ONTOGENY
OF T CELL MITOGEN RESPONSES
IN LEWIS RATS

by

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This dissertation is dedicated to my mother,

Mildred R. Snyder,

whose example has been my inspiration

and whose support has been my strength.

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STATEMENT OF THE PROBLEM

Investigation into the development of immune function has led to many basic insights. Division of the immune system into B cells and T cells was based in part on studies of the development of the bursa and thymus of chickens. The study of both the normal and defective development of the immune system has contributed to the dissection of the complex network of cell interactions during an immune response. The appearance of different functions at different stages allows one to characterize the components contributing to the response.

Since considerable evidence suggests that different mitogens preferentially activate distinct, although sometimes overlapping populations, knowledge of the ontogeny of cells responsive to various mitogens can provide a functional approach to learning in what sequence and within which organs T-cell subsets emerge, and how such development is regulated. Mitogenic lectins activate large cell populations, and do not require prior immunization for their activity. Some aspects of mitogen activity such as mitogen valency, capacity to induce factors and mitogen-induced cellular interaction mimic closely the activities of immune reaction to antigen. For these reasons we choose to use the mitogenic lectins Concanavalin A (Con A) and phytohemagglutinin (PHA) to study the development of the capacity for T cell activation.

Despite the availability of detailed studies on the ontogeny of immune functions in mice and humans, little is available on that important laboratory animal, the rat. Rats differ from mice immunologically, and may have immune characteristics similar to humans

but absent in mice. A most notable difference is the presence of a dominant population of adherent suppressor cells in rat spleens, similar to a population found in rabbits and guinea pigs, but not mice. This suggests that the ontogeny of mitogen responses and the ontogeny of the regulation of those responses in rats would be different from those of the mouse.

We decided to first of all trace carefully and in detail the development of mitogenic responses to Con A and PHA. This task included obtaining data for a wide age range, with sufficient data for each age tested that we would not have to combine data from widely disparate ages (for instance, combining responses of one week olds with responses of 2 week olds). We subsequently analyzed the development of a population of cells apparently responsible, in the presence of high doses of Con A, for suppression of the naturally high mitotic rate of juvenile cells. It is well known that neonatal spleen cells suppress division and differentiation of adult lymphoid cells induced by specific antigen. The suppression of lectin induced mitogenesis has not been so well documented. Therefore, we also investigated the ontogenic development of juvenile suppressors of lectin-induced mitogenesis.

RESPONSE TO MITOGENS AS A MODEL OF THE IMMUNE RESPONSE

The phenomenon of mitotic response to the plant lectin phytohemagglutinin (PHA) by peripheral blood lymphocytes (PBL) was discovered in 1960 by Nowell (Nowell, 1960). Since that discovery, mitogenic responses to lectins have proved useful in a number of ways.

As a model of biological control of cell activities, mitogen activation provides its own built in control, the resting cell. Lectin activation provides to those researchers dissecting biochemical sequences of activation a large pool of activated cells, circumventing an earlier problem of the low occurrence of spontaneous transformation in normal adult lymphoid cells (Pulvertaft and Pulvertaft, 1966). Finally, because immunized cells enter mitosis after contact with specific antigen (Elves et.al., 1963, Pearmain et.al., 1963), the response to lectins has been used as a major model for antigen-induced lymphocyte transformation (Andersson et.al., 1972, Bullock and Andersson, 1973).

In this study two lectins were used: phytohemagglutinin (PHA) from Phaseolus vulgaris, the common red kidney bean (Rigas and Osgood, 1955) and Concanavalin A (Con A) from Canavalia ensiformis, the jack bean (Lis and Sharon, 1973). Con A was shown to be mitogenic (Knight and Thorbecke, 1971, Powell and Leon, 1970, Stobo et.al., 1972) some 10 years after the demonstration that PHA induced mitosis. However, the complete chemical characterization of Con A (Edelman et.al., 1972) preceeded that of PHA, which was termed a "biochemical mystery" (Nowell, 1976). The binding and mitogenic activity of PHA can be inhibited by N-acetyl-D-galactosamine (Borberg et.al., 1968, Perles et.al., 1977), but less completely than the inhibition of Con A binding by alpha-methyl-mannoside (Lis and Sharon, 1973, Stobo et.al., 1972). PHA appears to activate a subset of helper/inducer T cells; whereas, Con A predominantly activates a population of T suppressor cells (Williams et.al., 1982, Nakayama et.al., 1980). Con A has also been shown to be both activating and mitogenic for resident peritoneal macrophages (Wang and Basch, 1979). Con A seems to differ from PHA in

that although they both trigger about the same number of blast cells, 4 to 5 times as many of the blast cells go on to DNA synthesis under the influence of Con A (Folch and Waksman, 1974a). However, the biochemical events preceeding mitosis after stimulation by either of these two lectins are similar.

A maximum of approximately 3×10^6 molecules of Con A per mouse lymphocyte (Gunther et.al., 1974) or 5×10^6 molecules of Con A per rat lymphocyte (Betel and Van den Berg, 1972) were bound to each cell 12 hours after stimulation with doses yielding maximum mitogenesis. Alpha-methyl-mannoside can be used as a competitive inhibitor of Con A binding; however, addition of alpha-methyl-mannoside to cells immediately after Con A was added reduced the number of molecules bound by only 60% (Gunther et.al., 1974). Although physical binding could not be completely blocked by the addition of alpha-methyl-mannoside, the mitogenic effects of Con A were blocked this way (Gunther et.al., 1974, Powell and Leon, 1970). Binding of Con A to as few as 6% of the Con A receptors on the cell surface was sufficient to induce transformation (Andersson et.al., 1972). A Scatchard plot of cells binding Con A revealed 2 affinity classes (Sitkovsky et.al., 1982) which may represent either different receptors on the lymphocyte surface or two separate cell types (Stobo et.al., 1972, Perles et.al., 1977).

The effects of mitogenic lectins on the mobility of cell surface receptors have provided rich experimental ground, yielding insight into the cytoskelton and the significance of valency in activation. It has been repeatedly observed that multivalency was necessary for cell activation. However, succinyl Con A, which is divalent, and native Con

A, which is tetravalent have different effects on both the cytoskeleton and the activation of cells at high mitogen doses. Patching is the phenomenon of clustering of cell surface proteins which were randomly distributed over the cell surface. It is often followed by capping, the movement of the clustered molecules to one pole of the cell. Native Con A is mitogenic at doses greater than 5 ug/ml, doses which have also been shown to inhibit the patching and capping of both Con A receptors and unrelated cell surface proteins such as the Ig receptor (Yahara and Edelman, 1972). These effects have been seen on lymphocytes in a number of studies; but in one comparative study, Con A had little effect on the mobility of surface components on macrophages (Henis and Elson, 1981). Peritoneal macrophages bound Con A in patches which were rapidly endocytosed, without the formation of caps (Pick, 1979). The ability of native Con A to cause cap inhibition is not localized to the region of Con A binding, but appears to affect the entire cell cytoskelton, as demonstrated by the inhibition of subsequent anti-immunoglobulin cap formation. Treatment with colchicine permitted the formation of caps and the induction of DNA synthesis by Con A, indicating that although the cytoskeleton was involved in the inhibition of cell surface mobility by Con A (Edelman et.al., 1973, Pick, 1979), the immobilization of cell surface receptors is not necessary for activation. Succinyl Con A, which is also mitogenic, but is only divalent, causes neither capping of its own receptors nor inhibition of capping of unrelated cell surface components, again suggesting that immobilization is not necessary for activation.

At high doses of mitogen (greater than 125 ug/ml) another

differential effect of succinyl Con A and native Con A is seen. Tritiated thymidine incorporation is greatly reduced by stimulation with high doses of native Con A, leading many authors to suggest that high doses of native Con A might be toxic. However, high Con A doses not only did not block cell activation as defined by blastogenesis, an increase in cell size, but actually increased the number of cells undergoing blastogenesis, even though subsequent DNA synthesis was inhibited (Waksman and Wagshal, 1978). Succinyl Con A, on the other hand, stimulated DNA synthesis even at extremely high doses, indicating that the higher valence of native Con A may indeed have an effect on some "turn-off" signal, or that immobilization of the cell surface may lead to a shut off of mitogenesis. In one study, drugs which interfered with microtubule assembly reduced lymphocyte response to doses of native Con A optimum for mitogenesis and increased the mitogenic response of lymphocytes to high doses which were normally inhibitory (Steen and Lindmo, 1979). Overall it appears that although most mitogenic lectins have effects on the microtubular system, those effects are not part of the necessary signal for mitogenesis. In addition, binding of lectin alone has been shown to be insufficient to trigger cells into mitosis. A complex series of events, which will be discussed below, involving factors produced by accessory cells and other T-cells is necessary for Con A induced activation of cells (Larsson and Coutinho, 1979, Wagner et.al., 1981). In this way mitogen activation mimics the multicellular activation network essential to antigen triggering of the immune response.

Biochemical events observed after mitogen binding have included a rise in cAMP levels by 15 minutes (Takigawa and Waksman, 1981), an

increase in membrane biosynthesis within 30 minutes (Broome and Jeng, 1973), the appearance of nuclear templates (Broome and Jeng, 1973), and the processing of RNA (Grunert and Schafer, 1982) by 2 hours, with a concomitant early rise in protein synthesis (Degen and Morris, 1980). A second rise in cAMP occurred at 24 hours (Takigawa and Waksman, 1981). RNA synthesis has been shown to increase later than protein synthesis, about 6 hours after culture initiation (Bettens et.al., 1982). RNA 5′ cap methylation increased within 30 hours, paralleling the increase in RNA synthesis (Grunert and Schafer, 1982). Con A stimulated cultures began DNA synthesis some 18-20 hours after initial Con A binding, then the rate of DNA synthesis plateaued by 48 hours (Gunther et.al., 1974).

It has been acknowledged almost since the original discovery of mitogenic responses to lectins that adherent cells were necessary for optimal responses (Gery et.al., 1971, Gery and Waksman, 1972). Although Con A bound to Sepharose was able to activate both T and B cells, only the response of T cells was macrophage dependent (Terukina and Arai, 1981). It is widely agreed that one critical function of the macrophage is the production of interleukin 1 (IL-1), originally known as lymphocyte-activating-factor. IL-1 binds to T cells to provide a second signal for production of a T cell growth-inducing factor called interleukin 2 (IL-2). Macrophages may serve other functions in mitogen activation, perhaps even requiring contact with the T cell as suggested for Ia-antigen interactions. Recently it was shown (Hunig et.al., 1983) that macrophages are required during Con A responses not only for the production of IL-2, but also for the induction of responsiveness to IL-2 in the activated T cell.

Mitogen activation not only induces division, it also induces cells to produce specific products. Wagner et.al. (Wagner et.al., 1981) briefly review the interactions of Con A stimulated cells. The binding of Con A to lymphocytes induces receptors for interleukins. Simultaneously, the production of IL-1 by macrophages is boosted by the stimulus of Con A binding. It appears that IL-1 and Con A then act together to cause the production of IL-2 by T lymphocytes. Other factors produced by stimulated T cells include B cell growth factors (Duncan et.al., 1982), interleukin 3 (IL-3) (Wagner et.al., 1981), migration inhibition factor and migration stimulation factor (MacSween et.al., 1982). These factors are translated both in vivo and in vitro into a variety of biological functions.

Activation by Con A also increases the production of a number of macrophage products such as plasminogen activator (Vassalli and Reich, 1977, Hamilton, 1981, Neumann and Sorg, 1981), prostaglandin E1 (PGE1) (Kurland et.al., 1979) and IL-1 (Meltzer and Oppenheim, 1977); however, it does not induce interferon production (Neumann and Sorg, 1981) by macrophages. Con A also was found to induce an oxidative burst in macrophages with increased release of O_2^- (Keisari, 1982, Holian and Daniele, 1982) and H_2O_2 (Keisari, 1982). However, Tomioka and Saito found (Tomioka and Saito, 1980) that Con A inhibited the release of H_2O_2 in macrophages responding to wheat germ agglutinin and phorbol myristic acetate (PMA). In control experiments these authors showed that Con A had the opposite effect on polymorphonuclear lymphocytes (PMN), as it enhanced PMA triggered H_2O_2 release. The action of Con A on macrophage can be partially accounted for by the finding that macrophage-activating-factor binds a mannose containing receptor

(Yamamoto and Tokunaga, 1981).

CON A ACTIVATION OF LYMPHOCYTES IN VITRO

The proportion of cells entering mitosis as a result of lectin activation is influenced by many variables, such as the number of cells in culture, media components, and serum source. In microplates the optimal number of rat lymphocytes has been reported to be from 2×10^5 cells per flat or round bottom well (Lindsay and Allardyce, 1979) to 4×10^5 cells in flat bottom wells (Holt et.al., 1981a). Round bottom wells probably increase the local cell density, leading to cell crowding and decreased responses. Brooks (Brooks, 1975) found that the optimal cell density for T-cell mitogen responses tended to be lower than the cell doses optimal for the mixed lymphocyte reaction (MLR) and responses to the B-cell mitogen, lipopolysaccharide (LPS) (Brooks, 1975). Stewart et.al. (Stewart et.al., 1975) have studied the increase in cell number due to mitosis after stimulation with PHA. The doubling time of human lymphocytes after exposure to PHA was 20 hours. In the first 24 hours there was a 2 fold drop in cell number, probably due to clumping or loss of damaged cells, then the cells increased to a maximum density of 5 to 10×10^6 viable cells per ml. A lower starting concentration of 10^5 cells/ml resulted in a lower initial cell loss than a starting concentration of 10^6 cells/ml, supporting the general observation that when too many cells are cultured, crowding decreases mitogenesis. In two separate studies (Stewart et.al., 1975, Distelhorst and Benutto, 1982) human peripheral blood lymphocytes continued to divide and increase their number up to 5 days after

initiation of culture with PHA. Holt et. al. (Holt et.al., 1981a) compared EHAA medium, a non-commercial medium formulated by Click for mouse cultures, and RPMI 1640, a standard medium for human cell cultures. After adjustment to rat osmolality, RPMI was found to be more effective in supporting antigen-induced cell division.

Serum is commonly added to lymphocyte cultures. Fetal calf serum is the most popular additive, but it has some unpredictable effects (Lindsay and Allardyce, 1979, Holt et.al., 1981a). It contains inhibiting (Splitter and Everlith, 1982, Ito et.al., 1982) and enhancing (Bullock and Moller, 1972, Mishell and Dutton, 1967) substances. In rat lymphocyte cultures, FCS gave both erratic mitogenesis and an overly high background (Holt et.al., 1981a). For antigen induced proliferation, 5% rat serum in RPMI was found to be effective (Holt et.al., 1981a); however, 5% rat serum in MEM in one study did not support mitogenic responses to Con A and PHA (Lindsay and Allardyce, 1979).

The use of two-mercaptoethanol (2-ME) in lymphocyte cultures has been studied fairly extensively. 2-ME promoted proliferation in both mouse (Broome and Jeng, 1973) and rat (Holt et.al., 1981) cultures. For the generation of cytotoxic cells 2-ME was needed during the first 2 days of culture. However, to boost mitogenesis, 2-ME needed to be present only during the first 24 hours (Parker et.al., 1982). If adherent cells were removed from thymocytes, 2-ME restored the blastogenic capability of those cells, but not the mitogenic capacity, for which Il-1 was needed (Bettens et.al., 1982). The rate of RNA synthesis in thymocytes depleted of macrophages was boosted by 2-ME (Bettens et.al., 1982). Although 2-ME promoted the survival of splenic

lymphocytes in culture (Broome and Jeng, 1973) there was no effect on thymic lymphocyte survival (Bettens et.al., 1982, Broome and Jeng, 1973). After rat thymocytes were separated with Ficoll-Hypaque, only the lighter (blastogenic) fractions responded to Con A with mitosis in the absence of 2-ME. If 2-ME was included, all fractions were able to respond mitogenically (Cartier et.al., 1982), suggesting that 2-ME provided a preparatory signal or protected cells from an inhibitory subpopulation (Aune and Pierce, 1981b).

Mitogen responses have a typical dose response curve. Low concentrations are unable to stimulate, higher concentrations have gradually increasing mitogenic effects, and at the highest concentrations there is a fall-off of mitogenic response. The effects of excessive doses of Con A have been fairly extensively studied. The hyperoptimal dose for Con A ranges from 20 ug/ml to greater than 125 ug/ml, depending on the culture system and mitogen source. Two hypotheses have been suggested for the decrease in thymidine uptake at high Con A doses; Con A is toxic at such high doses or Con A shuts off DNA synthesis, but does not kill the cells. Distelhorst and Benutto (Distelhorst and Benutto, 1982) argued against the concept that Con A is toxic by reporting that the decreased cell numbers seen following high-dose Con A stimulation were attributable to cell clumping, not death. Dispersion of the clumped cells with alpha-methyl-mannoside, followed by pronase digestion and centrimide lysis to release individual nuclei revealed no decrease in cell numbers. Steen and Lindmo (Steen and Lindmo, 1979) dispersed Con A activated cells with EDTA and alpha-methyl-mannoside, then evaluated cell size and viability by flow cytofluorometry. They found after 24 hours in culture that

actually more cells underwent blastogenesis with 100 ug/ml Con A than with 30 ug/ml despite the decrease in cell numbers in mitosis at the higher dose. Cell viability was over 80% with either dose over the four day culture period studied. However, they found that 200 ug/ml caused extensive cell death, apparently mostly among non-blastogenic cells. Cells dispersed with alpha-methyl-mannoside and centrimide gave similar results. Degen and Morris (Degen and Morris, 1980) found that at high Con A doses (>100 ug/ml) the induction of thymidine kinase was reversibly blocked. However, if alpha-methyl-mannoside was added 25 hours after culture initiation, the block to DNA synthesis was removed, and lymphocytes initiated division, indicating little immediate toxicity. Furthermore, these authors noted that as much as 150 ug/ml Con A, which in their system was highly suppressive of thymidine uptake, did not cause a loss of DNA from the cells in culture, implying that cell lysis was not occurring. However, Takigawa and Waksman (Takigawa and Waksman, 1981) using 200 ug/ml Con A found that even after using alpha-methyl-mannose to separate clumped cells, cell death could account for the decrease in DNA synthesis. Fewer than 25% of the cells survived for 24 hours. In contrast, control cultures without Con A, with 5 ug/ml Con A and with 200 ug/ml succinylated Con A yielded comparable cell survivals of >80% within the first 24 hours. Due to the observations that 200 ug/ml native Con A immobilizes cell surface receptors, shuts off cell division, and appears to kill the cells, where on the other hand succinylated Con A does not immobilize the cell surface, shut off cell division nor kill the cell, it has been suggested that immobilization of the cell surface by very high doses of Con A can lead directly to the turn-off of division and cell death

(Gunther et.al., 1973, McClain and Edelman, 1976, Wang and Edelman, 1978). Death at high Con A doses was not due to elevation of cAMP (Takigawa and Waksman, 1981) as it was shown that elevation of cAMP alone did not cause cell death. Furthermore, when lymph node cells were passed through nylon wool, there was no elevation of cAMP after exposure to hyperoptimal doses of Con A, but cell death occurred at the same rate as in unseparated cells.

In general the maximum incorporation of thymidine in response to mitogens, indicating maximum DNA synthesis, is reported to occur between 48 and 72 hours (Powell and Leon, 1970, Olding et.al., 1974), although sometimes maximum responses occurred as late as 4 days after culture initiation (Knight et.al., 1971). The kinetics of the mitogen response differ with age. For example, human infant peripheral blood lymphocyte (PBL) responses to PHA peaked at day 2, while the mother's responses peaked at day 3 (Olding et.al., 1974). Similarly, the kinetics of antigen responses also change with age. For example, the maximum thymidine uptake in the murine MLR occured after 6 to 7 days in culture for adults and 4 to 5 days for neonates (Mosier et.al., 1976).

Committment to mitosis may occur after as little as 5 minutes or as late as one hour after exposure to PHA (Naspitz and Richter, 1968, Kay, 1967). However, in these studies, cells were simply washed with fresh medium, a procedure which would not remove bound PHA. Therefore, one could not say that these cells were committed to mitosis in the absence of the mitogen. Committment to mitosis after stimulation with Con A appears to be delayed, however, these studies used a competitive inhibitor of Con A binding to release bound Con A. Powell and Leon showed that if tritiated thymidine uptake was measured during the first

round of division, addition of alpha-methyl-mannoside could block replication for up to 20 hours after culture initiation (Powell and Leon, 1970), implying that cells were not committed to DNA synthesis until later in culture. Once committed, however, DNA synthesis was independent of lectin.

The level of mitogen response is affected by a number of variables other than the culture conditions. The strain, sex and age of the animal have all been shown to be critical. The study of strain differences has led to considerable understanding of antigen responses, and should prove equally useful in studies with mitogen stimulation. When Lewis and Brown Norway (BN) rats were compared for mitogen responsiveness, an interesting pattern emerged. Lymph node (Williams et.al., 1973a) and peripheral blood cells (Newlin and Gasser, 1973) from Lewis rats were high responders to Con A and PHA, while the same cell types from BN rats were low responders. However, when the thymocyte responses to mitogen were compared, there was no difference between BN and Lewis (Williams et.al., 1973b). In addition skin graft rejection and graft versus host reactions of Lewis and BN rats were similar (Nielsen and Koch, 1975) . A later study (Raff and Hinrichs, 1977) showed that if Lewis and BN rats were treated in vivo with rabbit anti-rat T lymphocyte serum, the responses of Lewis splenocytes to PHA and Con A were depressed while responses of BN splenocytes were enhanced to become greater than the Lewis response.

The fact that female animals have higher antibody responses (Eidinger and Garrett, 1972) and a tendency to a higher incidence of auto-immune disease has been well documented. The effect of gender on T cell mitogen responses is less well established. Since it has been

demonstrated that testosterone induces depletion of cells from the thymus (Frey-Wettstein and Craddock, 1970) and orchidectomy causes a more rapid rejection of skin grafts (Frey-Wettstein and Craddock, 1970), the potential for modification of lymphocyte populations by sex hormones is clearly present. In mice, spleen cells from females had higher responses than spleen cells from males to both Con A and PHA, when using animals 3 weeks old (Krzych et.al., 1979) to 1 year old (Belisle and Strausser, 1981). Lymph node cells and thymus cells showed no difference between males and females. A preliminary study comparing mitogen responses of male and female rat lymph node cells reported, as in mice, no difference in mitogen responsiveness (Williams et.al., 1973a). It has also been shown that the splenic cell response of female mice to Con A, PHA and LPS can vary with the estrous cycle (Krzych et.al., 1978). In addition progesterone, estradiol, testosterone and cortisol have all been shown to inhibit lymphocyte transformation (Wyle and Kent, 1977). Of further interest is the converse of the above, specifically that the immune system has also been shown to affect sexual maturation. Neonatal thymectomy leads to a decrease in pituitary hormones and delayed sexual maturation in females (Pabst, 1980). The interaction of the endocrine and immune systems leads to a variety of questions, making it clear that considerably more research is needed in this area.

ONTOGENY OF MITOGEN RESPONSES

As first noted in 1966 (Pulvertaft and Pulvertaft, 1966), the division of unstimulated fetal and neonatal lymphocytes is higher than

the division of unstimulated adult lymphocytes. In humans, a high mitotic rate appeared in spleen, thymus and fetal liver cells by week 14 (Leino et.al., 1980), and persisted in peripheral blood cells up to the 3rd month (Alford et.al., 1976). In studies of murine thymocytes, labelled thymidine uptake was highest 3 to 9 days before birth and decreased gradually until day 18 after birth when typically low adult levels were observed (Mosier et.al., 1976). Thymic, splenic and peripheral lymphocytes from fetal, infant and adult guinea pigs when tested for RNA synthesis, rather than for DNA synthesis, by incorporation of radiolabelled uridine, had a uniformly low level of background activaion (Merikanto et.al., 1979).

Variation in background levels of mitosis critically complicates comparisons of cell responsiveness of different cell populations to mitogen stimulation. Data is reported three ways in the current literature, as the ratio of the stimulated counts to the background counts (stimulation index), as the difference between the stimulated counts and the background counts (delta cpm), and as the "raw" counts per minute. When possible, the maximum counts and background counts are both reported in tabular form. This method provides the reader with the maximum information, however, when dealing with large amounts of data it becomes unwieldy. Gutowski and Weksler (Gutowski and Weksler, 1982) advocated the use of the stimulation index (SI) because they felt that the variation in background made the delta cpm an "insufficient" way to compare responses. Alford et.al. (Alford et.al., 1976) also converted their data for Candida and SKSD induced transformation to SI in order to factor out the high background thymidine uptake. When the background was not accounted for, the raw

counts were high at birth, declined until the age of one year, then gradually increased with exposure to the antigen. Oosterom and Kater (Oosterom and Kater, 1981) reported that an apparent increase in mitogen response with age based on raw cpm disappeared when SI was used. However, none of these authors presented a direct comparison of delta cpm with stimulation index. The effect of using the SI may have been to minimize real, but relatively small changes in juvenile animals, and to exaggerate the importance of what may have been minimal increases in adult responses. This difficulty was considered by Stites et.al. (Stites et.al., 1974) who presented not only the SI but also the control and the stimulated counts, making possible the comparison of delta cpm to SI. The validity of the use of the stimulation index may be questioned since there are no data to indicate that the number of mitotic cells in an unstimulated population is in any way related to the number of potential mitogen responsive cells. Ling and Kay (Ling and Kay, 1975) suggest that the use of the SI is "unreal" because there is evidence that different stimuli are additive, not multiplicative. Therefore, it might be argued that delta cpm provides a more accurate approach to analyzing thymidine uptake from animals of different ages with differing background DNA synthesis rates.

Ontogeny of the murine mitogen response has been studied extensively using two organs, the spleen and the thymus. As these organs contain different cell populations, I will deal with each separately.

Early work indicated that both PHA and Con A responses of mouse spleen cells attained maximum thymidine incorporation levels by 3 to 4 weeks of age (Mosier and Cohen, 1975, Stobo and Paul, 1972). Krzych

et.al. (Krzych et.al., 1979) also found that murine spleen cells responded poorly to PHA at 1 and 2 weeks of age, but by 3 weeks of age the PHA response had increased abruptly to almost adult level. In the same study Con A responses were high from birth, and doubled to adult levels sometime between 3 and 8 weeks of age. Papiernik (Papiernik, 1976) found that the PHA responses of spleen cells began to rise in the 2nd week but, rather than attaining adult levels quickly, the response levels were still increasing at 6 to 8 weeks of age. In this study, Con A responses reached the adult level plateau at 4 weeks of age.

Mouse thymus cells respond differently than spleen cells. Mosier (Mosier et.al, 1976) found a "paradoxical" elevation of PHA response in fetal thymus, which dropped during the first two weeks after birth. In agreement, Stobo and Paul (Stobo and Paul, 1972) and Mosier (Mosier et.al., 1976) found that thymocyte PHA responses remained at near background levels throughout adulthood. In contrast, the Con A response of thymus cells rose to adult levels by 4 weeks postpartum (Stobo and Paul, 1972, Mosier et.al., 1976, Mosier and Cohen, 1975). Again in a conflicting report, Papiernik (Papiernik, 1976) showed that mouse thymus cells responded to both Con A and PHA in only the first 3 weeks after birth, subsequently dropping to extremely low levels of response by adulthood. Thus, even within the well studied mouse model considerable confusion exists as to the ontogeny of cells responsive to PHA and Con A.

Studies comparing human fetal or neonatal lymphocytic mitogen responses to those of adult cells have produced conflicting results. Some have found a pattern of maturation for human PBL responses to PHA to be similar to murine thymic responses to PHA. Carr et.al. (Carr

et.al., 1972) found that PHA stimulated thymidine incorporation of cord blood lymphocytes was consistently higher than that of adult PBL. However, the stimulation index for cord blood PBL was substantially lower than the adult stimulation index. Alford et.al. (Alford et.al., 1976) found that PHA stimulated thymidine uptake in PBL diminished with the age of the donor. Cord blood and PBL from infants up to 9 months old had significantly higher response levels than adults. However, these data have not been adjusted for the effect of background, which Alford showed in the same work to be higher in cord blood and infants up to 3 months old. The PHA response first appears in fetal peripheral blood by 14 to 15 weeks gestation (Stites et.al., 1974, Papiernik, 1970). According to Stites et.al. (Stites et.al., 1974) the response increased with fetal age, however, Papiernik (Papiernik, 1970) found no increase. Cord blood lymphocytes from premature infants have relatively low responses to both PHA and Con A, but by 4 to 13 days after birth the PBL responded as vigorously as PBL from full term infants (Leino et.al., 1981a). The study of human thymocytes and splenocytes has been largely confined to fetal material, however, despite the difficulties in obtaining adult thymic samples, one study (Oosterom and Kater, 1981) did compare the Con A and PHA responses of thymocytes from 6 month old infants to adult thymocytes. The maximum mitogen induced thymidine uptake of infant thymocytes was higher than adult thymocyte uptake, but when the stimulation index was used to "factor out" the background, infants were found to have mitogen response levels equivalent to adult. Human thymocytes first responded to PHA by 10-12 weeks gestation (Toivanen et.al., 1981, Leino et.al., 1980, Stites et.al., 1974) and to Con A by 13-15 weeks (Toivanen

et.al., 1981, Leino et.al., 1980). Human spleen cells first responded to PHA at fetal weeks 13-14 (Stites et.al., 1974, Leino et.al., 1980, Toivanen et.al., 1981) but not to Con A until 3 weeks later, at week 18 (Leino et.al., 1980).

Other animals for which the ontogeny of mitogen responses have been studied are guinea pigs, lambs and fishes. Merikanto (Merikanto et.al., 1979, Merikanto, 1979) has studied the guinea pig, an animal with an exceptionally long gestation period for its size (60 days) producing a relatively mature newborn. Even the earliest obtainable guinea pig PBL studied at 33 days of gestation, responded at a low level to both Con A and PHA. However, PBL responses to PHA and Con A were still below adult levels at birth, but rose to adult levels by 2 weeks. Guinea pig thymocytes responded to Con A at the first age studied (33 days of gestation) but were unable to respond to PHA until the fetuses were 48 days old. This is in contrast to mouse studies done by Mosier (Mosier et.al., 1976) who found that the earliest mitogen responses were to PHA. The development of guinea pig splenic responses followed the development of thymic responses, with the first significant Con A response by spleen cells at 38 days gestation, followed 20 days later by PHA responsiveness. Thymic and splenic mitogen responses were at adult level in newborn guinea pigs as opposed to the slower development of PBL noted above. In lambs, only the fetus has been tested, with no comparison to adults (Leino, 1978), however, the general pattern of the thymus maturing before the spleen was again observed. In fish, (Van Loon et.al., 1982), Ig+ thymus cells appear on day 14 after fertilization. These cells respond to both PHA and Con A and bear an antigen also found on fish brain cells, analogous to the

mouse Thy-1 antigen.

There are few reports on the development of mitogen responses in rats. Ranney and Oppenheim (Ranney and Oppenheim, 1972) reported that thymocytes from 5 day old rats responded to both PHA and Con A at the same level as 2 month and 8 month old rats, however cortisone resistant thymocytes from 5 day old rats failed to respond to PHA. Spleen cells from 5 day old rats were found to be totally unresponsive to both PHA and Con A. Folch and Waksman (Folch and Waksman, 1974a) compared the PHA responses of spleen cells from 2 week, 4 week, 8 week and 12 month old rats. Rats 2 weeks and 4 weeks old had about half as much thymidine uptake as adults in response to PHA. Holt et.al.(Holt et.al., 1981) found that spleen cells from suckling rat pups (less than 2 weeks old) had no response to PHA. The spleen cell PHA response data from 3 to 7 week old rats were reported as a pool, so it was impossible to detect developmental changes within this age range.

