 48 hours in the absence of additional protein or in the presence of 200 ug/ml of AFP or NMS. Cultures were performed and proliferation assayed as detailed in Materials and Methods.

Table 19. AFP induces proliferation in populations of T-lymphocytes of different origin.

| T-CELL SOURCE[a] | PROTEIN ADDED[b] | | |
|---|---|---|---|
| | NONE | AFP 200 ug/ml | NMS 200 ug/ml |
| | $^3$H-THYMIDINE INCORPORATION cpm ± S.E. | | |
| ADULT SPLEEN | 853 ± 142 | 20,185 ± 704 | 820 ± 43 |
| ADULT LYMPH NODE | 288 ± 92 | 177 ± 47 | 164 ± 13 |
| ADULT PBL | 401 ± 125 | 1,749 ± 404 | N.D. |
| ADULT THYMUS | 396 ± 75 | 323 ± 83 | 185 ± 50 |
| ADULT BONE MARROW | 8,168 ± 478 | 58,185 ± 2,932 | 12,751 ± 742 |
| NEWBORN SPLEEN | 8,790 ± 1,166 | 46,375 ± 1,915 | 18,802 ± 3,178 |

[a]250,000 Ig-anti-Ig affinity column passed cells from CBA/J mice were incubated for 48 hours in the absence of additional protein or in the presence of 200 ug/ml of AFP or NMS. Culture and assay of proliferation were performed as described in Materials and Methods.

(Figure 19), adult spleen (Figure 20), and newborn spleen (Figure 21). Cultures were performed in both serum-free medium (lefthand panels) and in medium supplemented with 0.5% NMS (righthand panels). Unfractionated cells (upper panels) as well as Ig-anti-Ig affinity column passed cells (lower panels) from the different sources were examined in these experiments. Cultures of cells obtained from adult bone marrow showed a response to AFP which was significant at a concentration of 10 ug/ml and increased in a linear fashion with increasing concentrations of AFP. NMS induced maximal proliferation of unmanipulated bone marrow cells in serum-free medium at 200 ug/ml but the response was not significantly greater than the level of reactivity resulting from a concentration of AFP 20 times less. On the other hand, comparable cells in serum supplemented medium did not respond to the addition of NMS. While showing a similar dose response curve, affinity column passed adult bone marrow cells demonstrated a reaction to AFP which was significantly higher in magnitude than that of the unselected cells. In cultures containing unfractionated adult spleen cells no significant increase in thymidine uptake was noticed upon the addition of less than 100 ug/ml of AFP. In fact, the presence of 10 to 50 ug/ml of AFP reduced the proliferation of whole adult spleen cells. AFP was seen to possess significantly more stimulatory activity for adult spleen cells than NMS at only 200 ug/ml, the highest level of protein added in these experiments. In agreement with the results for bone marrow cells, addition of NMS caused increased division of adult spleen cells cultured in serum-free medium but not in medium supplemented with serum. On the other hand, affinity purified adult splenic T cells responded with increased proliferation to the addition of 100 to 200 ug/ml of AFP while being refractory to the addition of NMS. No difference between the reactions of the T cells in

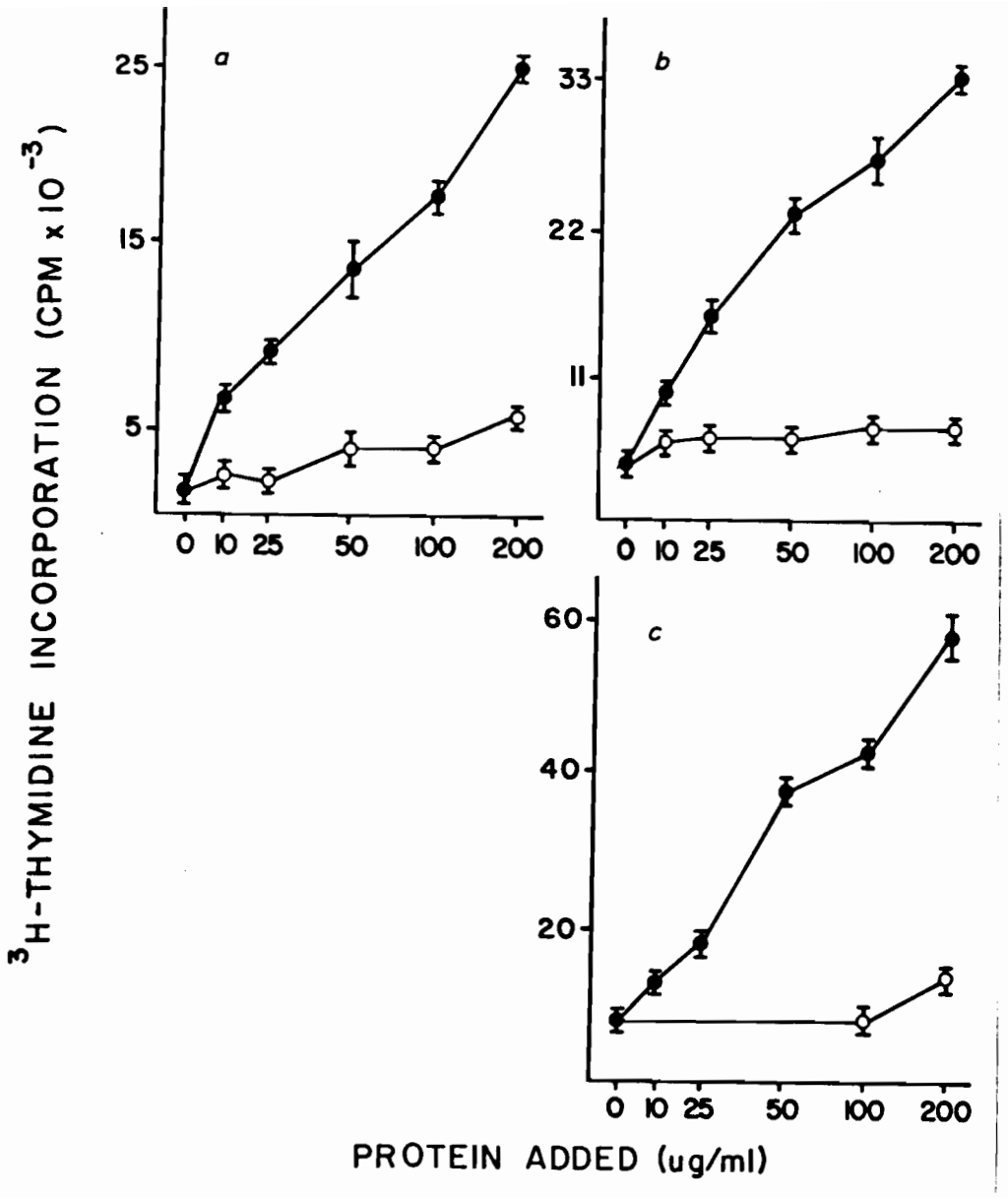



Figure 19. The stimulatory influence of AFP on untreated (panels a and b) and Ig-anti-Ig affinity column passed (panel c) adult bone marrow cells. 250,000 cells from the bone marrow of 12 week old CBA/J mice were incubated for 48 hours with various concentrations of AFP (●) and NMS (O) at which time the $^3$H-thymidine incorporation of the cells was determined. The cells were cultured in media without sera (panel a) or with a supplement of 0.5% NMS (panels b and c). Techniques are described in Materials and Methods.

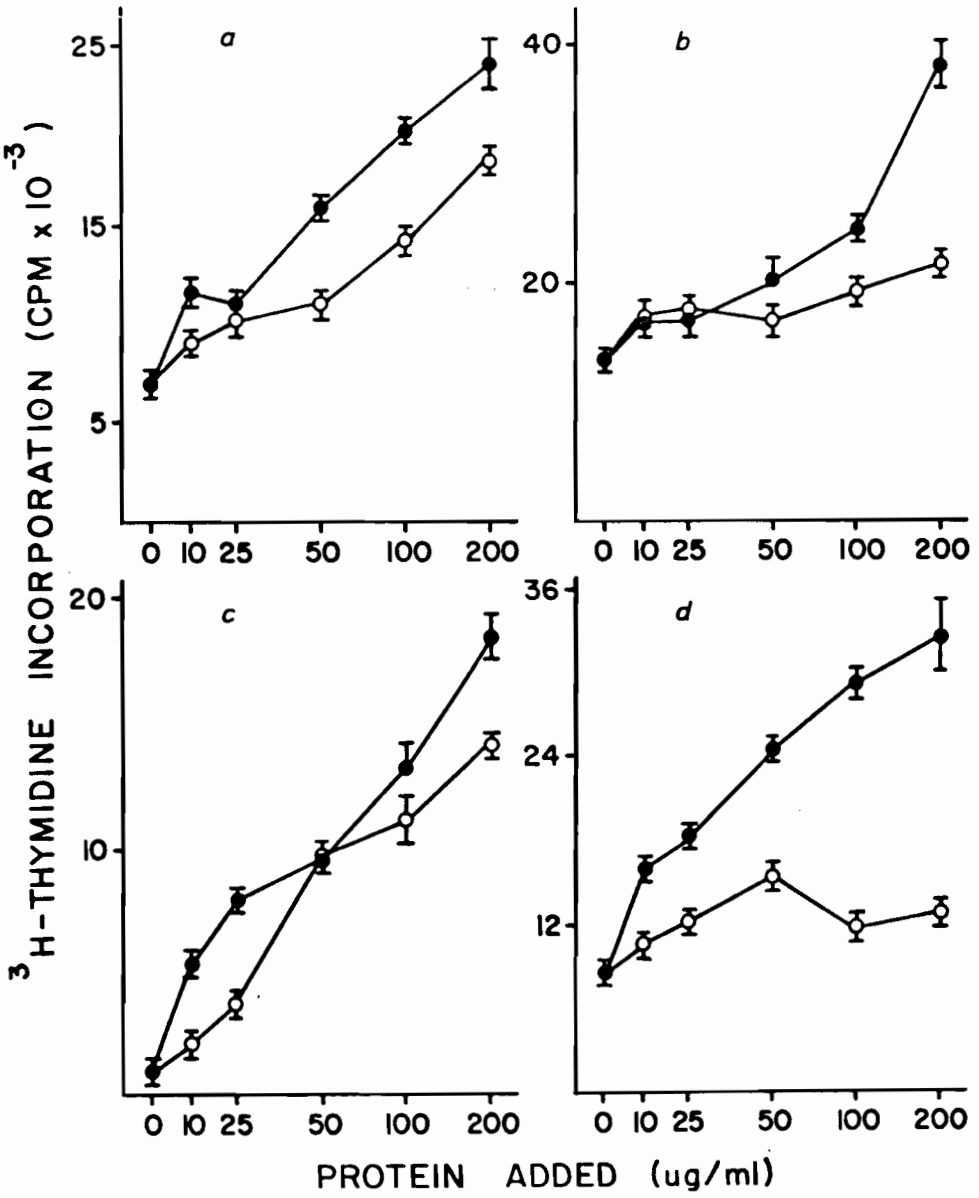



Figure 20. The stimulatory effects of AFP on adult CBA/J unselected spleen (panels a and b) versus purified splenic T cells (panels c and d). Whole spleen cells or Ig-anti-Ig affinity column isolated splenic T cells were cultured at a density of 250,000 cells per culture in serum-free medium (panels a and c) and in medium supplemented with 0.5% NMS (panels b and d). AFP (●) and NMS (O) were titrated into the cultures at the final concentrations indicated. Materials and Methods details the cell preparation, culture protocol, and the proliferative assay which was performed at 48 hours of culture.

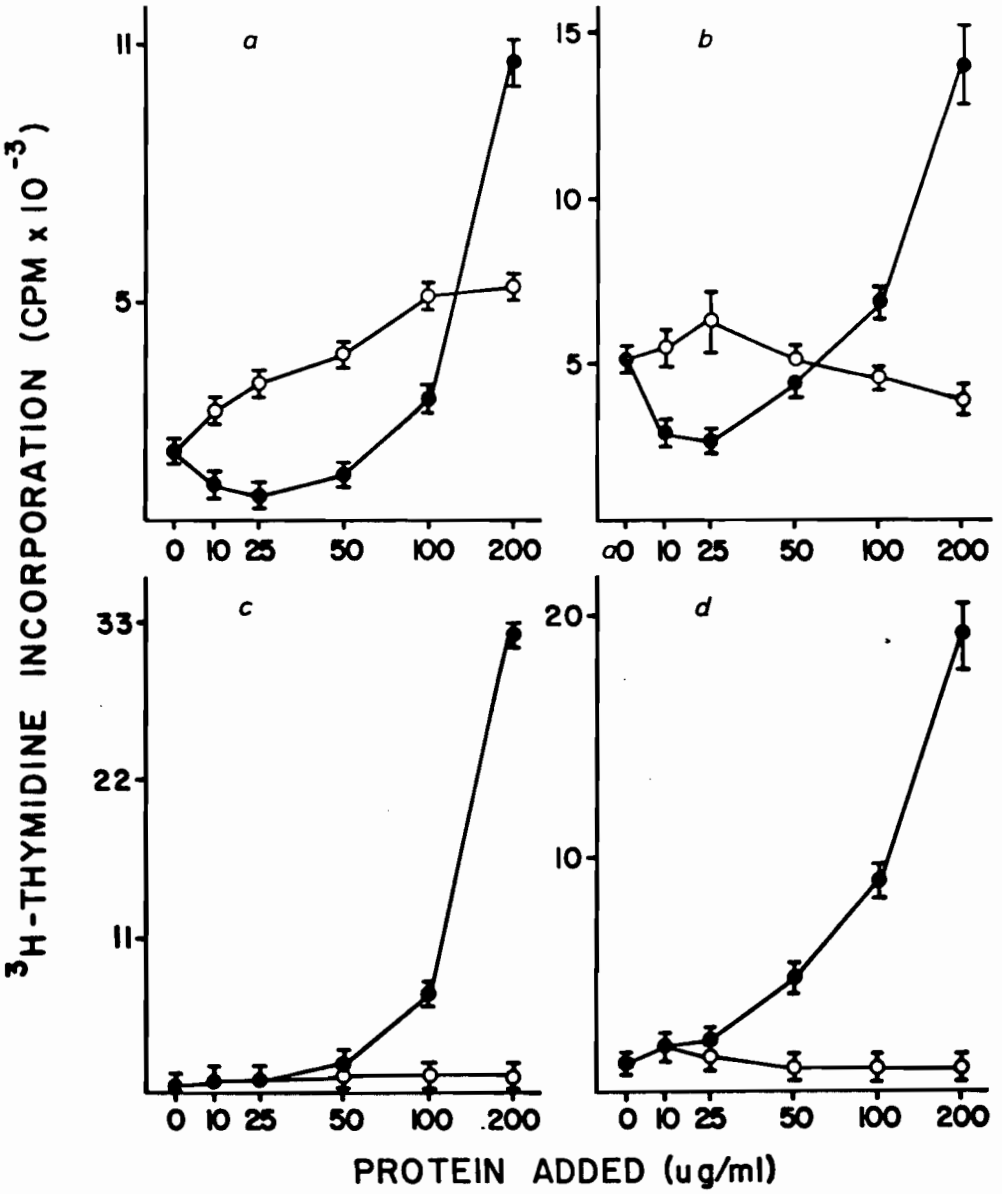



Figure 21. The stimulatory effects of AFP on neonatal CBA/J whole spleen (panels a and b) and splenic T cells (panels c and d). 250,000 unselected spleen cells or Ig-anti-Ig affinity column purified splenic T lymphocytes were cultured in the presence of the indicated final concentrations of AFP (●) or NMS (O) for 48 hours prior to assay of proliferation in the cultures. Cultures were carried out in serum-free medium (panels a and c) and in medium supplemented with 0.5% NMS (panels b and d). All protocols are described in Materials and Methods.

serum supplemented versus serum-free medium was noticeable. Newborn whole spleen cells and splenic T cells, when cultured in serum supplemented medium, showed significantly increased proliferation with added AFP and only minimal increases with similar additions of NMS. In contrast, both AFP and NMS induced proliferative responses of comparable magnitude in whole newborn spleen and splenic T cells in serum-free medium although AFP appeared marginally more stimulatory. These results indicate that AFP induces specific proliferative responses in cells from adult spleen and bone marrow as well as, to a lesser extent, newborn spleen. The increased AFP mediated proliferative responses of Ig-anti-Ig affinity column passed versus unselected cells from either adult cell population suggests that the reactive cells are not B cells or macrophages. These findings may therefore be related to the induction of suppressor T cells by AFP *in vitro* [99,163,164].