Since considerable data suggests that different mitogens activate different subclasses of cells, each with unique biological function, one would expect that the development of some functions would mature concurrently with specific mitogen responses. Mosier (Mosier et.al., 1976) showed that murine thymic PHA responses parallelled MLR activity with a high prenatal level, and a low adult level; whereas, Con A responses, low at birth and reaching adult levels by 7 to 9 weeks, parallelled cytotoxic T lymphocyte (CTL) activity. However, despite the concomitant development of PHA and MLR responses these activities probably reflect separate cell populations, based on the evidence that the mouse mammary tumor virus abrogates the MLR, but leaves the PHA response intact (Mosier et.al., 1976).

Other groups have compared development of the MLR in the spleen to its development in the thymus, with some additional comparisons to the development of CTL lymphocytes. Rodriquez et.al. (Rodriquez et.al., 1979) found strong alloreactivity in thymocytes from mice 1 day to 4 weeks old, but after week 4 the magnitude of the MLR decreased. In the same study, the splenic MLR appeared later, at 6-10 days, attained a maximum at 4 weeks, then decreased as the thymic responses did. However, Adler et.al. (Adler et.al., 1970) found that spleen cells from mice less than 3 weeks old did not respond to or stimulate an MLR. Wu found adult level thymic MLR from birth (Wu, 1978) and a significantly elevated splenic MLR which gradually increased to levels about 4 times higher by 4 weeks of age and remained high (Wu et.al., 1975). CTL responses in the same study of thymic responses (Wu, 1978) were detectable at birth, were maximum by 2 days of age, and were actually declining after 6 weeks. In contrast, Pilarski (Pilarski, 1977) found that CTL precursors (CTL-P) were absent in spleen cell preparations from 1-2 day old mice and did not appear until 2 to 3 days after birth. Widmer et.al. (Widmer et.al., 1981) found 3-19 CTL-P per 10^6 fetal or newborn thymus cells; 24 hours after birth that number had increased to 44-127 CTL-P per 10^6 thymocytes; and in adults, 300-400 CTL precursors per 10^6 thymus cells were detected.

Another function of T cells studied during ontogeny was the amplification by helper T cells of the responses of B-cells and other T cells. Thymus cells from 2 to 4 day old mice were already able to help mediate the switch from direct to indirect plaque forming cell responses (Haines and Siskind, 1980). Also as early as 8 days of age,

PNA, found that immature thymocytes could not respond to Con A even in the presence of Il-2. Ceredig et.al. (Ceredig et.al., 1982) corroborated these findings in a careful study using fluorescence-activated sorting of cells binding differentially to Lyt-2 and H-2 antigens. Neither cortisone sensitive, low H-2K cells, nor cortisone resistant, high H-2K, Lyt-2^- cells were able to mature to become cytotoxic when cultured with Il-2. Only cells of the phenotype widely agreed to be a "mature" phenotype for CTL cells (cortisone resistant, Lyt-2^+) gave rise to cytotoxic activity.

Because macrophages are required for T-cell mitogen responses, it is also important to understand the ontogeny of macrophage functions. Four major functions may be ascribed to macrophages; phagocytosis, antigen presentation, cytotoxicity and production of immunoregulatory factors such as Il-1. Antigen presentation and cytotoxicity appear to depend on Ia bearing macrophages, and interaction with histocompatible T cells. Lu and Unanue (Lu and Unanue, 1982) and Inaba et.al. (Inaba et.al., 1982) have shown that there are very few functional Ia^+ macrophages in the spleen and peritoneum of neonatal mice. This deficiency is reflected in an inability to perform functions requiring antigen presentation, such as help for antibody production (Nadler et.al., 1980, Inaba et.al., 1982) and T cell proliferation in response to antigen (Lu et.al., 1979, Lu and Unanue, 1982). However, after 2 weeks of age, the number of Ia^+ macrophages begins to gradually increase, and functional competence is simultaneously acquired (Inaba et.al., 1982). Phagocytic cells show an inverse relationship to Ia^+ accessory macrophages during ontogeny (Nakano et.al., 1978). High numbers of phagocytic cells are present at birth, which then decrease

with age. The 4th function, production of factors, is of interest because it is through factors that the mitogen response is assisted. Lu and Unanue (Lu and Unanue, 1982) found that both the spontaneous, and endotoxin initiated, production of Il-1 by neonatal macrophages was equivalent to the production of Il-1 by adult macrophages. In contrast, they found that in the production of Il-1 in response to cooperating T cells plus antigen the "neonate" (9-10 day old) was, as may be expected, deficient.

ADULT SUPPRESSOR CELL SYSTEMS

Normal rat spleens contain a dominant population of adherent suppressor cells. After elution from glass wool columns, spleen cells from BN and Lewis rats proliferated more strongly in response to mitogens and antigens than unseparated spleen cells (Raff and Hinrichs, 1977). Addition of macrophages from either strain suppressed the response of non-adherent cells. However, earlier studies (Folch and Waksman, 1974a) showed that the adherent suppressor cell activity was thymus dependent and could not be replaced by purified resident peritoneal macrophages. Thymic dependency was shown by thymectomizing and irradiating rats, then reconstituting them with bone marrow from rats which had been thymectomized 7 weeks earlier. Spleen cells from these rats lost the ability to suppress PHA responses of non-adherent normal rat spleen cells. On this basis Folch and Waksman proposed that the suppressor cell was a glass adherent T cell. Folch and Waksman also showed that rats thymectomized as adults had a transient fall in mitogen responsiveness (Folch and Waksman, 1973b). However, if

spleen cells from thymectomized rats were passed through glass wool before culture, the fall in mitogen response was eliminated (Folch and Waksman, 1973b), indicating that a population of adherent suppressor cells had been removed.

Additional studies indicate that the adherent cell population in rat spleens suppresses antibody production as well as T cell mitogen responses. Weiss and Fitch (Weiss and Fitch, 1978) showed that depletion of phagocytic and/or adherent cells by carbonyl iron from normal rat spleens allowed the production of antibody to sheep red blood cells (SRBC), while the same procedure applied to mouse spleen cells abrogated the ability of those cells to make anti-SRBC antibody.

Con A has been shown in mouse (Matsumato et.al., 1982, Aune and Pierce, 1981a and b, Pierce and Kapp, 1980), rat (Folch and Waksman, 1974a, Namba et.al., 1977, Waksman and Wagshal, 1978), rabbit (Redelman et.al., 1976), human (Davidsen et.al., 1982, Ratliff et.al., 1982, Fernandez and MacSween, 1980, Kishimoto et.al., 1979) and bovine (Smith et.al., 1981, Splitter et.al., 1981) lymphocyte culture to activate suppressor systems which are active against both T cell mitogen responses and B cell maturation and proliferation. PHA has also been shown to activate suppressor cells (Skoldstam et.al., 1982, Folch and Waksman, 1973a, Folch and Waksman, 1974a, Hayward and Kurnick, 1981), but is less commonly used than Con A. Skoldstam et.al. (Skoldstam et.al., 1982) found that Con A-induced suppression appeared before proliferation was measurable and that treatment with mitomycin C did not block suppressor function of Con A stimulated cells, however, high proliferation induced a still higher level of suppression. Prostaglandin E2, which inhibits T cell blastogenesis inhibited the

induction of Con-A-activated suppression (Skoldstam et.al., 1982), showing that blastogenesis was necessary for suppressive effects. Gullberg and Larsson (Gullberg and Larsson, 1982) defined the Con A activated suppressor cell as a nonadherent, radioresistant, Lyt-2^+ T cell, however the induction of suppression was radiosensitive. These suppressor cells were found to inhibit the production of Il-2 by fresh spleen cells. Suppressor T cells activated by Con A also block the proliferation, but not the induction, of antibody producing B cells (Redelman et.al., 1976).

The activity of Con A activated suppressor cells is at least in part mediated by soluble suppressor factors. One such factor, soluble immune response suppressor (SIRS), which has been widely studied, was originally described by Pierce and coworkers (Rich and Pierce, 1974, Tadakuma et.al., 1976). SIRS did not in itself suppress DNA synthetic responses to T cell mitogens such as PHA or Con A, but did affect DNA synthesis of B cells. However, it acted through the macrophage, causing the production of a suppressor factor which was later shown to inhibit T cell proliferation as well as antibody production (Aune and Pierce, 1981a,1981b). The suppression mediated by the macrophage suppressor factor could be reversed by high doses (5×10^{-4} M) of 2-ME in culture (Aune and Pierce, 1981b). Under conditions similar to those used for the production of SIRS from mouse spleen cells, rat spleen cells yielded an inhibitor of DNA synthesis (IDS) which was active on T cells as well as B cells (Namba and Waksman, 1975, Waksman and Wagshal, 1978). In further studies, (Namba et.al., 1977) IDS was shown to be produced by a spleen cell which was adherent to glass wool, or a thymocyte which was only weakly adherent to glass wool and

cortisone-sensitive. In vivo injection of BUDR blocked the production of IDS.

NEONATAL SUPRESSOR CELLS

Clear evidence indicates that antigen-induced responses are susceptible to neonatal suppresor cells. The earliest indication that poor neonatal responses resulted from suppression was Mosier's observation that antibody-producing cells in neonatal spleens were blocked by a subset of T cells (Mosier and Johnson, 1975). Subsequently spleen cells from newborn to 2 week old mice were also found to suppress both MLR (Pavia and Stites, 1979, Rodriquez et.al. 1979, Globerson and Umiel, 1978) and T-helpers for MLR (Argyris, 1978). Such suppression of antigen-specific reactions may not be due to T cells. Spleen cells from newborn nude mice, although lacking T cells, suppressed MLR as effectively as normal newborn spleen cells (Rodriquez et.al. 1979). Embryonic liver cells also suppressed the MLR, even when taken from fetuses without a thymus (Globerson et.al., 1975, Leino, 1981a, Monden et.al., 1982, Rabinovich et.al., 1979). Conversely, juvenile thymus cells completely failed to suppress the MLR (Argyris, 1979, Rodriquez et.al. 1979), supporting the suggestion that suppression is not initiated by thymus cells. The generation of CTL, appears to be independent of the MLR, as newborn spleen cells have been reported to both suppress (Gorczynski and MacRae, 1979) and not suppress (Pavia and Stites, 1979) the production of CTL, although the MLR was suppressed in both cases.

The question "do neonatal cells suppress the response of adult

cells to mitogen?" has not been as neatly resolved as the question of suppression of antigen responses. Spleens from 1-5 day old F1 mice failed to inhibit maternal strain spleen cell responses to Con A or PHA (Pavia and Stites, 1979), although they did inhibit the MLR. Spleen cells from somewhat older (2 weeks) mice were also unable to suppress PHA response, MLR activity or CTL generation in adult mouse spleens (Mosier, 1975), although they were still able to suppress PFC responses. However, some reports suggest that neonatal cells can suppress T-lymphocyte responses to mitogens. Liver cells from 19 day old mouse embryos suppressed adult spleen cell responses to both PHA and Con A (Rabinovich et.al., 1979), however due to the primarily hemopoietic nature of the fetal liver, the immunological significance of such activity is in some doubt. Human cord blood T lymphocytes inhibited the PHA responses of maternal lymphocytes (Olding and Oldstone, 1976), and spleen cells from 5 day old rats inhibited the PHA responses of adult and neonatal thymocytes (Ranney and Oppenheim, 1972). The suppression of Con A responses raises some interesting questions, as Con A itself promotes the division primarily of suppressor cells. One study has specifically traced the ontogeny of Con A activated suppressor cells in humans (Kishimoto et.al., 1979). The suppressor function was shown to be radiosensitive, however, more radioresistant in cord blood than in adult PBL, and sensitive to mitomycin C treatment. PBL from aging adults required 1 to 2 days longer in culture with Con A than did PBL from young adults for generation of suppressor activity. Although the kinetics of induction of Con A suppression was not studied in the newborn, one may infer that a shorter incubation with Con A might be required for maximal

suppression, such that in the routine 3 day culture, neonatal cells generate suppressors before the adult can respond.

Many attempts have been made to characterize the neonatal suppressor of T cell responses. Those attempts have led in 2 directions which appear to be converging on a theory incorporating the presently acknowledged interaction of lymphocytes and macrophages. Originally the thymus dependency of the suppression was taken almost for granted. Olding and Oldstone (Olding and Oldstone, 1976) showed that human cord blood T cell-enriched populations suppressed PHA induced proliferation of maternal PBL, whereas B and macrophage populations did not suppress. A number of workers (Argyris, 1978, Pavia and Stites, 1979, Gutowski and Weksler, 1982) have shown that neonatal splenic suppressor cells in mice were sensitive to anti-Thy 1 plus complement. Argyris (Argyris, 1979) also found that the murine neonatal suppressor activity was cortisone resistant and radiation resistant. The rat splenic suppressor of adult thymocyte responses to PHA was also shown to be radiation resistant (Ranney and Oppenheim, 1972). In later work, Argyris (Argyris, 1982) thymectomized mice at birth, and showed that the ability to suppress the adult MLR was lost more rapidly in thymectomized mice than in control mice. The suppressor activity was further ascribed to a $Lyt1^+$ and $I\text{-}J^+$ cell. Murgita et.al. (Murgita et.al., 1981) found that the murine neonatal splenic suppressor of antibody production was also a $Thy\text{-}1^+$, $Lyt1^+$, $I\text{-}J^+$ cell, leading Argyris to suggest that the same cell was responsible for both activities.

Two groups have challenged the T cell nature of the mouse neonatal spleen cell suppressor activity. Rodriquez et.al. (Rodriquez et.al.

1979) found that spleens from mice less than 6 to 7 days of age contained a suppressor of MLR activity which was resistant to treatment with anti-Thy 1 plus complement and could be found in the spleens of nude mice. The suppression could be removed by passage of the spleen cells through a nylon wool column, but not by passage through a column of autologous serum-coated glass beads. The second group arguing against T cells as the cause of neonatal suppressor activity (Piquet et.al., 1981) studied the suppression of antibody production; but based on the combined observations of Murgita et.al. and Argyris it may be legitimate to discuss this work in the context of the suppressor of T cell responses. Piguet et.al. (Piquet et.al., 1981) provide evidence that the suppressor cells in newborn mouse spleens are macrophages. They found that after 4 days in culture, neonatal spleen cells had 5 to 10 times as many macrophages as cultured adult spleen cells. If adult spleen cells were cultured on a monolayer of neonatal adherent cells, PFC responses were suppressed. However, neither monolayers of adult adherent cells nor purified neonatal splenic T cells suppressed adult spleen cell PFC responses. The suppressive effects of the neonatal adherent cells could be relieved by treatment with silica or prostaglandin inhibitors such as indomethacin or aspirin.

The synthesis of these two approaches was first suggested by Folch and Waksman (Folch and Waksman, 1973a) in a discussion of a suppressor of PHA induced proliferation found in normal adult rat spleens. This suppressor was glass wool adherent but could not be reconstituted by purified resident peritoneal macrophages. To resolve this dilemma, Folch and Waksman tentatively proposed that suppression was due to a macrophage-T-cell complex, although Waksman appears to discard this

hypothesis in later papers. In a subsequent paper (Folch and Waksman, 1974a) they showed that removal of the glass wool adherent cells from 2 week old rat spleen cells did not improve the low PHA responses, but by 4 weeks removal of adherent cells allowed a 3 fold increase to maximum adult levels. The work on macrophage-T interaction in suppression of T lymphocyte mitogen responses was carried further by Holt et.al. (Holt et.al., 1981b), who demonstrated that the suppression of spleen cell proliferation by macrophages was dependent on the presence of a subset of T cells in the replicating population. Several further lines of investigation have shown that T cell suppressor activity is dependent on the presence of macrophages (Raff et.al., 1978, Hebert et.al., 1980).

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I. ONTOGENY OF T-CELL MITOGEN RESPONSE IN LEWIS RATS: CULTURE CONDITIONS AND DEVELOPMENTAL PATTERNS

ABSTRACT

Spleen and thymus cells from female Lewis rats 2 to 220 days old were cultured with either of the T cell mitogens, phytohemagglutinin (PHA) or Concanavalin A (Con A). Initial studies established that optimal conditions for lymphocyte mitogenesis included 5% rat serum and 5×10^{-5} M 2 mercaptoethanol (2-ME) with 2.5 to 5.0 x 10^5 cells per 0.2 ml culture medium. The optimal dose of Con A for mitogenesis ranged from 1 ug/ml for suckling rats to 125 ug/ml for adult rats. Thymus cells differed from spleen cells in that they required 72 hours for maximum thymidine incorporation; whereas spleen cells attained maximum incorporation within 48 hours. Thymus cells also never attained the high levels of incorporation attained by spleen cells. Thymidine uptake in unstimulated spleen cell cultures was high in the neonate and decreased with age. Due to the inflating effect of the decreasing denominator in the commonly used ratio of maximum uptake to background (stimulation index), we elected to analyze our data using the difference between maximum uptake and background (delta cpm). Maximum splenic cell responses to Con A increased from very low levels in the 2 day old to adult levels by 3 weeks. Maximum PHA responses of splenocytes matured more slowly, reaching adult levels by 8 weeks, and were consistently lower than Con A responses. Thymus cell responses to Con A and PHA were mature from birth, but also showed a lower response to PHA than to Con A. We concluded that the thymus became immunocompetent before the spleen, that cells responding to Con A matured more rapidly than cells responding to PHA and that neonatal

cells were more sensitive to Con A than were adult cells.

INTRODUCTION

Mitogen responses are frequently used as a convenient model for studying the ontogeny of immunocompetent cells. The ontogeny of mitogen responses has been intensively studied in mice (1-4) and humans (5-10). More limited data have been reported using lymphocytes from guinea pig (11) and fetal lamb (12). Each of these species presents a valuable developmental pattern which is distinct; each offers a unique insight into the process of immune development. In spite of the major role of rats in laboratory studies, little is known of the ontogeny of mitogen responsive cells in rats, although it has been shown that in the rat, thymocytes responsive to concanavalin A (Con A) are present during the first postnatal week (13).

Because the published data on culture conditions for rat lymphocytes are not in agreement (14-16), we initially examined the influence of different concentrations of 2 mercaptoethanol (2-ME), serum source, culture duration and cell concentration on the mitogen response. After establishing culture conditions, we next asked at what age can rat spleen or thymus cells respond to the T-cell mitogens Con A and phytohemagglutinin (PHA). We found that in rat spleen cells a dramatic (10-20 fold) increase occurred in Con A responsiveness coincident with weaning; whereas, the PHA response emerged later and more slowly, peaking after sexual maturity (60-80 days). Although the optimum PHA dose remained constant throughout the 110 day age range studied, the optimum Con A dose varied with age. Older Lewis rat spleen cells responded to much higher doses of Con A than did younger

rats. Thymic responses to both PHA and Con A, on the other hand, were at adult levels from birth, and were maximum for the same dose of mitogen at all ages.

MATERIALS AND METHODS

Animals: Adult Lewis rats were obtained from Microbiological Associates (Walkersville, Md.) and Charles River (Wilmington, Ma.) and bred in our laboratory. Only female offspring were used. Adults were defined as animals over 10 weeks old.

Media and reagents: Unless otherwise noted, all media and reagents were obtained from Flow Laboratories Inc., Inglewood, Ca. or Gibco Laboratories, Grand Island, NY. Culture medium was prepared by supplementing MEM with 100 units/ml Penicillin and 100 mcg/ml Streptomycin, 0.1mM nonessential amino acids, 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 2.0 mg/ml sodium bicarbonate, 2×10^{-5} M 2-mercaptoethanol (2-ME, M-6250, Sigma Chemical Company, St.Louis, Mo.) and 2.5% heat-inactivated Lewis rat serum. Rat serum was obtained from rats exsanquinated just prior to organ removal. The blood was allowed to clot at room temperature for 15 to 30 minutes, then refrigerated for 1 to 2 hours. Solid elements were removed by centrifugation at 1000xg for 15 minutes at room temperature, then the serum was kept at $4^{o}C$ until used. In one series of experiments 2.5% heat-inactivated fetal calf serum (FCS, lot #405-6012, Flow Laboratories) was used instead of rat serum.

Cell culture: Rats were anaesthetized by intra-peritoneal injection with 0.2 to 0.6 mls of Equi-Thesin (8 mg/ml pentobarbital, 34 mg/ml chloral hydrate and 17 mg/ml magnesium sulfate in a 28% propylene glycol, 12% alcohol base), exsanguinated by cardiac puncture, and

killed by cervical dislocation. Spleen cell suspensions were prepared by gently pressing cells out of the splenic capsule with a spatula and dispersing the cells in approximately 20 mls minimal essential medium (MEM, Flow # 12-104-54, without $NaHCO_3$) at 4^oC by trituration. Thymus cell suspensions were prepared by teasing the cells from the thymic lobes with two 22 g needles into about 20 mls of MEM at 4^oC. Cells were pooled such that each culture represented cells from 2 to 4 adult or 4 to 8 juvenile animals all of the same age. The debris was allowed to settle from each cell suspension for 10 minutes at 4^oC. The cell suspension was withdrawn and the cells were recovered by centrifugation at 1000xg for 15 minutes at 4^oC and washed 3 times with 50 mls of cold MEM. Washed cells were suspended in cold, fully supplemented MEM and, after hemocytometer counting of an aliquot diluted in 1% acetic acid, were adjusted to 2.5×10^6 cells per ml.

Five $\times 10^5$ cells (except where otherwise noted) in 0.2 mls supplemented MEM were added to each well of a Falcon (Becton, Dickinson and Co., Cockeysville, Md.) Microtest II flat bottom well culture plate containing 0.05 ml of mitogen or medium control. Cultures were incubated at 37^oC in 5% CO_2, 95% air in a humidified culture box. After 48 hours, except where noted, 0.1 uCi of <methyl-^{3}H> thymidine (22 Ci/mM, ICN Pharmaceuticals, Inc., Plainview, NY) in 0.01 ml MEM was added to each well. Cells were harvested 18 hours later with an automated harvester constructed in our lab, and counted in 80% toluene, 20% methanol with 4 gm/liter Omnifluor (New England Nuclear, Boston, Ma.).

Mitogens: A stock solution containing 2 mg/ml of Concanavalin A (Con A, Pharmacia, Inc., Piscataway, NJ.) was prepared and stored at

$4^{o}C$ in phosphate buffered saline (PBS). Purified phytohemmagglutinin (PHA, Burroughs-Wellcome, Beckenham, England) was reconstituted to 625 ug/ml in MEM and stored at $-20^{o}C$. Lipopolysaccharide (LPS, Bacto 3946-25 from S. typhosa, Difco Laboratories,) was reconstituted to 1 mg/ml in PBS and stored at $-20^{o}C$. All mitogens were diluted serially fivefold from 625 ug/ml to 1 ug/ml in culture medium just prior to use. Fifty ul of each dilution or control medium was added to each of six wells just prior to addition of 0.2 mls cell suspension.

Data analysis: The mean counts of tritiated thymidine uptake per minute, and the standard deviation were calculated from 6 replicate cultures at each mitogen dose. Stimulation indices were calculated as the ratio of the counts per minute of ^{3}H thymidine incorporated by stimulated cultures to the counts per minute incorporated by control cultures. The difference between the counts per minute in stimulated and unstimulated cultures is designated as the delta cpm. The significance of the difference between means was calculated using the Mann-Whitney U test. Lines were fitted using the least squares method. Pearson's R (correlation coefficient) and the significance of correlation were calculated using the formulae and tables in the CRC Handbook of Tables for Probability and Statistics, 2nd ed. 1974.

RESULTS

RAT CELL BLASTOGENESIS

INFLUENCE OF SERUM ON RAT SPLEEN CELL MITOGEN RESPONSES:

We compared the influence of autologous rat serum and fetal calf serum on mitogen stimulation of rat spleen cells. As shown in figure 1 very strong responses were supported by both sera. Autologous serum alone was much less mitogenic than FCS alone (7092 +/- 958 versus 18056 +/- 1207), but at the optimum dose of Con A (5 ug/ml) significantly more ^{3}H thymidine was incorporated in the presence of rat serum ($p<.001$). At higher doses of Con A, responses in the presence of FCS were much lower than responses in autologous serum. In contrast, with rat serum the response to PHA was slightly lower at every mitogen dose except the optimum, which was not significantly affected by the choice of serum. B-cell stimulation by LPS was significantly greater with FCS at all mitogen doses tested. Because autologous serum was similar to or better than FCS in supporting optimum mitogenesis, had lower mitogenicity and more closely approximated in vivo conditions, all further experiments used rat serum.

EFFECTS OF 2-ME ON RAT SPLEEN CELL CULTURES STIMULATED WITH CON A.

Pilot studies to establish credible culture responses repeatedly produced poor responses to Con A until 2-ME was added to the culture medium. Following addition of 2-ME to cultures supplemented with

autologous rat serum, striking responses were obtained as shown in figure 2. Without 2-ME the maximum ^{3}H thymidine incorporation was only 5,000 cpm. At the optimum dose of 2-ME (2×10^{-5} M), ^{3}H thymidine incorporation increased over 10 fold, to 70,000 cpm. Lower doses of 2-ME did not support such vigorous blastogenesis, nor did higher doses, which may have been toxic. Two-mercaptoethanol at this dose of 2×10^{-5} M was used in all subsequent cultures of both spleen and thymus cells.

RAT THYMUS CELL RESPONSE TO THE T-CELL MITOGENS PHA AND CON A

Figure 3 shows the responses of adult thymus cells to PHA and Con A in the presence of autologous serum. Tritiated thymidine incorporation into adult thymus cells at the optimal concentrations of PHA and Con A was about 20% of that for spleen cells tested in the same experiment. Thymus cells responded comparably in the presence of either FCS or autologous rat serum to both Con A and PHA (not shown). PHA induced lower incorporation than Con A, paralleling similar findings with spleen cells.

TIME IN CULTURE VS CELL CONCENTRATION.

Two initial experiments emphasized the importance of determining the effects of cell concentration and culture duration (figure 4). In experiment A, the response of 2.5×10^{5} cells to the optimal dose of 25 ug/ml Con A dropped slightly between 48 and 72 hours of culture. When 5×10^{5} cells were cultured instead, at the optimum Con A dose the drop from 48 to 72 hours was more pronounced, appearing as a flattening of the dose response curve by 72 hours. In experiment B, with 2.5×10^{5}

cells, a shift in the dose response curve, as well as a decrease in response occurred between 48 and 72 hours of culture. Again, when twice as many cells were used, the dose response curve was altered by 72 hours revealing a distinctive drop at that Con A dose which had in fact been optimum 24 hours earlier. We therefore investigated in more detail the role of both cell concentration and culture period.

KINETICS OF CON A STIMULATION OF SPLEEN CELLS AND THYMUS CELLS IN VITRO.

Spleen or thymus cells were cultured alone or with Con A and harvested at 24, 48, 72 and 96 hours after initiation of culture. Figure 5 shows the results of this experiment. After one day in culture very little label was incorporated into Con A stimulated spleen cells or thymus cells. However the thymic cell background incorporation was quite high at 24 hours compared to both the later time points and the spleen background incorporation. After two days in culture the splenic response peaked at about 95,000 cpm with an optimum dose of 25 ug/ml Con A. Thymus cells still showed a low response, although higher than at 24 hours. On the third day of culture, the splenic response, although comparable in magnitude to the response at 48 hours, occurred with 5 fold less Con A, whereas the thymic response attained its peak. The shift in the Con A dose response of spleen cells at 72 hours creates a graph with a bimodal response. This bimodal dose response was seen in 25% of the spleen cell cultures done in early studies, and in 50% of the spleen cell cultures in more recent studies. The bimodal dose response never appeared in thymus cell cultures. Because the drop always appeared in cultures which 24 hours

earlier had shown maximum responses, we hypothesize that the effect may be due to a) the induction of suppressor cells by the vigorously proliferating lymphocytes, b) exhaustion of cells or nutrients, or c) buildup of toxic waste products due to the more active metabolism of these cells. Direct toxicity of Con A is unlikely because the response to higher doses of Con A did not drop at 72 hours. By the fourth day of culture incorporation was down to background levels in both spleen and thymus.

RELATIONSHIP BETWEEN CELL CONCENTRATION AND MITOGENIC RESPONSE.

Spleen cells from adult and 2 week old rats were cultured with Con A for a total of 48 or 72 hours (figure 6). After 48 hours in culture the response to Con A increased with the number of cells in culture, although not in direct proportion. After 72 hours in culture 5×10^5 cells showed a drop in response, with a "sag" at that Con A dose which had been optimum at 48 hours, similar to the results in figures 4 and 5. The response of 2.5×10^5 cells also dropped slightly from the level at 48 hours, but the response of 1.25×10^5 and 0.6×10^5 actually improved with longer culture. After 72 hours in culture, 1×10^6 cells (not shown) had a lower response than 5×10^5 cells, suggesting that this cell dose caused crowding or suppression.

Since our primary interest was in ontogeny of the capacity to respond to mitogens, we examined the correlation between cell density and ^3H thymidine uptake of 'juvenile' cells stimulated with Con A. As can be seen, 2 week old spleen cells responded poorly on both day 2 and day 3. Increasing or decreasing the number of cells in culture had little effect on maximum cpm. Five $\times 10^5$ cells did not respond to the

two higher doses of Con A, nor did 2.5×10^5 cells on day three.

ONTOGENY OF RESPONSIVENESS TO T CELL MITOGENS

INFLUENCE OF AGE ON THE DOSE OF CON A NEEDED TO INDUCE MAXIMUM SPLEEN CELL BLASTOGENESIS

Experiments comparing the Con A responsiveness of adult spleen cells with juvenile cells made it apparent that the optimum dose for Con A mitogenesis varied with age. Figure 7 displays 30 separate experiments (each experiment comparing an adult to a juvenile rat) showing the relationship between age and optimum dose of Con A for mitogenesis, i.e. that dose giving maximum thymidine uptake. At no time did any animal respond maximally to 0.2 ug/ml Con A. Fifty percent of the rats younger than 21 days (suckling rats) had maximum ^3H thymidine responses to 1.0 ug/ml Con A, but it was rare for an older animal to respond maximally at this dose. Most of the older rats, and the remainder of the suckling rats responded maximally to 5.0 or 25.0 ug/ml Con A. The rats which responded maximally to 125 ug/ml Con A were, with only 3 exceptions, older than 80 days. In contrast, high doses of Con A frequently drove the response of spleen cells from suckling rats well below background, suggesting that suppression may have occurred. By the Mann-Whitney U test, the age of the rats responding maximally to 1.0 ug/ml was significantly different from the age of the rats responding maximally to 125.0 ug/ml ($p<.001$). In contrast, thymus cells from animals of all ages responded maximally to only one dose of Con A, 25 ug/ml.

RELATIONSHIP OF DONOR AGE TO BACKGROUND ^{3}H THYMIDINE UPTAKE.

When the level of ^{3}H thymidine uptake in unstimulated spleen cell cultures was plotted against the age of the spleen cell donor (figure 8), it became clear that lymphocytes from young animals had a 10 fold increased ^{3}H thymidine uptake in culture without mitogen, indicating a higher intrinsic rate of metabolism or division. Thymus cells did not show a comparable relationship of background activation and age. At all ages tested the background mitogenesis of thymocytes varied between 100 and 300 cpm.

RELATIONSHIP OF SPLEEN CELL DONOR AGE TO CON A STIMULATION INDEX.