### iv. Regulation of Mitogen Induced T Cell Proliferation by AFP.

Early studies indicated that the response of murine spleen cells to T cell mitogens could be only partially inhibited by AFP in concentrations of up to 200 ug/ml [168]. However preliminary experiments in this investigation suggested that both CON A and PHA reactions of CBA/J thymocytes could be virtually ablated by 25 to 50 ug/ml of AFP. The experiment outlined in Figure 22 was performed to determine whether differences in the concentrations of mitogens employed could effect the susceptibility to inhibition by AFP of the reactions of thymocytes versus spleen cells. Adult spleen cells (Fig. 22 a & b) as well as functionally mature $PNA^-$ thymocytes (Fig. 22 c & d) were stimulated with different concentrations of CON A (Fig. 22 a

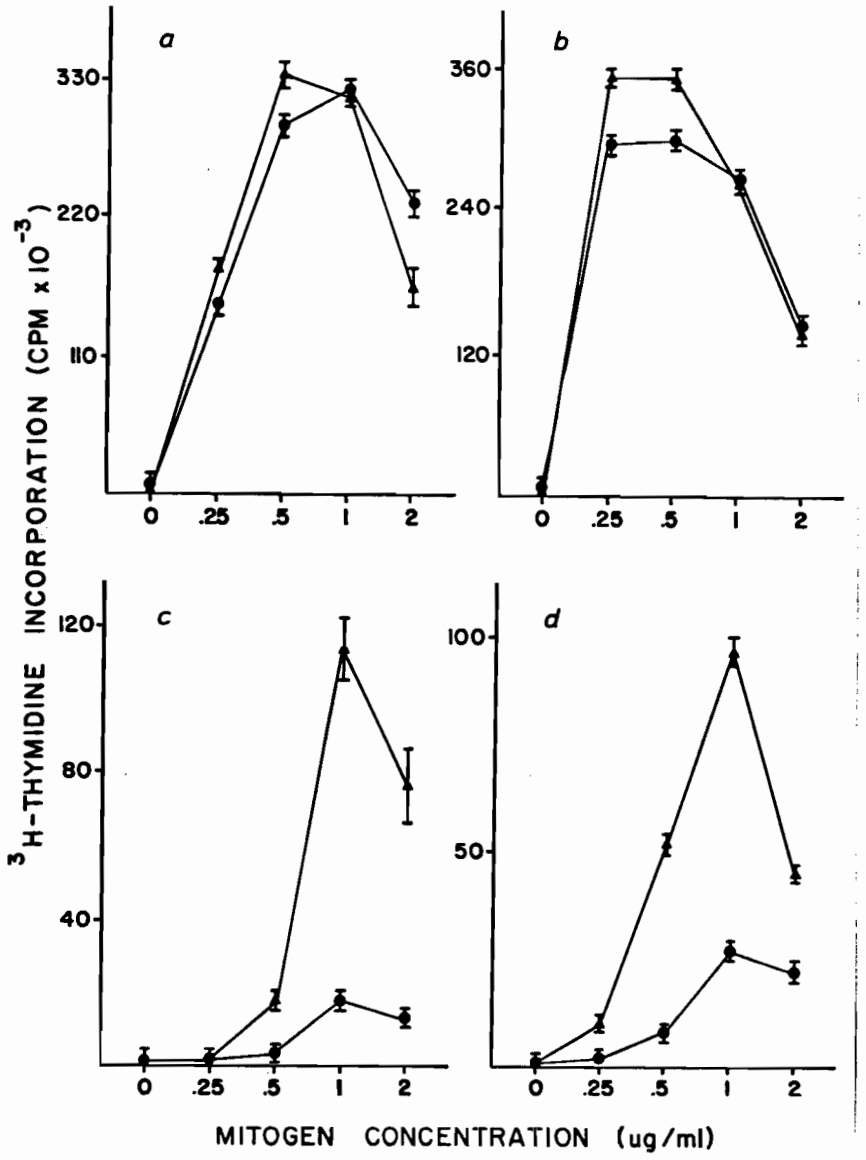
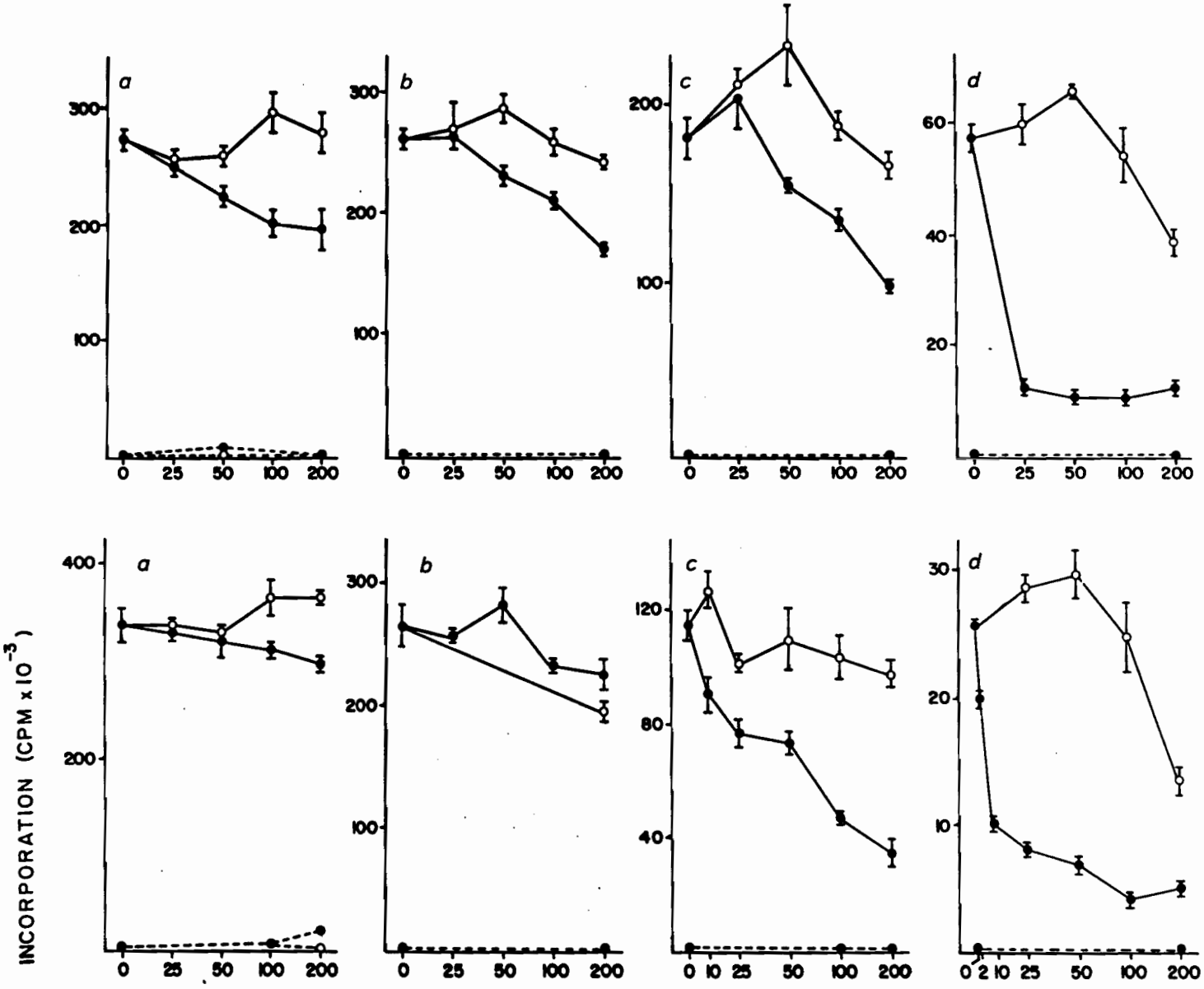



Figure 22. T cell mitogen concentration versus the influence of AFP. 250,000 Ig-anti-Ig affinity purified T cells from adult CBA/J spleen (panels a and b) and 250,000 PNA negative cells from adult CBA/J thymus (panels c and d) were cultured without (▲) or with 200 ug/ml AFP (●) in various concentrations of CON A (panels a and c) or PHA (panels b and d). Proliferation was assayed at 48 hours. Background proliferation was in both cases under 500 cpm and is not shown. Cells were manipulated and cultured as described in Materials and Methods.

& c) and PHA (Fig. 22 b & d) in the presence and absence of 200 ug/ml of AFP. Throughout the range of mitogen concentrations which elicited proliferative responses in the cell populations, the thymocytes remained susceptible while the spleen cells continued to be resistant to the inhibitory effects of AFP. The peak reactions of the $PNA^-$ thymocytes to both CON A and PHA occurred at mitogen concentrations of 1 ug/ml and were inhibited by 70 to 80% by the addition of AFP. On the other hand, peak reactivity of the spleen cells occurred at CON A concentrations of 0.5 and 1 ug/ml and at concentrations of PHA from .25 to 1 ug/ml. The addition of AFP to maximally stimulated spleen cells resulted in an approximately 10% reduction in the PHA response but no significant inhibition of the CON A reaction. Thus AFP may have more intense suppressive effects on the proliferative responses of thymocytes versus spleen cells. In addition, the regulatory properties of AFP on T cell mitogen responses appear to be unrelated to the concentration of mitogens employed. These results indicate that AFP probably inhibits mitogen reactions through direct anti-proliferative actions on the susceptible cells rather than by acting in a competitive manner with either CON A or PHA.

As a consequence of the above findings, a study was undertaken to determine whether AFP has the property of selectively inhibiting the mitogen reactivity of T cells from particular lymphoid organs. Figure 23 shows the results of the titration of AFP versus NMS into CON A stimulated cultures consisting of unseparated cells (upper panels), purified T cells (middle panels), and isolated Lyt $1^+23^-$ T-cells (lower panels) from the indicated adult CBA/J lymphoid organs. CON A activated cultures of unselected spleen or peripheral blood cells plus mitogen were suppressed by less than 30% in the presence of 200 ug/ml of AFP. The CON A responses of T cells as well as Lyt $1^+23^-$ T cells from these


a
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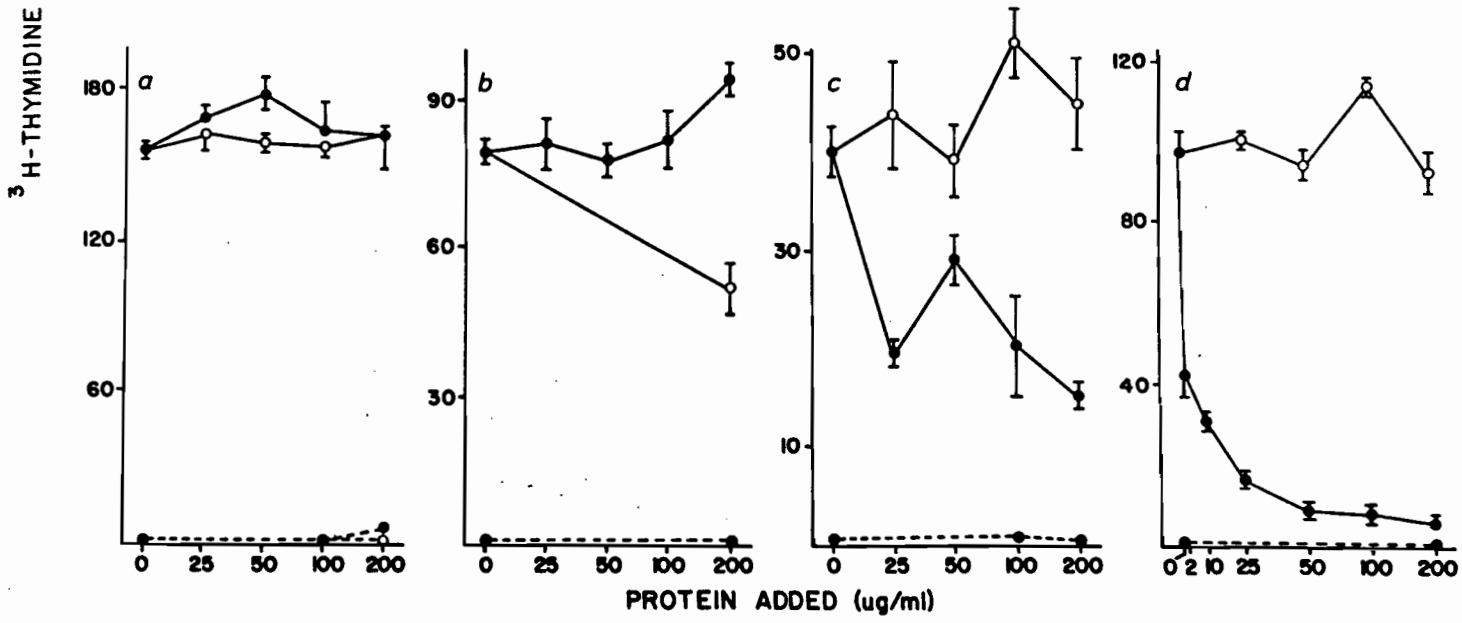


Figure 23. The effect of AFP on the CON A response of various populations of adult CBA/J lymphoid cells from spleen (a panels), peripheral blood (b panels), lymph node (c panels), and thymus (d panels). 250,000 unselected cells (upper panels), purified T cells (middle panels), or isolated Lyt $1^+23^-$ cells (lower panels) from each tissue were cultured for 48 hours in the presence of CON A (1 ug/ml) and various concentrations of AFP (●) or NMS (O). The backgrounds are shown by the broken lines. T cells were prepared by affinity purification while Lyt $1^+23^-$ cells were obtained by anti-Lyt 2.1 plus complement treatment of T cell preparations. Cell manipulations, cultures, and assay of proliferation were performed as described in Materials and Methods.

two sources were not significantly inhibited by AFP. On the other hand, the CON A reactions of unfractionated cells, T cells, and Lyt $1^+23^-$ T cells from lymph node were all suppressed by approximately 50% to 65% by 200 ug/ml of AFP. However the CON A induced proliferation of the three comparable preparations of adult thymocytes showed considerably greater sensitivity to AFP. Mitogen stimulated unselected, affinity purified, as well as isolated Lyt $1^+23^-$ thymocytes were all suppressed by over 80% in the presence of as little as 25 ug/ml of AFP. While the mitogen reactions of the other cell populations were maximally inhibited by 200 ug/ml of AFP, the response of thymocytes exhibited peak inhibition at AFP concentrations of 50 to 100 ug/ml. It is noteworthy that the proliferative responses to CON A of the various Lyt $1^+23^-$ cells were within the same order of magnitude. This indicates that the susceptibility to inhibition by AFP is not related to the extent of the mitogen reaction. Therefore CON A reactive Lyt $1^+23^-$ adult thymocytes are more sensitive to inhibition by AFP than are comparable cells from lymph node, spleen, and peripheral blood.

We next sought to determine whether the same pattern of sensitivity to AFP is shown by Lyt $1^+23^-$ cells isolated from the various organs and stimulated with PHA. The results of a typical experiment are shown in Figure 24. Only the proliferation of PHA-reactive Lyt $1^+23^-$ cells from thymus was significantly inhibited by the addition of AFP versus NMS. The suppression of the Lyt $1^+23^-$ thymocyte PHA reaction by AFP closely resembled the inhibition of the response of the same cells to CON A, with over 80% of the reaction being removed by 100 ug/ml of AFP. In contrast, Lyt $1^+23^-$ cells from spleen, peripheral blood, as well as lymph node showed PHA reactivity which was relatively unaffected by the addition of AFP.

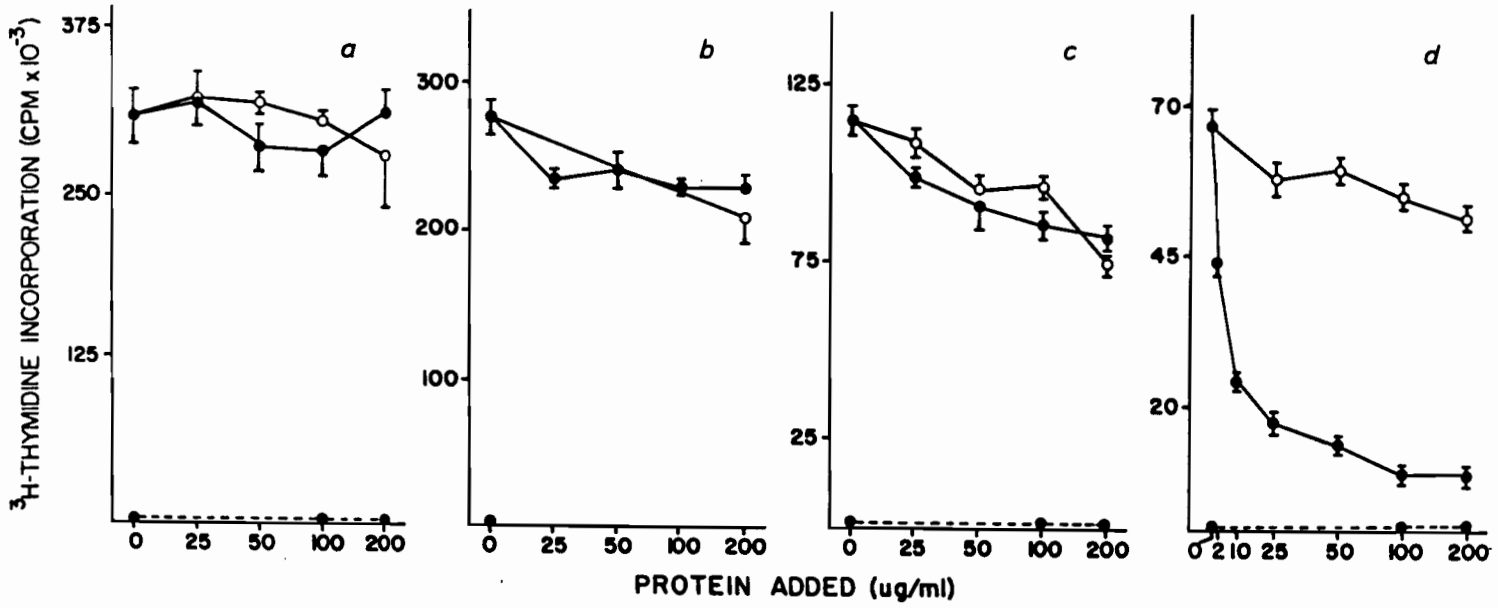


Figure 24. The effect of AFP on the response to PHA of adult CBA/J Lyt $1^+23^-$ T lymphocytes isolated from spleen (panel a), peripheral blood (panel b), lymph node (panel c), and thymus (panel d). Cultures were performed in the presence of various concentrations of AFP (●) or NMS (○). Backgrounds are shown by the broken lines. Cells were prepared and cultured as outlined in the legend to Figure 23 with the exception that the mitogen employed was PHA (1 ug/ml).

The above results suggest that the two sub-populations of adult lymph node T cells that proliferate when stimulated with CON A and PHA may differ in their sensitivity to AFP. On the other hand, the populations of adult thymocytes that respond to the two mitogens appear to be equally susceptible to inhibition by AFP. To determine whether this is indeed the case we examined the inhibitory effects of AFP on parallel cultures of T cells (upper panels) and isolated Lyt $1^+23^-$ cells (lower panels) from adult lymph node (Figure 25) and thymus (Figure 26) stimulated with CON A (lefthand panels) and PHA (righthand panels). Both the reactions of unselected T cells and isolated Lyt $1^+23^-$ cells from lymph node to CON A were significantly inhibited by AFP with a maximal suppression of approximately 60% obtained at an AFP concentration of 200 ug/ml. In contrast, the responses of the same populations of cells to PHA were not affected by AFP when compared with cultures containing equivalent concentrations of NMS. However CON A and PHA stimulated whole or Lyt $1^+23^-$ adult thymocytes exhibited virtually identical susceptibility to inhibition, with approximately 80% suppression being achieved in each of the various culture combinations by the presence of 100 ug/ml of AFP. We therefore conclude that CON A and PHA reactive lymph node cells differ in their sensitivity to inhibition by AFP while thymocytes responding to the two mitogens do not.

We have also studied the effects of AFP on the CON A (lefthand panels) and PHA (righthand panels) reactions of T cells (upper panels) and Lyt $1^+23^-$ cells (lower panels) from newborn CBA/J thymus (Figure 27) and spleen (Figure 28). The neonatal thymocyte experiments were conducted in parallel with those of the adult shown in Figure 26. The patterns obtained for the inhibition of CON A and PHA responses versus the concentrations of AFP added were virtually identical for newborn and adult whole thymocytes. The CON A

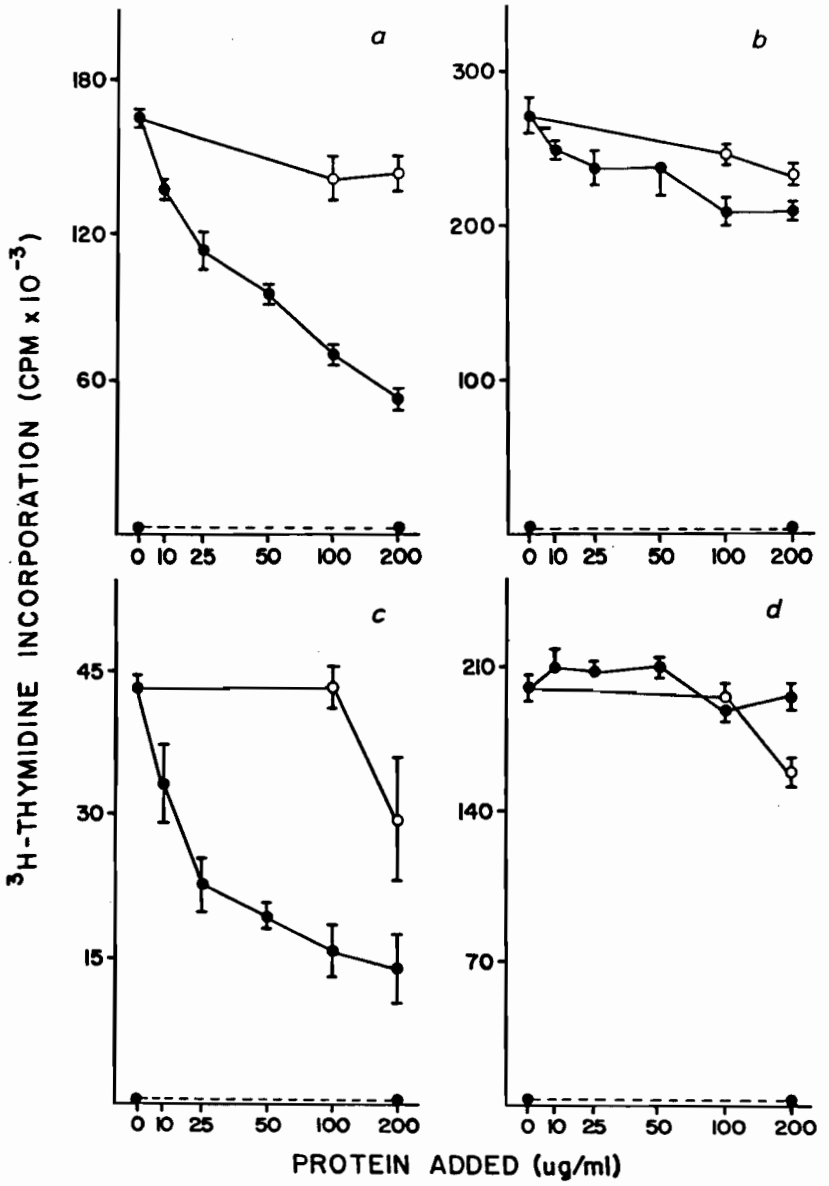


Figure 25. Comparison of inhibition by AFP of the response to CON A versus PHA of adult CBA/J whole T and Lyt $1^+23^-$ T cells from lymph node. Purified T cells plus CON A (panel a) and PHA (panel b) as well as Lyt $1^+23^-$ cells plus CON A (panel c) and PHA (panel d) were cultured in the presence of either AFP (●) or NMS (O) in the concentrations indicated. Backgrounds are shown by the broken lines. Cells were prepared and cultured and the 48 hour proliferative response assayed as described in the legends to Figures 23 and 24.

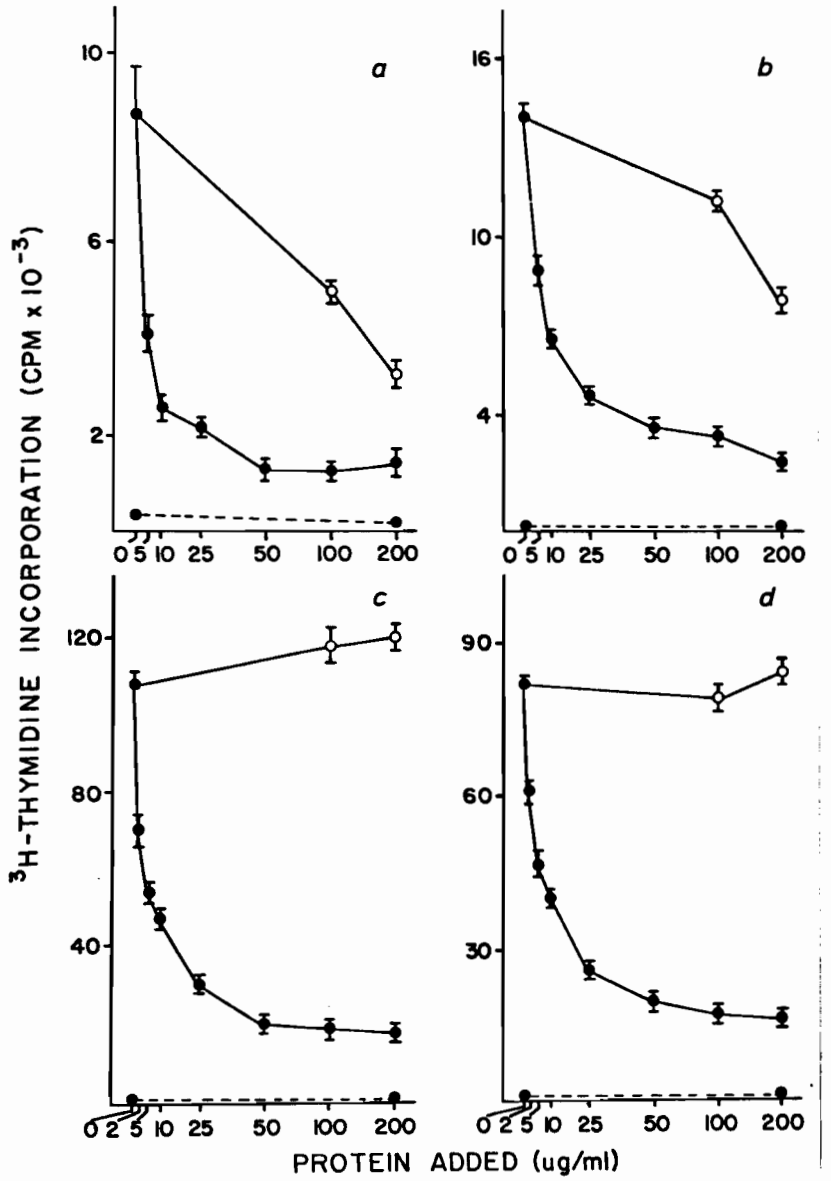


Figure 26. Inhibition by AFP of unselected and Lyt $1^+23^-$ T lymphocytes from CBA/J adult thymus responding to CON A and PHA. Parallel cultures of whole thymocytes stimulated with CON A (panel a) and PHA (panel b) as well as Lyt$1^+23^-$ thymocytes stimulated with CON A (panel c) and PHA (panel d) were performed in the presence of various concentrations of AFP (●) or NMS (O). Backgrounds are shown by the broken lines. Cells were prepared and cultured and the 48 hour assay of proliferation carried out as described in the legends to Figures 23 and 24.

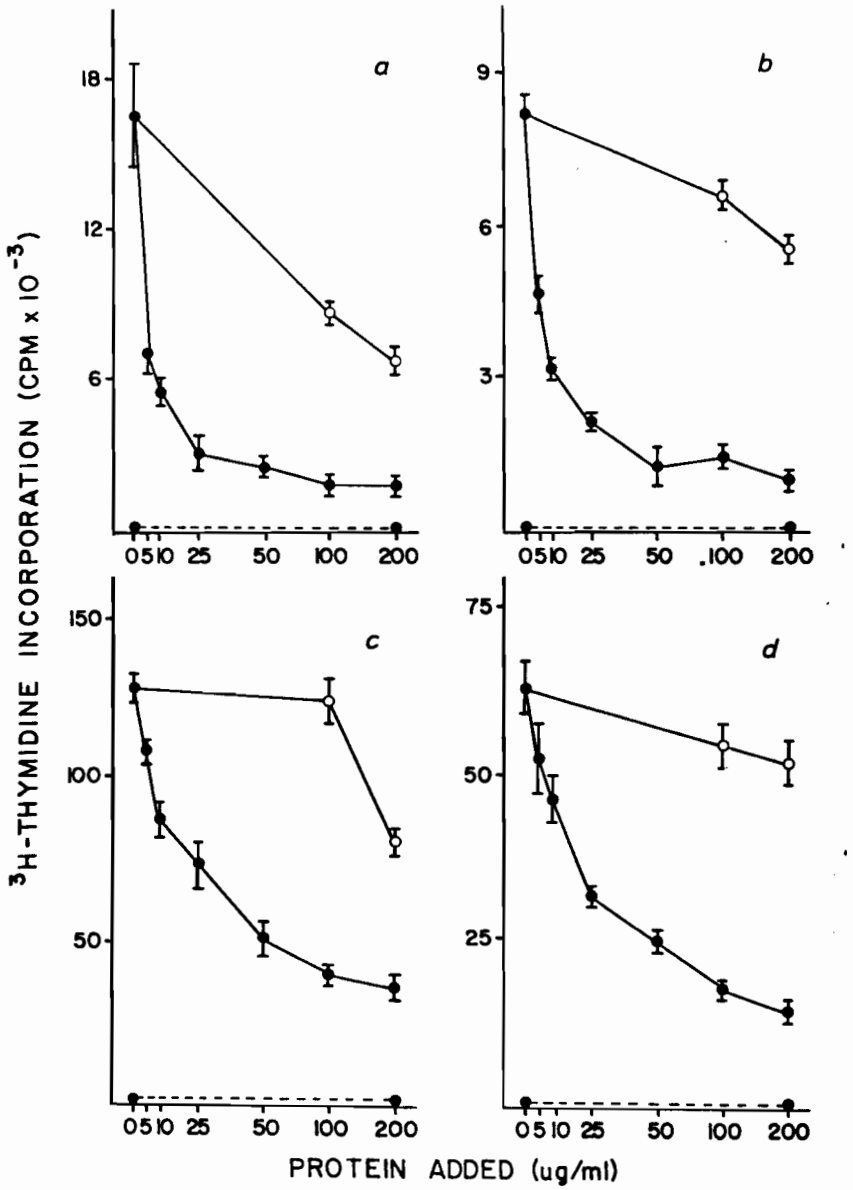


Figure 27. AFP mediated suppression of CON A and PHA reactions of unselected and Lyt $1^+23^-$ thymocytes from newborn CBA/J mice. Parallel cultures of whole thymocytes stimulated with CON A (panel a) and PHA (panel b) as well as Lyt$1^+23^-$ thymocytes stimulated with CON A (panel c) and PHA (panel d) were carried out in the presence of various concentration of AFP (●) or NMS (O). Backgrounds are shown by the broken lines. All manipulations were performed as detailed in the legend to Figure 26.