To determine the ontogeny of the spleen cell response to Con A, spleen cells from female Lewis rats ranging in age from 4 to 220 days old were cultured over a broad Con A dose range (0.2 - 125 ug/ml). As previously shown in figure 7, the optimum Con A concentration (that inducing maximum stimulation) varied widely with age. In order to represent the response potential at each age tested, we plotted the mean maximum response for each cell pool, regardless of which dose induced that response, rather than the response to a preselected Con A dose. We first plotted the resulting ^{3}H thymidine uptake as the stimulation index (SI) versus the age of the donor animal. The result was roughly a right rectangular hyperbola with a large amount of scatter among the responses of older animals (figure 9). A log/log plot clearly indicates that the stimulation index increased with age. However, since the background incorporation decreases with age (figure 8), the use of the stimulation index exaggerates the difference between young and old animals. We believe that the use of delta cpm (figure

10) may provide a more accurate indication of the ontogeny of response to mitogen.

RELATIONSHIP OF SPLEEN CELL DONOR AGE TO CON A INDUCED DELTA CPM.

When the same data described in figure 9 were recalculated and plotted as delta cpm against age it appeared that experiments completed in two separate series two years apart were distinct (figure 10); whereas, in figure 9 (SI), the points from each series (not indicated) appeared to be randomly mixed. However, statistical analysis of the data before and after conversion to log scores indicated no significant difference ($p>.05$) between the correlation coefficients of the two series individually or combined. The 'hyperbolic' relationship found when plotting stimulation indices disappears, and the age vs. delta cpm graph is now linear ($r = .553$, $p<.001$), albeit with a large amount of scatter. After conversion to a log/log plot r becomes .833.

Spleen cells from male rats 7 to 140 days old were cultured and the responses were analyzed as were the females (figure 11). Although the sample is too small for statistical significance the trend appears to be the same as in female rats. Responses are low prior to weaning, and highly variable after weaning and in adults.

APPEARANCE OF MATURE RAT SPLEEN CELL RESPONSES TO CON A.

As can be clearly seen in figures 9, 10, and 11 there is a dramatic shift in Con A responsiveness between the ages of 19 and 21 days, when the rats are weaned. We separated the data into two groups representing suckling rats and weaned animals (figure 12). Statistical analysis of the correlation between age and delta cpm established that

r was significant for suckling animals ($p<0.05$), but not significant for weaned animals ($p>0.05$), indicating that there is no relationship of age and magnitude of mitogenic response after weaning.

ONTOGENY OF SPLEEN CELL RESPONSES TO PHA.

Spleen cells from female Lewis rats 4 to 110 days old were cultured in 10 separate experiments with 0-25.0 ug/ml PHA as in figure 1. The response to the optimum dose is plotted here (figure 13); however, unlike responses to Con A, optimum responses to PHA rarely deviated from 5.0 ug/ml regardless of the age of the donors. Prior to 21 days of age the responses to PHA were clustered below 5,000 cpm. In contrast to the dramatic rise following weaning in Con A stimulated cultures, the responses to PHA increased gradually as the animals matured, and did not achieve maximum responses until 60 to 70 days of age.

In order to compare the maturation of Con A and PHA responses directly, 3 sets of litter matched donors were tested for Con A and PHA responsiveness simultaneously (figure 14). During the first two weeks after birth, Con A and PHA responses were barely above the background. By 19 days, the Con A response had started to increase, becoming maximum by day 33, agreeing with the earlier pooled Con A data in figure 10. However, the PHA response didn't start to increase until day 33, and then it continued to increase gradually with increasing age, again confirming the earlier conclusion from the pooled PHA data in figure 13.

ONTOGENY OF THE THYMIC RESPONSE TO CON A AND PHA.

Thymus cells from female Lewis rats 4 to 110 days old were cultured in 10 separate experiments without mitogen or with 0.2-125 ug/ml Con A or 0.2-25 ug/ml PHA as in figure 3 (figure 15). In all experiments, maximal thymidine incorporation occurred at 25 ug/ml Con A and 5.0 ug/ml PHA. Thymic responses to Con A were already at adult levels at the earliest age tested, day 4, but as with adult splenic responses there was a large amount of scatter. In adults, thymic responses to Con A were consistently lower than splenic responses, however, as the earlier maturation of thymic cells would suggest, thymic cells from suckling animals often gave higher responses to Con A than spleen cells from the same animals. PHA responses of thymus cells also attained adult levels of response by 4 days of age, but the thymidine incorporation was about half of the maximum attained by cells responding to Con A. It may be noted that the lowest responses in the thymus to both PHA and Con A occurred from days 14-35. These three experiments were carried out with simultaneous adult thymus cell cultures which gave typical adult responses.

DISCUSSION

Activation of lymphocytes by mitogenic lectins has been used as a method of evaluating lymphocyte immunological maturity, and as a tool to evaluate the immunocompetence of individuals. The acquisition of the mitogen response has been correlated with the ability to function in a mixed lymphocyte reaction with the generation of cytotoxic T lymphocytes (2), or the ability to initiate a graft versus host reaction (3). This study was undertaken to provide information on the development of mitogen response in the rat, an animal model largely ignored for such studies. In this paper we first define the optimum culture parameters for activation by mitogenic lectin, then test a large number of animals over a broad age range to map the stages of development of the ability to respond immunologically in the rat.

Fetal calf serum (FCS) is a major supplement for tissue culture studies, and is frequently used when autologous sera are ineffective. Previous investigators have argued for (15) and against (14,16) the use of FCS in rat lymphocyte cultures. A major objection to the use of FCS is its frequent mitogenic activity (14,17), which we also observed. As shown in figure 1, we found homologous rat serum to be as effective as or more effective than FCS while insuring lower background stimulation.

Although two-mercaptoethanol (2-ME) has been used to enhance the proliferation of mouse lymphocytes since the early 1970s (18) no study of 2-ME dose response for rat lymphocytes has been reported. Holt et.al. (14) found that 2×10^{-4} 2-ME enhanced proliferation in rat lymph node lymphocytes only 2 to 3 fold over cultures without 2-ME.

Since most investigators of murine cells use 1-5 x 10^{-5} M 2-ME, we tested a broad dose range of 2-ME concentrations on rat lymphocyte cultures (figure 2). In accord with the murine culture data, we found 2 x 10^{-5} M was also optimal for rat splenic lymphocytes, producing up to a 20 fold increase in mitogen stimulation over cultures without 2-ME.

The mitogen dose response of lymphocytes is determined by such variables as the strain (19-21), serum concentration (22) and source of reagents (21,22). In our system, doses lower than 0.2 ug/ml Con A failed to stimulate mitogenesis. Most maximum responses were attained with 5 and 25 ug/ml Con A. However, younger rats responded maximally most often to 1 ug/ml. In contrast, Mosier, in an early paper (2), reported that young mice responded better to slightly higher doses of Con A than were optimal for the old mice. Of particular interest in our work was the new finding that many older (>80 days) adult rats responded maximally to 125 ug/ml. In most reports "high" doses of Con A produce poor stimulation. It was recently reported that an apparent loss of human lymphocytes following 24 hours of culture with 100 ug/ml Con A was actually due to non-damaging aggregation of the cells by the lectin, rather than toxicity (23). Our findings, in support of the above data, suggest that high doses of Con A can actually stimulate optimum mitosis in cells from older animals. Ultimately, however, increasing Con A dose can result in cell toxicity. Takigawa and Waksman reported that 200 ug/ml Con A was toxic to rat lymph node lymphocytes (24). Viability was determined in the presence of alpha-methyl-mannose to break up aggregates. Although high doses of Con A can induce cytotoxic macrophages (25), the above loss in viable

cells also occurred in the absence of macrophages.

The effects of cell concentration and time in culture have not been examined in detail for adult rats. Knight et.al. (13) found that after 3 days of culture 2 x 10^6 cells in one ml responded maximally to doses of Con A between 20 and 100 ug/ml. Lindsay and Allerdyce (16) determined that 3.5 days was optimum for both Con A and PHA cultured with 1-2 x 10^5 cell per 0.2 ml well, however, doses of Con A greater than 20 ug/ml were not tested. Keller et. al.(15) tested PHA responses only after 3 days in culture, and with 1 x 10^6 cells per .2 ml culture. All of these studies of rat culture conditions have tested only one cell dose, and in only one study (16) was the time in culture examined. In this study we have tested both the effects of number of cells in culture and time in culture. We determined that 2.5 and 5 x 10^5 cells gave maximal responses at both 48 and 72 hours. Most cells in culture have undergone little thymidine incorporation by 24 hours, but by 48 hours responses are high even with as few as 1 x 10^5 cells per culture. By 72 hours the responses of high cell concentration cultures are beginning to drop, while the responses of cells at lower cell density are still increasing. Although not shown, 1 x 10^6 cells per culture in 3 separate experiments gave lower responses at 72 hours than 5 x 10^5 cells.

Thymidine uptake in unstimulated cultures was greater with splenocytes from young rats than with splenocytes from old rats. Similarly, it has been shown with human cord blood lymphocytes (6) and murine thymocytes (2) that unstimulated neonatal cells incorporate more ^3H thymidine than do unstimulated adult cells. Although juvenile rat spleen cells in our study had a high spontaneous background, juvenile

rat thymus cells had a high background at 24 hours which dropped to a constant low background level of thymidine incorporation by 48 to 72 hours (not shown). The variation of background thymidine uptake with age is an important consideration for ontogeny studies since the use of the stimulation index may exaggerate the difference between young and old animals, possibly leading to false conclusions (6). Therefore, we suggest that the use of delta cpm under these conditions provides a more accurate indication of the ontogeny of the response to mitogen.

The ontogeny of splenocyte mitogen responses has been examined by a number of investigators. Mosier and Cohen reported that mouse spleen cell reponses to PHA and Con A reached adult levels between 3 and 4 weeks of age (4). More extensive data of Stobo and Paul indicated that Con A response of spleen cells peaked early, reaching adult levels at 3 weeks; whereas, PHA responses rose more slowly, reaching adult levels at 8 weeks (1). Guinea pig splenic responses to these mitogens peak much earlier, reaching a maximum at one week after birth (11). As can be seen in figures 9 and 10 our data parallel the mouse studies of Stobo and Paul (1), although in rats the 10 to 20 fold increase in Con A response occurs precipitously directly after weaning at 3 weeks. However, if adherent suppressor cells were removed prior to culture, the response of 10-20 day old animals was improved (paper in preparation) so that the increase in Con A reactivity more closely approximated that of the mouse. Because of this dramatic loss of suppression following weaning, it may be useful to speculate that nursing, in addition to providing passive immunity, may in some manner affect the process of lymphocyte maturation. Walford et.al.(26) suggest that the high fat diet associated with nursing may suppress

mitogenic responses.

It has been reported that a population of mitogen responsive lymphocytes accumulates earlier in the thymus than in the spleen in humans (5), guinea pigs (11), mice (2), and, in this report, in rats. Thymic responses to Con A were already at adult levels at the earliest age tested, day 4, but as with adult splenic responses there was a large amount of scatter. In adults, thymic responses to Con A were consistently lower than splenic responses, however as the earlier maturation of thymic cells would suggest, thymic cells from suckling animals often gave higher responses to Con A than spleen cells from the same animals. PHA responses of thymus cells also attained adult levels of response by 4 days of age, but the thymidine incorporation was about half of the maximum attained by cells responding to Con A. This lower response to PHA than to Con A has been observed with cells from mice, but not guinea pigs (11) or humans (5). Although the majority of experimental values did not change with age, the lowest responses in the thymus to both PHA and Con A occurred from days 14-35. It is possible that some shift in the thymic population may occur during this interval, coincident with the dramatic change in splenic responses to Con A. More extensive testing of thymus cells from animals within this age range is needed to establish this observation.

This report represents an examination of the ontogeny of mitogen responses in the rat, and culture conditions for rat lymphocyte mitogenesis. We found that rat spleen cells responded equally well after 48 and 72 hours of culture, required 2-ME in culture, and responded well and reproducibly with autologous serum in culture. The optimal dose of Con A was low (1-5 ug/ml) for spleen cells from

unweaned rats, and high (25-125 ug/ml) for spleen cells from aging (>80 days old) rats. Splenic responses to Con A matured rapidly coincident with weaning at 21 days, then stayed high but variable. Splenic responses to PHA matured more slowly, reaching a peak between 40 and 60 days of age, then also became variable. Thymic responses to both Con A and PHA were at adult levels from birth.

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Figure 1. A comparison of the influence of autologous Lewis rat serum and fetal calf serum on the mitogenic response of adult Lewis rat spleen cells. Spleen cells from 3 eight week old female Lewis rats were pooled, and 5 x 10^5 cells were added to each well as described in the methods. The cells were cultured with 2 x 10^{-5} M 2-ME and either A) autologous rat serum or B) fetal calf serum, without stimulant or with 0.2 to 125.0 ug/ml Con A (●——●), PHA (○---○) or LPS (Δ•••Δ). Cultures were pulsed with ^{3}H thymidine at 48 hours and harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

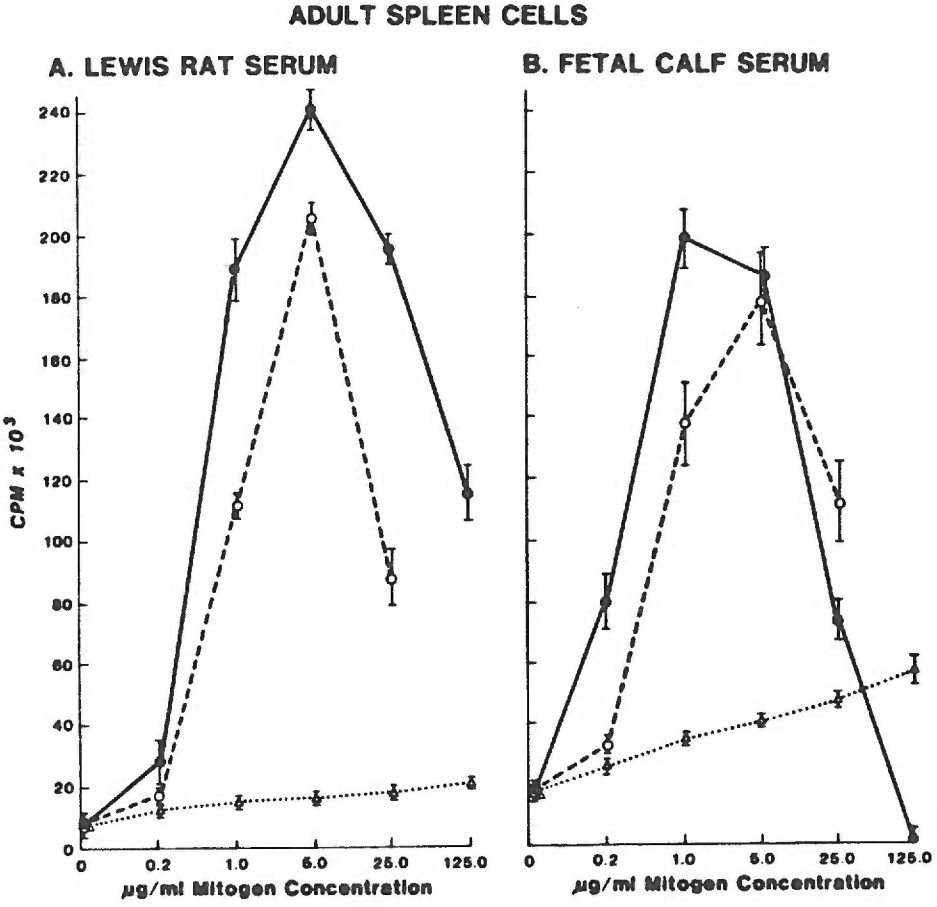

ADULT SPLEEN CELLS
A. LEWIS RAT SERUM
B. FETAL CALF SERUM
CPM x 10³
240
220
200
180
160
140
120
100
80
60
40
20
0
0
0.2
1.0
5.0
25.0
125.0
µg/ml Mitogen Concentration

Figure 2. Effect of 2-ME on the response of Lewis rat spleen cells to Con A. Five x 10^5 spleen cells were cultured in autologous rat serum with the indicated concentrations of 2-ME plus 0-125 ug/ml Con A as shown. Cultures were pulsed with ^3H thymidine at 48 hours and harvested 18 hours later. Each point represents the mean of 6 replicate cultures.

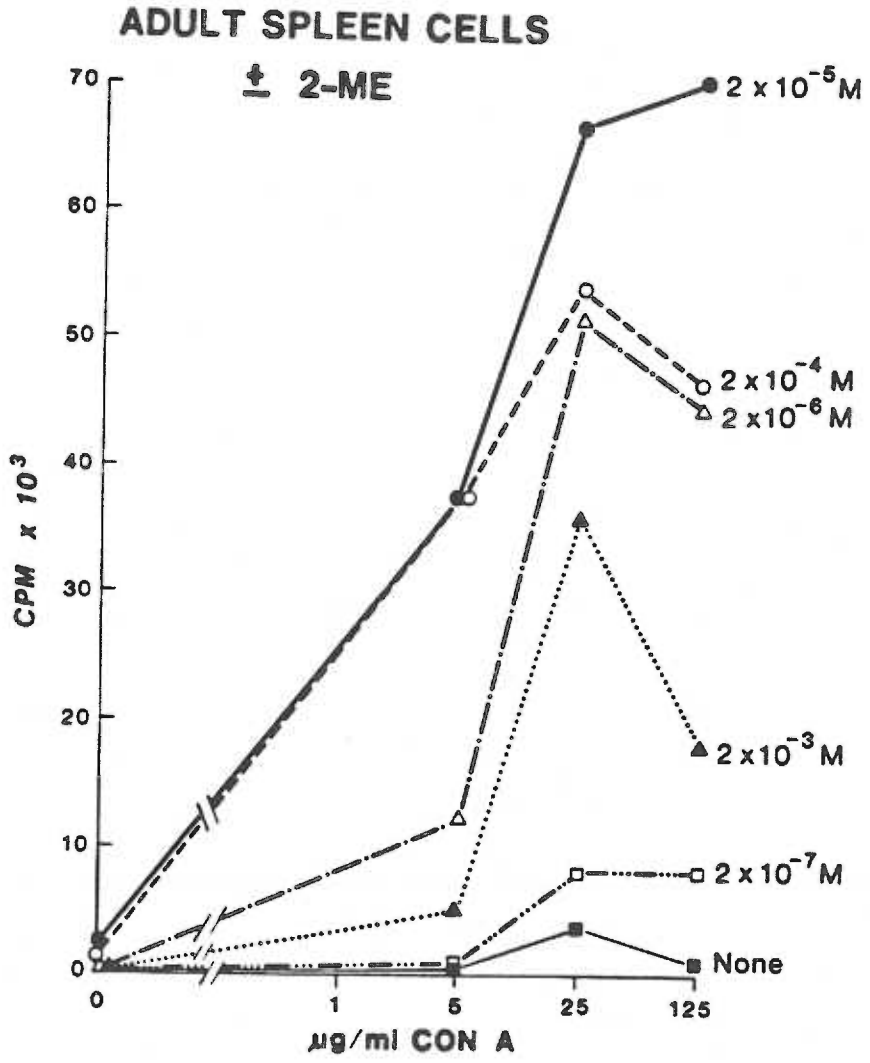

ADULT SPLEEN CELLS
± 2-ME
70
60
50
40
30
20
10
0
CPM x 10³
0
1
5
25
125
µg/ml CON A
2 x 10⁻⁵M
2 x 10⁻⁴ M
2 x10⁻⁶ M
2 x10⁻³ M
2 x10⁻⁷M
None

Figure 3. Response of Lewis rat thymus cells to T cell mitogens. Five x 10^5 thymus cells were cultured with 0 to 125 ug/ml Con A (●——●) or 0 to 25 ug/ml PHA (O---O) After 48 hours the cultures were pulsed with ^3H thymidine, then harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

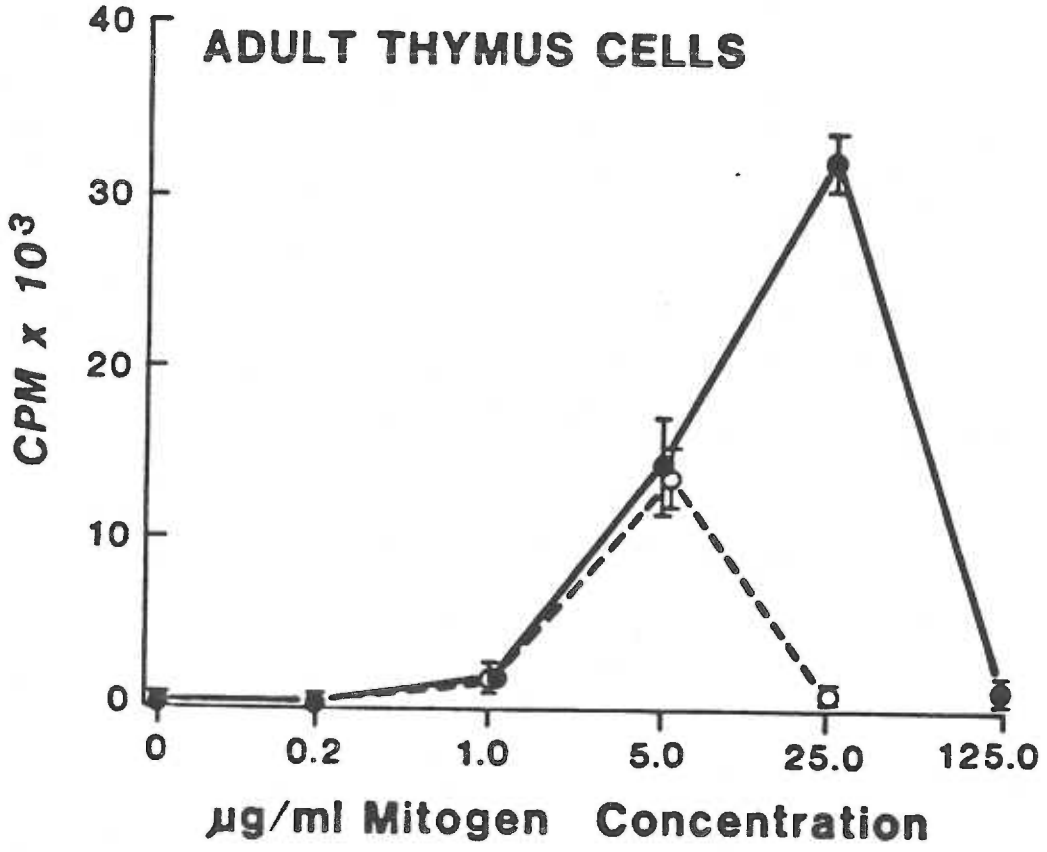

ADULT THYMUS CELLS
CPM x 10³
40
30
20
10
0
0
0.2
1.0
5.0
25.0
125.0
µg/ml Mitogen Concentration

Figure 4. Influence of cell concentration on Con A dose response. The top panels represent one experiment, the bottom panels another. Two and five tenths x 10^5 spleen cells (left side) and 5 x 10^5 spleen cells (right side) from adult Lewis rats were cultured with 1-125 ug/ml Con A, pulsed with ^3H thymidine during the last 18 hours before harvest, then harvested at 48 (●——●) and 72 (○---○) hours. Each point represents the mean of 6 replicate cultures.

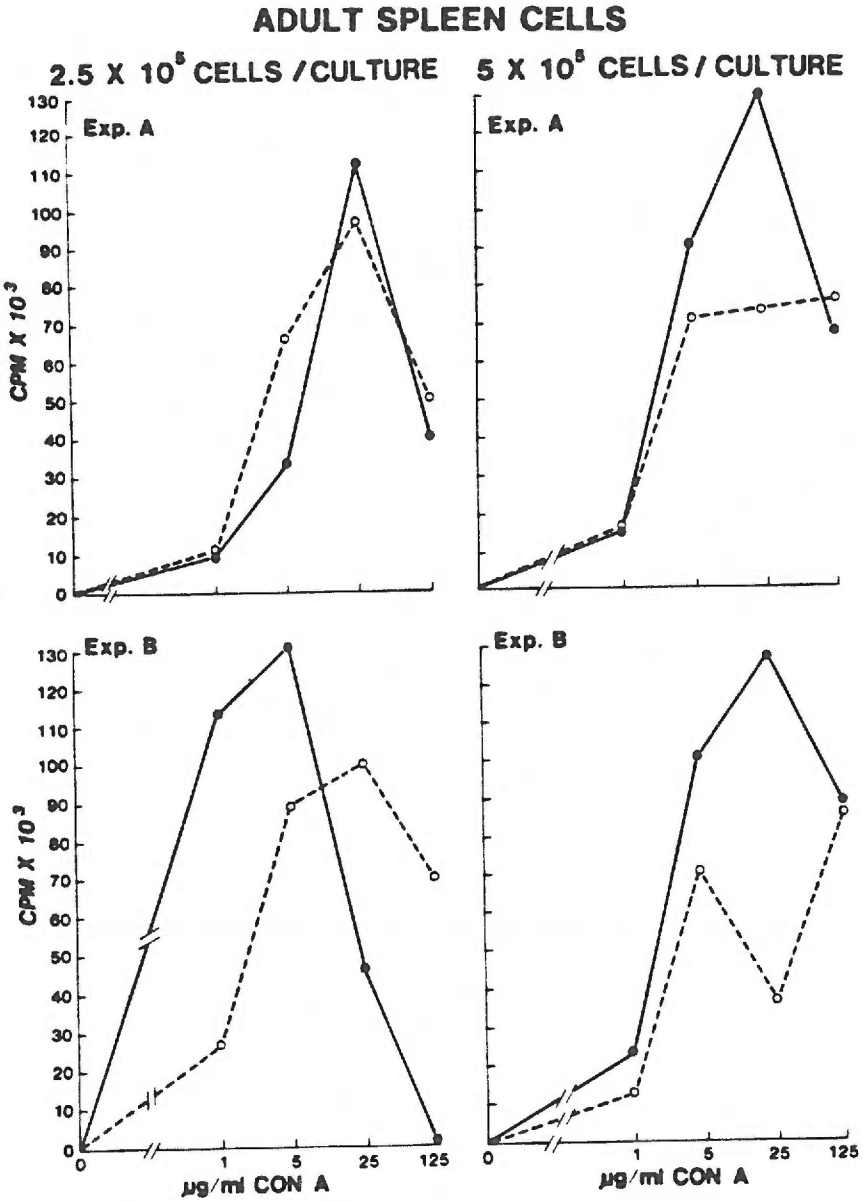

ADULT SPLEEN CELLS
2.5 X 10⁵ CELLS / CULTURE
5 X 10⁵ CELLS / CULTURE
Exp. A
Exp. A
Exp. B
Exp. B
CPM X 10³
CPM X 10³
0
10
20
30
40
50
60
70
80
90
100
110
120
130
0
1
5
25
125
µg/ml CON A
µg/ml CON A

Figure 5. Relationship of culture duration to the ^{3}H thymidine incorporation of adult spleen and thymus cells responding to Con A. Five x 10^{5} spleen or thymus cells were cultured alone or with 1-125 ug/ml Con A and harvested at 24 (▲···▲), 48 (○---○), 72 (●——●), and 96 (△-·-△) hours after initiation of culture. Each culture was pulsed with ^{3}H thymidine for the last 18 hours. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

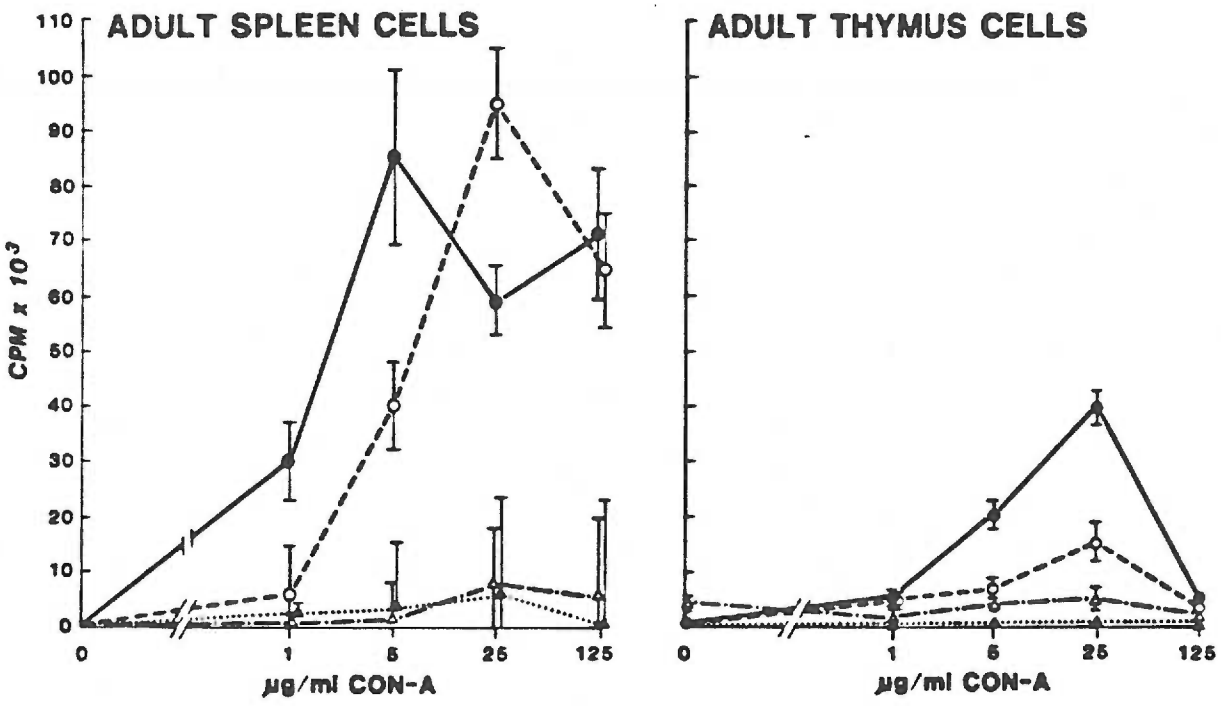

ADULT SPLEEN CELLS
ADULT THYMUS CELLS
CPM x 10³
110
100
90
80
70
60
50
40
30
20
10
0
0
1
5
25
125
µg/ml CON-A
µg/ml CON-A

Figure 6. The influence of cell number and time in culture on Con A dose response in adult and juvenile Lewis rat spleen cells. Spleen cells from adult (top) and 2 week old (bottom) female Lewis rats were cultured with 0 to 125 ug/ml Con A for 30 hours (left) or 54 hours (right), pulsed with ^{3}H thymidine, then harvested 18 hours later. Figure legend: 5 x 10^5 cells (●——●), 2.5 x 10^5 cells (○---○), 1.25 x 10^5 cells (△-·-△) and 0.6 x 10^5 cells (▲···▲). Each point represents the mean of 6 replicate cultures.

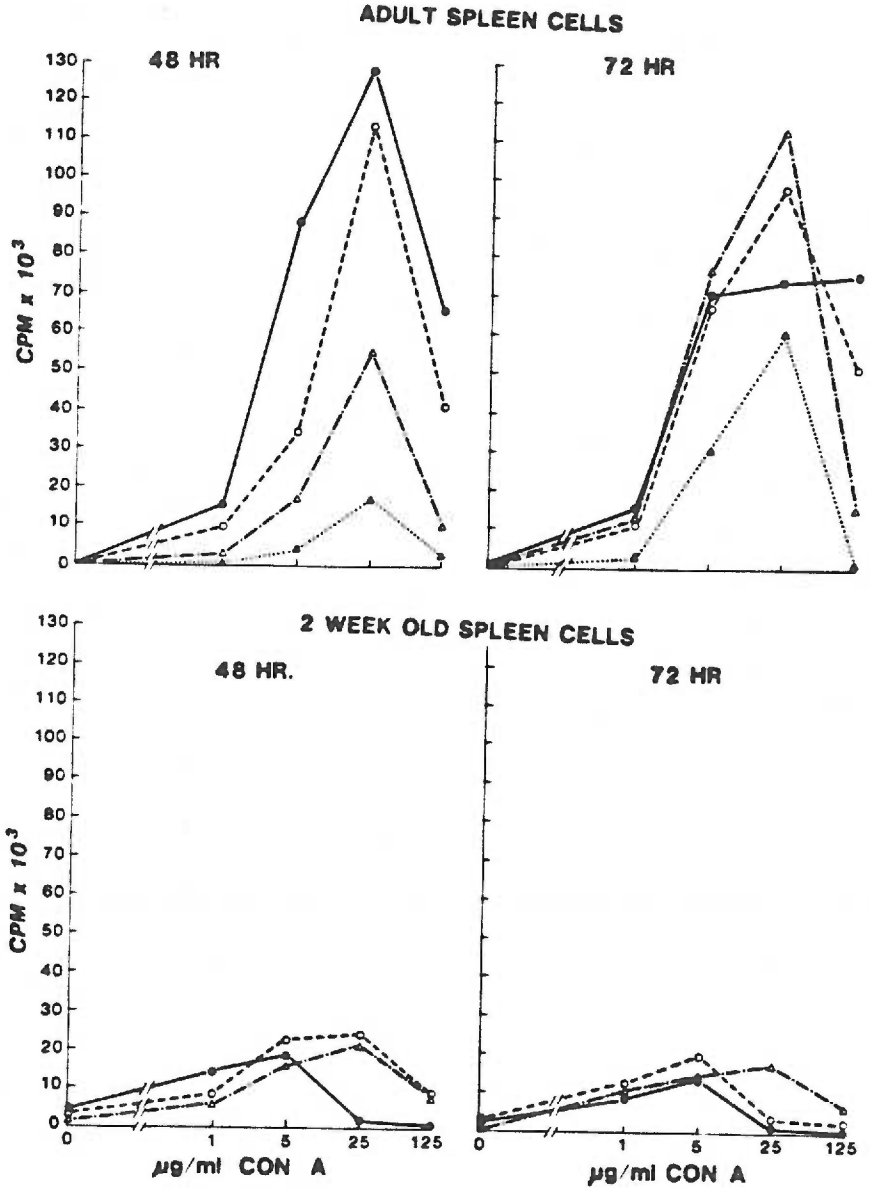

ADULT SPLEEN CELLS
48 HR
72 HR
CPM x 10³
2 WEEK OLD SPLEEN CELLS
48 HR.
72 HR
CPM x 10³
0
1
5
25
125
µg/ml CON A
µg/ml CON A

Figure 7. Influence of donor age on the optimal dose of Con A for spleen cell blastogenesis. Spleen cells from female Lewis rats 4 days old to 220 days old were cultured in 30 separate experiments (each experiment tested cells from an adult at same time as cells from any animal under 60 days old) at a concentration of 5×10^5 cells per culture in 6 replicate cultures, each of which contained either 0, 0.2, 1, 5, 25 or 125 ug/ml Con A. The cells were cultured for 48 hours, pulsed with ^3H thymidine and harvested 18 hours later. Each point represents the mean of 6 replicate cultures at the dose of Con A which produced the maximum response within each experimental group. The horizontal hatched line represents the average age of animal which responds optimally at the given dose of Con A. The age of the animals responding to 1.0 ug/ml is significantly different from the age of the animals responding to 125.0 ug/ml ($p<.001$).

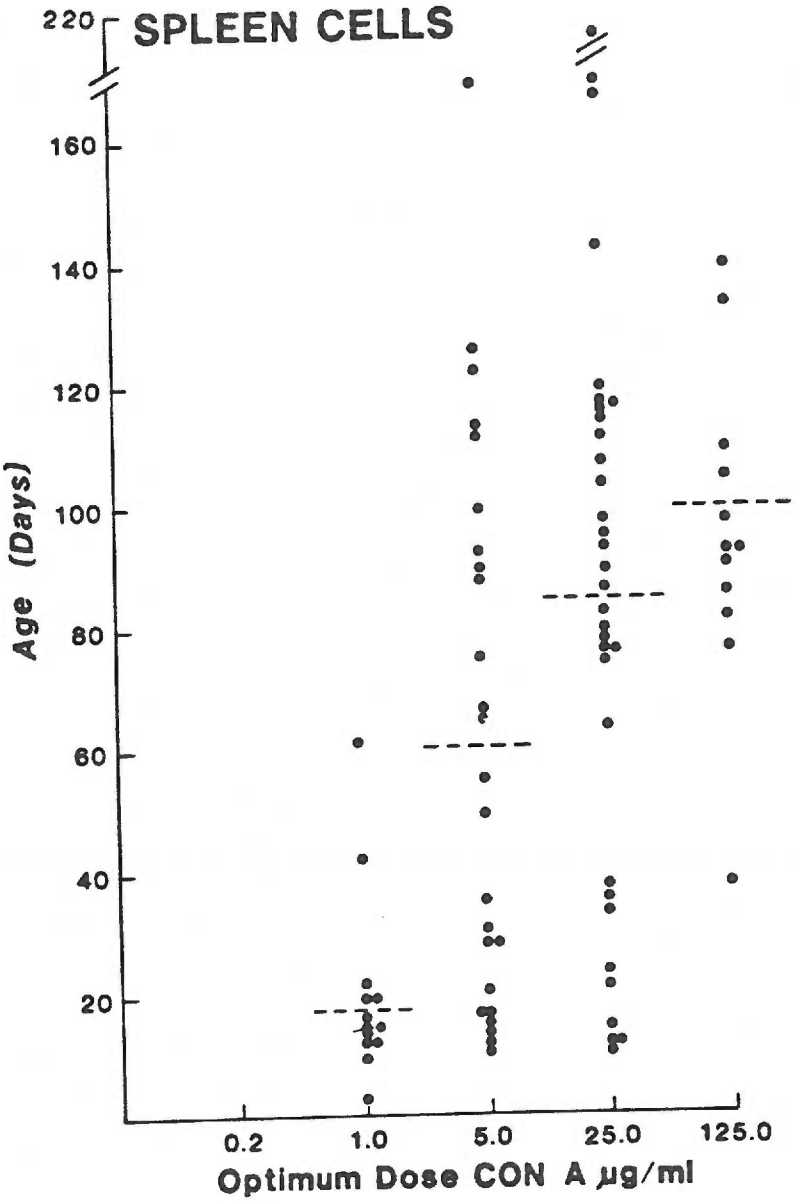

SPLEEN CELLS
220
160
140
120
100
80
60
40
20
Age (Days)
0.2
1.0
5.0
25.0
125.0
Optimum Dose CON A µg/ml

Figure 8. Relationship of donor age to amount of background ^{3}H thymidine uptake. Spleen cells from female Lewis rats 4 to 220 days old were cultured in 30 separate experiments as in figure 7. Each point represents the mean response of 6 replicate unstimulated cultures. The inset presents the logarithm of each point plotted against the logarithm of the age of the donor animals.

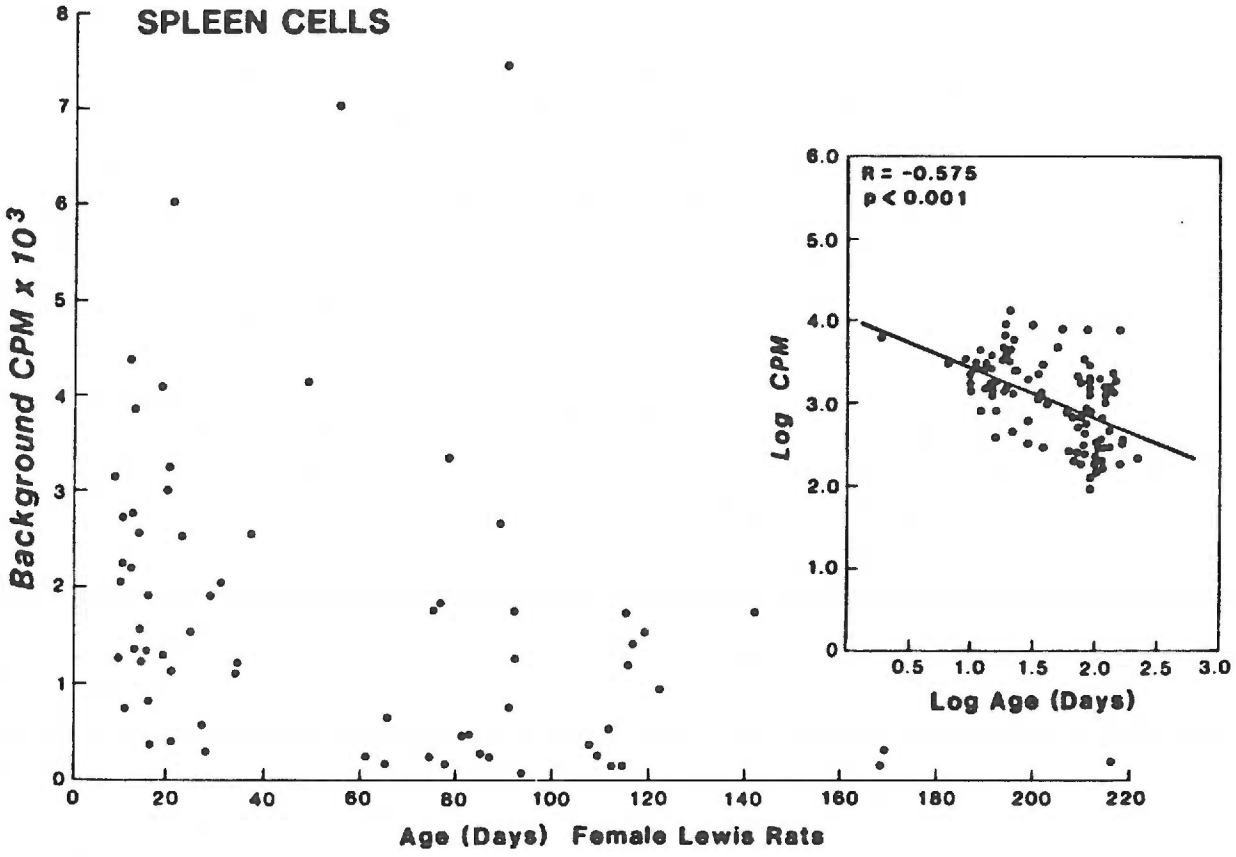

SPLEEN CELLS
Background CPM x 10³
Age (Days) Female Lewis Rats
R = -0.575
p < 0.001
Log CPM
Log Age (Days)

Figure 9. Relationship of spleen cell donor age to Con A stimulation index. Spleen cells from female Lewis rats ranging in age from 4 to 220 days old were cultured as in figure 7. Each point represents the mean response of 6 replicate cultures at the optimum dose of Con A divided by the mean response of 6 cultures of control cells from the same group of animals. In the inset the logarithm of each point is plotted against the logarithm of the age of the donor animals.

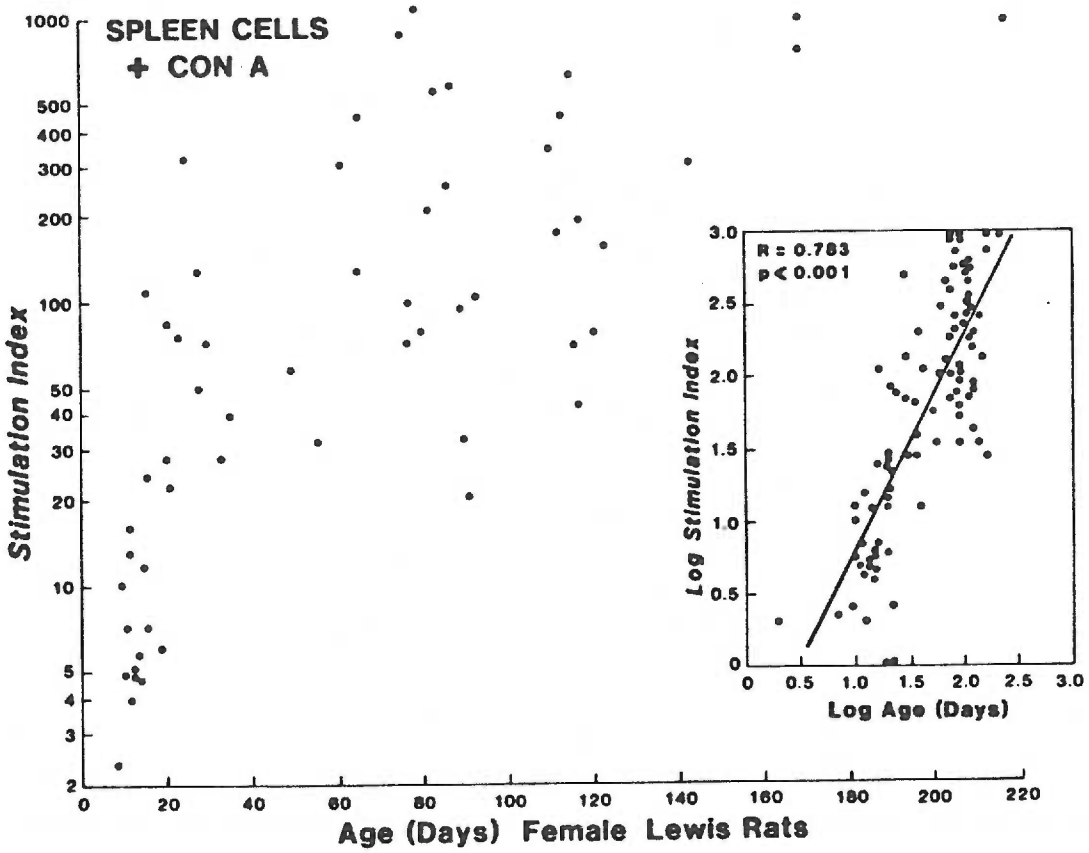

SPLEEN CELLS
+ CON A
Stimulation Index
1000
500
400
300
200
100
50
40
30
20
10
5
4
3
2
0
20
40
60
80
100
120
140
160
180
200
220
Age (Days) Female Lewis Rats
R = 0.783
p < 0.001
Log Stimulation Index
3.0
2.5
2.0
1.5
1.0
0.5
0
0.5
1.0
1.5
2.0
2.5
3.0
Log Age (Days)

Figure 10. Relationship of Con A induced ^{3}H thymidine uptake to age of spleen cell donor. Spleen cells from female Lewis rats 4 days old to 220 days old were cultured as in figure 7. Each point represents the mean of 6 replicate cultures at the optimum dose of Con A minus the mean of 6 control cultures from the same cell pool. Closed points represent a series of experiments done in 1979-80, open points represent a series run in 1982. The inset plots the logrithm of each point against the logrithm of the age of the donor animals.

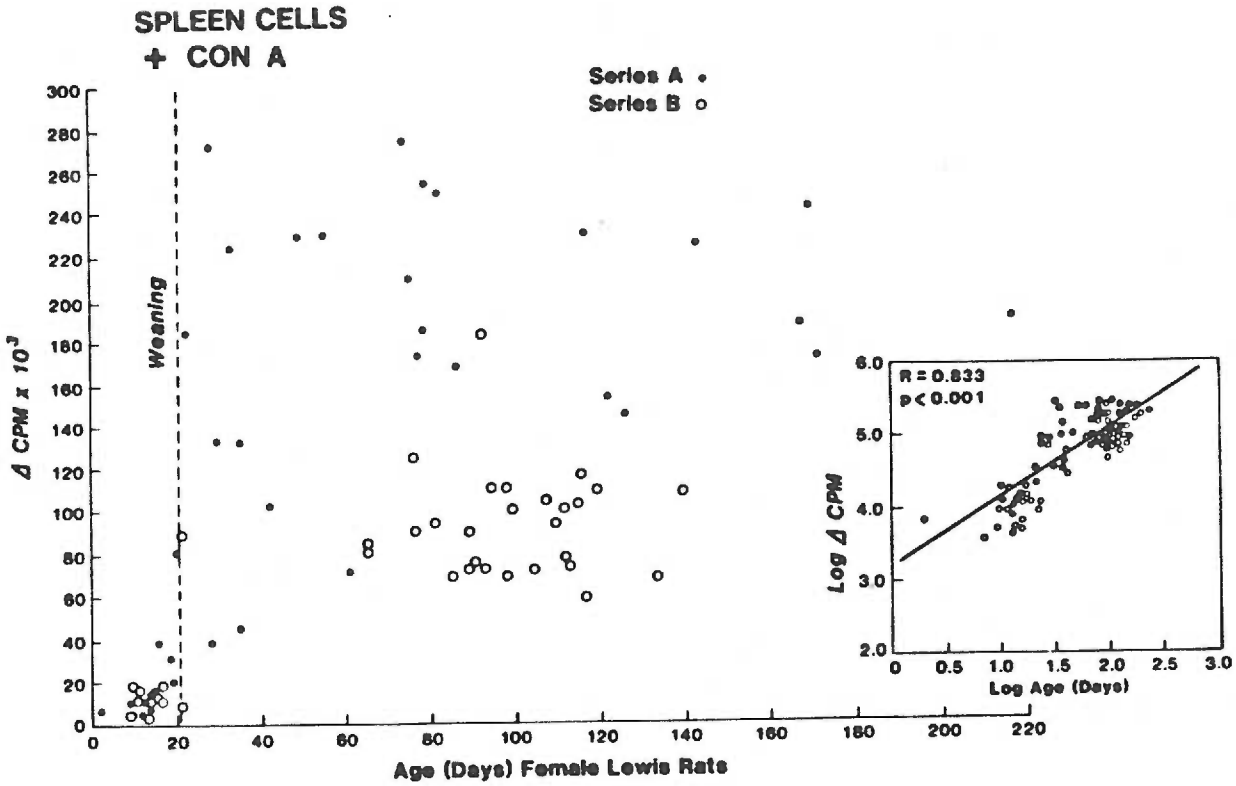

SPLEEN CELLS
+ CON A
Series A •
Series B ○
Δ CPM x 10³
Weaning
Age (Days) Female Lewis Rats
R = 0.833
p < 0.001
Log Δ CPM
Log Age (Days)

Figure 11. Relationship of Con A induced ^{3}H thymidine uptake to age of spleen cell donor. Spleen cells from male Lewis rats 7 days old to 140 days old were cultured as in figure 7. Each point represents the mean of 6 replicate cultures at the optimum dose of Con A minus the mean of 6 control cultures from the same cell pool. The inset plots the logrithm of each point against the logrithm of the age of the donor animals.

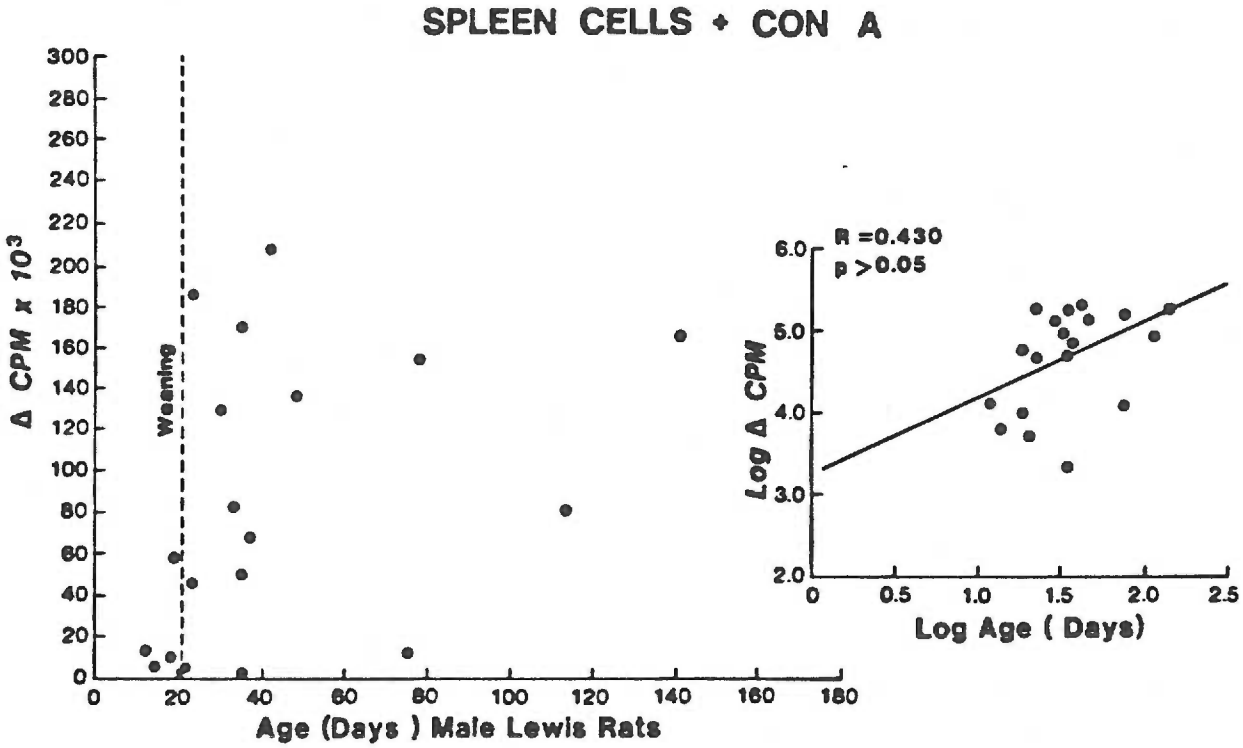

SPLEEN CELLS + CON A
Δ CPM x 10³
Weaning
Age (Days) Male Lewis Rats
R =0.430
p >0.05
Log Δ CPM
Log Age (Days)

Figure 12. The correlation between age and level of Con A induced blastogenesis changes coincident with weaning. Spleen cells from female Lewis rats 4 to 21 and 22 to 220 days old were cultured as in figure 7. Each point represents the mean of 6 replicate cultures at the optimum dose of Con A minus the mean of 6 control cultures from the same cell pool. Closed points represent a series of experiments done in 1979-80, open points represent a series of experiments done in 1982.

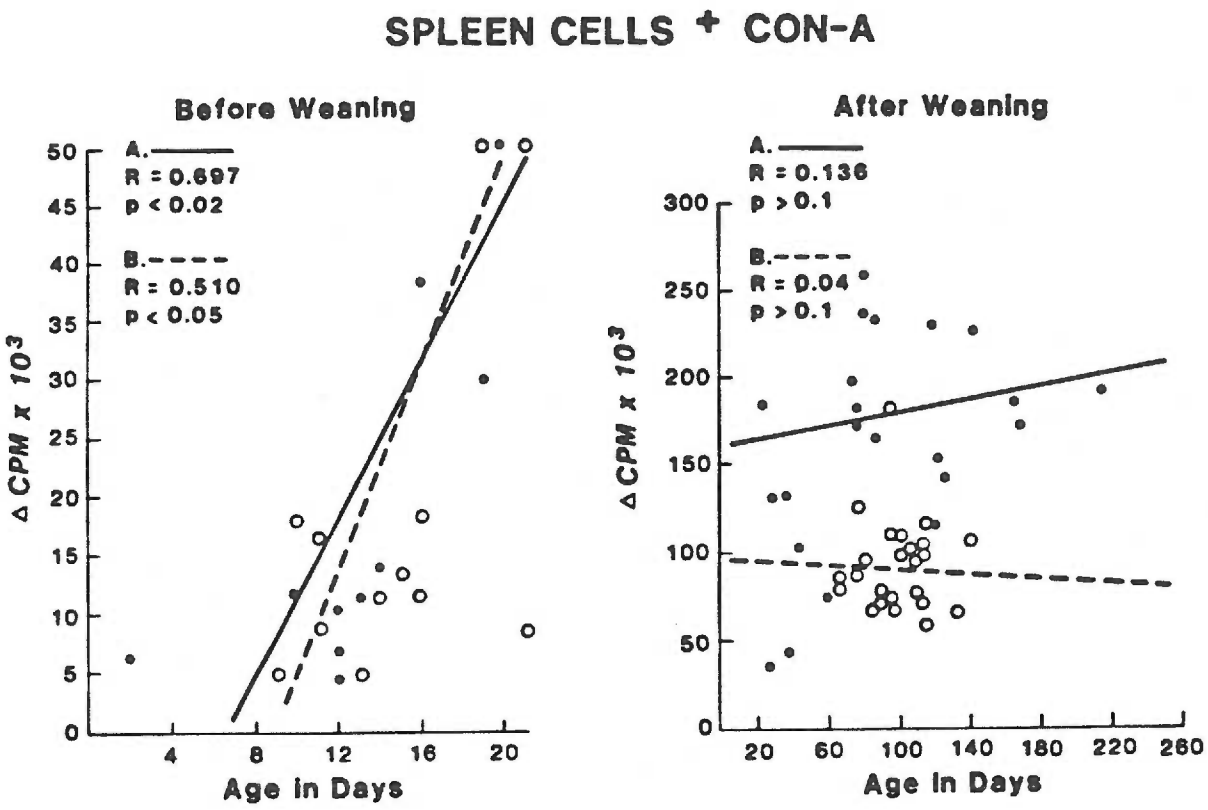

SPLEEN CELLS + CON-A
Before Weaning
A.
R = 0.697
p < 0.02
B.
R = 0.510
p < 0.05
Δ CPM x 10³
Age in Days
After Weaning
A.
R = 0.136
p > 0.1
B.
R = 0.04
p > 0.1
Δ CPM x 10³
Age in Days

Figure 13. Relationship of donor age and spleen cell response to PHA. Spleen cells from female Lewis rats 4 to 110 days old were cultured in 10 separate experiments at a concentration of 5×10^5 cells per culture with 0 to 25.0 ug/ml PHA (as in figure 1) for 48 hours, pulsed with ^3H thymidine, and harvested 18 hours later. Each point represents the mean response of 6 replicate cultures at the optimal dose of PHA minus the mean response of 6 control cultures from the same cell pool.

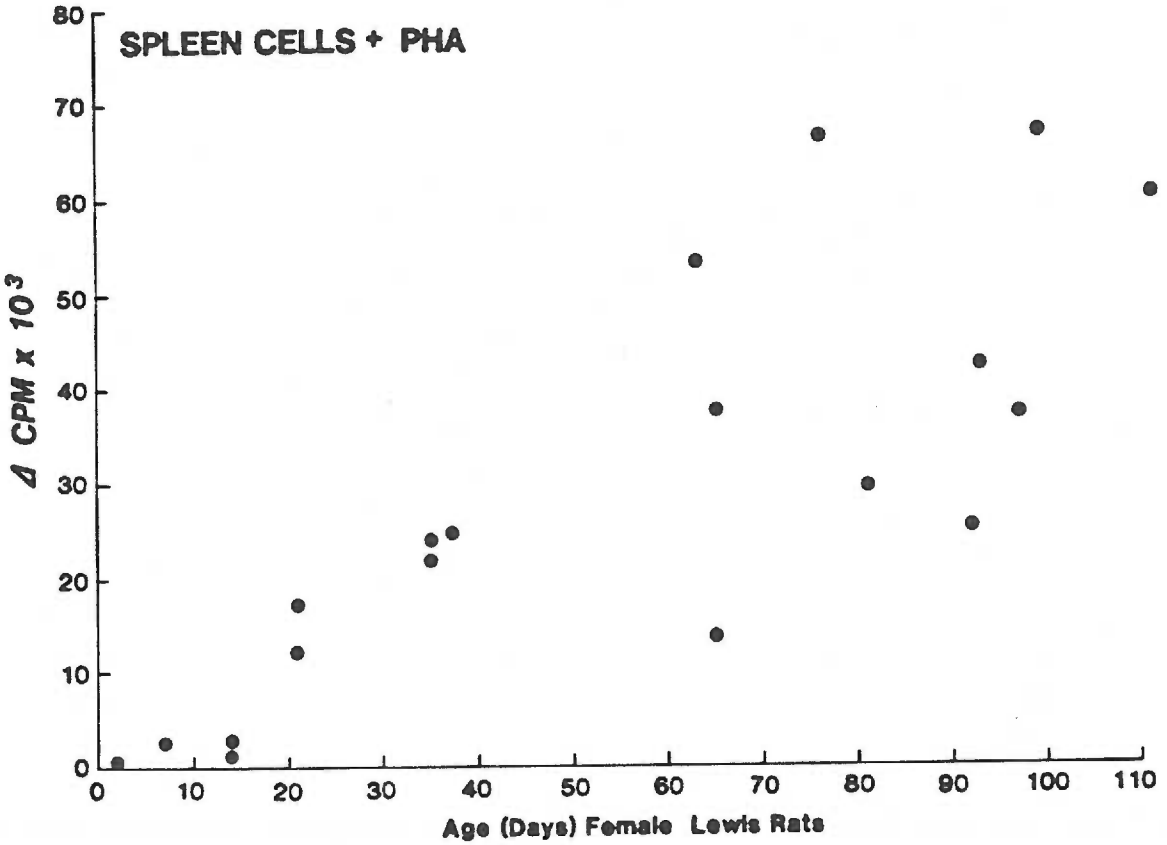

SPLEEN CELLS + PHA
Δ CPM x 10³
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
Age (Days) Female Lewis Rats

Figure 14. A comparison of the age-related response of spleen cells to PHA and Con A. The pups from 2 litters born on October 16, 1979 were pooled, and 2-3 sacrificed at 2, 12, 19 and 33 days of age. Animals from one litter born on July 29, 1980 and one litter born on July 8, 1980 were sacrificied at 49, 55 and 82 days old. The spleen cells were cultured with 0.2 to 25.0 ug/ml PHA, 0.2 to 125 ug/ml Con A, and no mitogen for 48 hours, pulsed with ^{3}H thymidine, then harvested 18 hours later. Each bar represents the mean response of 6 replicate cultures at the optimum dose of each mitogen +/- the standard deviation. The dashed line represents the background in each culture.