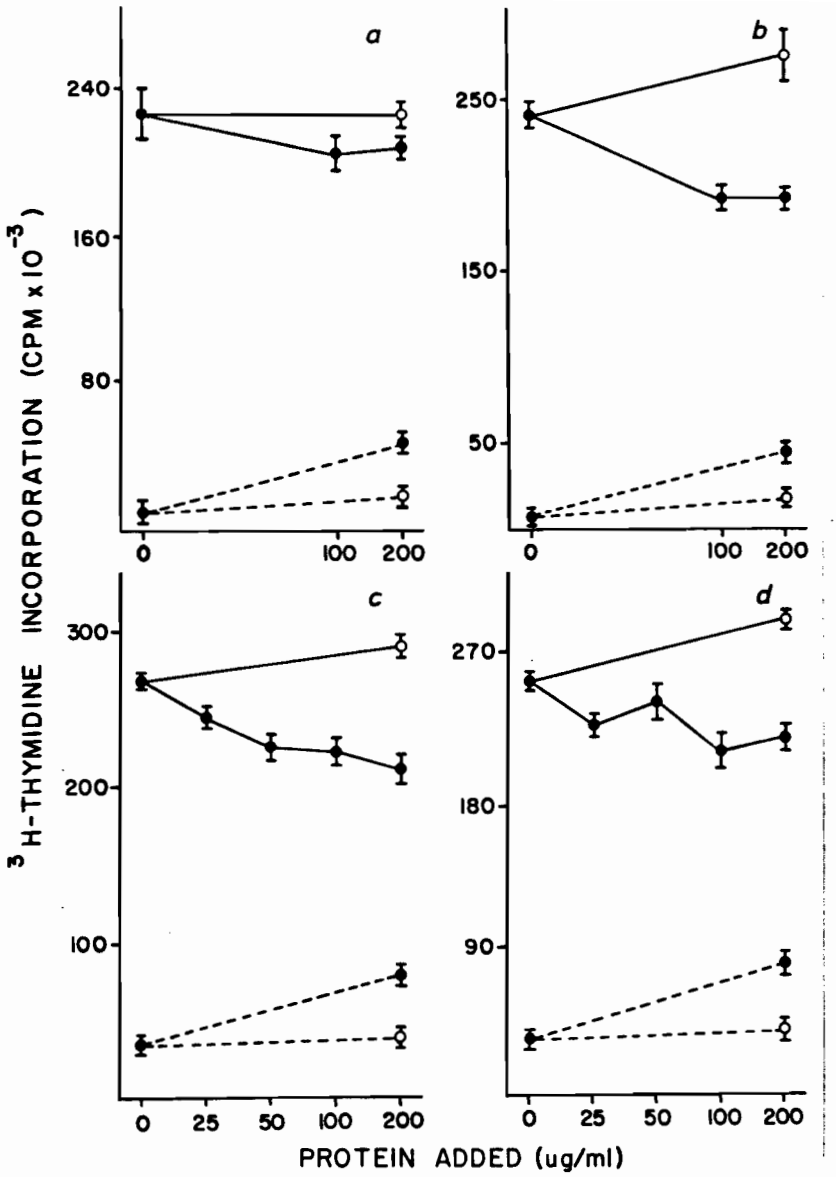


Figure 28 The effects of AFP on the response to CON A and PHA of purified T and isolated Lyt $23^-$ cells from newborn CBA/J spleen. T cells cultured with 1 ug/ml of CON A (panel a) or PHA (panel b) or Lyt $23^-$ T cells cultured with CON A (panel c) or PHA (panel d) in the presence of various concentrations of AFP (●) or NMS (O). Backgrounds are shown by the broken lines. T cells were affinity purified from newborn spleen following d $H_2O$ lysis of erythroid cells and Lyt $23^-$ cells were isolated from purified T cells by anti Lyt 2 plus RC treatment. The proliferative assay was performed at 48 hours. All the techniques employed are detailed in Materials and Methods.

and PHA induced proliferative reactions of unselected newborn thymocytes were equally suppressed by the addition of AFP with over 80% inhibition seen in the presence of 50 ug/ml. On the other hand, the responses of neonatal Lyt $1^+23^-$ thymocytes to the two mitogens appeared to be slightly less susceptible to regulation by AFP than the comparible reactions of the other thymocyte populations, including unselected cells from both adult and newborn thymus as well as isolated Lyt $1^+23^-$ adult thymocytes. Neonatal Lyt $1^+23^-$ thymocytes responding to CON A or PHA were suppressed by less than 60% through the addition of 50 ug/ml of AFP while maximum inhibition of less than 75% was achieved at 200 ug/ml. The CON A and PHA induced proliferation of affinity purified T cells and isolated Lyt $1^+23^-$ cells from newborn spleen was noticeably less susceptible to suppression by AFP than that of neonatal thymocytes. A maximum inhibition of less than 20% in the presence of 200 ug/ml of AFP was seen in the cultures of mitogen stimulated newborn spleen T cell populations. It is therefore apparent that the mitogen reactive T cell populations which are the most sensitive to the anti-proliferative effects of AFP are thymocytes from both the newborn and adult. This contrasts with the relative insensitivity to AFP mediated inhibition of mitogen reactive T cells in neonatal and adult spleen.

v. Regulation of MLC Induced Proliferation by AFP.

Previous investigations have established that murine AFP inhibits conventional allogeneic MLRs by selectively interfering with proliferation induced by genetic differences across the I region of the major histocompatibility complex [170,186, reviewed in 171]. Having determined that neonatal thymocytes respond in the Type I AMLR against syngeneic Ia-bearing stimulator cells [see Section VIII. i. p.65], we

sought to ascertain whether AFP could suppress this autologous interaction. As shown in Figure 29, the titration of AFP into the neonatal AMLR resulted in statistically significant suppression of the proliferative response at AFP concentrations as low as 5 ug/ml. Maximum inhibition, approximately 95% of the control response, was achieved by the addition of 100 ug/ml of AFP. Thus the Type I AMLR appears to be exquisitely sensitive to regulation by AFP.

We next examined the ability of AFP to inhibit the restimulation of AMLR activated newborn thymocytes. As demonstrated in Figure 30, AMLR primed cells, restimulated with fresh adult spleen cells, mediate a secondary autologous reaction with more rapid kinetics and a greater peak proliferation than the primary response. 200 ug/ml of AFP added to the secondary AMLR caused a 60% reduction in the response, while significant inhibition was obtained by the addition of as little as 10 ug/ml. This indicates that the sensitivity to AFP of the autologous responders is maintained irrespective of initial exposure to stimulation in the absence of the regulatory protein.

Since the AMLR showed notable susceptibility to inhibition by AFP we decided to evaluate the sensitivity of autologous versus allogeneic MLRs to the regulatory effects of AFP. Initially, a comparison was made of the effects of AFP on the neonatal CBA/J Type I AMLR (Figure 31a) versus a conventional one-way allogeneic MLR consisting of adult CBA/J ($H\text{-}2^k$, $Mls^d$) spleen cells responding to mitomycin inactivated Balb/cJ ($H\text{-}2^d$, $Mls^b$) spleen cells (Figure 31b). Proliferation in the AMLR (panel a) was abrogated by 100 and 200 ug/ml of AFP while the same concentrations suppressed the allogeneic reaction (panel b) by less than 50%. Furthermore, the addition of 10 ug/ml of AFP inhibited the AMLR by 40 to 50% without significantly affecting the allogeneic reaction.

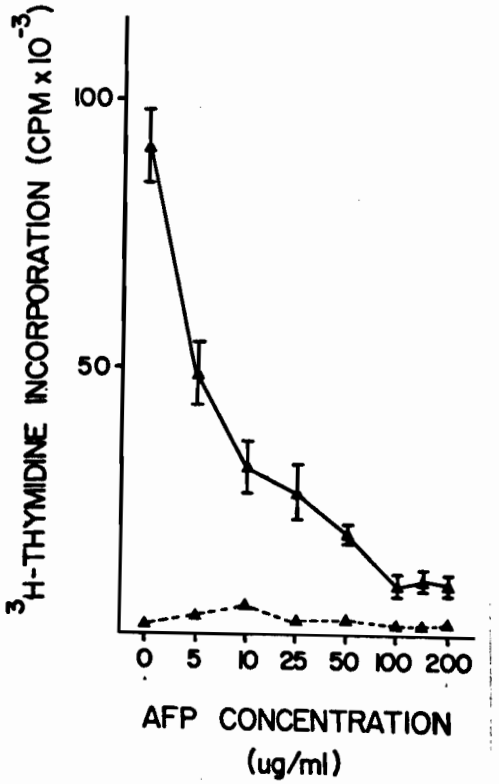


Figure 29. Titration of AFP into the neonatal AMLC. 250,000 CBA/J newborn thymocytes were cultured with an equal number of syngeneic adult spleen cells in the presence of the indicated concentrations of AFP (▲). Peak proliferation of the cultures occurred at 120 hours of culture and was assayed by $^3$H-thymidine incorporation as described in Materials and Methods. Backgrounds are shown by the broken lines.

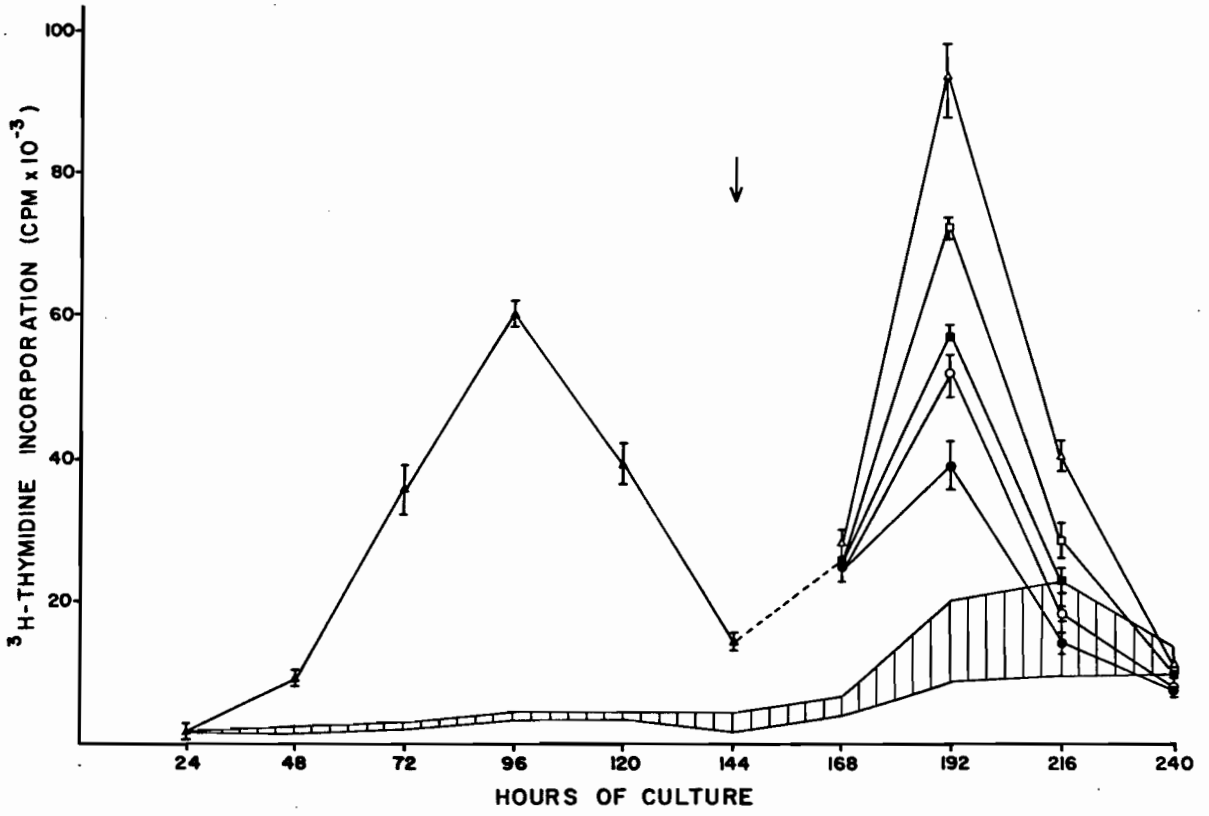


Figure 30. The inhibitory influence of AFP on the secondary AMLR. Newborn thymocytes were primed in AMLR as described in Materials and Methods with the exception that the cultures were performed in 250 ml flasks. At the times indicated 200 ul samples were removed from the cultures, placed in microtitre wells, and the proliferation assayed. This primary reaction is designated by (▲). At 144 hours the viable cells remaining in the flask cultures were washed, counted, and 250,000 cells cultured with an equal number of fresh adult spleen stimulator cells in fresh medium. Secondary cultures were carried out in the absence of AFP (△), or in the presence of AFP in final concentrations of 200 ug/ml (●), 100 ug/ml (○), 50 ug/ml (■), and 10 ug/ml (□). The added background activities of stimulators and responders cultured separately are represented within the hatched area.

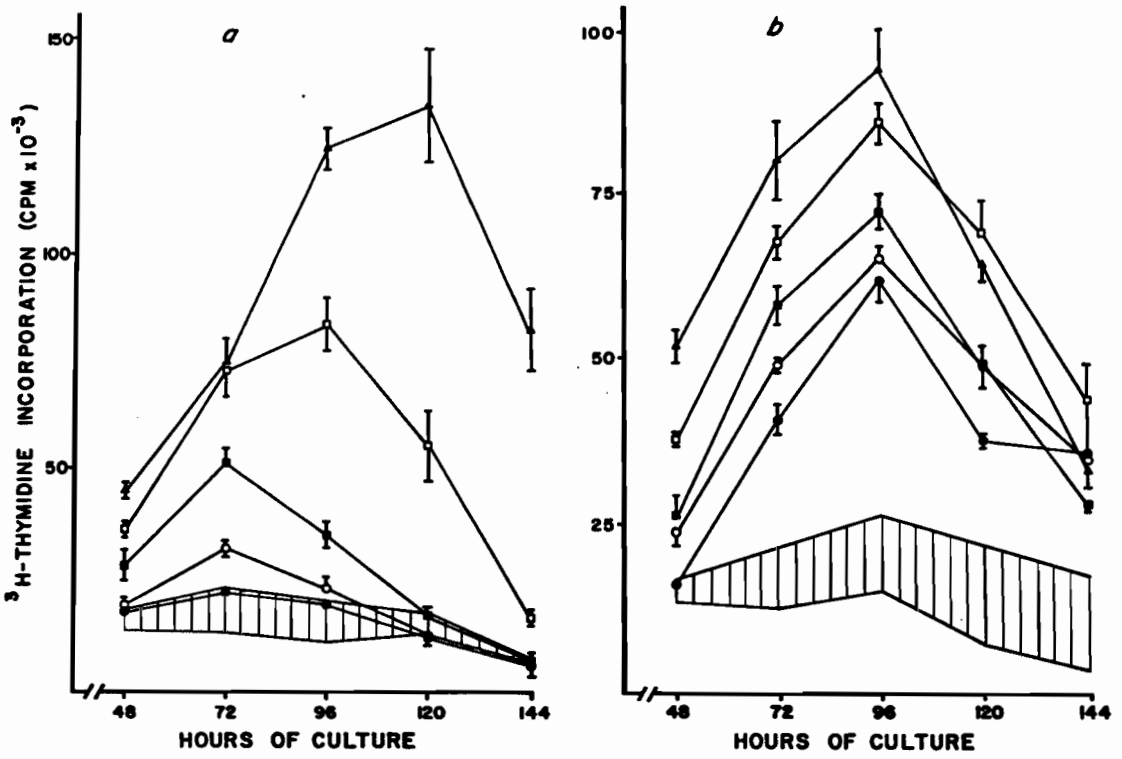


Figure 31. Effect of AFP on newborn autologous (panel a) versus adult allogeneic (panel b) MLR. AFP was added at the initiation of cultures in final concentrations of 200 ug/ml (●), 100 ug/ml (○), 50 ug/ml (■), and 10 ug/ml (□). Cultures without added protein are designated (▲). AMLCs consisted of 250,000 newborn thymocytes cultured with an equal number of syngeneic adult spleen cells. Adult allogeneic MLCs consisted of 250,000 adult CBA/J spleen cells plus 500,000 mitomycin C inactivated adult Balb/cJ spleen cells. The added background proliferative activities of the responder and stimulator populations cultured separately were within the hatched areas. Cell culture and assay of proliferation were performed as detailed in Materials and Methods.

These results clearly indicate that newborn thymocytes responding in the autologous MLC possess enhanced sensitivity to regulation by AFP over that of adult spleen cells reacting in allogeneic MLCs initiated by MHC plus non-MHC histoincompatibilities.

The knowledge that Lyt $1^+23^-$ cells are responsible for most of the proliferation observed in Type I AMLRs (see Section VIII. i. p. 57) led us to investigate the possibility that AFP mediated suppression is directed preferentially against autoreactive versus alloreactive cells of the Lyt $1^+23^-$ subset. Our findings, shown in Figure 32, indicate that this is indeed the case. The inhibitory effects of AFP, tested over a concentration range from 10 to 100 ug/ml, were significantly greater on isolated newborn CBA/J Lyt $1^+23^-$ thymocytes responding in AMLR against T-depleted syngeneic adult spleen cells (Fig. 32a) than on the same thymocyte population reacting against T-depleted Balb/c spleen cells (Fig. 32b). Purified adult splenic Lyt $1^+23^-$ T cells stimulated by T-depleted Balb/c spleen cells (Fig. 32c) were least susceptible to the regulatory properties of AFP. Through a number of replicate experiments these relative differences in the sensitivity of Lyt $1^+23^-$ cells responding in AMLRs versus allogeneic MLRs remained highly consistent and independent of the magnitude of the proliferative responses.