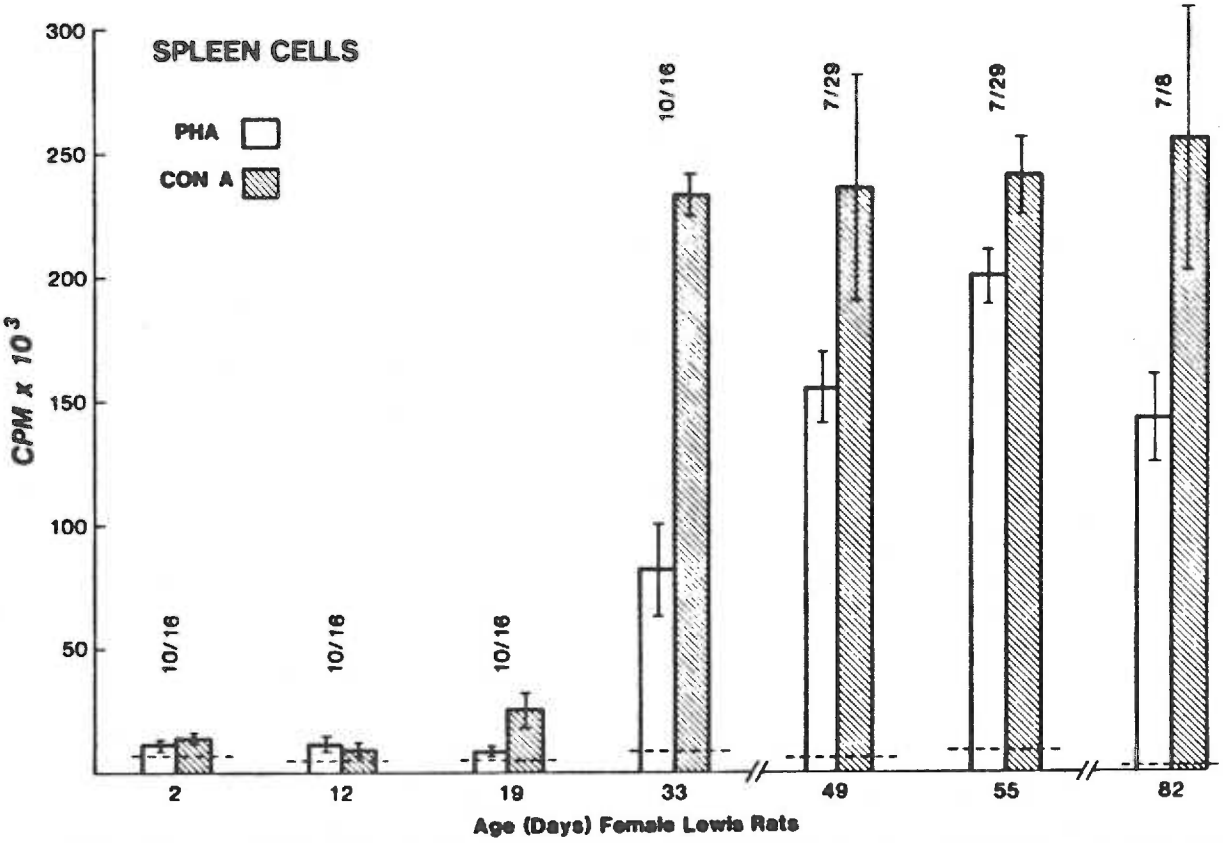
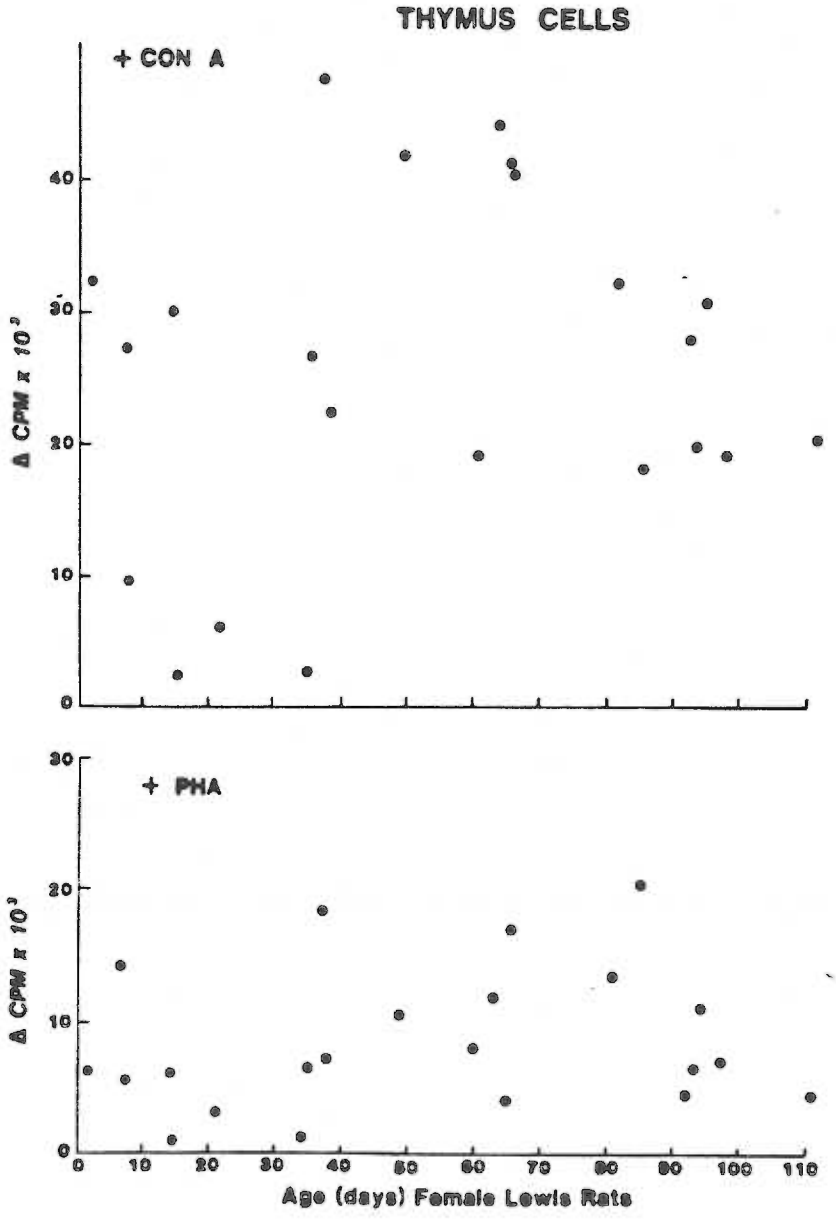

SPLEEN CELLS
PHA
CON A
CPM x 10^3
300
250
200
150
100
50
10/16
10/16
10/16
10/16
7/29
7/29
7/8
2
12
19
33
49
55
82
Age (Days) Female Lewis Rats

Figure 15. Relationship of donor age and thymus cell response to Con A and PHA. Thymus cells from female Lewis rats 4 to 110 days old were cultured in 10 separate experiments at a concentration of 5 x 10^5 cells per culture without mitogen or with 0.2 to 125.0 ug/ml Con A or 0.2 to 25.0 ug/ml PHA (as in figure 3) for 48 hours, pulsed with ^3H thymidine, and harvested 18 hours later. Each point represents the mean response of 6 replicate cultures at the optimal dose of mitogen minus the mean response of 6 control cultures from the same cell pool. In each experiment, cells from animals under 60 days old were cultured at the same time as cells from animals over 60 days old.


THYMUS CELLS
+ CON A
Δ CPM x 10³
40
30
20
10
0
+ PHA
Δ CPM x 10³
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100 110
Age (days) Female Lewis Rats

II. ONTOGENY OF T-CELL MITOGEN RESPONSE IN LEWIS RATS: EARLY APPEARANCE AND LOSS OF SUPPRESSOR ACTIVITY

ABSTRACT

Spleen cells from rats 2 to 132 days old were cultured with 1 - 125 ug/ml Concanavalin A (Con A). At high doses of Con A, the high spontaneous thymidine uptake of spleen cells from rats 15 to 21 days old was suppressed, whereas spleen cells from younger rats showed no suppression of spontaneous mitogenesis at equally high Con A doses. Removal of either plastic-, nylon wool-, or carbonyl iron (cFe) adherent cells not only removed suppression of background by high Con A doses, but also allowed mitogenic responses at adult levels in normally unresponive 15 to 21 day old pups. Low doses of X-irradiation did not cause a similar loss of suppression. We suggest that although there is an influx of Con A responsive cells into the rat spleens at 15 to 16 days, the mitogen responses of these cells are suppressed by an adherent cell population which is activated by high doses of Con A.

INTRODUCTION

In the preceeding paper (1) we reported that spleen cells from rats younger than 21 days old have a low response to Con A and PHA. In this paper we present the observation that the normally high spontaneous blastogenesis of younger rats was suppressed at doses of Con A which were optimal for blastogenesis in adult animals. Since Mosier (2) found that a subset of T cells in neonatal spleens prevented B cells from producing antibody the presence of suppressor cells in neonatal spleen has been an accepted dogma. However, the nature of those suppressor cells as well as their role in ontogeny has been hotly contested (3-14). After observing the phenomenon of suppression of background blastogenesis in suckling rats, we became interested in characterizing and tracing the ontogeny of this population of suppressor cells.

Folch and Waksman (12) presented evidence that the suppressor present in 2 week old rat spleens was glass adherent, but not plastic adherent, and susceptible to anti-Thy 1 treatment. In an earlier paper (13) Folch and Waksman had suggested that the suppression of mitogen responses in adult rat spleens might be caused by the interaction of a T cell and a macrophage. Other authors (14-16) have also suggested that neonatal suppression might be due to the interaction of a T cell and a macrophage. There is ample evidence that suppression may be mediated by the action of macrophages and T cells in cooperation (14-21). To find out whether the suppression of background blastogenesis could be attributed to either or both of

these cell types, we decided to treat the neonatal suppressors with methods designed to remove macrophages, such as treatment with carbonyl iron and plastic adherence, and with methods which have been found to remove suppressor T cells, specifically passage through nylon wool and low dose irradiation.

We found that Con A in doses optimal for blastogenesis of adult spleen cells caused suppression of background blastogenesis only in rats between the ages of 12 to 21 days. When adherent cells were removed by carbonyl iron treatment, nylon wool or plastic adherence, spleen cells from rats in this developmental period were able to respond to Con A at adult levels without the suppression of spontaneous blastogenesis. Irradiation did not boost the responses of suckling rats implying that the adherent suppressor cells were resistant to low dose irradiation.

MATERIALS AND METHODS

Animals: Adult Lewis rats were obtained from Microbiological Associates (Walkersville, Md.) and Charles River (Wilmington, Ma.) and bred in our laboratory. Suckling rats are 0-21 days old. Juvenile rats were defined as 0 to 6 weeks old, and adults were defined as animals over 10 weeks old.

Media and reagents: Unless otherwise noted, all media and reagents were obtained from Flow Laboratories Inc., Inglewood, Ca. or Gibco Laboratories, Grand Island, NY. Culture medium was prepared by supplementing MEM with 100 units/ml Penicillin and 100 mcg/ml Streptomycin, 0.1mM nonessential amino acids, 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 2.0 mg/ml sodium bicarbonate, 2×10^{-5} M 2-mercaptoethanol (2-ME, M-6250, Sigma Chemical Company, St.Louis, Mo.) and 2.5% heat-inactivated Lewis rat serum. Rat serum was obtained from rats exsanquinated just prior to organ removal. The blood was allowed to clot at room temperature for 15 to 30 minutes, then refrigerated for 1 to 2 hours. Solid elements were removed by centrifugation at 100xg for 15 minutes at room temperature, then the serum was kept at 4^{o}C until used.

Cell suspensions: Rats were anaesthetized by intra-peritoneal injection with 0.2 to 0.6 mls of Equi-Thesin (8 mg/ml pentobarbital, 34 mg/ml chloral hydrate and 17 mg/ml magnesium sulfate in a 28% propylene glycol, 12% alcohol base), exsanguinated by cardiac puncture, and killed by cervical dislocation. Spleen cell suspensions

were prepared by gently pressing cells out of the splenic capsule with a spatula and dispersing the cells in approximately 20 mls minimal essential medium (MEM, Flow # 12-104-54, without $NaHCO_3$) at 4^oC by trituration. Cells were pooled as noted in the figure legends. The debris was allowed to settle from each cell suspension for 10 minutes at 4^oC. The cell suspension was withdrawn, then the cells were recovered by centrifugation at 100xg for 15 minutes at 4^oC and washed 3 times with 50 mls of cold MEM. Washed cells were suspended in cold, fully supplemented MEM and, after hemocytometer counting of an aliquot diluted in 1% acetic acid, were adjusted to 2.5×10^6 cells per ml.

Carbonyl iron treatment: Freshly washed cells (1×10^8) were resuspended in 10 mls MEM plus 10% fetal calf serum (Flow Laboratories), then 100 mg washed and sterilized carbonyl iron (cFe, C04765 Pfaltz and Bauer, Inc., Stamford, Ct.) was added. Cells and cFe were poured into a bacteriological grade plastic petri dish (Falcon) and incubated at 37^oC for 1 hour. The cFe and cFe/cell complexes were removed by 2 applications of a 5 pound pull magnet. Remaining cells were washed 1 time, counted and adjusted to 2.5×10^6 cells per ml.

Nylon wool treatment: The technique detailed in (Mishell and Shiigi) was used. In brief, nylon wool (Fenwall Laboratories) was washed, dried, packed into 12 ml plastic syringes and sterilized by autoclave. Fresh, washed cells were resuspended at 5×10^7 cells/ml in MEM supplemented with 5% heat-inactivated fetal calf serum (FCS). After rinsing the column with warmed FCS-MEM, 1×10^8 cells were added, and allowed to incubate at 37^oC for one hour. Non-adherent cells were eluted with 20 mls of 37^o FCS-MEM, centrifuged at 200 x g

for 10 minutes, then resuspended in culture medium at 2.5 x 10^6 cells per ml.

Plastic adherence: Two mls of 50% heat-inactivated FCS in MEM were added to 25cm^2 tissue culture flasks, which were then kept at 4°C overnight. Before use, the flasks were rinsed twice with 10 mls fresh cold MEM. Fresh, washed cells were suspended at a concentration of 10^7 cells/ml in cold MEM plus 5% heat inactivated FCS, then 10 mls were added to each 25 cm^2 flask. Flasks were incubated at 37°C for one hour. Non-adherent cells were decanted and held on ice until culture.

Irradiaton: Cells were suspended in 5% FCS-MEM at a concentration of 1-5 x 10^7 cells/ml and held on ice for transport to the irradiation facility. Cells were irradiated for the doses designated in the figure on a rotating table irradiator with a cesium source emitting 203 rads per minute.

Cell culture: Five x 10^5 cells (except where otherwise noted) in 0.2 mls supplemented MEM were added to each well of a Falcon (Becton, Dickinson and Co., Cockeysville, Md.) Microtest II flat bottom well culture plate containing 0.05 ml of mitogen or medium control. Cultures were incubated at 37°C in 5% CO_2, 95% air in a humidified culture box. After 48 hours 0.1 uCi of <methyl-^{3}H> thymidine (7.2 uCi/mM, ICN Pharmaceuticals, Inc., Plainview, NY) in 0.01 ml MEM was added to each well. Cells were harvested 18 hours later with an automated harvester constructed in our lab, and counted in 80% toluene, 20% methanol with 4 gm/liter Omnifluor (New England Nuclear, Boston, Ma.).

Mitogens: A stock solution containing 2 mg/ml of Concanavalin A

(Con A, Pharmacia, Inc., Piscataway, NJ.) was prepared and stored at $4^{o}C$ in phosphate buffered saline (PBS). Con A was diluted serially fivefold from 625 ug/ml to 1 ug/ml in culture medium just prior to use. Fifty ul of each dilution or control medium was added to each of six wells just prior to addition of 0.2 mls cell suspension.

Data analysis: The mean counts of tritiated thymidine uptake per minute, and the standard deviation were calculated from 6 replicate cultures at each mitogen dose. The significance of the difference between means was calculated using the Mann-Whitney U test.

RESULTS

SUPPRESSION OF JUVENILE BACKGROUND BLASTOGENESIS BY HIGH DOSES OF CON A

We previously reported (1) that spleen cells from suckling rats (less than 21 days old) responded poorly to Con A and PHA, although the thymidine uptake of unstimulated juvenile cells was significantly higher than the uptake of unstimulated adult cells. We also observed that splenocytes from young animals responded optimally to lower doses of Con A than did older animals (1). Concentrations of Con A optimal in adults not only failed to stimulate suckling rat splenocytes but also dramatically suppressed spontaneous thymidine incorporation, often to less than 10% of the background level. Figure 1 presents a typical experiment comparing the dose response curves for young and adult rats. One ug/ml stimulated splenocytes from both adult and 12 day old rats. However, higher doses of Con A (5,25 and 125 ug/ml) stimulated only the adult cells, and suppressed the spontaneous blastogenesis of young cells by more than 90%. We have also observed (unpublished) that high mitogen dose suppression was maximum when cells were cultured at higher concentrations, i.e. 2.5 and 5 x 10^5 cells per culture as compared to 1 x 10^5. Since as little as 5 ug/ml suppressed background cpm, and since 25 times this dose was a potent stimulant of adult cells, non-specific toxicity appears to be an unlikely explanation for this suppression. However, juvenile cells could be more sensitive to Con A toxic effects. To determine if all

juvenile cells displayed such sensitivity we asked "at what ages does high mitogen dose suppression occur?".

HIGH DOSE CON A SUPPRESSES SPONTANEOUS SPLEEN CELL BLASTOGENESIS ONLY FROM 12 TO 21 DAYS OF AGE

In order to describe the development of high mitogen dose suppression, we examined the effect of 125 ug/ml Con A on the spontaneous blastogenesis of spleen cells from 2 day old to 37 day old rats. As shown in table I, high dose suppression of background level thymidine uptake was limited to a particular "window" of development. Between 12 and 15 days of age 125 ug/ml Con A suppressed the spontaneous thymidine uptake of 5 out of 10 cultures by more than 60%. Suppression of background was the rule between days 15 and 19, with 7 out of 7 experiments showing suppression (mean suppression = 77%). Before day 12, background was high, but high doses of Con A did not suppress the spontaneous mitosis, a point which argues against a heightened susceptibility of juvenile cells to Con A toxicity. After weaning at day 21, background blastogenesis dropped to low levels, usually less than 1000 cpm, making the suppression of spontaneous blastogenesis an inappropriate assay for the presence of suppressors activated by Con A.

REMOVAL OF CELLS ADHERENT TO CARBONYL IRON BOOSTS RESPONSE TO CON A IN SUCKLING RAT SPLEEN CELLS.

To test whether young animals are prevented from displaying

"adult level" Con A responses by the presence of suppressor cells, we attempted to remove suppressor cells by adsorption to carbonyl iron. Carbonyl iron treatment, as described in the methods, removes primarily phagocytic (macrophage) and "sticky" cells (B cells). Additionally, in order to trace the ontogeny of these suppressor cells we tested rats of various ages (figure 2). No significant change in background counts, indicated by the dashed line, occurred due to removal of adherent cells with cFe. Removal of adherent cells from spleen cells of animals 10 to 14 days old resulted in a limited improvement of the response to 25 ug/ml Con A in 3 out of 5 experiments, but had little effect on the responses to 5 ug/ml Con A. However, by day 16 and continuing through day 19, removal of adherent cells allowed an adult level of response to Con A at both mitogen doses. This development of ability to respond at adult levels coincides with the peak of the appearance of the Con A induced suppression of background blastogenesis (cf. Table I). After day 23, at which time the response of unseparated cells was at adult levels, treatment with carbonyl iron had variable effects, sometimes improving low level responses, sometimes reducing a high response level. It appears that in mature rats, the level of cFe adherent suppressor cells is in a state of balance which may vary with environmental conditions. Depletion of adherent cells below a critical level appears to diminish mitogen responses, in agreement with the finding that macrophages are required for Con A responses (23).

MALE AND FEMALE LEWIS RAT SPLEEN CELLS RESPOND EQUALLY WELL AFTER CARBONYL IRON TREATMENT

We have previously shown that males are slower to develop the ability to respond with DTH to ABA-T than females (24), however, T cell mitogen responses of male and female rat spleen cells develop approximately in parallel (1, 25, and unpublished observations). We asked whether treatment with carbonyl iron would also reveal equivalent response levels between male and female rats, especially at the critical transition point between 19 and 23 days (figure 3). As shown, cFe treatment dramatically enhanced the spleen cell responses of both male and female 19 day old rats to approximately the same levels. By 23 days cFe no longer enhanced, but depressed both male and female responses.

REMOVAL OF NYLON WOOL ADHERENT CELLS BOOSTS CON A RESPONSES OF SUCKLING RAT SPLEEN CELLS

Passage of cells through nylon wool holds back populations of adherent macrophages, B cells and T suppressor cells. In all three experiments shown in figure 4 nylon wool treatment enhances the Con A response of suckling rat spleen cells. However, similar to results obtained with cFe, cells from 9 and 10 day old animals were unable to respond at high levels, whereas cells from 19 day old animals could respond at adult levels (110,000 cpm). It should be pointed out that, unlike the results obtained with cFe, nylon wool seems to selectively remove the high dose suppressors. There is no drop in response level at 125 ug/ml Con A in the 19 day old animal. However, this is insufficient evidence to suggest that a different population of cells

is being removed.

REMOVAL OF PLASTIC ADHERENT CELLS REVEALS ADULT LEVEL RESPONSES BY 16 DAY OLD RATS

Within the context of an experiment planned to evaluate the effect of juvenile adherent cells added back to adult spleen cell cultures (to be published), we tested the effect of removing plastic adherent cells (primarily macrophages) from an immature spleen cell population. Pooled spleen cells from young pups were incubated on plastic flasks for 1 hour at 37°C. Non-adherent cells were decanted, and adherent cells were removed with EDTA. As shown in figure 5, the response of 16 day old animals was boosted to adult levels by the removal of plastic adherent cells. This result was consistent with the results for cFe and nylon wool, but cannot be assumed to demonstrate removal of the same cell population which suppresses spontaneous blastogenesis during only a brief developmental window.

JUVENILE SUPPRESSOR CELLS ARE RESISTANT TO LOW DOSES OF RADIATION

In contrast to the results obtained with cFe, nylon wool, and plastic adherence, irradiation of suckling cells did not improve responses. In all three experiments, irradiation with the low dose of 200 rads depressed mitogenic responses slightly, indicating that the suppressor cell is radiation resistant. Higher doses of radiation (800 rad) obliterated the mitogenic response of juvenile and adult (not shown) cells, however the juvenile cells retained the ability to

suppress adult mitogenic responses (to be published).

DISCUSSION

We have previously reported (1) that spleen cells from suckling rats (less than 21 days old) responded poorly to Con A and PHA. However, the thymidine uptake of unstimulated suckling rat splenocytes (background) was actually much higher than the thymidine uptake of unstimulated adult splenocytes. We found in this study that in rats between the ages of 12 to 21 days, the addition of Con A in doses optimal for blastogenesis of adult spleen cells caused suppression of background blastogenesis. When adherent cells were removed by carbonyl iron treatment, nylon wool or plastic adherence, spleen cells from rats in this developmental period were able to respond to Con A at adult levels with the suppression of spontaneous blastogenesis abolished. Background blastogenesis was high in spleen cells from rats 2 days to 12 days old, but the addition of Con A did not enhance or suppress blastogenesis. Furthermore, the response to Con A in this age of rat could not be substantially improved by the removal of cFe or nylon wool adherent cells.

Suppression of neonatal spontaneous thymidine uptake by mitogen was also noted by Holt (15) who found that addition of PHA lowered the level of DNA synthesis below unstimulated levels in rats less than 2 weeks old. These results differ from ours in that we found suppression of background only in rats 12 days to 19 days old. Holt did not examine the effect of removal of adherent cells on background blastogenesis. We found that removal of cFe adherent cells had no effect on levels of background blastogenesis, but allowed responses to doses of Con A which were suppressive with unseparated spleen cells.

Adherence to glass or plastic is often used as evidence that one is working with a macrophage or monocyte. However, there is some evidence that glass adherent cells may carry T cell determinants and be thymus dependent. In a series of papers Waksman and colleagues (12,13,26) established the existence in rat spleens of an adherent suppressor of mitogen responses. The suppressor activity was lost if rats were thymectomized or irradiated and reconstituted with bone marrow from thymectomized rats. The suppressor cell was also considered distinct from macrophages because its activity could not be reconstituted with purified splenic or resident peritoneal macrophages. Furthermore, the activity of the suppressor appeared to be modulated by a thymic factor. Unfortunately, most of this evidence was indirect, and did not exclude the possibility of interaction between a macrophage and a T cell. Holt et.al. (15) provided more direct evidence for an adherent suppressor T cell by treating the glass wool adherent fraction of rat spleen cells with anti-thymocyte serum and showing that suppressor activity was lost. This adherent T cell was resistant to radiation, indomethacin and mitomycin C, but sensitive to cyclophosphamide. A glass wool adherent suppressor T cell has also been isolated from mouse spleens (18). Helper T cells may be adherent as well. Sopori et.al. (19) have shown that rat spleen cells contain an adherent, radiation resistant, anti-thymocyte serum sensitive helper population that enhances CTL responses.

However, simply showing that the T cells themselves are adherent does not deny the possibility that a macrophage-T-cell interaction occurs. Holt's work (15) established that the adherent T cell regulated the cytostatic activities of macrophages. Webb and

Nowowiejski (18) found that a macrophage activated the adherent suppressor T cells from mouse spleens via a prostaglandin dependent pathway. Gutowski and Weksler (16) showed that either depletion of adherent cells or treatment with anti-Thy 1 plus complement abolished the ability of 1 week old spleen cells to suppress the syngeneic MLR. They went a valuable step further, in showing that neither purified T cells nor purified macrophage from 1 week old rat spleens could mediate suppression alone.

The interaction of T cells and macrophages is of interest to us because it is possible that the juvenile spleen suppressor activity which we removed by adherence techniques may be due to either a single adherent cell, or a cooperative interaction between macrophages and T cells. Folch and Waksman (12) found that removal of adherent cells had variable effects depending on age. Two week old rat spleen cells responded equally poorly to PHA whether adherent cells were present or removed. However, by 4 weeks of age, removal of adherent cells allowed PHA responses to attain maximum adult levels. The addition of resident adherent peritoneal cells did not cause suppression of the Con A response, however, this may be due to the difference in activation state between induced and resident peritoneal macrophages. Holt et.al. (15) found that 3-4 week old rat spleen cells were susceptible to suppression by induced peritoneal exudate cells (PEC) to a greater extent than were spleen cells from older animals. These results fit with ours in that by 15-19 days of age, removal of adherent cells allowed a strong, adult level mitogen response. However, prior to 12 days of age, rat spleen cells were refractory to stimulation by mitogen in both the presence and absence of adherent

cells.

The refractory state of the 2-12 day old cells is probably due to either an intrinsic immaturity or an absence of the responding T cell. Migration of T-cells into the spleen may occur after the 12th postnatal day. In early work, Stobo and Paul (28) found that Thy-1 bearing cells were missing from mouse spleens during the first week of life, and had only attained 50% of adult levels by 2 weeks. We found in preliminary work that even after the addition of Con A supernatants containing growth factors and interleukins, very young suckling rat spleen cells still could not respond to Con A. Likewise, the addition of irradiated adult spleen cells did not allow responses (unpublished observations). Stutman and Ishizaka (27) found similarly that the deficient IL-2 production of 1-14 day old mouse spleens could not be remedied by the addition of IL-1 or adult macrophages.

Low dose (100-200 rad) irradiation has been shown to effectively block suppression by some T cell subsets (29), but to be ineffective against macrophage mediated suppression (30). Ranney and Oppenheim (31) observed that 1000 rad irradiated spleen cells from 5 day old rats but not from adult rats suppressed isologous thymocyte background 80-94%. Holt et.al. (15) also reported that adherent splenic cells which cooperate with alveolar macrophages to suppress PHA responses are resistant to 1-450 rad. We also detected a radiation resistant suppressor cell. As shown here 100-800 rad completely failed to enhance the Con A response of spleen cells from 7-13 day old rats, in contrast to the increased Con A responses obtained from 4-13 day old rat spleen cells after treatment with cFe. Such irradiated cells retain the capacity to suppress adult Con A response (Middleton and

Bullock, submitted for publication). It is possible that similar radiation treatment of older suckling rats might reveal a radiation sensitive suppressor, but those experiments are still in progress.

In summary, we found that between days 12 and 21, high doses of Con A appeared to activate a suppressor cell population not present in younger animals, and not active in unstimulated cultures. This suppressor activity has been characterized as removable by cFe, plastic adherence and nylon wool, but resistant to irradiation.

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TABLE I.

EFFECT OF 125μg/ml CON A ON BACKGROUND CPM

AGE (Days)	2	7	9	10	11	12	13	14	15	16	19	20	21	23	28	30	37
No. of Experiments with Suppressed[a] Background	0/1	0/1	0/1	0/3	0/3	1/2	0/2	3/5	1/1	2/2	4/4	2/3	2/4	0/1	0/2	0/1	0/1
Mean: $\frac{\text{Stim. CPM}}{\text{Background CPM}}$	0.62	1.34	1.41	3.45	1.66	1.02	2.51	1.17	0.11	0.18	0.29	0.37	2.85	50.60	18.2	47.30	191.10

[a]Suppressed by > 60%

Figure 1. Suppression of juvenile background blastogenesis by high doses of Con A. Spleen cells were pooled from four 12 day old male and female rat pups (O---O) and from two 112 day old females (●—●). Five x 10^5 cells per well were cultured as described in the methods, without stimulant or with 0.2 to 125.0 ug/ml Con A. Cultures were pulsed with ^3H thymidine at 48 hours and harvested 18 hours later. The responses of splenocytes from 12 day old and 112 day old rats are presented with separate ordinates due to the different magnitude of the responses. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

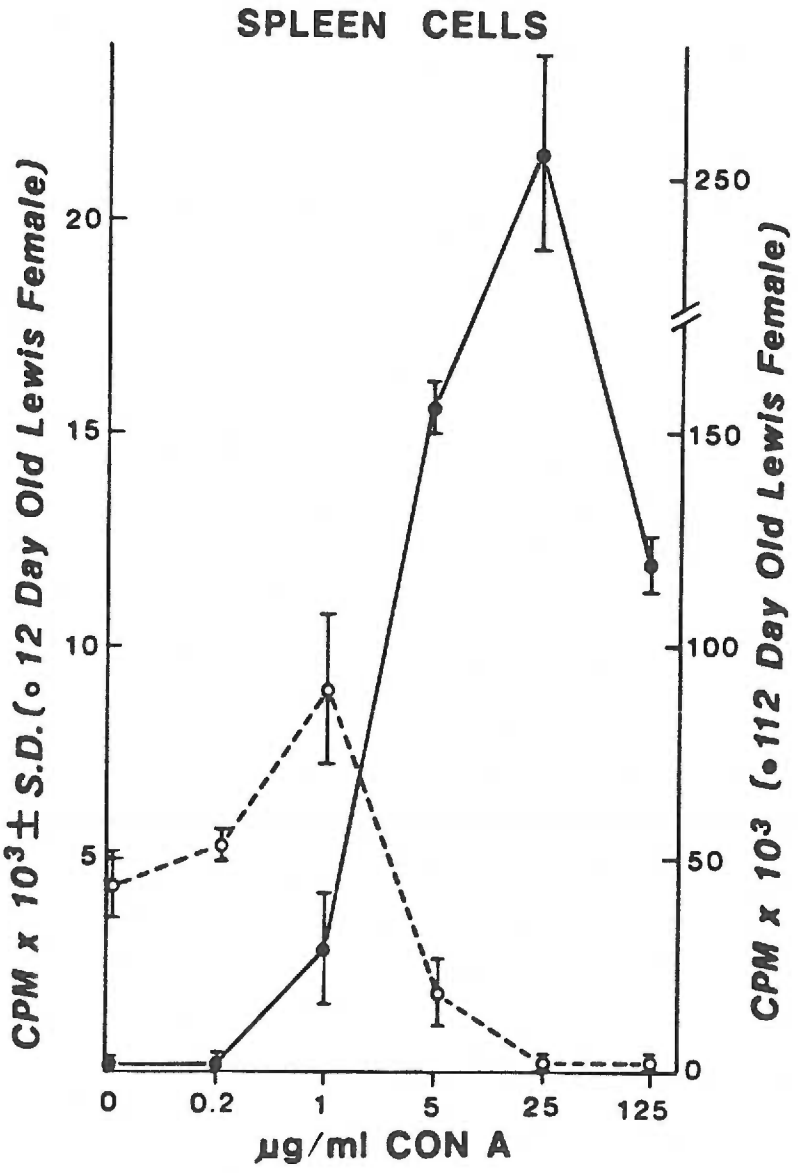

SPLEEN CELLS
CPM x 10³ ± S.D. (○ 12 Day Old Lewis Female)
CPM x 10³ (● 112 Day Old Lewis Female)
20
15
10
5
250
150
100
50
0
0
0.2
1
5
25
125
µg/ml CON A

Figure 2. Removal of cells adherent to carbonyl iron (cFe) boosts responses to Con A in suckling rat spleen cells. The data from 13 separate experiments are presented. Spleen cells pooled from two to four 10 day to 132 day old female Lewis rats were cultured either without cFe treatment (□) or after removal of cFe adherent cells (▨). Treated and untreated cells were then cultured separately at 5 x 10^5 cells/ml without stimulant (dashed line), or with 25 (A) or 5 (B) ug/ml Con A. Each culture was pulsed with ^3H thymidine at 48 hours. Cultures were harvested 18 hours later. Each column represents the mean of 6 replicate cultures +/- the standard deviation.