We have demonstrated that the autoreactive Lyt $1^+23^-$ thymocytes found during early ontogeny appear to be more susceptible to regulation by AFP than alloreactive Lyt $1^+23^-$ T cells. However adult T cells from various organ sources are capable of responding in Type II AMLCs [30,70,83,85,194, 229 and see Fig. 8a]. Thus we were able to determine whether adult autoreactive cells are also sensitive to inhibition by AFP. As seen in Figure 33, the addition of AFP into AMLCs consisting of adult CBA/J lymph node cells responding to

Figure 32. Comparative effects of AFP on proliferating Lyt $1^{+}23^{-}$ cells in newborn autologous MLR (panel a), newborn allogeneic MLR (panel b), and in adult allogeneic MLR (panel c). Cultures were carried out in the absence of additional protein (▲), in the presence of 100 ug/ml mouse serum albumin (◊), or in the presence of AFP in final concentrations of 100 ug/ml (O), 50 ug/ml (■), 25 ug/ml (△), and 10 ug/ml (□). Newborn autologous and allogeneic assay MLCs consisted of 75,000 neonatal CBA/J Lyt $1^{+}23^{-}$ thymocytes responding against 250,000 T-depleted spleen cells from adult CBA/J and Balb/cJ mice respectively. The adult allogeneic assay MLC consisted of 75,000 Lyt $1^{+}23^{-}$ splenic T cells from adult CBA/J mice responding to 250,000 T-depleted Balb/cJ spleen cells. The adult T cells were isolated from whole spleen by Ig-anti-Ig affinity column passage. Lyt $1^{+}23^{-}$ cells were prepared from T cells by treatment with anti-Lyt 2.1 plus complement. Splenic stimulator cells were depleted of T cells by negative selection with rabbit anti-mouse brain associated T serum plus complement. Balb/cJ cells were inactivated with mitomycin C. Cell manipulations, culture, and assay of proliferation were performed as detailed in Materials and Methods.

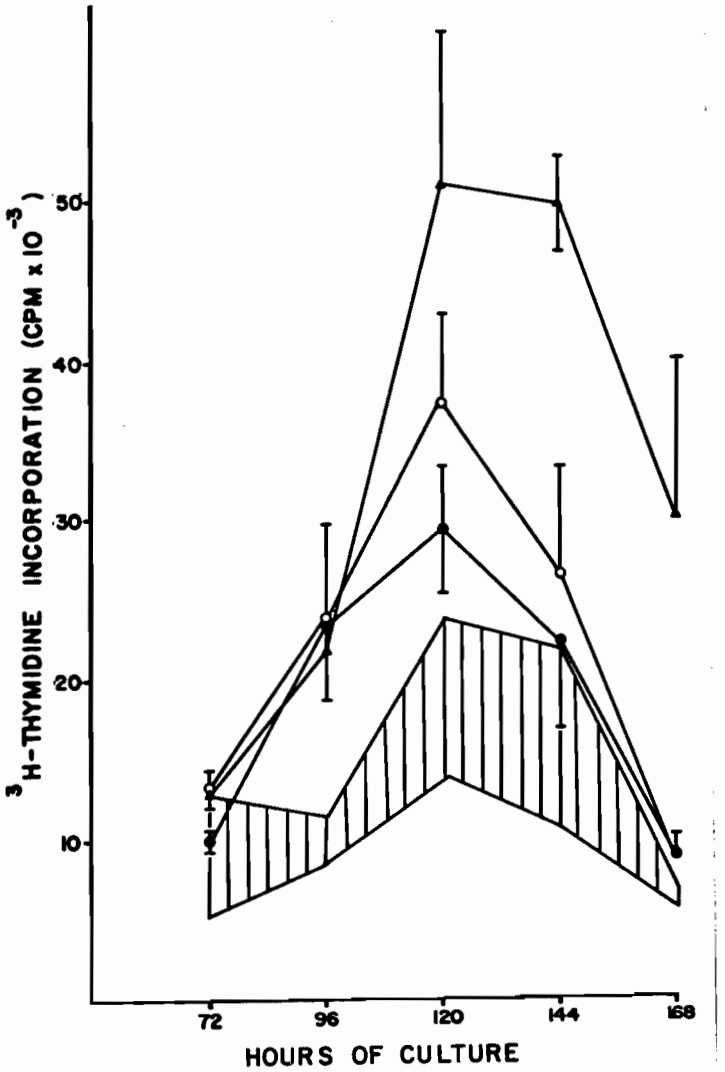


Figure 33. Inhibition of the adult AMLR by AFP. 250,000 adult CBA/J lymph node cells were cultured with 250,000 autochthonous spleen cells in the absence of additional protein (▲) and in the presence of 200 ug/ml (●) and 100 ug/ml (O) of AFP. The added background proliferation of responder and stimulator cells cultured separately with and without AFP was within the hatched area. Cultures were performed and the proliferation assayed as detailed in Materials and Methods.

autochthonous spleen cells indicates that 200 ug/ml of the protein can reduce the reaction to virtually background levels of proliferation. This suggests that adult autoreactive cells may be as sensitive to regulation by AFP as the neonatal thymocyte autologous responder.

While newborn spleen cells are poor (relative to adult spleen cells) stimulators of AMLRs at least in part because of the presence of suppressor cells (see Section VIII. ii. p.70), it is possible to demonstrate a significant AMLR between populations of neonatal thymocytes and spleen cells. Thus newborn Lyt $1^+23^-$ thymocytes can respond to autochthonous T-depleted spleen cells and the susceptibility of this reaction to inhibition by AFP is contrasted with that of an adult lymph node versus spleen AMLR in Table 20. Both types of autologous reaction were significantly suppressed by as little as 10 ug/ml of AFP. A concentration of 200 ug/ml of AFP inhibited the control neonatal and adult AMLRs by 68 and 98% respectively. These results indicate that AFP has the ability to suppress proliferation induced in AMLRs between autochthonous responders and stimulators.

All mammalian species that have been examined for the presence of AFP have been shown to possess this protein [1,3]. In addition, immunochemical relationships between AFPs from various species have been demonstrated [108]. An examination of the cross-reactivity, in the AMLC, of the immunoregulatory properties of AFP originating from several different species was therefore undertaken (Table 21). At a concentration of 100 ug/ml, mouse, rat, and human AFP were all capable of significantly suppressing a murine AMLR. The inhibitory effect of human AFP, in this system, was both less efficient and diluted out more rapidly than those of the equivalent mouse or rat proteins. The phylogenetically more closely related mouse and rat AFPs showed essentially the same ability to regulate the murine AMLR. Thus the capacity

Table 20. Suppression of newborn and adult autochthonous AMLRs by AFP.

| PROTEIN ADDED TO AMLR (ug/ml) | AMLR TYPE | |
|---|---|---|
| | NEWBORN --> NEWBORN[a] | ADULT --> ADULT[b] |
| | $^3$H-THYMIDINE INCORPORATION cpm[c] ± S.E. (% Suppression) | |
| NONE | 5,412 ± 214 | 40,076 ± 2,921 |
| NMS 200 | 5,186 ± 500 (4) | 36,389 ± 9,992 (9) |
| AFP 200 | 1,717 ± 347 (68)* | 965 ± 5,443 (98)* |
| AFP 100 | 1,807 ± 1,377 (67)** | 2,388 ± 2,797 (94)* |
| AFP 50 | 3,950 ± 497 (27)** | 15,475 ± 3,405 (61)** |

[a]The newborn --> newborn AMLC consisted of 75,000 Lyt $1^+23^-$ thymocytes cultured with 250,000 T-depleted spleen cells prepared from the same neonatal CBA/J mice. Cell treatment, culture, and the proliferative assay were performed as described in Materials and Methods.

[b]The adult --> adult AMLC consisted of 250,000 lymph node cells cultured with an equal number of spleen cells prepared from the same adult CBA/J mice. The cells were prepared, cultured, and the resulting proliferation assayed as detailed in Materials and Methods.

[c]cpm was calculated according to the formula of Ting and Ranney [242]: cpm = cpm AMLC - cpm responder alone - cpm stimulator alone.

*The response is significantly inhibited in comparison with the control response without added protein; $p \leq 0.001$.

**The response is significantly inhibited in comparison with the control response without added protein; $p \leq 0.03$.

Table 21. The effect of heterologous AFPs on newborn murine autologous MLR.

| CONCENTRATION OF PROTEIN ADDED (ug/ml)[a] | $^3$H-THYMIDINE INCORPORATION (cpm) | | | |
|---|---|---|---|---|
| | MOUSE ALBUMIN | MOUSE AFP | RAT AFP | HUMAN AFP |
| 100 | 34,419 | 4,582* | 1,143* | 14,078† |
| 50 | 31,589 | 5,191* | 1,262* | 28,315 |
| 25 | 29,172 | 6,755* | 2,837* | 25,066 |
| 10 | 29,248 | 15,459† | 8,219* | 31,831 |
| 5 | 28,779 | 35,839 | 28,024 | 38,053 |

[a]Protein was added to cultures of 75,000 Lyt $1^+23^-$ newborn CBA/J thymocytes plus 250,000 T-depleted syngeneic adult spleen cells inactivated with mitomycin C. Proliferation was assayed at 120 hours of culture. $^3$H-thymidine incorporation was 31,647 $\pm$ 3,360 cpm in control cultures without added protein while background incorporation of the responder and stimulator cells cultured separately was a total of 687 $\pm$ 171.

*The inhibition is significant at $p \leq 0.005$ when compared to control cultures without added protein.

†The inhibition is significant at $p \leq 0.01$ when compared to control cultures without added protein.

to inhibit autologous reactivity is preserved in AFPs from different species, perhaps indicating the teleological importance of this regulatory activity.

Several criteria have been met that may serve to indicate that AFP has a specialized function in vivo during early ontogeny to prevent the expression of potentially harmful autoreactive processes: 1) AFP shows a selective ability to inhibit autologous versus allogeneic MLRs; 2) autochthonous AMLRs between neonatal thymocytes and spleen cells are sensitive to AFP mediated suppression; and 3) the capacity to regulate AMLRs is phylogenetically preserved in AFPs from different species. Should AFP be involved in the prevention of deleterious autoreactivity in vivo during early life it might be expected that high serum levels of AFP would persist throughout the period of ontogeny where there is demonstrable autoreactive potential in the thymus. We thus examined the ability of thymocytes from mice of different ages to respond in the AMLR and simultaneously analyzed the serum AFP concentrations of the corresponding animals. The results of this study are shown in Figure 34. Thymocytes taken from mice on the day of birth were found to possess the greatest reactivity in AMLCs. These animals also had the highest serum AFP concentration, which was measured at approximately 5 mg per ml. Decreases in both AMLR responder capacity and serum AFP levels were noticeable by three to four days of age and both parameters were reduced to less than 50% of the peak measurements by seven to eight days postpartum. This parallel decline in thymic AMLR responder capacity and serum AFP levels continued until the end of the second week of life when no significant autologous reaction was detected and serum AFP approached the low concentration found in adult CBA/J mice. Throughout the period of early life when thymocytes were found to possess a substantial capacity to respond in the AMLR, serum AFP levels were

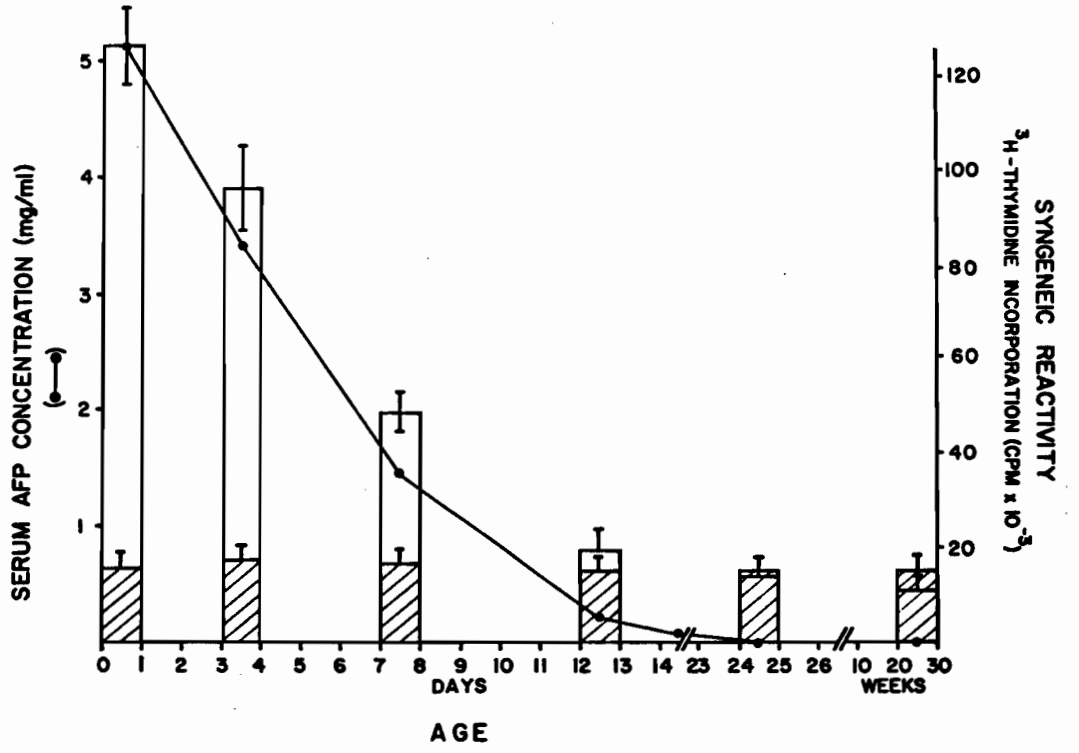


Figure 34. Comparison of serum AFP levels versus the ability to respond in AMLR at different ages after birth. Thymocytes (250,000) from mice of different age groups were cultured with an equal number of adult spleen stimulator cells. Peak $^3$H-thymidine incorporation occurred at approximately 120 hours of culture and is expressed by the open bars. The hatched portion of the bars represents the combined background proliferation of responder and stimulator cells cultured separately. Levels of AFP in sera pooled from the different groups (5 to 15 mice per group) of thymocyte donors were determined by rocket electrophoresis and are expressed by (●).

considerably higher than the 100 ug/ml necessary to block the AMLR *in vitro*. Therefore AFP is present *in vivo* in concentrations sufficient to control the proliferation of autoreactive neonatal thymocytes during development.

## IX. DISCUSSION

### i. Phenotypic and Functional Characterization of Neonatal Thymus and Spleen Cell Populations.

The initial phase of this investigation concerned the definition of lymphocyte populations in the thymus and spleen of neonatal mice. Our phenotypic analysis of Ig-anti-Ig affinity purified T cells confirms observations by others [91,238] that a large proportion of newborn splenic T cells do not possess the levels of Thy 1 determinants found on adult splenic T cells. While 90% of the adult affinity column-passed spleen cells were sensitive to negative selection with monoclonal anti-Thy 1, less than 20% of the similarly prepared spleen cells from newborn mice were killed by equivalent dilutions of anti-Thy 1 plus RC. Antiserum directed against MICG, a second antigenic system prominent on cells of the T lineage [20], eliminated more than 90% of adult splenic T cells over a wide range of concentrations. However most Ig-anti-Ig affinity column filtered neonatal spleen cells were killed by only high concentrations of anti-MICG serum (1/2). This indicates that a large percentage of newborn splenic T cells may express MICG determinants but at a lower density than adult splenic T cells. Neonatal and adult affinity column passed spleen cells also showed differences in sensitivity to treatment with anti-T serum plus RC. Over 90% of the adult cells were lysed by all the dilutions of anti-T ranging from 1/2 to 1/128 while the newborn cells showed increases in susceptibility which paralleled the increasing concentrations of anti-T serum employed in the cytotoxic protocol. Up to 90% of the neonatal spleen T cells were killed by the higher concentrations (1/2 to 1/8) of anti-T in the presence of RC. These results also suggest that the majority of newborn spleen cells which did not adhere to Ig-anti-Ig affinity columns may express antigenic products associated with T

lymphocytes at lower density than adult splenic T cells. The increased susceptibility of neonatal splenic T-like cells to complement dependent lysis with anti-T serum versus anti-Thy 1 or anti-MICG sera may be a reflection of the specificity of anti-T serum for multiple T cell antigens and/or its known specificity for stem cells [48,88,190]. Whereas anti-Thy 1 [206] and anti-MICG [20] sera are each directed against a single T cell antigen, anti-T serum contains specificities for up to four T cell antigens [48,88,190]. Absorption of the anti-T serum employed in this study with adult thymocytes and splenic T cells removed the ability of the antiserum to kill newborn splenic T cells in the presence of RC (not shown). On the basis of their susceptibility to be lysed with anti-MICG as well as anti-T plus RC, most Ig-anti-Ig affinity column passed newborn spleen cells would therefore seem to be of the T cell lineage. We have also confirmed the earlier report of Haaijman et. al. [91] that relatively few (compared to the adult) neonatal spleen cells possess Lyt 1 determinants. While 80% of affinity purified adult splenic T cells were sensitive to anti-Lyt 1 plus RC, we were able to kill less than 20% of similarly prepared newborn spleen T cells. Approximately 20% of neonatal splenic T cells show adult levels of sensitivity to negative selection with anti-Thy 1, anti-MICG, anti-Lyt 1, and anti-T sera. The remainder of the newborn spleen T cells are more resistant to cytotoxic treatment with these anti-T cell reagents and, as a result, may be relatively immature phenotypically. Hauptman has demonstrated that during fetal development pre-T cells develop first cytoplasmic MICG and then surface bound MICG before expressing Thy 1 [20]. Thus it is conceivable that the Ig-anti-Ig affinity column non-adherent neonatal spleen cells which are resistant to negative selection with anti-Thy 1 but sensitive to cytotoxic treatment with high concentrations of anti-MICG are in fact pre-T cells.

While there are notable phenotypic differences between newborn and adult splenic T cells, thymocyte populations from neonates and adults have been demonstrated to possess more comparable surface antigenic phenotypes [20,91,114]. Our results indicate that cytotoxic treatment with anti-Thy 1, anti-MICG, or anti-T sera can eliminate virtually all neonatal as well as adult thymocytes. At the higher antiserum dilutions tested neonatal thymocytes appeared to be slightly more resistant to negative selection than adult thymocytes. This suggests that a minor fraction of the newborn thymocyte population may express lower than adult levels of T cell antigens. Analogous proportions of cells possessing Lyt $1^+2^+$, Lyt $1^+2^-$, and Lyt $1^-2^+$ surface antigenic phenotypes have been reported for newborn versus adult thymocytes [114]. These findings are generally supported by our observations. However we have found that 20% of neonatal thymocytes are resistant to negative selection with both anti-Lyt 1 and anti-Lyt 2 while virtually 100% of adult thymocytes are killed by the same treatment. This indicates that a portion of the newborn thymocyte population does not express adult levels of Lyt antigens. It is therefore evident that both newborn spleen and thymus contain T cells that are relatively deficient (with respect to adult T cells) in certain T cell antigens.

Previous investigations have concluded that neonatal thymocytes do not possess an adult capacity to respond to CON A and PHA [4,35,38,105,154,155,209,232,238]. In addition, earlier studies showed that spleen cells from neonates do not respond to these mitogens [105,232,238]. However we have determined that whole or isolated Lyt $1^+2^-$ newborn and adult thymocytes proliferate with comparable magnitude in the presence of T cell mitogens. Moreover, we have found that unselected newborn spleen cells have a somewhat limited but still significant ability to respond to CON A and PHA.

Presumably, these findings are the result of improved culture conditions in our system and more fastidious culture requirements of neonatal versus adult T lymphocytes for optimal stimulation. While newborn spleen cells appeared to possess a relatively poor capacity to respond to T cell mitogens, we discovered that purified T cells from the spleens of mice as young as four days of age showed an ability to proliferate in response to T cell mitogens which was equivalent to that of purified adult splenic T cells. This is particularly suprising in view of the fact that the newborn Ig-anti-Ig column passed spleen cell population consists of only 20% mature T cells on the basis of Thy 1 and Lyt 1 antigen expression. The majority of cells in this neonatal spleen population are apparently pre-T cells as they possess MICG but not Thy 1 determinants [20]. These findings indicate that during early postnatal ontogeny there are mature CON A and PHA reactive T lymphocytes in both the thymus and spleen. Nevertheless neonatal T cells are less responsive to mitogenic stimulation than adult T cells when an unselected whole newborn spleen population is tested. This raises the intriguing possibility that neonatal Non-T spleen cells may be capable of suppressing the response of T cells to mitogens. It is interesting to note in this regard that our comparison of the LPS reactivity of newborn versus adult whole and T-depleted spleen cells showed deficient responsiveness of the newborn cells to this B cell mitogen. This suggests that the depressed reactivity of newborn spleen cells to both T and B cell mitogens may be at least in part due to regulation by cells within the Non-T population.

It has been shown that the thymus of the neonate contains a population of cells capable of responding vigorously in AMLCs. Syngeneic adult spleen cells are normally employed as the source of autologous stimulator cells for neonatal responder thymocytes in this Type I AMLC

[19,27,28,29,101,102,103,104]. A recent study in the human system has concluded that the proliferation in AMLCs is the result of stimulation from heterologous proteins, such as FCS, included in the culture medium [106]. We have determined that this is clearly not the case for murine AMLRs. Thus, we obtained significant proliferation upon co-culture of untreated CBA/J neonatal thymocytes and adult spleen cells in AMLCs lacking any heterologous serum additives. Although serum-free AMLCs supported relatively weak reactions, strong proliferation was seen in AMLCs supplemented with 0.5% adult CBA/J NMS. Significant AMLRs were also observed in cultures containing 1% NHS as well as 1% of a selected batch of FCS. It is notable here that FCS generally does not support the AMLR as well as the other serum additives. Moreover we have found that only selected batches of FCS are capable of supporting increased proliferation in the AMLC (data not shown). These findings show that heterologous serum additives are by no means a necessary requirement for the generation of AMLRs. Conversely autologous serum supplements promote very strong AMLRs.

We were able to characterize the populations of cells interacting in the Type I AMLR in a culture system free of potential contributions from heterologous serum additives. The adult lymphocytes that respond in Type II AMLCs have been identified as Lyt $1^+23^-$ T cells [19,30,85,127]. We have determined that neonatal thymocytes of the Lyt $1^+23^-$ phenotype are also responsible for the proliferation in Type I AMLRs. These newborn AMLC responder cells possess MICG determinants and relatively low levels (in comparison with most neonatal and adult thymocytes) of Thy 1 antigens but do not express Ia antigens. On the other hand, considerable evidence indicates that Ia antigens are expressed by the stimulator populations in both Types I and II AMLRs [19,70,

83,85,127,195, and see Table 4 and Figure 6]. Earlier data suggested that the stimulatory cells in the AMLRs are Ig-bearing B cells [19,27,103,195]. However more recent results indicate that Ia-positive dendritic cells are particularly efficient inducers of these reactions [177]. In addition it is likely that cell types capable of stimulating Type II AMLRs can also stimulate Type I reactions [19,70, and see Section VI.ii. a) p.7]. We have shown that in vivo anti-mu treatment, which has been demonstrated to result in the loss of Ig bearing B cells [90,128,142,166] as well as other cell subpopulations [110], removes the ability of adult spleen cells to stimulate syngeneic newborn thymocytes. Thus it is possible that B cells, or their products (including immunoglobulin), may be involved in the stimulation of Type I AMLRs. Alternatively, in vivo anti-mu treatment may, through some as yet uncharacterized mechanism, remove Ia-positive Type I AMLR stimulator cells which are not B cells. In any case, masking of Ia antigens in Type II AMLCs by titrating in anti-Ia serum has been shown to inhibit the autologous response [26,127,194]. We have revealed that the addition of monoclonal anti-Ia serum can also block proliferation in the Type I AMLR (Figure 6). These results directly implicate the Ia antigen, or a structure situated in close proximity on the cell membrane, in the stimulation of both Types I and II AMLRs. Therefore in both of these classes of autologous MLR Lyt $I^{+}23^{-}$ cells respond against syngeneic Ia-bearing cells.

It is noteworthy that in vivo anti-mu treatment results not only in the removal of AMLR stimulator cells but also in the premature disappearance of the neonatal thymocyte auto-responder. The process leading to this accelerated loss of AMLC reactive cells in the newborn thymus by in vivo anti-mu treatment at birth is, at present, unknown. However Janeway has shown that the development of a particular population of helper T cells is sensitive to such treatment [110]. These

findings indicate that there is an as yet uncharacterized mechanism through which in vivo anti-mu treatment can affect certain T cell subpopulations.

Neonatal thymocytes have been shown to proliferate in allogeneic as well as autologous MLCs [19,27,28,29,87,101, 102,103,104,105,154]. Our comparison of the magnitude of proliferation induced in preparations of newborn thymocytes by genetically identical in contrast with H-2 plus Non-H-2 disparate stimulator cells indicates that neonatal thymocytes may possess an enhanced ability to react against auto versus allo-antigens. This preferential reactivity against syngeneic stimulation in the MLC is also seen in populations of isolated Lyt $1^+23^-$ newborn thymocytes (see Figure 32). This tends to support speculation that distinct subpopulations of newborn thymocytes may respond in autologous versus allogeneic MLCs. However we have been unable to discern any phenotypic differences between auto and alloreactive neonatal thymocytes. In addition Elliott et. al. have demonstrated that alloreactive T cells bind syngeneic Ia antigens [64] and therefore presumably possess the capacity to interact with autologous stimulator cells. Thus we cannot conclude at present whether the auto and alloreactive newborn thymocytes are in fact separate subpopulations of cells or, alternatively, if they are representatives of a single cell population which is capable of responding against self as well as allogeneic antigenic stimulation.

Our experiments have also shown that populations of adult whole T and isolated Lyt $1^+23^-$ T cells from various source organs can respond significantly to stimulation from syngeneic T-depleted adult spleen cells. When compared directly with the proliferative reaction of the adult cells in AMLRs, equal numbers of unselected or Lyt $1^+23^-$ newborn thymocytes showed a response of greater magnitude. This

indicates that the thymus of the neonate contains a significantly higher autoreactive potential, as measured by the capacity to react in AMLCs, than adult lymphoid organs. Of all the cell populations tested adult whole and Lyt $1^{+}23^{-}$ thymocytes possessed the least ability to respond in AMLCs. This is consistent with our findings that thymocytes from mice of increasing age gradually lose their capacity to react in AMLCs and are unable to do so by 2 weeks after birth. Other investigators have reported that AMLC responder cells do not appear in peripheral lymphoid organs until approximately 6 weeks of age [19,195]. Therefore autoreactive cells are lost from the thymus during ontogeny but do not appear elsewhere in the immune system for up to 4 weeks. It has also been shown that the proportions of cells of the various Lyt phenotypes migrating from the thymus are equivalent during neonatal life and adulthood [224]. Taken together these findings suggest that the thymus ceases to produce AMLC responder thymocytes during early life. There would also appear to be a mechanism present during the first weeks of life that either eliminates, or prevents the detection of, autoreactive cells in the peripheral lymphoid organs of the young mouse.

As mentioned above, earlier studies had concluded that newborn spleen cells are unable to respond in allogeneic MLCs [6,105]. We have confirmed the more recent observations of Wu et. al. [256] that newborn spleen cells can in fact proliferate in MLCs in response to allogeneic stimulation but with a lower magnitude than equal numbers of adult spleen cells. We established that isolated neonatal splenic T cells proliferate more strongly in one-way allogeneic MLCs than unselected spleen cells. We found that equivalent purification of adult T cells from spleen does not result in a comparable increase in their MLC reactivity. Rodriguez et. al. had made the former observation but neglected to perform

the latter control. Our results therefore suggest that the Non-T population within newborn spleen may be capable of regulating the response of T cells to alloantigens. However we determined that an equal number of adult splenic T cells respond significantly better in allo-MLCs than do the neonatal spleen T cells. This latter finding is indicative of either a defect in the ability of the neonatal T cells to respond to allogeneic stimulation or an inhibitory mechanism operative within this T cell population.

There is thus no doubt that significant numbers of functional allo and autoreactive T cells are present within the lymphoid organs of mice during the first few days of life. The absence of pathological processes due to both the maternal-fetal transfer of potentially alloreactive [62,251] as well as the resident autoreactive cells suggests the existence of efficient regulatory mechanisms active within the neonate and/or the absence of stimulatory antigens during early life. Newborn spleen cells are indeed poor stimulators in allogeneic [5] as well as autologous [27,28,103] MLCs. However the Ia antigens implicated in the stimulation of both allogeneic and autologous reactions [19,26,70,83,84,85,127, 194] are detectable early in gestation [57]. Although Ia bearing B cells are scarce or absent during early postnatal ontogeny [94,116] Ia-positive macrophages and dendritic macrophages are present in mice at birth [107,134]. Ia-bearing dendritic macrophages, which have been shown to stimulate both allogeneic [236] and autologous [177] MLRs, are found in significant numbers particularly within the thymus of the neonate [134]. Lu et. al. have shown that during early ontogeny Ia-positive macrophages appear in the thymus before appearing in the spleen [134]. Therefore the thymus of the newborn contains both the responder cells capable of mediating proliferative responses against autologous antigens <u>in vitro</u> as well as cells which bear the

presumed stimulatory antigens. We have shown that isolated neonatal Lyt $1^{+}23^{-}$ thymocytes can in fact respond with low but significant levels of proliferation to autochthonous stimulator cells prepared from the spleens of the thymus donors (Table 20). In the neonate *in vivo* it is therefore likely that either the majority of the antigens that stimulate AMLRs are not expressed appropriately and thus are not immunogenic for the autoreactive thymocytes, and/or inhibitory processes prevent activation of the auto-responsive cells.

ii. Regulation of MLC Induced Proliferation by Newborn Splenic Inhibitory Cells.

Previous investigations which have examined naturally occuring neonatal suppressor cells capable of inhibiting cellular proliferation *in vitro* have been primarily concerned with the ability of newborn cells to suppress the response of adult cells in allogeneic or semi-allogeneic MLCs [12,13,14, 16,18,100,178,179,184,210]. Presumably such inhibitory cells would be active in the prevention of maternal anti-fetal host versus graft or maternal anti-newborn graft versus host-like reactions. These reactions could result from the observed leakage of potentially alloreactive parental cells across the placental barrier from the maternal to fetal circulation [62,251]. In the absence of regulatory mechanisms maternal lymphocytes would be capable of reacting against the foreign paternal histocompatibility antigens expressed on the fetal cells. However there are other cellular immune processes which could possibly have harmful effects during fetal and early neonatal life. As discussed above, the newborn (in comparison with the adult) has a relatively large population of autoreactive cells capable of responding in AMLCs. In addition, the self Ia antigens presumed to stimulate AMLCs are also present during early ontogeny. The phenotype of

these autoreactive cells is Lyt $1^{+}23^{-}$ which is identical to that of T cells shown to mediate HVG reactions [140]. Moreover, T cell growth factors are elaborated during the AMLR [126,255]. Such factors could possibly cause the induction of cytotoxic cells with specificity for self antigen as has been shown for allogeneic effect factor [9]. Human AMLRs have also been demonstrated to result in the activation of effector cells including cytotoxic [149,225, 248,253] and suppressor [54,71,219,253] cells. There are thus a number of hazardous functions that could be initiated by autoreactive cells in vivo in addition to the obvious problem of potentially uncontrolled proliferation directed against self-antigens. Evidence is also accumulating that maternal lymphocytes may be stimulated by embryonic antigens expressed by fetal cells [51]. Therefore regulation of autoreactive cell populations may be a critically important function of the immune system during early neonatal life. In accordance with these observations we have studied the effects of naturally occuring newborn suppressor cells on both autologous and allogeneic MLCs.

In this investigation we have shown that two distinct cell populations from newborn spleen are capable of inhibiting both allogeneic and Type I autologous MLCs. One class of neonatal splenic suppressor cell is non-adherent to Ig-anti-Ig glass bead affinity columns and lacks a receptor for the B cell specific lectin SBA. Since these inhibitory cells are relatively resistant to cytotoxic treatment with anti-Thy 1 as well as anti-Lyt 1 and 2 sera it is likely that they are not phenotypically mature T cells. The inability or only partial ability of anti-Thy 1 plus RC to kill newborn splenic MLC T-inhibitory cells has been demonstrated previously [12,13,16]. In our experiments we were able to specifically remove most of the suppressive effects on MLCs of these cells by negative selection with anti-MICG or anti-T

cell sera. This indicates that the Ig-anti-Ig affinity column passed, but Thy 1 negative newborn spleen cells inhibitory for MLC reactions are evidently cells of the T lineage and possibly pre-T cells. The low density or absence of Thy 1, Lyt 1, and Ia antigens clearly distinguishes the MLC suppressor cells from the neonatal spleen T cells that inhibit T-dependent PFC responses in vitro [99,163,165].

We have shown that in addition to suppressing the allogeneic MLC reactivity of syngeneic adult spleen cells, the newborn splenic MLC suppressor T cells are also able to inhibit both the allogeneic and autologous MLRs of thymocyte responders obtained from the same pool of neonatal mice (see Figure 10 and Table 7). This potential for self-regulation suggests that these inhibitory T cells may be responsible for the relatively poor alloreactivity seen in populations of purified newborn versus adult splenic T cells.

Although there is no direct evidence to suggest that the newborn splenic T cells suppressive for allogeneic MLCs are distinct from those inhibitory for AMLCs, we have observed that the ability to inhibit the allogeneic reaction disappears between two and three weeks earlier during ontogenetic development than the capacity to suppress AMLRs. This is particularly surprising since low numbers of newborn splenic T cells appear to be more effective in suppressing allogeneic versus autologous MLCs. The differential loss of inhibitory ability in autologous versus allogeneic responses may reflect the appearance during early ontogeny of increasing proportions of alloreactive but not autoreactive T cells in the spleen. This hypothesis is supported by observations that Type II AMLR responders do not appear in the peripheral lymphoid organs until six weeks or more after birth [19,195] while allogeneic MLR reactive cells are present in spleen as early as the day of birth [256]. It is also noteworthy that we did not find any significant

phenotypic differences in the populations of newborn splenic T cells suppressing autologous versus allogeneic MLRs.