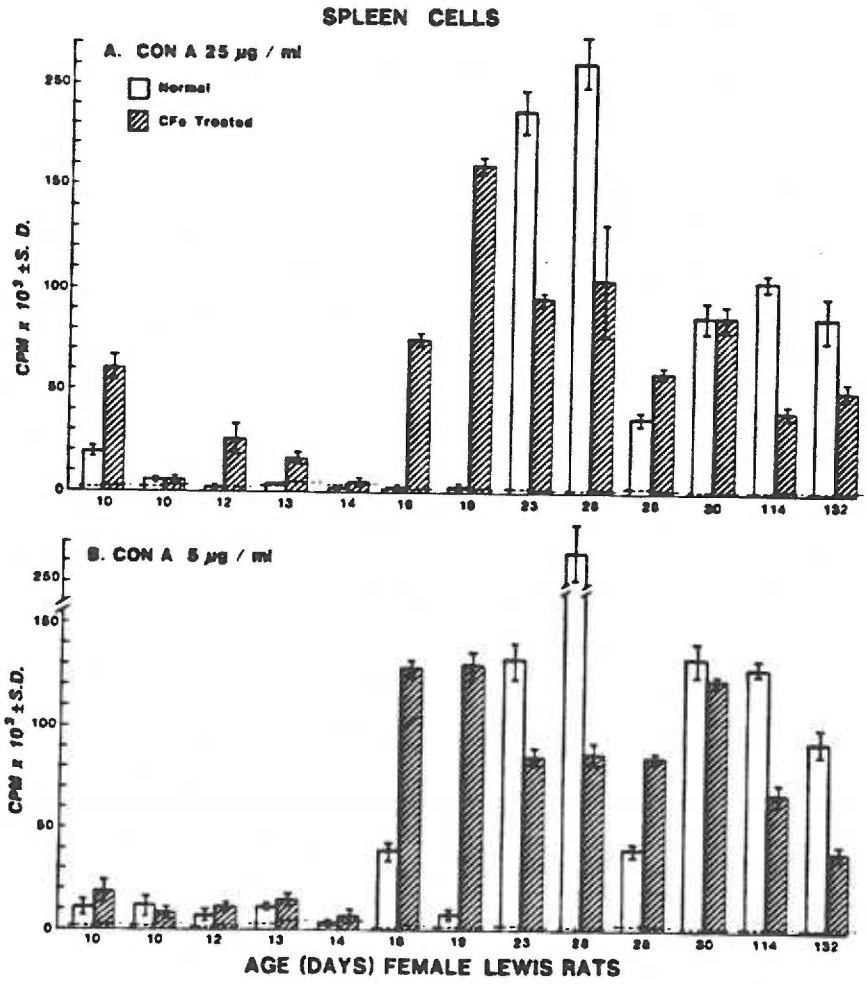

SPLEEN CELLS
A. CON A 25 µg / ml
Normal
CFe Treated
CPM x 10³ ±S. D.
250
150
100
50
0
10
10
12
13
14
16
19
23
28
28
30
114
132
B. CON A 5 µg / ml
CPM x 10³ ±S.D.
AGE (DAYS) FEMALE LEWIS RATS

Figure 3. Male and female Lewis rat spleen cells respond equally well after carbonyl iron treatment. Two experiments are presented which use spleen cells pooled by sex from 3 male and 3 female pups from the same litter. The top panels represent one experiment using 19 day old rats, the bottom panels represent another using 23 day old rats. Spleen cells were either untreated (●—●) or depleted of adherent/phagocytic cells with carbonyl iron (○--○) before being cultured at 5 x 10^5 cells per well with 0 to 125 ug/ml Con A. Each culture was pulsed with ^3H thymidine at 48 hours. Cultures were harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

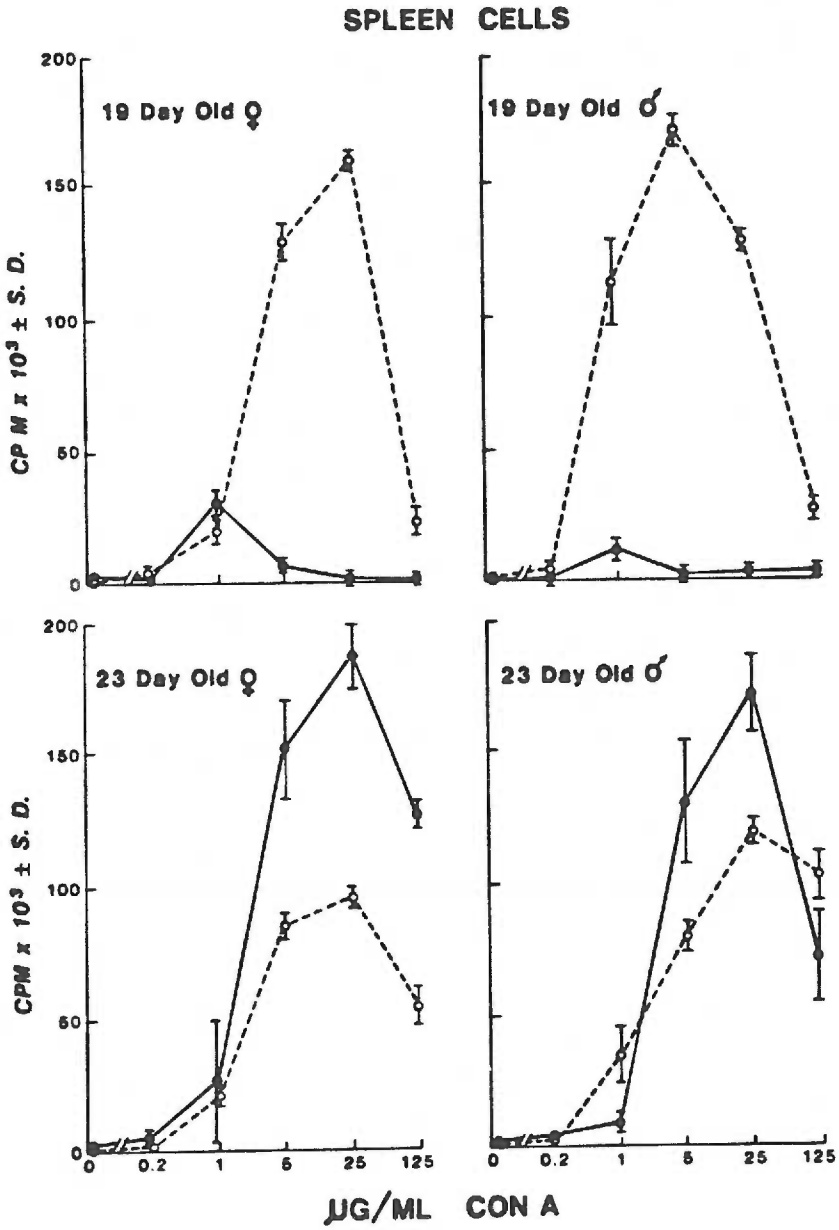

SPLEEN CELLS
19 Day Old ♀
19 Day Old ♂
23 Day Old ♀
23 Day Old ♂
CPM x 10³ ± S. D.
CPM x 10³ ± S. D.
200
150
100
50
0
0
0.2
1
5
25
125
µG/ML CON A

Figure 4. Removal of nylon wool adherent cells boosts responses of spleen cells from suckling rats. Three experiments using either 9,10 or 19 day old female Lewis rat spleen cells are presented. Cells were either untreated (O--O) or passed through a nylon wool column (●—●) before culture. Cells were cultured at a cell density of 5 x 10^5 cells per well with 0 to 125 ug/ml Con A. Cultures were pulsed with ^3H thymidine after 48 hours in culture, and harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

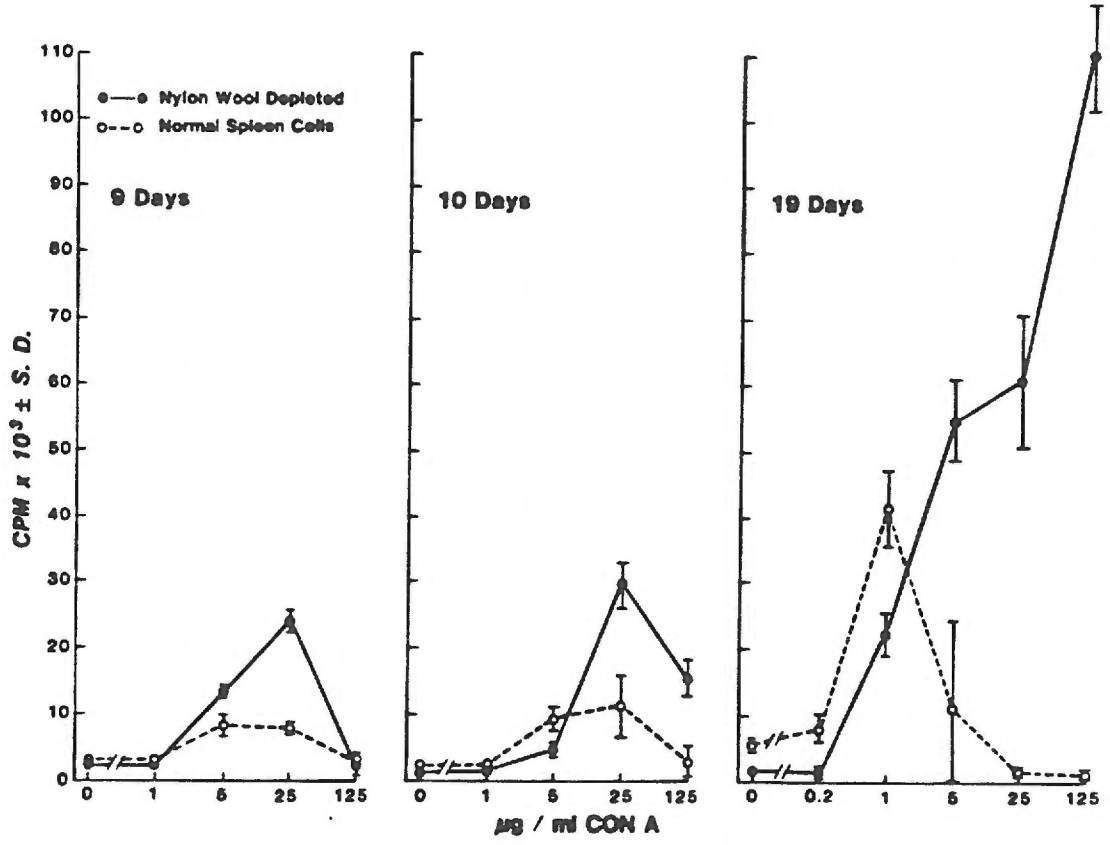

Nylon Wool Depleted
Normal Spleen Cells
9 Days
10 Days
19 Days
CPM x 10³ ± S. D.
µg / ml CON A
0
1
5
25
125
0.2

Figure 5. Removal of plastic adherent cells reveals adult level responses in 16 day old female rats. Nonadherent spleen cells (O--O) from 16 day old rat pups were decanted from plastic dishes after 1 hour at 37°C; unseparated cells (●—●) were held in tubes on ice for 1 hour before culture. Cells were cultured at a cell density of 5 x 10^5 cells/ml with 0 to 125 ug/ml Con A. After 48 hours cultures were pulsed with ^3H thymidine, then harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

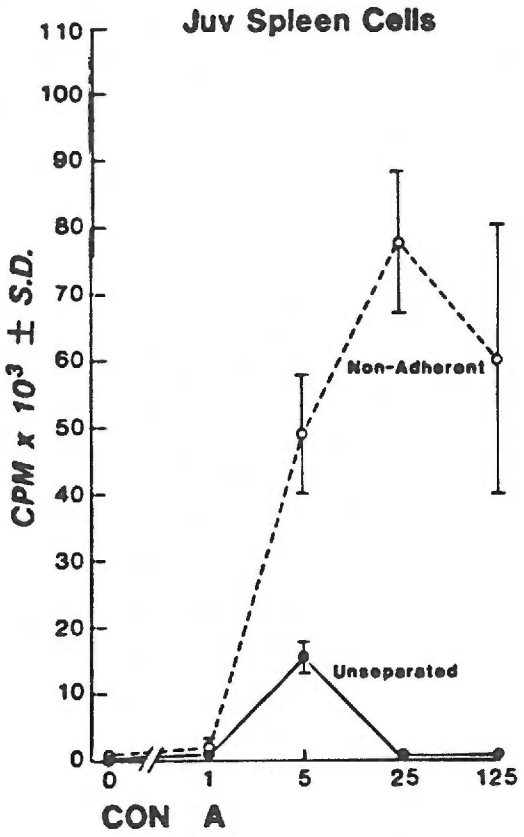
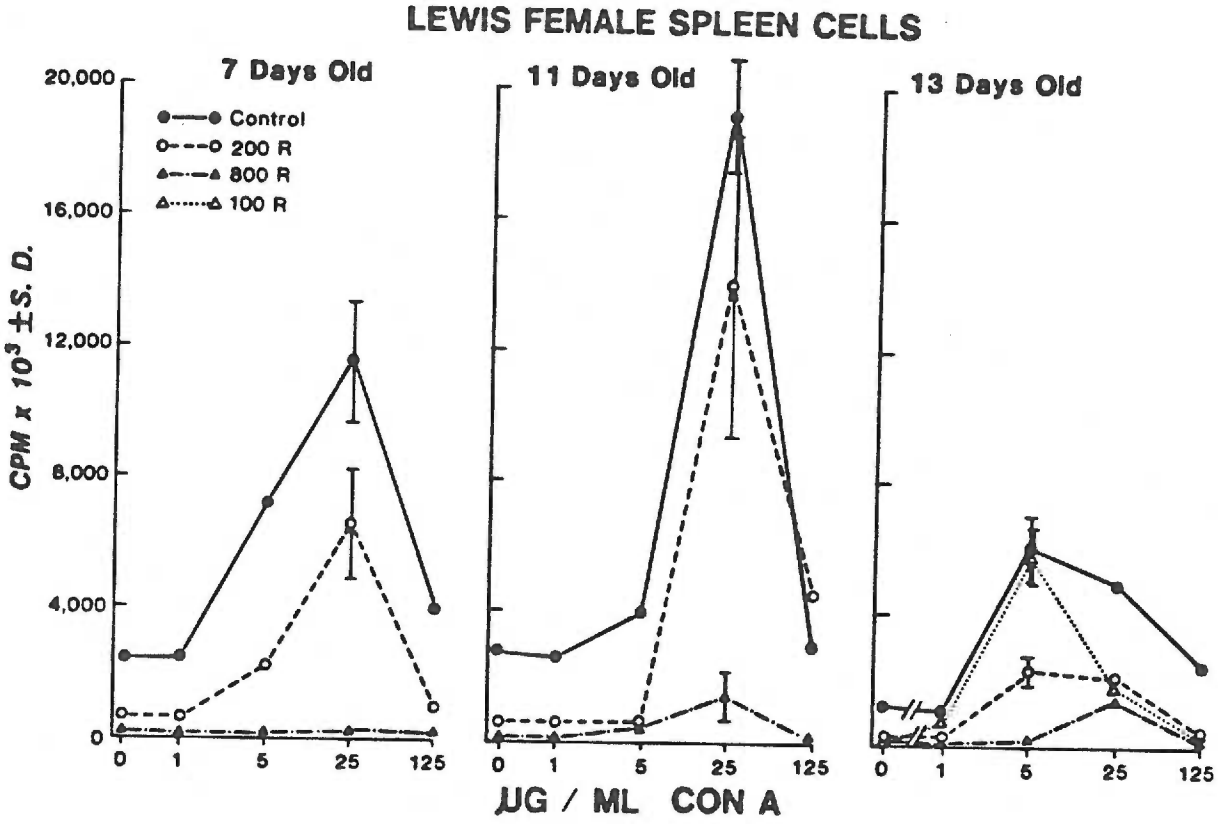

Juv Spleen Cells
CPM x 10³ ± S.D.
110
100
90
80
70
60
50
40
30
20
10
0
Non-Adherent
Unseparated
0
1
5
25
125
CON A

Figure 6. Juvenile suppressor cells are resistant to low doses of radiation. Three separate experiments with spleen cells from 7, 11 and 13 day old Lewis pups are presented. Cells were cultured at 5 x 10^5 cells per well without treatment (●—●) or following irradiation with 100 (Δ···Δ), 200 (○--○), or 800 (▲-·-▲) rad. Cells were cultured with 0-125 ug/ml Con A for 48 hours, pulsed with ^3H thymidine and then harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.


LEWIS FEMALE SPLEEN CELLS
7 Days Old
11 Days Old
13 Days Old
Control
200 R
800 R
100 R
CPM x 10³ ±S. D.
20,000
16,000
12,000
8,000
4,000
0
0 1 5 25 125
JUG / ML CON A

III. ONTOGENY OF T-CELL MITOGEN RESPONSE IN LEWIS RATS: JUVENILE ADHERENT SUPPRESSOR CELLS BLOCK ADULT MITOGEN RESPONSES

ABSTRACT

Spleen cells from suckling female Lewis rats (4 to 20 days old) were able to suppress mitogenic responses to Concanavalin A (Con A) and phytohemmagglutinin (PHA) of spleen or thymus cells from adult female Lewis rats and thymus cells from suckling Lewis rats. Thymus cells from suckling rats were unable to suppress adult spleen cell mitogenic responses to Con A. Removal of carbonyl iron (cFe), plastic or nylon wool adherent cells removed the suppressive action of juvenile spleen cells, however, irradiation did not. Separated plastic-adherent spleen cells from suckling animals suppressed adult mitogenic responses to Con A. At optimal Con A doses 2-mercaptoethanol (2-ME, 2×10^{-5} M) abolished the suppressive effect of juvenile cells, however, at the hyperoptimal dose of Con A (125 ug/ml) even higher doses of 2-ME did not relieve suppression by juvenile cells. These suppressor cells in suckling pups were affected by early weaning which decreased suppression, resulting in enhanced mitogenic responses of juvenile cells and removal of the ability to suppress adult mitogenic response.

INTRODUCTION

As previously reported (1,2) spleen cells from immature rats (less than 21 days old) responded poorly to the T cell mitogens phytohemagglutinin (PHA) and concanavalin A (Con A). Between the ages of 15 and 21 days the low response was due to the presence of adherent suppressor cells. Removal of adherent cells with carbonyl iron (cFe), plastic or nylon wool allowed adult level responses to occur in the remaining cells. However, removal of adherent cells did not improve responses prior to 15 days of age, probably due to the lack of a cell able to respond to mitogen. We wanted to investigate the possibility that suppressor cells might be present at this early stage, so we conducted experiments mixing spleen cells from suckling rats with responsive adult spleen cells.

The interaction of adherent cells and T cells in suppression has been well documented (3-6). In a previous paper we presented evidence that T cells able to respond to Con A at adult levels are present in rat spleen from day 15 (2). The appearance of the T cell responsiveness coincides with the appearance of the suppression of background mitogenesis by high doses of Con A. The suppressive effect can be removed by techniques depending on adherence. These techniques are frequently used to remove macrophages, a cell population whose ontogeny has not been well studied in rats. Inaba et.al. (7) and Lu and Unanue (8) have shown that Ia^+ macrophages are deficient in mice until about 2 weeks of age, but phagocytosis and IL-1 production are high in younger animals.

In this paper we present evidence that an adherent cell is present in rat spleens from at least the 2nd day of life, and probably in cooperation with another cell the adherent cell suppresses mitogenic responses of T cells. The dominance of the adherent suppressor activity wanes by the third week, coincident with weaning, suggesting that suppressor activity may be affected by the weaning process.

MATERIALS AND METHODS

Animals: Adult Lewis rats were obtained from Microbiological Associates (Walkersville, Md.) and Charles River (Wilmington, Ma.) and bred in our laboratory. Adults were defined as animals over 10 weeks old.

Media and reagents: Unless otherwise noted, all media and reagents were obtained from Flow Laboratories Inc., Inglewood, Ca. or Gibco Laboratories, Grand Island, NY. Culture medium was prepared by supplementing MEM with 100 units/ml Penicillin and 100 mcg/ml Streptomycin, 0.1mM nonessential amino acids, 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 2.0 mg/ml sodium bicarbonate, $2x10^{-5}$ M 2-mercaptoethanol (2-ME, M-6250, Sigma Chemical Company, St.Louis, Mo.) and 2.5% heat-inactivated Lewis rat serum. Rat serum was obtained from rats exsanquinated just prior to organ removal. The blood was allowed to clot at room temperature for 15 to 30 minutes, then refrigerated for 1 to 2 hours. Solid elements were removed by centrifugation at 100xg for 15 minutes at room temperature, then the serum was kept at $4^{o}C$ until used.

Cell suspensions: Rats were anaesthetized by intra-peritoneal injection with 0.2 to 0.6 mls of Equi-Thesin (8 mg/ml pentobarbital, 34 mg/ml chloral hydrate and 17 mg/ml magnesium sulfate in a 28% propylene glycol, 12% alcohol base), exsanguinated by cardiac puncture, and killed by cervical dislocation. Spleen cell suspensions were prepared by gently pressing cells out of the splenic capsule with

a spatula and dispersing the cells in approximately 20 mls minimal essential medium (MEM, Flow # 12-104-54, without $NaHCO_3$) at 4°C by trituration. Thymus cell suspensions were prepared by teasing the cells from the thymic lobes with two 22 g needles into about 20 mls of MEM at 4°C. Cells were pooled as noted in the figure legends. The debris was allowed to settle from each cell suspension for 10 minutes at 4°C. The cell suspension was withdrawn, and the cells were recovered by centrifugation at 100xg for 15 minutes at 4°C and washed 3 times with 50 mls of cold MEM. Washed cells were suspended in cold, fully supplemented MEM and, after hemocytometer counting of an aliquot diluted in 1% acetic acid, were adjusted to 2.5×10^6 cells per ml.

Carbonyl iron treatment: Freshly washed cells (1×10^8) were resuspended in 10 mls MEM plus 10% fetal calf serum (Flow Laboratories), then 100 mg of washed, sterile carbonyl iron (cFe, C04765 Pfaltz and Bauer, Inc., Stamford, Ct.) was added. Cells and cFe were poured into a bacteriological grade plastic petri dish (Falcon) and incubated at 37°C for 1 hour. The cFe and cFe/cell complexes were removed by 2 applications of a 5 pound pull magnet. Remaining cells were washed 1 time, counted, and adjusted to 2.5×10^6 cells per ml.

Nylon wool treatment: The technique detailed in (Mishell and Shiigi) was used. In brief, nylon wool (Fenwall Laboratories) was washed, dried, packed into 12 ml plastic syringes and sterilized by autoclave. Fresh, washed cells were resuspended at 5×10^7 cells/ml in MEM supplemented with 5% heat-inactivated fetal calf serum (FCS). After rinsing and incubating the column at 37° C for 1 hour with warmed FCS-MEM, 1×10^8 cells were added, and allowed to incubate at

37^{o}C for one hour. Non-adherent cells were eluted with 20 mls of 37^{o} FCS-MEM, centrifuged at 200 x g for 10 minutes, then resuspended in culture medium at 2.5×10^{6} cells per ml.

Plastic adherence: Two mls of 50% heat-inactivated FCS in MEM were added to $25cm^{2}$ tissue culture flasks, which were then kept at 4^{o}C overnight. Before use, the flasks were rinsed twice with 10 mls fresh cold MEM. Fresh, washed cells were suspended at a concentration of 10^{7} cells/ml in cold MEM plus 5% heat inactivated FCS, then 10 mls were added to each 25 cm^{2} flask. Flasks were incubated at 37^{o}C for one hour. Non-adherent cells were decanted and held on ice until culture. Adherent cells were washed vigorously three times with warm MEM without FCS. Flasks were incubated at room temperature for 10 to 20 minutes with four mls 0.2% EDTA in 5% FCS-MEM. Adherent cells were removed from the plastic surface with vigorous shaking, and rinsed twice more with cold MEM. Adherent cells were centrifuged at 200 x g for 10 minutes, and resuspended in fresh culture medium three times to remove EDTA.

Irradiaton: Cells were suspended in 5% FCS-MEM at a concentration of $1\text{-}5 \times 10^{7}$ cells/ml and held on ice for transport to the irradiation facility. Cells were irradiated for the doses designated in the figure on a rotating table irradiator with a cesium source emitting 203 rads per minute.

Cell culture: Five $\times 10^{5}$ cells (except where otherwise noted) in 0.2 mls supplemented MEM were added to each well of a Falcon (Becton, Dickinson and Co., Cockeysville, Md.) Microtest II flat bottom well culture plate containing 0.05 ml of mitogen or medium control.

Cultures were incubated at 37°C in 5% CO_2, 95% air in a humidified culture box. After 48 hours 0.1 uCi of <methyl-^{3}H> thymidine (22 Ci/mM, ICN Pharmaceuticals, Inc., Plainview, NY) in 0.01 ml MEM was added to each well. Cells were harvested 18 hours later with an automated harvester constructed in our lab, and counted in 80% toluene, 20% methanol with 4 gm/liter Omnifluor (New England Nuclear, Boston, Ma.).

Mitogens: A stock solution containing 2 mg/ml of Concanavalin A (Con A, Pharmacia, Inc., Piscataway, NJ.) was prepared and stored at 4°C in phosphate buffered saline (PBS). Purified phytohemmagglutinin (PHA, Burroughs-Wellcome, Beckenham, England) was reconstituted to 625 ug/ml in MEM and stored at -20°C. All mitogens were diluted serially fivefold from 625 ug/ml to 1 ug/ml in culture medium just prior to use. Fifty ul of each dilution or control medium was added to each of six wells just prior to addition of 0.2 mls cell suspension.

Data analysis: The mean counts of tritiated thymidine uptake per minute, and the standard deviation were calculated from 6 replicate cultures at each mitogen dose. The significance of the difference between means was calculated using the Mann-Whitney U test. Lines were fitted using the least squares method. Pearson's R (correlation coefficient) and the significance of correlation were calculated using the formulae and tables in the CRC Handbook of Tables for Probability and Statistics, 2nd ed. 1974.

RESULTS

JUVENILE SPLEEN CELLS SUPPRESS ADULT SPLEEN CELL RESPONSES TO BOTH CON A AND PHA

To find out whether juvenile spleen cells were capable of suppressing adult spleen cell responses as well as self, and to determine whether animals younger than 14 days also have suppressor cells, we conducted a series of experiments in which we mixed adult and juvenile cells in culture with mitogen. Figure 1 shows a representative experiment. Adult spleen cells alone gave very high responses to both Con A (left panel) and PHA (right panel). When mixed with spleen cells from 12 day old rats, mitogenesis was almost completely suppressed. The juvenile cells effectively suppressed both Con A and PHA stimulated mitogenesis of adult spleen cells, indicating that although activation may be necessary for the expression of suppression, the suppressors are not limited to stimulation by Con A. In 22 out of 23 experiments, cells from rats 2 days to 21 days old were able to suppress adult mitogenesis by more than 85%. Loss of response was not due to the use of half as many adult cells in the mixed cultures since 2.5×10^5 cells responded as vigorously as 5×10^5 cells when cultured without the addition of juvenile cells.

JUVENILE SPLEEN CELLS SUPPRESS BOTH JUVENILE AND ADULT THYMOCYTE RESPONSES TO CON A AND PHA

We next wished to learn whether thymus cell responses were also susceptible to suppression by juvenile spleen cells. Since juvenile thymus cell response to Con A was as high as the adult thymus cell response, it was also of interest to test for differences in susceptibility of adult and juvenile thymus cells to suppression. Figure 2 shows the data from one experiment. Juvenile thymus cells alone respond strongly to Con A, and to a reasonable degree to PHA. Juvenile spleen alone gave virtually no response to either mitogen. When mixed with juvenile spleen cells, the response of juvenile thymus to both Con A and PHA was blocked. When adult thymus was mixed with juvenile spleen, analogous results were obtained.

JUVENILE THYMUS CELLS ARE UNABLE TO SUPPRESS THE CON A RESPONSE OF ADULT SPLEEN CELLS

A logical next question was "would juvenile thymus cells stimulated with Con A be able to suppress adult spleen responses?". This question was of particular interest considering that thymic responses were usually much lower than splenic responses to T cell mitogens. Figure 3 presents one experiment showing clearly that although juvenile spleen cells vigorously suppressed adult spleen cell response to high doses of Con A, juvenile thymus cells effected little suppression. Male and female juvenile spleen cells produced comparable suppression, and both male and female thymus cells failed to suppress.

REMOVAL OF ADHERENT/PHAGOCYTIC CELLS WITH CARBONYL IRON RELIEVES

SUPPRESSION BY JUVENILE SPLEEN CELLS

Treatment with cFe removes a mixed population of phagocytic and adherent cells including macrophages and B cells. To characterize the suppressor cell population, untreated and cFe treated juvenile spleen cells were mixed with adult spleen cells (figure 4). Adult spleen cell responses to Con A were high; whereas, the juvenile cell responses were low, about 10% of the adult response. When adult and juvenile spleen cells were mixed, the response was about 20% of the adult response alone at 25 ug/ml Con A. However, if the juvenile spleen cells were first treated to remove cFe adherent cells, the response of the mixture of adult and treated juvenile cells increased to about 80% of the adult alone. These results indicate that most, but not all of the suppressors in juvenile spleens are removed by a single treatment with cFe. In this experiment, treatment with cFe also improved the response of juvenile cells alone about 20 fold (not shown). The loss of suppression of self and adult simultaneously may indicate that a common cell type is responsible for both types of suppression.

PASSAGE OF SPLEEN CELLS THROUGH NYLON WOOL COLUMNS RELIEVES SUPPRESSION BY JUVENILE SPLEEN CELLS

We next wished to determine if a traditional approach to removing T-suppressor cells would also remove juvenile suppression of adult spleen cell responses to Con A. As in earlier experiments, spleen cells from adult and juvenile Lewis rats were cultured alone or mixed,

both before and after passage of the juvenile cells through nylon wool. As shown in figure 5, juvenile spleen cells dramatically suppressed the adult Con A response, particularly at high Con A concentrations. However, after passage of juvenile cells through nylon wool, suppression of adult response to Con A was almost completely relieved. Although not shown, passage through nylon wool also increased the response of juvenile cells alone.

PLASTIC ADHERENT CELLS FROM JUVENILE RAT SPLEENS ARE SUPPRESSIVE FOR ADULT SPLEEN CELLS

Both the cFe technique (10) and nylon wool (11,12) remove mixed populations of cells. In order to determine the contribution of macrophage type adherent cells, we separated juvenile spleen cells by allowing macrophages to adhere to serum coated plastic flasks, decanting the non-adherent cells, then removing the macrophages with EDTA. As shown in figure 6, unseparated juvenile cells suppressed adult spleen cells by over 94% at 25 ug/ml Con A, and at 125 ug/ml Con A by almost 100%. Non-adherent juvenile spleen cells, however, failed to suppress adult spleen cell response to Con A and in addition displayed adult level responses. Because of low adherent cell recovery, we used 10^4 adherent cells mixed with 5×10^5 adult spleen cells. This number of juvenile cells (10% of adult) caused 75% suppression of the adult response at 125 ug/ml Con A. Lower numbers of adherent cells failed to cause suppression of adult spleen cell mitogenic responses (not shown).