The second population of MLC-suppressor cells in newborn spleen that we have examined possesses a number of characteristics which provide for a clear distinction from the inhibitory cells of the T cell lineage discussed directly above. These Non-T inhibitory cells are retained by Ig-anti-Ig glass bead affinity columns and yet are not plate or Sepadex G10 adherent and do not phagocytize carbonyl iron. Since the Non-T suppressor cells are not adherent to glass or plastic plates, they are probably retained by Ig-anti-Ig affinity columns through surface Ig and/or Fc receptors. Moreover they are resistant to complement dependent cytotoxic treatment with anti-T cell sera. When added to adult murine spleen cell preparations the selected batches of SBA employed in our studies specifically agglutinates only B lymphocytes [208]. We have determined that SBA agglutinates newborn spleen cells in a similar discriminatory manner and that the Non-T suppressor cells are $SBA^+$ (see Figures 12 and 13). It has previously been demonstrated that during early postnatal ontogeny murine spleen contains both surface $IgM^+$ and $IgM^-$ B cells [112]. We have in fact been able to separate newborn $SBA^+$ spleen cells into two subpopulations based on differences in Ig expression. This conclusion is supported by observations of distinct differences in the staining of the separated neonatal $SBA^+$ spleen cells with fluorescein conjugated anti-IgM. Nevertheless, the subpopulations of newborn $SBA^+$ spleen cells both bearing and lacking (or expressing relatively low levels) surface IgM were found to possess roughly equivalent MLR-inhibitory capabilities. We have also shown that detectable numbers of other markers present on mature B lymphocytes, including Fc and C3 receptors as well as Ia antigens, are absent on the newborn Non-T suppressor cells. This latter finding is consistent

with the well established fact that during ontogenetic development these structures do not appear on the surface of B lymphocytes in significant numbers until several weeks past the neonatal stage [7,75,94,116]. In our experiments a minor proportion of newborn splenic MLC-suppressor cells were isolated as Fc receptor or C3 receptor positive. This suggests that the Non-T MLC inhibitors may develop these receptors during maturation. It would therefore appear that the newborn splenic Non-T MLC-suppressor population consists of cells of the B lineage that express a range of levels of surface IgM and probably represent an immature cell population linked to the B cell lineage.

In comparison with adult spleen cells newborn spleen cells react poorly in allogeneic MLCs [6,105,256]. Our findings (Figure 9) as well as those of others [210] indicate that splenic Non-T cells are responsible for this low reactivity since purification of T cells from neonatal spleen results in a significant increase in their ability to respond in the allogeneic MLC. We have shown that addition of the Non-T cells back to purified neonatal splenic T cells in the allogeneic MLC does in fact suppress their proliferative response to a level consistent with that of unselected spleen cells. This represents a direct demonstration of self-regulation where Non-T cells inhibit the allogeneic MLC of T cells from the same preparation of newborn spleen. Thus it is evident that Non-T cells are responsible for at least part of the poor alloreactivity of neonatal spleen cells.

Our analysis of the changes during development of the inhibitory capacity of Non-T spleen cells showed that the abilities to suppress both autologous and allogeneic MLRs begin to disappear approximately two weeks after birth. The inhibitory effects of the Non-T cells on both classes of MLC are lost at equal rates (see Figure 15). In addition, no

differences were noted in the phenotypic characterization of newborn Non-T spleen cells suppressing autologous versus allogeneic reactions. Although newborn Non-T spleen cells suppress autologous reactions more efficiently than allogeneic responses there is thus no evidence to suggest that these inhibitory activities are carried out by different sub-populations of cells.

We have demonstrated that newborn spleen cells of both T and B lineages can inhibit in vitro autologous and allogeneic mixed lymphocyte reactivity. A number of previous investigations have shown that newborn murine spleen contains cells capable of suppressing allogeneic MLRs [12,13,14,16,18, 178,179,184,210]. However considerable disagreement exists as to the exact nature of the MLC-suppressor cell. Various studies have concluded that either T lymphocytes [12,13,16, 36,37,55,137,156,157,184,211], Non-T lymphoid cells [210], or cells of the monocyte line [191] are the sole inhibitory population present within neonatal spleen. This controversy can largely be explained by our findings that at least two distinct classes of MLC-suppressor cells are present in the spleen of the neonate. The possibility exists that preceeding investigators have either failed to separate the two inhibitory populations or have been satisfied with demonstrating the existence of a single population and have overlooked a second class of suppressor cell. It is noteworthy that several investigations examining newborn splenic T-inhibitory cells have indicated that the cells were difficult to lyse with anti-T cell reagents plus complement [12,13,16,36,37,137,156,157,211]. This could perhaps result from the apparent immaturity of the T suppressor of the MLC as shown in our study, or alternatively may indicate the presence of the Non-T inhibitor. On the other hand, Rodriguez et. al. [210], who have concluded that the neonatal splenic MLC suppressor is a Non-T cell, failed to detect the

weaker but still significant inhibitory effects of T cells. Although these authors showed that separation of newborn splenic T cells from the Non-T inhibitory population increases allogeneic responsiveness [210], they failed to indicate whether the neonatal splenic T cells achieved the level of alloreactivity of adult spleen T cells. We have found that isolated newborn spleen T cells do not in fact proliferate as well as adult splenic T cells in comparable allogeneic MLCs. Moreover we have also shown that newborn splenic T cells can inhibit both allogeneic MLCs and AMLCs. The most conclusive evidence that neonatal suppressor cells are at least partly responsible for the weak MLC responsiveness of newborn spleen cells is the fact, demonstrated in this investigation, that these inhibitory cells possess the potential for self-regulation.

The existence of suppressor macrophages has been established in a number of systems [63,121,132,202,230,245, 246]. In our investigation we have observed inhibition of primarily the allogeneic MLR by plate adherent newborn spleen cells. Minor inhibitory effects, which diluted out rapidly, were also seen in AMLCs. However it is not known to what extent the neonatal adherent cell preparations were contaminated with lymphoid cells. Moreover adult adherent, but not non-adherent, spleen cells also suppressed the MLRs. This suggests that the addition of large numbers of macrophages to MLCs, whether newborn or adult, can inhibit the proliferative response and tends to de-emphasize the possibility that neonatal macrophages possess a specialized ability to suppress MLCs. We have shown that depletion of newborn whole or Non-T spleen of macrophages through their adherence or phagocytic abilities did not decrease but actually increased, in most instances, the capacity of the remaining cells to inhibit MLRs. Thus, the nature of the two MLC-suppressor cell populations in newborn spleen appears to

be lymphoid rather than monocytic. Piguet et. al. have studied cells from the spleens of neonates that seem to have the capacity to inhibit TD PFC responses [191]. They have characterized these inhibitors as monocyte precursors largely on the basis of partial sensitivity of the cells to silica treatment [191]. However, with respect to adult macrophages, neonatal splenic macrophages are defective in presenting antigen in an immunogenic form [107,125,134,135,173] and may as a result interfere with the generation of an immune response if present in sufficient numbers. With the knowledge that macrophages can bind [67,68,199,240] and transmit [198,203] regulatory factors it is also possible that newborn macrophages may appear to inhibit certain reactions by carrying suppressive factors produced by neonatal spleen [15,184] or $SBA^+$ spleen cells [Hooper, D.C. and Murgita, R.A., unpublished observations] or by intercepting helper factors. Recently evidence has been presented that macrophages may be involved in the induction of suppressor T cells by AFP [188]. Thus it is possible that neonatal macrophages may seem to have inhibitory capacities without being suppressor cells.

The presence of inhibitory cells in unstimulated newborn spleen is generally taken as indicating that these cells are naturally occurring and do not require activation in vitro or in vivo like adult suppressor T cells activated by conventional antigens [42,60,150]. Nevertheless there have been studies indicating that neonatal cells must be cultured in vitro under specified conditions to develop certain regulatory properties [212,214]. Since we and others [210] have demonstrated that newborn T cells can in fact respond in MLCs, the possibility exists that these cells added to control MLCs are stimulated to acquire inhibitory properties during the early phase of the MLR. In many of our experiments, and particularly in the allogeneic MLCs,

neonatal splenic T cells added to control cultures at their initiation did not become noticeably inhibitory until up to 96 hours later. This delay before the observation of significant inhibition due to newborn splenic T cells is sufficient to allow the activation of conventional suppressor cells [60,150]. The addition of newborn splenic T cells to MLCs often boosted the proliferative response at 48 hours of culture. This contrasts with the addition of neonatal Non-T cells to MLCs. The Non-T inhibitory population was generally suppressive at the beginning the MLR. We have observed that newborn splenic T cells respond significantly better in allogeneic MLCs than in AMLCs (not shown). We have also shown that low numbers of newborn splenic T cells suppressed allogeneic but not autologous reactions. These two facts taken together perhaps suggest that neonatal spleen T cells do indeed require activation to express inhibitory properties and that the allogeneic MLC provides a more potent stimulus than the AMLC. It is conceivable then that early events in an MLC may be responsible for activation of the newborn T MLC-inhibitory cell.

We have determined that the neonatal splenic Lyt $123^-$ T cells capable of suppressing MLRs can be readily distinguished from the Lyt $1^+23^-$ cells inhibitory for T-dependent antibody responses on the basis of their surface antigenic phenotypes (see Tables 12 and 13). However this fact does not exclude the possibility that the MLC-suppressor T cells can inhibit PFC responses. Because of its high efficiency the TD PFC-inhibitory cell has been studied most often at a ratio of 1:20 with the adult responder cells. Our results in the MLC indicate that inhibition due to the two different classes of MLC-suppressor cells disappears at ratios between 1:4 and 1:8. At a ratio of 1:20 the regulatory effects of the MLC-suppressor cells may not have been detectable on the PFC response. Therefore it is

unknown at present whether the T and B MLC-inhibitory populations can suppress PFC responses when added to cultures in a ratio higher than 1:20.

iii. The Regulatory Effects of Alpha-fetoprotein On Proliferation

Purified AFP of both murine and human origin has been demonstrated to possess highly selective immunoregulatory properties in vitro [79,101,102,162,163,164,165,167,168,169, 181,186,187,188, and reviewed in 170]. In this investigation we have examined the effects of fetal derived AFP on the proliferation of various populations of murine lymphocytes stimulated by T cell mitogens, alloantigens, and autoantigens. During the course of these experiments we found that certain cell populations, showing minimal DNA turnover when cultured in the absence of known stimulatory substances, proliferate vigorously when AFP is added. Evidence that AFP possesses stimulatory properties has been previously presented [46]. We have determined that the stimulatory effects of AFP are directed primarily at Ig-anti-Ig affinity column non-adherent cells present in adult bone marrow and spleen, as well as to a lesser extent newborn spleen (see Tables 18,19 and Figures 19,20,21). Thus adult bone marrow and T cells from adult or newborn spleen, but not cells from thymus, lymph node, peripheral blood, or peritoneal exudates can consistently respond to AFP. This AFP induced cell division is dependent upon the concentration of the protein added and is not mimicked by the addition of the same concentrations of NMS. Analysis of the time course of AFP induced proliferation indicates that this is a rapid and short termed event occurring between 24 and 72 hours of culture which becomes undetectable shortly afterwards. Previous investigations have shown that AFP induces an inhibitory Lyt $1^{+}23^{-}$ T cell in vitro from preparations of

adult splenic T cells [163,164,165]. It is conceivable that the proliferation we have observed in spleen T cells may be related to the induction of these inhibitory cells. The possibility also exists that AFP is capable of stimulating the development of inhibitory cells from bone marrow precursors. The increased cell division caused by AFP is indicative that the purified protein does not have cytotoxic effects. Direct microscopic observation of various cell populations cultured in the presence of AFP corroborate this lack of cytotoxicity.

While AFP has apparently mitogenic effects on specific cell populations, earlier studies have shown that AFP can also suppress mitogen induced proliferation [168,169]. We have confirmed and extended these results by demonstrating that AFP selectively inhibits the response of T lymphocytes from particular source organs to CON A and PHA. Our findings indicate that both adult and newborn unselected and isolated Lyt $1^{+}23^{-}$ thymocytes responding to either CON A or PHA are exquisitely sensitive to inhibition by AFP. In contrast we have determined that the CON A and PHA reactions of cells from adult peripheral blood as well as adult and newborn spleen are largely refractory to the inhibitory capabilities of AFP. Both the suppressive effects of AFP on the mitogenesis of thymocytes, and their absence on that of spleen cells, were determined to be unrelated to the concentrations of mitogens employed. While the other lymphoid populations studied showed similar sensitivity (or lack of) to AFP in CON A versus PHA mitogen reactions, adult lymph node cells showed a clear difference. Unselected, purified T, and isolated Lyt $1^{+}23^{-}$ cells from lymph node stimulated by CON A were susceptible to inhibition by AFP, although to a lesser extent than comparable populations of CON A reactive thymocytes. On the other hand, identical lymph node cell preparations responding to PHA were not

suppressed significantly more by AFP than by equivalent concentrations of NMS. Several inferences may be drawn from this comparison of the susceptibility to inhibition by AFP of the CON A and PHA responsive Lyt $1^+23^-$ cells from the various organ sources. Although these cells are all phenotypically identical and respond with roughly comparable $^3$H-thymidine incorporation to the two mitogens, it is evident that immunosuppressive AFP can target certain functionally distinct cell subsets. Thus Lyt $1^+23^-$ cells in both the newborn and adult thymus differ from the majority of peripheralized Lyt $1^+23^-$ T cells responding to CON A and PHA in that they are sensitive to inhibition by AFP. Moreover the differences in the suppression by AFP of the CON A versus PHA responses of lymph node cells may indicate that within the unselected as well as Lyt $1^+23^-$ T cell populations in lymph node separate subpopulations of cells respond to the different mitogens. Alternatively, should a single population of cells be reacting to both mitogens the above findings might suggest that the proliferation induced by PHA can become refractory to AFP before that triggered by CON A. While the other lymphoid populations all showed comparable levels of $^3$H-thymidine incorporation when stimulated by the two mitogens, we found that whole and isolated Lyt $1^+23^-$ T lymph node cells showed significantly higher incorporation induced by PHA versus CON A. This fact tends to support the suggestion that, at least in lymph node, there may be a distinction between the Lyt $1^+23^-$ cells responding to the different mitogens. In any case it is readily apparent that the differential sensitivity to inhibition by AFP offers a clear distinction between the Lyt $1^+23^-$ thymocytes and phenotypically identical peripheral T cells that respond to CON A and PHA in both neonates and adults. This supports the contention that the majority of mitogen reactive T cells may lose their sensitivity to AFP coincidental with migration

from the thymus.

Prior analysis in allogeneic MLCs has indicated that the proliferation induced by Ia antigenic differences between the responder and stimulator cell donor strains is the most susceptible to the inhibitory effects of AFP [186]. In contrast proliferative reactions occurring between lymphocytes from strains possessing the same Ia determinants but allogeneic K, D, or Non-H2 antigens exclusive of those encoded by the M locus, were found to be relatively resistant to suppression by AFP [186]. We have determined that the lymphocytes responding in AMLCs are also exquisitely sensitive to inhibition by AFP. Initial comparisons were made of the suppression by AFP of neonatal CBA/J thymocyte Type I AMLRs versus conventional adult CBA/J spleen allogeneic reactions against inactivated H-2 and Non H-2 distinct Balb/cJ. The results indicated that the autologous MLR could be eliminated by the addition of as little as 100 ug/ml of AFP while the allogeneic response was decreased by a maximum of less than 50% by 200 ug/ml of the protein. We then compared the sensitivity to inhibition by AFP of the proliferation of isolated Lyt $1^+23^-$ T cells from neonatal thymus and adult spleen, the former stimulated by adult T-depleted syngeneic as well as H-2 and Non H-2 disparate allogeneic spleen cells and the latter by the allogeneic stimulators. Our findings indicate that newborn Lyt $1^+23^-$ thymocytes stimulated by syngeneic cells are more susceptible to AFP mediated suppression than those stimulated by allogeneic cells. Moreover, the alloreactive neonatal Lyt $1^+23^-$ thymocytes were found to be significantly more sensitive to inhibition by AFP than isolated Lyt $1^+23^-$ adult splenic T cells responding to the same allogeneic stimulator cells. Since we have demonstrated that self Ia antigens may be the stimulatory structures in the Type I AMLC these results support the previous conclusions that AFP

preferentially inhibits proliferative responses directed against Ia antigens. The differences in the susceptibility to AFP mediated suppression of the newborn versus adult Lyt $1^+23^-$ cells reacting against allogeneic stimulator cells suggests that the neonatal thymocytes may be more restricted than phenotypically identical adult splenic T cells in the ability to respond to various alloantigens. We therefore speculate that neonatal Lyt $1^+23^-$ thymocytes may react preferentially, and more exclusively than adult Lyt $1^+23^-$ T cells, against Ia antigens (both self and foreign) while the adult cells respond to Ia as well as other H-2 and Non H-2 lymphocyte activating determinants.

We have also examined the susceptibility to regulation by AFP of autologous Type II MLRs composed of lymph node cells responding to spleen cells both prepared from the same pool of adult mice. Like the Type I reactions Type II AMLRs are highly sensitive to inhibition by AFP. This contrasts with the relative resistance to AFP mediated suppression of adult peripheral T cells reacting against allogeneic stimulators in conventional allogeneic MLCs [186]. Therefore AMLC reactive cells from both newborns and adults possess an enhanced susceptibility to inhibition by AFP in comparison with cells of the same origin responding in allogeneic MLCs. We have demonstrated that the thymus of the newborn contains a population of cells whose autoreactive potential, as measured in the AMLC, exceeds that of adult cell populations. We have also determined that these newborn thymocytes can in fact respond in AMLCs to autochthonous neonatal splenic stimulator cells and that this reaction is inhibited by AFP. As discussed in Section VI. ii. a), it is possible that dendritic cells present in the thymus at birth can provide the autologous stimulus for autoreactive newborn thymocytes. Thus it seems likely that the neonate must be capable of regulating the activity of autoresponders within the thymus

as well as in peripheral lymphoid organs. However we have been unable to identify any population of cells in newborn thymus that can suppress AMLCs (data not shown). This suggests that a soluble factor, such as AFP, may control autoreactive processes in the thymus during early ontogeny. We have shown that AFP fulfills the functional requirements for such an activity in that the inhibitory effects of AFP are enhanced in autologous versus allogeneic MLCs. Thus AFP may allow potentially beneficial reactivity against invading foreign agents while selectively controlling autoimmunity. Moreover our comparitive measurements in mice of different ages of serum AFP levels versus the ability of thymocytes to respond in AMLCs indicate that elevated concentrations of AFP are present throughout the perinatal period wherein unselected thymocytes can respond in the AMLC. The concentrations of AFP measured during this developmental interval are in each case many times higher than the 50 ug/ml required to virtually abrogate the AMLR. We therefore conclude from these findings that AFP may have a specialized regulatory function to selectively control autoreactivity during early ontogeny.

The ability of AFP to differentiate between mixed lymphocyte reactions involving primarily Ia versus other lymphocyte activating determinants has raised a question concerning the cellular target(s) of this regulatory protein. The most obvious argument is that AFP interacts in some way with the Ia-bearing stimulator cells and prevents Ia antigen recognition by Ia-reactive T cells. The demonstrated induction by AFP of Lyt $1^+23^-$ I-$J^+$ suppressor cells for T-dependent antibody responses [165] neither supports nor denies this interpretation. However, the fact that proliferation induced in particular populations of unselected and isolated Lyt $1^+23^-$ T cells by CON A or PHA is highly sensitive to inhibition by AFP confirms that AFP has direct

anti-proliferative effects for T cells. We have been unable to find any involvement of Ia antigens in the AFP sensitive mitogen reactions by either attempted blocking with anti-Ia serum or by cytotoxic pretreatment of the responder cells with anti-Ia plus complement [Hooper, D.C. and Murgita, R.A., unpublished observations]. In addition we would suspect that the mitogen reactions of thymocytes versus splenic T cells, for example, have equal requirements for Ia antigens. Yet the responses of thymocytes versus spleen cells to CON A and PHA show considerable differences in sensitivity to inhibition by AFP. Thus it is evident that AFP can directly inhibit the proliferation of T cells in the seeming absence of a requirement for Ia antigen involvement. The mechanism enabling AFP to apparently distinguish between subpopulations of T cells stimulated in MLRs by Ia versus Non-Ia antigenic determinants is therefore likely to be the result of a basic difference(s) between the Ia and Non-Ia reactive cells themselves. AFP may discriminate cells on the basis of the presence or absence of surface structures capable of binding and/or internalizing the protein. It is unlikely that the Lyt antigenic phenotype is involved since we have demonstrated in mitogen reactions distinct subsets of T cells possessing the Lyt $1^{+}23^{-}$ phenotype which are both sensitive and resistant to the inhibitory effects of AFP. On the other hand plausible candidates for surface structures involved in the mediation of sensitivity to AFP are Ia antigen receptors. As discussed above, the cells responding against Ia antigens in MLCs, and therefore possessing Ia receptors, show an enhanced susceptiblity to inhibition by AFP over cells responding against other antigens. In addition newborn thymocytes, which are more sensitive to inhibition by AFP than adult splenic T cells when responding in allogeneic MLCs, are also highly susceptible to AFP mediated suppression in CON A and PHA reactions. This might indicate that the

AFP-sensitive populations of mitogen reactive cells may have a specificity in allogeneic MLCs that is more restricted to Ia determinants than mitogen reactive cells that are relatively insensitive to AFP. It is then conceivable that all AFP-sensitive cells possess receptors for Ia antigens and that these, or structures closely related either by physical proximity and/or genetic linkage, are involved in the selectivity of the inhibitory effects of AFP. It is noteworthy that there is no evidence to suggest that AFP can distinguish anti-self Ia from pure anti-allogeneic Ia interactions. If AFP acts through the anti-Ia receptor this may indicate that there are basic similarities between the receptors for self versus foreign Ia antigens on the cells of a particular strain of mouse.

Alternative explanations, distinct from those involving specific receptors, exist for the selective inhibitory effects of AFP. For example, the selectivity may be the result of differences in the metabolic pathways leading to proliferation after stimulation in AFP-susceptible versus refractory T cell populations. Thus AFP would be capable of inhibiting only cells expressing certain metabolic features such as particular alterations in cyclic nucleotide concentrations [89]. This implies that either prior to or shortly following the initiation of a response, mitogen reactive thymocytes and Ia-reactive T cells should have some metabolic properties in common that are distinct from other T cell populations and can lead to inhibition of proliferation upon the addition of AFP. It should be noted that selective surface binding and/or transport across the cell membrane of AFP does not exclude the possibility for restricted effects of the protein on metabolic processes. Conceivably susceptible cells could show selectivity at both levels of possible interactions with AFP.

The direct anti-proliferative effects of AFP are

apparently reversible. The neonatal thymocytes used throughout this investigation as mitogen and MLC responders were prepared from mice whose naturally occurring serum AFP levels were in the mg/ml range. These cells are presumably bathed in a concentration of AFP in vivo that is up to 100 times the 50 ug/ml necessary to virtually block their AMLR and mitogen reactivity in vitro. In comparison with adult thymocytes those of the newborn demonstrate no apparent deficit in responding to mitogens and possess an enhanced ability to respond in autologous and allogeneic MLCs. Thus these cells have not been irreversibly inactivated by exposure to AFP in vivo. Preliminary experiments indicate that, in certain systems, the direct anti-proliferative effects of AFP in vitro can in fact be reversed simply by washing out the AFP [Hooper, D.C., and Murgita, R.A., unpublished observations]. These findings perhaps suggest that AFP is mediating inhibitory effects by interacting with a cell surface component/receptor with low affinity. Alternatively, the rate of turnover of the AFP molecule in a suppressed cell may be high, necessitating a continual supply of AFP in the surrounding milieu to replace the inactivated protein and maintain the inhibited state of the cell.

iv. General Discussion.

It is apparent from our results that newborns within a few days of birth possess significant numbers of phenotypically and functionally mature T cells. In agreement with the previous literature [20,114] we have found approximately equivalent proportions of Thy 1, MICG, Lyt 1, and Lyt 2 bearing cells in neonatal and adult thymus. Our observations tend to confirm reports that few spleen cells in early ontogeny possess Thy 1 antigens [91,238]. However we have demonstrated that the majority of Ig-anti-Ig affinity column non-adherent neonatal spleen cells may be of the T

cell lineage in that they express MICG as well as determinants detected by anti-T serum.

A number of preceeding investigations have concluded that neonatal thymocytes do not respond with adult levels of proliferation to the T cell mitogens CON A and PHA [4,35,38,105,154,155,209,232,233,238]. We have not detected any such difference in reactivity between whole or isolated Lyt $1^+23^-$ newborn versus adult thymocytes. We have also been able to show, in contrast with others [105,233,238], that unselected neonatal spleen cells can respond significantly to CON A and PHA. Moreover, we have determined that preparations of T cells isolated from newborn spleen approach more closely the ability of adult splenic T cells to respond to these mitogens.

Thymocytes from neonatal mice are known to proliferate in MLCs in response to autologous [19,27,28,29,101,102,103, 104] as well as allogeneic [28,87,105,154] stimulator cells. We have determined that newborn thymocytes at a restricted period during early ontogeny can respond against autologous Ia-positive stimulator cells in cultures free of heterologous serum additives. The proliferation observed in the Type I AMLC is apparently due entirely to Lyt $1^+23^-$ $Ia^-$ neonatal thymocytes with low Thy 1 density. We have compared the autologous reactivity of newborn whole and Lyt $1^+23^-$ thymocytes with phenotypically similar T cell populations from various adult lymphoid sources and concluded that the thymus of the neonate contains elevated numbers of autoreactive cells. Our results indicate that neonatal thymocytes respond with equivalent or better $^3H$-thymidine incorporation to autologous versus allogeneic stimulation in MLCs. In contrast with some [6,105] but not all [256] previous studies we have observed that unselected neonatal spleen cells can respond in allogeneic MLCs. We have also determined that purified neonatal splenic T cells respond

with increased proliferation in allogeneic MLCs versus the unselected spleen cells from which they were prepared. Nevertheless we have found that isolated newborn splenic T cells do not respond in allogeneic MLCs with the magnitude of adult spleen T cells. These latter two findings suggest the presence of both T and Non-T suppressor cells in neonatal spleen.

Thus within a few days of birth significant numbers of mature MLC-reactive T cells are resident within both murine thymus and spleen. In the case of the autoreactive thymocyte population, it seems likely that the stimulatory antigens are also present in vivo during early life [57], apparently within the thymus itself [134]. We have shown that the hyporeactivity of the newborn mouse in tests of cell mediated immunity is at least partly due to the presence of several distinct naturally occurring inhibitory mechanisms. As discussed above, the regulatory activity of newborn spleen cells in various in vitro systems has been previously described. However a controversy exists in the literature concerning the identity of the inhibitory cell population [12,184,191,210 for example]. While numerous investigators have concluded that the suppressors are T cells [12,13,16,36, 37,55,137,156,157, 184,211], others suggest that they are not [191,210]. Our results indicate that there are several valid explanations that may tend to explain why such discrepancies exist in the literature concerning the identification of the inhibitory cells resident within newborn spleen. A readily apparent reason based on the results of the present investigation is that there is more than one type of newborn splenic inhibitory cell. In most cases the activities of neonatal suppressor cells have been examined in different in vitro assays of immune reactivity which may lead to the preferential observation of only one type of regulatory cell. For example, in this laboratory we

have described Thy $1^+$ Lyt $1^+23^-$ I-$J^+$ T cells in newborn spleen that significantly inhibit in vitro TD PFC responses [99,165]. Removal of this class of inhibitory cell from a preparation of neonatal splenic T cells has no effect on the ability of the remaining cells to inhibit either allogeneic or autologous MLC induced proliferation. Moreover the suppression of TD PFC reactions can be ablated by the removal of the I-$J^+$ PFC inhibitory cells even though the residual newborn splenic T cell population contains at least five times as many MLC-suppressor cells as the eliminated PFC inhibitors. Thus within newborn spleen T cells there are distinct inhibitory cell populations that preferentially act upon different immunological processes. In addition, we have described two separate classes of cells in newborn spleen that can suppress the same immune reactions (MLCs). Because of the relatively immature characteristics of both of these cell types, it is conceivable that previous investigations have been unable to distinguish that there are in fact two types (and possibly more) of MLC-suppressor cells in newborn spleen. These cells, apparently of T and B lineages, inhibit both autologous and allogeneic MLRs. We have also demonstrated that the T and B suppressors can inhibit the MLC induced proliferation of not only adult but also newborn responder cells. Thus these regulatory cells are likely to be responsible for at least part of the observed hyporeactivity of neonatal spleen cells in MLCs. It has previously been reported than autoreactive newborn thymocytes cannot respond against autochthonous spleen cells [28]. From our data it appears that this may be at least in part due to the presence of inhibitory cells within the neonatal spleen.

We have also studied the potent immunosuppressive effects of purified murine AFP on selected T cell populations responding to mitogens, alloantigens, and autoantigens. AFP

effectively inhibits the CON A and PHA induced proliferation of whole and isolated Lyt $1^{+}23^{-}$ thymocytes. On the other hand AFP has less substantial effects on purified T or isolated Lyt $1^{+}23^{-}$ cells from spleen, peripheral blood, or lymph node responding to the same mitogens. This does not necessarily indicate that only thymocytes and CON A-responsive lymph node cells are sensitive to AFP mediated suppression. It implies that the majority of mitogen reactive peripheral T cells, in contrast with most thymocytes, are resistant to the inhibitory effects of AFP. Interestingly, the same distinction in sensitivity to AFP is seen in newborn mitogen reactive thymocytes versus spleen cells. Apparently most T cells migrating from the thymus, whether in neonatal or adult life, either are in the process of losing or have already lost their susceptibility to the anti-proliferative effects of AFP. However not all peripheral T cells are refractory to suppression by AFP. The T lymphocytes that respond in MLCs against relatively pure Ia antigenic differences as well as those that respond against self-Ia in AMLCs are also highly susceptible to inhibition by this protein. Newborn thymocytes proliferating in AMLCs and, to a lesser extent, in allogeneic MLCs are also suppressed by AFP. These findings taken together suggest that mitogen reactive thymocytes, when cultured in MLCs, may be restricted to reacting primarily against Ia antigens. Acquisition of the ability to respond to Non-Ia antigens (including self-Ia plus other determinants) may be a developmental step coincidental with migration from the thymus and the loss of sensitivity to AFP.

We have demonstrated that neonates harbor a population of autoreactive thymocytes whose proliferative activity in the AMLC is greater than that of autoreactive adult T cells from various organ sources and that these cells are sensitive to the inhibitory effects of AFP. As the level of AMLC

responsiveness decreases in the thymus during postnatal ontogeny the serum concentration of AFP also subsides suggesting a causal relationship between neonatal autoreactivity and the presence of AFP. Within the newborn, it is therefore conceivable that AFP has a primary regulatory function in the prevention of autoreactive T cell proliferation induced within the thymus by self-Ia antigens. It is interesting to note that tolerance is most readily induced during early development prior to the onset of mature immunological responsiveness [24,32]. Perhaps during early ontogeny AFP is involved in the acquisition of lasting tolerance in T cells to self antigen by allowing interaction with but no proliferation against autologous determinants.

The presence of efficient, and apparently antigenically unrestricted, suppressors of MLC induced proliferation in the spleens of newborns may be an indication of the necessity for regulatory mechanisms protecting the fetus and newborn from damage from cellular elements of the maternal immune system. Maternal lymphocytes are capable of reacting against paternal and perhaps embryonic antigens on fetal/neonatal cells. Moreover, maternal lymphocytes have been detected within the circulation of neonates indicating that maternal-fetal transfer of immunologically competant cells may occur [62,251]. The developing animal may protect itself from maternal anti-fetal reactions by a combination of AFP and suppressor cells. It is readily apparent that while the two types of inhibitory mechanisms have overlapping immunoregulatory functions, the suppressor cells act on proliferative reactions which are relatively unaffected by AFP. The occurrence of two distinct types of MLC-inhibitory cells in newborn spleen may be the result of some as yet unconfirmed specialization in their modes of activity. This is apparently the case for the newborn splenic T cells capable of inhibiting MLC versus TD PFC responses.

Alternatively, this may reflect the need for different classes of suppressor cells in the various locations of the immune system where either T or B lymphocytes are the prevalent cell type.

Thus we have characterized regulatory mechanisms in the neonate which are capable of inhibiting in vitro assays of lymphoid cell proliferation. We have also determined that neonatal T cells possess considerably more reactivity to mitogens and in MLCs, particularly against autologous stimulator cells, than has been previously demonstrated. Therefore AFP may be instumental in both establishing tolerance to self antigens and, in co-operation with suppressor cells, protecting the developing animal from maternal immune aggression.

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