IRRADIATION OF JUVENILE RAT SPLEEN CELLS DOES NOT AFFECT SUPPRESSION

Table 1 presents the effects of 800 rads on both adult and juvenile spleen cells. Greater than 90% depression of the Con A induced mitogenic response occurred in both cell populations. Doses of irradiation as low as 150-450 rad have also been previously shown to destroy suppresor cells in neonatal rats (13). Adult and juvenile cells were irradiated with 800 rad and mixed with mitogen responsive adult spleen cells (figure 7). As shown in the left hand panel, addition of irradiated adult spleen cells to unirradiated adult spleen cells did not cause any decrease in the mitogenic response. This data indicates that 1) irradiation at this dose did not create toxic products which suppress mitogenic responses and 2) cell "crowding" may not be invoked as an explanation for suppression. We have, throughout this study, chosen to use a cell concentration which is on the plateau of a cell dose/response curve so that equivalent thymidine uptake levels could be expected from both 2.5 and 5 x 10^5 cells per ml. In the majority of experiments, adult cells cultured at 2.5 x 10^5 and 5 x 10^5 cells per ml produced comparable responses. In the right hand panel of figure 7 it can be seen that both irradiated and unirradiated juvenile cells suppress the adult response by about 75% at 25 ug/ml and 100% at 125 ug/ml Con A. In other experiments up to 2000 rad did not abolish the ability of juvenile spleen to suppress adult spleen responses, indicating that the juvenile suppressor in this system is highly radiation resistant.

SUPPRESSION BY JUVENILE SPLEEN CELLS IS PARTIALLY REVERSIBLE WITH 2-ME

Adherence and radiation resistance are major characteristics of macrophages, cells which have been shown to possess suppressive capacity in mice and rats, both adult and neonatal. A recent series of reports suggests that Con A induced, macrophage-mediated suppression can be abrogated by the presence of 2-ME (14-16). We therefore asked whether the suppression produced by juvenile spleen cells could be abolished with 2-ME. Spleen cell suspensions from 7 and 99 day old Lewis rats were cultured separately, and mixed, as shown in figure 8. Presented are the results of stimulation with 5, 25 and 125 ug/ml Con A in the presence of 0 and 2×10^{-7} to 2×10^{-4} molar 2-ME. Optimal responses were obtained at 2×10^{-6} M 2-ME at all three doses of Con A. At 125 ug/ml of Con A, juvenile cells effected strong suppression regardless of the amount of 2-ME added, although in a separate experiment 2-ME overcame suppression even at this dose. However, at 5 and 25 ug/ml of Con A, the suppression by juvenile spleens was completely abrogated by 2×10^{5} M 2-ME. Con A and 2-ME may be dose-dependent antagonists with increasing Con A causing suppression, and increasing 2-ME blocking suppression.

THE ONTOGENY OF JUVENILE SPLENIC SUPPRESSOR CELLS

To analyse the development of the suppressor cells which we have partially characterized in this paper, we compiled, from 25 experiments, the percent suppression of adult Lewis rat spleen cells by spleen cells from Lewis rat pups 2 to 28 days old. Figure 9 presents the percent of predicted response at four doses of Con A,

where the distance below the predicted response (dashed line) represents the amount of suppression effected by the neonatal cells, and distance above represents enhancement. At 1 ug/ml and 5 ug/ml Con A considerable scatter exists with the regression for all 25 experiments falling very close to the 100% line, indicating that at these doses of Con A, juvenile spleen cells do not suppress adult responses. However, at 25 ug/ml Con A a significant ($p < 0.05$) trend emerges. Animals younger than 20 days old suppress more strongly and more consistently than older animals. At 125 ug/ml Con A 20 out of 23 suckling animals suppressed adult responses dramatically, whereas after the age of 21 days (weaning), no animal suppressed. Also indicated in the figure as open circles and a hatched regression line is the effect of treatment with either nylon wool or cFe. These treatments were able to relieve some or all of the suppression at all ages tested, reinforcing the hypothesis that the adherent cell is critical in the suppressive action of juvenile spleen cells.

EFFECT OF EARLY WEANING ON SUPPRESSOR CELL ACTIVITY INDUCED BY HIGH DOSE CON A STIMULATION

As already shown in figure 7, adult spleen cells are a poor source of suppressor cells, and as indicated in figure 9, high suppressor activity was lost coincident with weaning at 21 days. It is known that mother's milk may have effects on the immune system of the suckling animal. Therefore we next asked if weaning per se affected suppressor activity. The effect of weaning rats 1 to 3 days prematurely is shown in table II. Early weaning enhanced the response

of juvenile cells alone from 192% to 8980%, and reversed the suppression of adult cells by 157% to 349%. Such effects of early weaning could be due to the loss of suppressive factors or cells provided by a milk diet, or perhaps to the stress engendered by removal of the mother.

DISCUSSION

As previously reported (1,2) spleen cells from immature rats (<21 days old) respond poorly to the T cell mitogens PHA and Con A. We showed that between the ages of 14 and 21 days the low response was due to the presence of adherent, radioresistant suppressor cells; since following removal of adherent cells, adult level responses were obtained. However, before the age of 14 days no responses were revealed by the removal of adherent cells. In order to detect a suppressor present prior to 14 days, we conducted a series of experiments in which we studied the suppression of adult lymphoid cells by spleen cells from rats 2-32 days of age. In 22 out of 23 experiments, cells from rats 2-21 days old suppressed adult mitogenesis by more than 85%. After the age of 21 days (time of weaning), little suppression was detected. However, lower levels of suppressors probably continue on into adulthood (17). Since treatment with either nylon wool or cFe abrogated suppression at all ages tested, it is likely that the adherent cells which suppressed adult responses are also the cells which suppressed juvenile spleen cell "background" division. It should also be noted that spleen cells from animals as young as 2 days old suppressed effectively. This is of interest because Con A induced suppression of background cpm does not appear until day 14, coincident with the appearance of adult-level response capability. Thus there appear to be two requirements for suppression, a set of adherent suppressor cells present from birth, and another cell type appearing later in parallel with the influx of

Con A-reactive lymphocytes into juvenile spleen.

As shown here, adherent suppressor cells, present in 2 day old pups, were highly radiation resistant, up to 2000r. Irradiated adult spleens failed to suppress the response to Con A of autologous unirradiated splenocytes indicating that irradiation at this dose did not create toxic products which suppress mitogenic responses. Also, it is clear that cell crowding may not be invoked as an explanation for suppression after mixing of adult and juvenile cells. Supporting this is the additional finding that putative crowding by thymus cells also fails to suppress adult spleen cell responses to mitogen. Ranney and Oppenheim, using an approach similar to ours, also found that spleen cells from 5 day old BN rats suppressed autologous thymus cell response to Con A even after irradiation of the spleen cells with 1000 rad (18).

Much of the early work assumed that a single, adherent T dependent suppressor cell was responsible for neonatal suppression of the mitogen response. Mitomycin C (17) and irradiation resistance described above were often misleading since suppressor spleen populations were routinely mixed with non-treated responding populations which probably already contained an adherent suppressor T-cell or "second arm" of the suppressor mechanism. However, in at least one experiment macrophages were added to 400 rad irradiated, adherent suppressor T cells, and suppression was obtained (3). Even if future findings support these studies, they suggest only that division is not required for function, since such treatment is not markedly cytotoxic. Since these adherent suppressor T cells are large blast cells (3,19) often associated with mitotic activity, and the

cells are sensitive to *in vivo* cyclophosphamide (3), they may actually be dividing *in situ*. If true, the stimulus for division may be lost following removal from the host since these adherent blast suppressor cells are rapidly lost within the first 24 hours of culture (3).

The inability of neonatal thymus cells to suppress adult spleen cell responses to T-cell mitogens reported here suggests the absence of a major suppressive component, probably a macrophage. Macrophages must be considered as important mediators of suppression. Holt et.al. (3) demonstrated that adherent T-dependent suppressor cells suppress due to the presence of particular "activated" macrophages, i.e. induced peritoneal macrophages, but not resident peritoneal macrophages. Resident alveolar macrophages do assist in suppression, perhaps because they are environmentally activated, or possibly programmed for suppressor function. It should be noted that both germ free rat PEC and T-depleted rat PEC worked well (3,21). Macrophages pretreated with indomethacin were equally effective.

A possible role for macrophages in this system was recently presented by Aune and Pierce who demonstrated that Con A activated, Lyt 2^+ mouse T cells secrete a suppressor factor termed soluble immune response suppressor (SIRS) (14,15). This factor is modified by H_2O_2 secreted by "activated" macrophages resulting in a product which shuts down DNA synthesis in a variety of cell types. (16). This product, macrophage suppressor factor (MØ-SF), is similar to another Con A induced DNA synthesis inhibitor, IDS, found in stimulated rat lymphocyte cultures (16). Since MØ-SF activity is subject to inactivation by 2-ME, it was reasonable to ask if neonatal suppressor cell activity in rats was also sensitive to 2-ME. As presented here

2-ME appears able to reverse Con A induced suppression, at least when lower doses of Con A were used.

Adult spleen cells are a poor source of suppressor cells, and as indicated in the results, high suppressor activity was lost coincident with the time at which Con A responses of whole spleen cells reached maximum levels, at weaning. It is known that mother's milk has effects on the immune system of the suckling animal. Therefore we asked if weaning per se affected suppressor activity. The effect of weaning rats 1 to 3 days prematurely as shown in table II strongly suggests that such is the case. Early weaning enhanced the response of juvenile cells alone and reversed the suppression of adult cells. Such effects of early weaning could be due to the loss of suppressive cells or factors provided by a milk diet, or perhaps to the stress engendered by removal of the mother. The stress of early weaning could increase corticosteroid levels, which have been shown to both alleviate suppression (17) and to increase it (18). Milk products have been shown to be capable of suppressing T-cell mitogen responses (23,24). Ogra et.al.(25), observing poor mitogen responses following culture of resident human T cells in colostrum, suggested that colostrum could contain suppressor factors or suppressor cells. Clearly additional study is needed in this critical area.

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TABLE I.

EFFECT OF IRRADIATION ON CON A RESPONSE

Rads	Adult CPM	Juvenile CPM
0	120,000 ± 2000	19,000 ± 2000
800	15,000 ± 3000	1,400 ± 900
% Decrease	88%	93%

TABLE II.

EFFECTS OF EARLY WEANING ON SUPPRESSOR CELL ACTIVITY INDUCED BY HIGH DOSE CON A STIMULATION

Exp.	Weaned Early	JUV Cells Alone	% Increase	JUV Cells & Adult Cells	% Increase
A	–	449 ± 93	–	49,900 ± 19993	–
	+	7,023 ± 3414	1564	88,007 ± 13222	176
B	–	614 ± 192	–	31,286 ± 18829	–
	+	55,143 ± 13913	8980	109,297 ± 12050	349
C	–	134 ± 40	–	22,438 ± 10302	
	+	258 ± 272	192	35,174 ± 11399	157
D	–	789 ±	–	22,438 ± 10302	–
	+	4,656 ± 2619	590	49,007 ± 12071	218

A – 19 Day Weaned, Day 20 Assay
B – 18 Day Weaned, Day 19 Assay
C – 18 Day Weaned, Day 21 Assay
D – 19 Day Weaned, Day 21 Assay

Figure 1. Juvenile spleen cells suppress adult responses to both Con A and PHA. Spleen cells pooled from two 112 day old Lewis females were cultured alone (●——●) or mixed in a 1:1 ratio with spleen cells pooled from four 12 day old male and female Lewis rat pups (○---○) at a final density of 5×10^5 cells/well. Cells were cultured with 0 to 125 ug/ml of Con A (left panel), or 0 to 25 ug/ml PHA (right panel). Cultures were pulsed with ^3H thymidine at 48 hours and harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation. The maximum responses of juvenile spleen cells in this experiment were 8,000 cpm to Con A and 10,000 cpm to PHA.

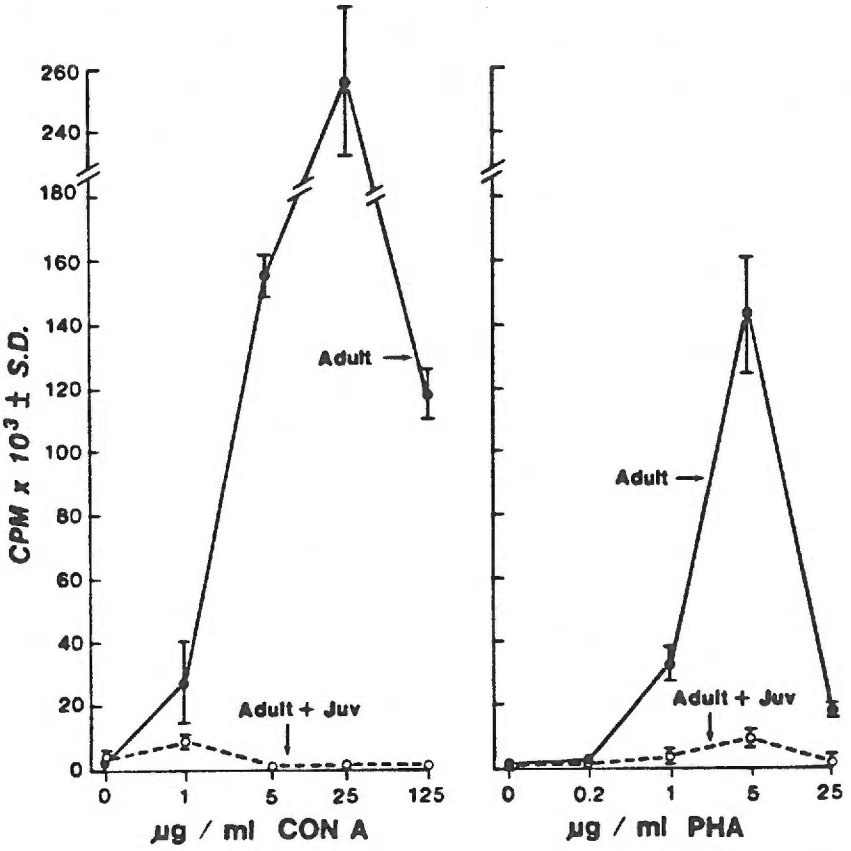

260
240
180
160
140
120
100
80
60
40
20
0
CPM x 10³ ± S.D.
Adult
Adult + Juv
0
1
5
25
125
µg / ml CON A
Adult
Adult + Juv
0
0.2
1
5
25
µg / ml PHA

Figure 2. Juvenile spleen cells suppress both juvenile and adult thymocyte responses to Con A and PHA. Spleen (JUV SPL) and thymus (JUV THY) cells from four 19 day old Lewis pups were cultured separately, and mixed (MIX), in a 1:1 ratio at a final density of 5 x 10^5 cells/well in all cultures. Thymus cells pooled from two 119 day old Lewis female were also cultured, either alone (ADULT THY), or mixed 1:1 with juvenile spleen cells (MIX), again at a final density of 5 x 10^5 cells/well in all cultures. Cells were cultured without stimulant (incorporation level indicated by the hatched line) or with 25 ug/ml Con A (top panels) or 5 ug/ml PHA (bottom panels). Cultures were pulsed with ^3H thymidine at 48 hours and harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

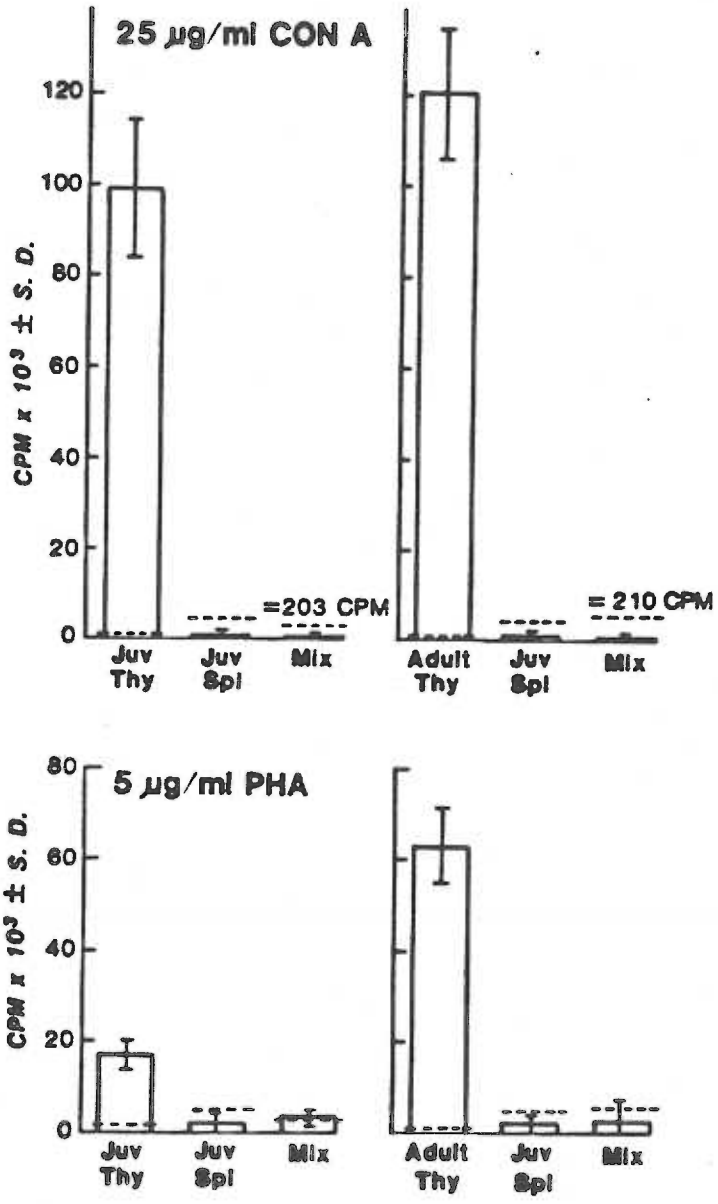

25 µg/ml CON A
CPM x 10³ ± S. D.
120
100
80
60
40
20
0
=203 CPM
= 210 CPM
Juv Thy
Juv Spl
Mix
Adult Thy
Juv Spl
Mix
5 µg/ml PHA
CPM x 10³ ± S. D.
80
60
40
20
0
Juv Thy
Juv Spl
Mix
Adult Thy
Juv Spl
Mix

Figure 3. Juvenile thymus cells are unable to suppress the Con A response of adult spleen cells. Spleen cells from a 100 day old Lewis female were cultured alone at 5 x 10^5 cells/well (△•••△) or mixed with 1:1 with thymus cells (○---○,●——●) or spleen cells (□---□,▲-•-▲) from male (○---○,□---□) or female (●——●,▲---▲) 14 day old Lewis rat pups. Cells were cultured with 0 to 125 ug/ml Con A, pulsed with ^3H thymidine at 48 hours and harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation. Juvenile spleen cells alone (not shown) did not exceed 9,000 cpm, and juvenile thymus cells alone (not shown) did not exceed 30,000 cpm.

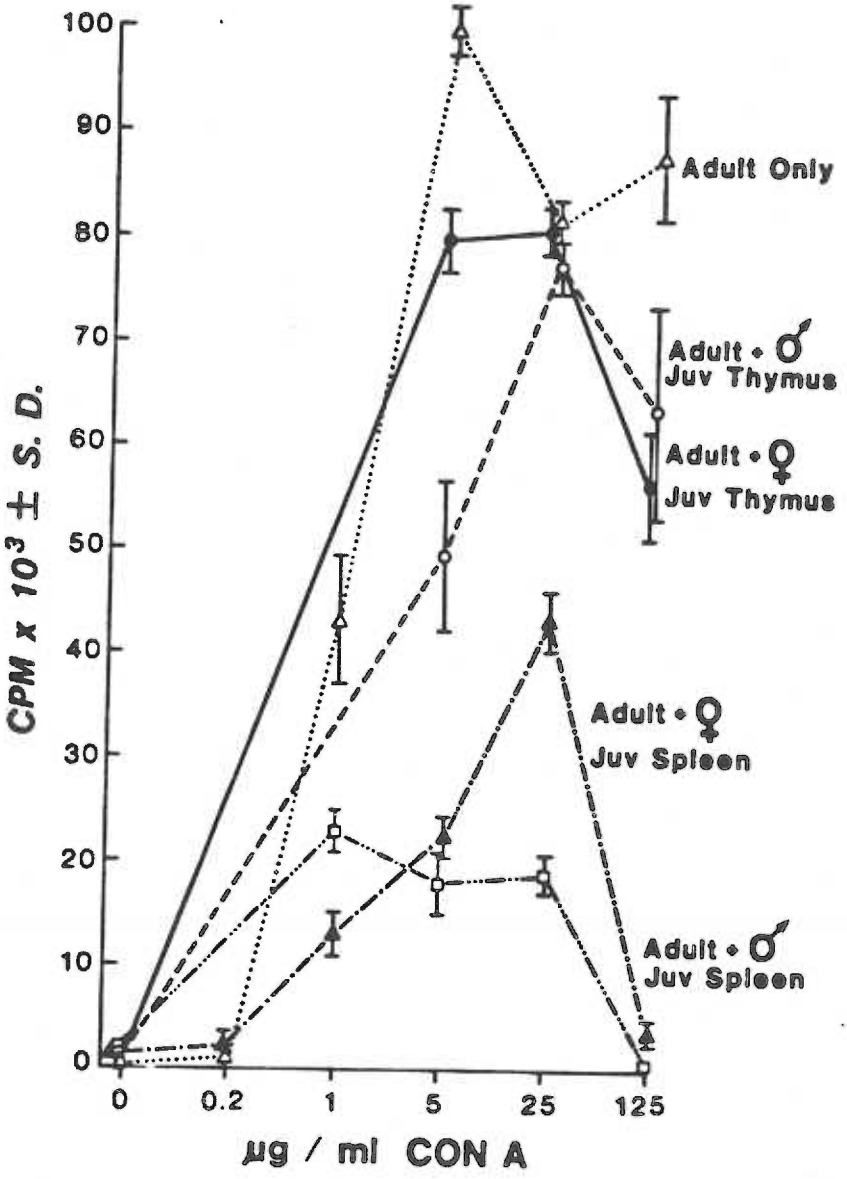

100
90
80
70
60
50
40
30
20
10
0
CPM x 10³ ± S. D.
0
0.2
1
5
25
125
µg / ml CON A
Adult Only
Adult • ♂
Juv Thymus
Adult • ♀
Juv Thymus
Adult • ♀
Juv Spleen
Adult • ♂
Juv Spleen

Figure 4. Removal of adherent/phagocytic cells with carbonyl iron (cFe) relieves suppression by juvenile spleen cells. Spleen cells from four 14 day old Lewis rat pups were pooled, then a portion was treated with cFe as described in the methods. Spleen cells from two adults aged 162 and 254 days were pooled, then cultured either alone (▲---▲) or mixed in a 1:1 ratio with untreated (●——●) or cFe treated (△···△) juvenile spleen cells. Untreated juvenile spleen cells were also cultured alone (○-·-○). Carbonyl iron treated juvenile cells cultured alone gave a maximum response of 26,000 cpm (not shown). All cultures had a final cell density of 5×10^5 cells/well. Cells were cultured with 0 to 125 ug/ml Con A, pulsed with ^3H thymidine at 48 hours and harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

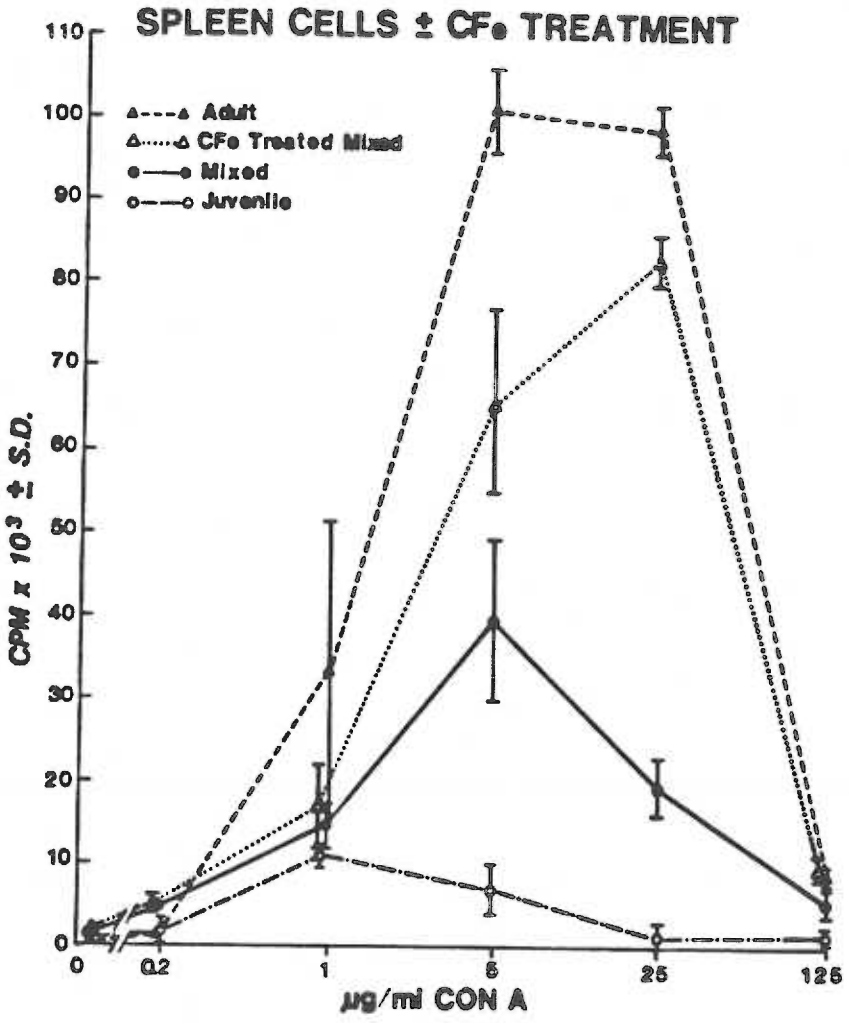

SPLEEN CELLS ± CFe TREATMENT
Adult
CFe Treated Mixed
Mixed
Juvenile
CPM x 10³ ± S.D.
110
100
90
80
70
60
50
40
30
20
10
0
0
0.2
1
5
25
125
µg/ml CON A

Figure 5. Passage of spleen cells through nylon wool columns relieves suppression by juvenile spleen cells. Spleen cells from twelve 10 day old Lewis rat pups were pooled, then all but an aliquot was put through a nylon wool column. Spleen cells pooled from three 112 day old Lewis females were cultured either alone (●---●) or with untreated (▲——▲) or nylon wool passaged (○---○) juvenile spleen cells in a 1:1 ratio. Untreated juvenile spleen cells were also cultured alone (△-•-△). All cultures had a final cell density of 5×10^5 cells/well. The maximum response of nylon wool passaged juvenile spleen cells when cultured alone was 29,000 cpm. Cells were cultured with 0 to 125 ug/ml Con A, pulsed with ^3H thymidine at 48 hours and harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

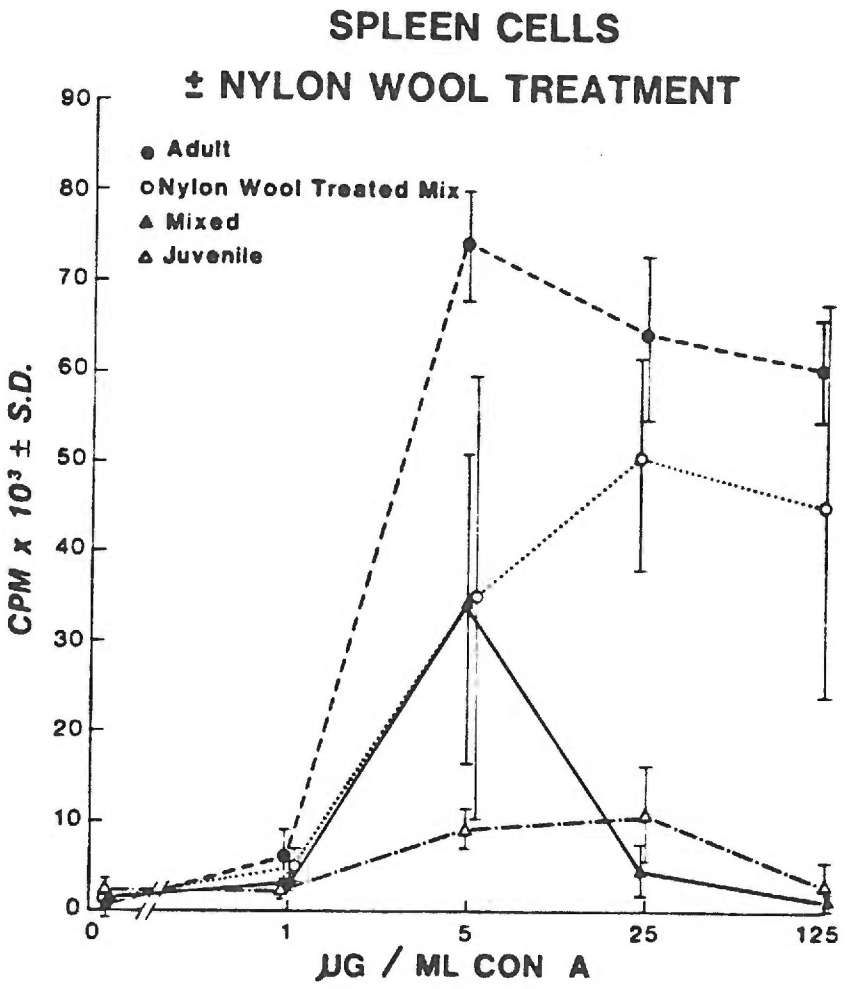

SPLEEN CELLS
± NYLON WOOL TREATMENT
● Adult
○ Nylon Wool Treated Mix
▲ Mixed
△ Juvenile
CPM x 10³ ± S.D.
90
80
70
60
50
40
30
20
10
0
0
1
5
25
125
µG / ML CON A

Figure 6. Plastic adherent cells from juvenile rat spleens are suppressive for adult spleen cells. Spleen cells from twelve 15-16 day old Lewis pups were pooled, and all but an aliquot was incubated on fetal calf serum coated plastic culture dishes for one hour at 37°C. The nonadherent cells were decanted, and the adherent cells were removed with EDTA. Spleen cells pooled from three 114 day old Lewis females were cultured alone (△---△) at a density of 2.5×10^5 cells/well, or 2.5×10^5 cells per culture were mixed with 2.5×10^5 nonadherent (○---○), 2.5×10^4 adherent (▲-·-▲) or 2.5×10^5 unseparated (●——●) juvenile spleen cells to give a final density of 5×10^5 cells/culture. The mitogen dose response curve for 5×10^5 adult cells per culture almost exactly overlaid the curve for 2.5×10^5 cells/well used here. In the right panel are the results for unseparated and nonadherent juvenile cells cultured alone. Insufficient cell numbers were obtained for culture of adherent cells alone. Cells were cultured with 0 to 125 ug/ml Con A, pulsed with ^3H thymidine at 48 hours and harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

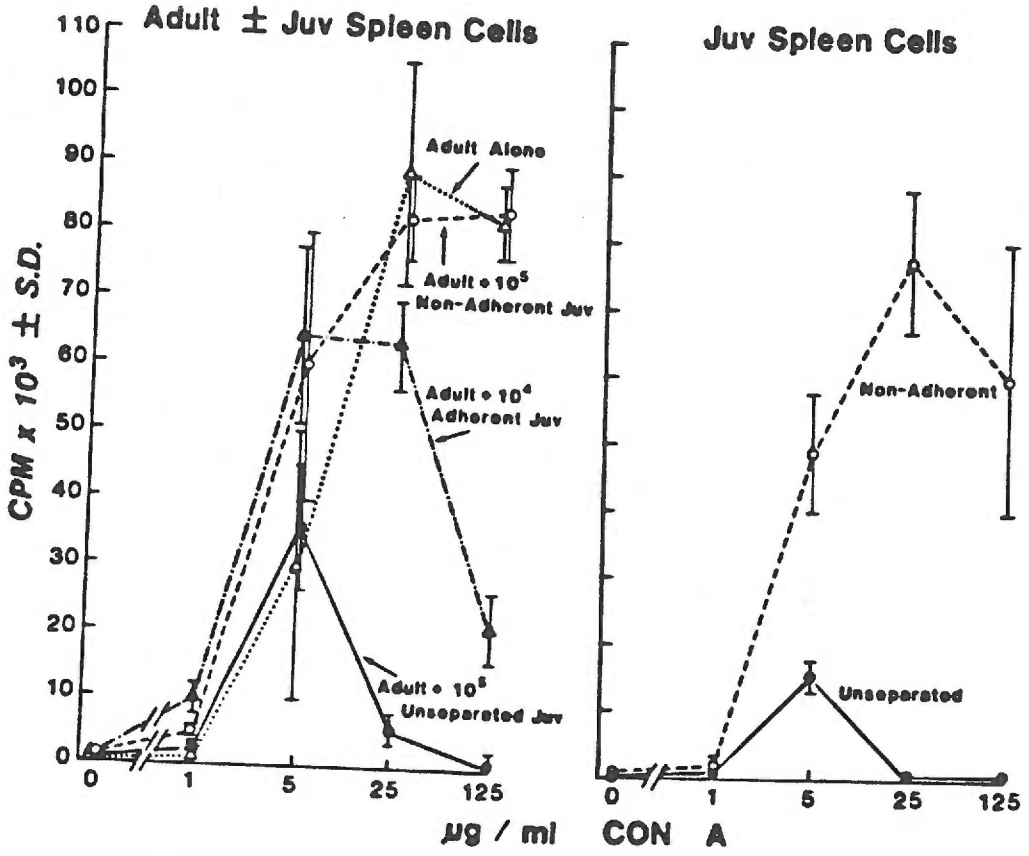

Adult ± Juv Spleen Cells
Juv Spleen Cells
CPM x 10³ ± S.D.
110
100
90
80
70
60
50
40
30
20
10
0
Adult Alone
Adult • 10⁵
Non-Adherent Juv
Adult • 10⁴
Adherent Juv
Adult • 10³
Unseparated Juv
Non-Adherent
Unseparated
0
1
5
25
125
µg / ml
CON A

Figure 7. Juvenile spleen cells which suppress adult spleen cell responses to Con A are radiation resistant. Spleen cells pooled from two 109 day old female Lewis rats or nine 11 day old Lewis rat pups were untreated, or irradiated with 800 rad as described in the methods. The left panel shows the result of culturing 2.5 x 10^5 (○-·-○), or 5 x 10^5 (●——●) adult cells per well, or of mixing 2.5 x 10^5 untreated adult spleen cells with 2.5 x 10^5 irradiated adult spleen cells (▲---▲). The right panel shows again the response of 5 x 10^5 untreated adult spleen cells (●——●) and the response of 2.5 x 10^5 untreated adult spleen cells plus 2.5 x 10^5 untreated (○-·-○) or 2.5 x 10^5 irradiated (▲---▲) juvenile spleen cells. Irradiated adult spleen cells alone did not exceed 6,000 cpm in response to Con A, and juvenile spleen cells alone did not exceed 6,000 cpm with or without stimulation or irradiation (not shown). Cells were cultured with 0 to 125 ug/ml Con A, pulsed with ^3H thymidine at 48 hours and harvested 18 hours later. Each point represents the mean of 6 replicate cultures +/- the standard deviation.

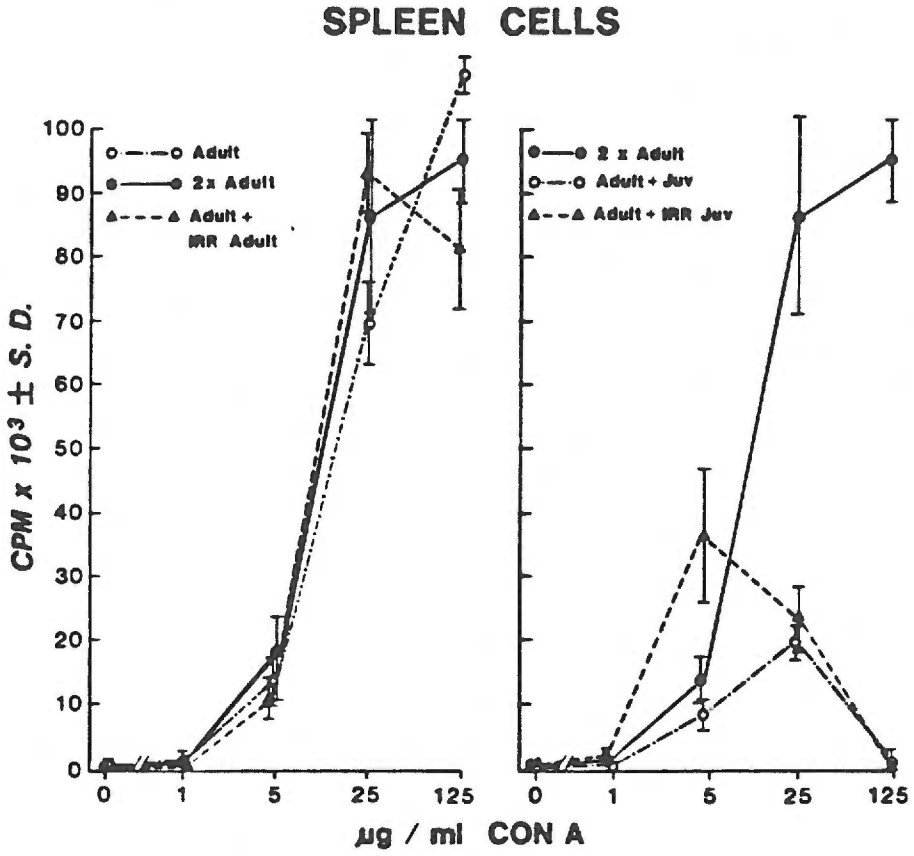

SPLEEN CELLS
Adult
2x Adult
Adult + IRR Adult
2 x Adult
Adult + Juv
Adult + IRR Juv
CPM x 10³ ± S. D.
0
10
20
30
40
50
60
70
80
90
100
0
1
5
25
125
µg / ml CON A

Figure 8. Suppression by juvenile spleen cells is partially reversible with 2-ME. Spleen cells pooled from seven 11 day old Lewis rat pups (dashed line) or three 99 day old Lewis females (open bar) were cultured with 5, 25, or 125 ug/ml Con A separately or mixed (hatched bar) in a 1:1 ratio at a final density of 5×10^5 cells in all cultures. To some cultures, 2-ME was added at molar concentrations from 2×10^{-7} to 10^{-4}. Cultures were pulsed with ^3H thymidine at 48 hours and harvested 18 hours later. Each bar represents the mean of 6 replicate cultures at the optimum dose of Con A minus the mean of 6 replicate cultures without stimulant. Standard deviation is indicated by the vertical line at the top of each bar.

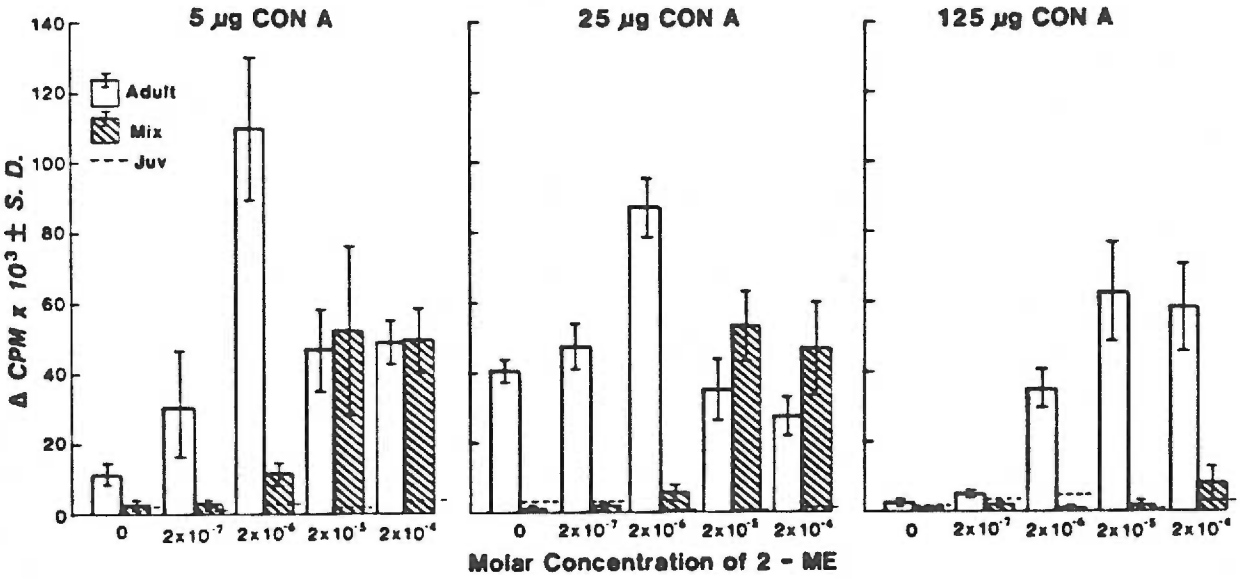
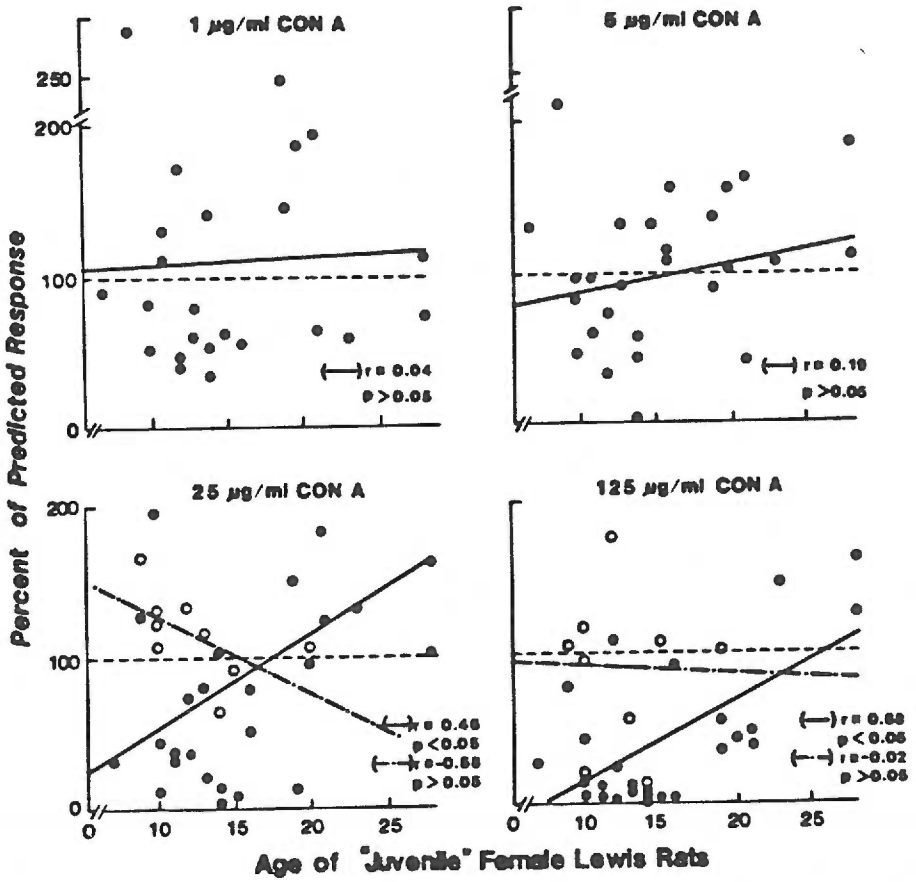

5 µg CON A
25 µg CON A
125 µg CON A
Adult
Mix
Juv
Δ CPM x 10³ ± S. D.
140
120
100
80
60
40
20
0
0
2x10⁻⁷
2x10⁻⁶
2x10⁻⁵
2x10⁻⁴
Molar Concentration of 2 - ME

Figure 9. The ontogeny of the juvenile splenic suppressor cell. This figure presents the maximum suppression attained in each of 25 experiments using spleen cells from Lewis rats 2 to 30 days old mixed with adult spleen cells. Juvenile and adult spleen cells were cultured separately or mixed as in figure 1. Predicted response was calculated as the adult response at 5×10^5 cells/well divided by 2 plus the juvenile response at 5×10^5 cells/well divided by two. The suppression effected by juvenile spleen cells is shown for untreated cells (solid circles) and cells depleted of adherent cells by either cFe or nylon wool (open circles). The linear regression lines for suppression by untreated (solid line) and nonadherent (dashed line) juvenile spleen cells are also presented.


1 µg/ml CON A
5 µg/ml CON A
25 µg/ml CON A
125 µg/ml CON A
Percent of Predicted Response
Age of "Juvenile" Female Lewis Rats
250
200
100
0
10
15
20
25
r = 0.04
p > 0.05
r = 0.19
p > 0.05
r = 0.46
p < 0.05
r = -0.58
p > 0.05
r = 0.68
p < 0.05
r = -0.02
p > 0.05

DISCUSSION AND CONCLUSIONS

Few studies have detailed the ontogeny of immune function in rats. Although much has been done with mice, it would not be correct to simply extrapolate those findings to rats. Rats are immunologically in some ways more like humans than mice are, making them a valuable tool in laboratory studies relevant to human function. Rats possess a dominant adherent suppressor cell in their spleens, similar to a population found in rabbits and quinea pigs, but not mice. These aspects of immunity in the rat make the ontogeny of mitogen responses in spleen and thymus an attractive topic for study. Initially we established the optimal conditions for the mitogenic response. We then performed a series of cultures testing mitogen responses in a large number of rats, covering a broad age range and testing 2 to 3 day intervals in 2 to 21 day old rats, a period during which rapid development occurs. In the course of those studies we observed that at high doses of Con A some of the suckling animals routinely had lower thymidine uptake than in unstimulated cultures. We investigated the course of development of the suppressor population responsible for this phenomenon, and concurrently studied the development of a similar suppressor population capable of blocking adult responses to mitogenic lectins.

CELL CULTURE

The effects of cell concentration, time in culture, and their inter-related effects have not been examined in detail for either adult or neonatal rats. Knight et.al. (1973) found that after 3 days of culture 2×10^6 cells in one ml responded maximally to doses of Con A between 20 and 100 ug/ml. Lindsay and Allardyce (1979) determined

that 3.5 days was optimum for both Con A and PHA cultured with 1-2 x 10^5 cell per 0.2 ml well, however, doses of Con A greater than 20 ug/ml were not tested. Keller et.al.(1982) tested PHA responses only after 3 days in culture, and with 1 x 10^6 cells per .2 ml culture. All of these studies of rat culture conditions have tested only one cell dose, and in only one study (Lindsay and Allardyce, 1979) were the effects of time in culture examined. In paper I we tested the effects of varying both the number of cells in culture and the period of incubation. We determined that 2.5 and 5 x 10^5 cells gave maximal responses at both 48 and 72 hours. We found that most cells in culture underwent little thymidine incorporation by 24 hours, but by 48 hours responses were high even with as few as 1 x 10^5 cells per culture. By 72 hours the responses of cells cultured at high concentrations were beginning to drop, while the responses of cells at lower cell density were still increasing. Very high cell concentrations were suppressive since, although not shown, 1 x 10^6 cells per culture in 3 separate experiments gave lower responses at 72 hours than 5 x 10^5 cells.

Fetal calf serum (FCS) is a major supplement for tissue culture studies, and is frequently used when autologous sera are ineffective. Previous investigators have argued for (Holt et.al., 1981a) and against (Knight et.al., 1973, Keller et.al., 1982) the use of FCS in rat lymphocyte cultures. A major objection to the use of FCS is its frequent mitogenic activity, observed here and previously reported by others (Knight et.al, 1973, Linday and Allardyce, 1979, Bullock and Moller, 1972). In paper I we found homologous rat serum to be as effective as or more effective than FCS while insuring lower

background stimulation. Although not reported, we also determined that use in culture of serum from either neonatal or old, male or female rats did not change the level of stimulation.

Two-mercaptoethanol (2-ME) has been used to enhance the proliferation of mouse lymphocytes since the early 1970s (Broome and Jeng, 1973). Proliferation in rat cultures was also shown to be enhanced by 2-ME (Holt et.al., 1981a). Parker et.al. (1982) found that for the generation of cytotoxic cells, 2-ME was needed during the first 2 days of culture, however, to boost mitogenesis, 2-ME needed to be present only during the first 24 hours. Two-mercaptoethanol was found to boost the rate of RNA synthesis by thymus cells depleted of macrophages (Bettens et.al., 1982). Although 2-ME promoted the survival of splenic lymphocytes in culture (Broome and Jeng, 1973) there was no effect on thymic lymphocyte survival (Bettens et.al., 1982, Broome and Jeng, 1973). After rat thymocytes were separated with Ficoll-Hypaque, only the lighter (blastogenic) fractions were found to respond to Con A with mitosis if 2-ME was not included in the medium. If 2-ME was included, all fractions were able to respond mitogenically (Cartier et.al., 1982), suggesting that 2-ME provided a preparatory signal or protected cells from an inhibitory substance (vide infra Aune and Pierce, 1981b). We tested a broad dose range of 2-ME concentrations on rat lymphocyte cultures (paper I). In accord with murine culture data, we found 2×10^{-5} M was optimal for rat splenic lymphocytes, producing up to a 20 fold increase in mitogen stimulation over cultures without 2-ME.

A possible role for 2-ME in the neonatal suppressor system was recently suggested by work of Aune and Pierce (1981a,1981b). Con A

activated, Lyt 2^+ mouse T cells secrete a suppressor factor termed soluble immune response suppressor (SIRS). SIRS is modified by the H_2O_2 produced by "activated" macrophages, resulting in a factor (MO-SF) which shuts down DNA synthesis in a variety of cell types. (Aune and Pierce, 1981c). Since MO-SF is subject to inactivation by 2-ME, it was reasonable to ask if neonatal suppressor cell activity in rats was also sensitive to 2-ME. As presented in paper III 2-ME appears able to reverse Con A induced juvenile spleen cell mediated suppression, when doses of Con A optimum for mitogenesis of adult spleen cells were used. Although the mechanism whereby 2-ME enhances lymphocyte culture has been largely unexplained, the possibility that it may inactivate a suppressor factor is attractive.

ONTOGENY

The ontogeny of splenocyte mitogen responses has been examined by a number of investigators. Mosier and Cohen (1975) reported that mouse spleen cell reponses to PHA and Con A reached adult levels between 3 and 4 weeks of age. More extensive data of Stobo and Paul (1972) indicated that Con A responses of spleen cells peaked early, reaching adult levels at 3 weeks; whereas, PHA responses rose more slowly, reaching adult levels at 8 weeks. Guinea pig splenic responses to these mitogens peaked much earlier, reaching a maximum at one week after birth (Merikanto et.al., 1979). As described in paper I, our data parallel the mouse studies of Stobo and Paul (1972), although in rats the 10 to 20 fold increase in Con A response occurs precipitously just after weaning at 3 weeks.

Normally mitogen responsiveness appears earlier in thymus cells than in spleen cells, as has been reported in humans (Toivanen et.al.,

1981), guinea pigs (Merikanto et.al., 1979), mice (Mosier, 1974), and in rats (Middleton and Bullock, paper I). Thymocyte responses to Con A were already at adult levels at the earliest age tested, day 4, but as with adult splenic responses there was a large amount of scatter. In adults, thymic responses to Con A were consistently lower than splenic responses, presumably due to the large number of "immature" T cells found normally in adult thymus. However, thymic cells from suckling rat pups often gave higher responses to Con A than spleen cells from the same animals, as might be expected, given that mitogen responsive cells appear in thymus before they appear in spleen. PHA responses of thymus cells also attained adult levels of response by 4 days of age, but the thymidine incorporation was about half of the maximum attained by cells responding to Con A. Lymphocytes from mice, but not guinea pigs (Merikanto et.al., 1979) or humans (Toivanen et.al., 1981) have also been shown to have lower mitogenesis in response to PHA than to Con A.

The mitogen dose response of lymphocytes has been tested for such variables as the strain (Rozing and Vaessen, 1979, Williams et.al., 1973a and 1973b), serum concentration (Coutinho et.al., 1973) and age (Stites et.al., 1972, Mosier, 1974). Mosier (1974) reported that spleens from young mice responded better to slightly higher doses of Con A than were optimal for old mice. Stites et.al. (1972) found that human cord blood lymphocytes responded optimally to lower doses of PHA than did human adult peripheral lymphocytes. In our study, we examined the effect of age on rat spleen mitogen dose response. Doses lower than 0.2 ug/ml Con A failed to stimulate mitogenesis in rats of all ages. Frequently, 1 ug/ml Con A was optimal for juvenile

responses, however it was rarely optimal for responses by adult spleen cells. Most adult maximum responses were attained with 5 and 25 ug/ml Con A. Of interest was the new finding that many older (>80 days) adult rats responded maximally to the high dose of Con A, 125 ug/ml. In most reports "high" doses of Con A produce poor stimulation. It was recently reported that an apparent loss of human lymphocytes following 24 hours of culture with 100 ug/ml Con A was actually due to non-damaging aggregation of the cells by the lectin, rather than toxicity (Distelhorst and Benutto, 1982). Our findings suggest that higher doses of Con A can actually stimulate optimum mitosis in cells from older animals. However, younger rats responded maximally most often to 1 ug/ml.

SUPPRESSION

Concentrations of Con A optimal in adults not only failed to stimulate splenocytes from suckling rats but also dramatically suppressed spontaneous thymidine incorporation, often to less than 10% of the background level. Since as little as 5 ug/ml suppressed background cpm, and since 25 times this dose was a potent stimulant of adult cells, non-specific toxicity appears to be an unlikely explanation for this suppression.

In line with our primary interest in ontogeny, we examined the changes in the dose response curve with age. As reported in paper I, we found that spontaneous blastogenesis (background) was high in spleen cells from rats 2 days to 12 days old. The addition of Con A at this early age did not suppress the background blastogenesis, nor did it stimulate the cells. Furthermore, the response to Con A in

this age of rat could not be substantially improved by the removal of cFe or nylon wool adherent cells or addition of Con A supernatant. However, in rats between the ages of 12 to 21 days, the addition of Con A in doses optimal for blastogenesis of adult spleen cells caused marked suppression of background blastogenesis. When adherent cells were removed by carbonyl iron treatment, nylon wool or plastic adherence, spleen cells from rats in this developmental period were able to respond to Con A at adult levels with the suppression of spontaneous blastogenesis abolished. Removal of cFe adherent cells had no effect on levels of background blastogenesis, unless mitogen was added (paper II). Then, at higher doses of Con A, the spontaneous mitogenesis was suppressed. This suggests that suppression was dependent on activation. Suppression of neonatal spontaneous thymidine uptake by mitogen was also noted by Holt et.al. (1981b) who found that addition of PHA lowered the level of DNA synthesis below unstimulated levels in rats less than 2 weeks old. This work differs from ours in that we found suppression of background only in rats older than 2 weeks. Holt et.al. (1981b) did not examine the effect on background mitogenesis of removal of adherent cells.

The lack of mitogen response in the 2-12 day old cells is most probably due to an intrinsic immaturity or absence of the responding T cell within the spleen. We found in preliminary work that even after the addition of Con A supernatants, a commonly used source of IL-2, very young suckling rats still could not respond to Con A. We were also unable to elicit a response in spleen cells from 12 day old rats after the addition of irradiated adult cells (unpublished observations). Stutman and Ishizaka (1982) found similarly that the

deficient IL-2 production of 1-14 day old mouse spleens could not be remedied by the addition of IL-1 or adult macrophages. In early work, Stobo and Paul (1972) found that Thy-1 bearing cells were missing from mouse spleens during the first week of life, had only attained 50% of adult levels by 2 weeks, and were at adult levels by 3 weeks. As Thy 1 is a marker of mature T cells, this is further evidence that neonatal spleens are deficient in mature T cells.

Other investigators have found, as we have, that removal of adherent cells improves the mitogen responses of young rat spleen cells, but the ontogenetic pattern differs from the one we found. Folch and Waksman (1974) found that removal of adherent cells did not improve the responses of two week old rat spleen cells to PHA. However, by 4 weeks of age, removal of adherent cells allowed PHA responses to attain maximum adult levels. Holt et.al. (1981b) found that 3-4 week old rat spleen cells were more susceptible to suppression by peritoneal exudate cells than were adult spleen cells. We found that by 15-19 days of age, removal of adherent cells allowed a strong, adult level mitogen response and that by 3 weeks of age, rat spleen cells could respond at a high level even in the presence of adherent cells.

Having detected a suppressor cell which was able to suppress neonatal responses between the ages of 12 to 21 days, we next asked whether the 2 to 12 day old cells contained a suppressor which could function in the presence of adult responding cells. In paper III we examine the suppression of adult lymphoid cells by spleen cells from rats 2-32 days of age. In 22 out of 23 experiments, cells from rats 2-21 days old suppressed adult mitogenesis by more than 85%. After

the age of 21 days (weaning), little suppression was detected. However, lower levels of suppressors probably continue on into adulthood (Folch and Waksman, 1974a and 1974b). Since treatment with either nylon wool or cFe abrogated suppression at all ages tested, it is likely that the adherent cells which suppressed adult responses are also the cells which suppressed juvenile spleen cell "background" division. It should also be noted that spleen cells from animals as young as 2 days old suppressed adult mitogenic responses effectively (paper III). This is of interest because Con A induced suppression of background cpm does not appear until adult-level response capability around day 14.

Suppressor activity was lost coincident with the time at which Con A responses of whole spleen cells reached maximum levels, at weaning. It is known that mother's milk may have effects on the immune system of the suckling animal. Therefore we asked if weaning per se affected suppressor activity. The effect of weaning rats 1 to 3 days prematurely as shown in table II strongly suggests that such is the case. Early weaning enhanced the response of juvenile cells alone and reversed the suppression of adult cells. Such effects of early weaning could be due to the loss of suppressive substances or cells provided by a milk diet, or perhaps to the stress engendered by removal of the mother. The stress of early weaning could increase corticosteroid levels, however, such treatment has been shown both to alleviate suppression (Folch and Waksman, 1974a), and to increase it (Ranney and Oppenheim, 1972). Milk products have been shown to be capable of suppressing T-cell mitogen responses (Diaz-Jouaneu and Williams, 1974, Parmley et.al., 1976). Ogra et.al.(1978), observing

poor mitogen responses following culture of resident human T cells in colostrum, suggested that colostrum could contain suppressor factors or suppressor cells. Clearly additional study is needed in this critical area.

Low dose (100-400 rad) irradiation has been shown to effectively block suppression by some T cell subsets (McCullagh, 1975, Chiorazzi et.al., 1977), but to be ineffective against macrophage mediated suppression (Gorczynski et.al., 1971). Ranney and Oppenheim (1972) observed that irradiated spleen cells from 5 day old rats but not from adult rats suppressed isologous thymocyte background 80-94%. Holt et.al. (1981b) also reported that adherent splenic cells most active in the neonatal period, which cooperate with alveolar macrophages to suppress PHA responses are resistant to 1-450 rad. We also detected a radiation resistant suppressor cell. As shown in paper II 100-800 rad completely failed to enhance the Con A response of spleen cells from 7-13 day old rats, in contrast to the slightly increased Con A responses obtained from 4-13 day old rat spleen cells after treatment with cFe. It is possible that similar treatment of older suckling rats might reveal a radiation sensitive suppressor, but those experiments are still in progress.

The suppression of adult mitogenic response, present in 2 to 14 day old pups, was also resistant to radiation up to 2000 rad. As shown in paper III irradiated adult spleen cells failed to suppress the response to Con A of autologous unirradiated splenocytes indicating that irradiation at this dose did not create toxic products which suppress mitogenic responses, and that cell crowding was probably not an explanation for suppression after mixing of adult and

juvenile cells. The finding that juvenile thymus cells also fail to suppress adult spleen cell responses to mitogen is further support for the argument that cell crowding is not the cause of suppression.

INTERACTION OF T CELL AND MACROPHAGE

Adherence to glass or plastic and radiation resistance are often taken to indicate that one is working with a macrophage or monocyte. However, resistance to irradiation simply means that the suppressor activity does not depend on DNA synthesis. There is also evidence that adherent cells may carry T cell determinants and be thymus dependent. Holt et.al. (1981b) provided direct evidence for an adherent suppressor T cell by treating the glass wool adherent fraction of rat spleen cells with anti-thymocyte serum and showing that suppressor activity was lost. This adherent T cell was resistant to radiation, indomethacin and mitomycin C, but sensitive to cyclophosphamide. A glass wool adherent suppressor T cell has also been isolated from mouse spleens (Webb and Nowowiejski, 1981). Helper T cells may be adherent as well. Sopori et.al. (1981) have shown that rat spleen cells contain an adherent, radiation resistant, anti-thymocyte serum sensitive helper population that enhances CTL responses.

Simply showing that the T cells themselves are adherent does not deny the possibility that a macrophage-T-cell interaction occurs, although some early work assumed that a single, adherent T dependent suppressor cell was responsible for neonatal suppression of the mitogen response (Folch and Waksman, 1974a). Holt's work (1981b) established that an adherent T cell regulated the cytostatic

activities of macrophages. Macrophages were added to 400 rad irradiated adherent suppressor T cells, and suppression was obtained. Webb and Nowowiejski (1981) found that a macrophage activated the adherent suppressor T cells from mouse spleens via a prostaglandin dependent pathway. Of interest here are the findings of Malkovsky et.al. (1983) who recently reported that a cyclophosphamide-sensitive $I\text{-}J^{+}$ mouse splenic T-cell can be "armed" by specific T-cell factors induced by antigen to secrete a non-specific suppressor of DNA synthesis stimulated by Con A. It was suggested in this report that macrophage activity may be involved. Namba et.al. (1977) also reported that adherent T-dependent rat splenic suppressor cells produced an inhibitor of Con A induced DNA synthesis. These cells were sensitive to in vivo BUDR treatment implying that division might be necessary for their activity in vivo. Gutowski and Weksler (1982) showed that either depletion of adherent cells or the treatment with anti-Thy 1 plus complement abolished the ability of 1 week old spleen cells to suppress the syngeneic MLR. They went a valuable step further, in showing that neither purified T cells nor purified macrophage from 1 week old rat spleens could mediate suppression alone.

The interaction of T cell and macrophage is of interest to us because we believe that the suppression in neonatal rat spleens is the result of cooperative interaction of an adherent cell unique to or dominant in neonatal spleen, and a mature T cell. Our evidence for this is as follows. First, neonatal rat thymus cells do not suppress adult spleen responses. Thymus cells of neonatal rats are immunocompetent, as shown by ability to respond to Con A. Therefore,

the neonatal T cell alone or in combination with macrophages present in adult spleens does not suppress. Second, when spleens from 2 to 13 day old rats were mixed with juvenile or adult thymus cells, then stimulated with PHA or Con A, thymidine incorporation was depressed significantly below the background incorporation of either the spleen or the thymus (paper III.). It should be noted that at this age, addition of Con A to spleen cells alone did not depress blastogenesis below background. Third, neonatal spleens gained the ability to suppress their own spontaneous blastogenesis only after an influx of immunocompetent T cells at about 2 weeks of age. However, they were capable of suppressing adult spleen and thymus cell mitogen responses from birth. Last, both suppressive activities were removable by adherence techniques, and were radiation resistant. As pointed out, although these suggest a macrophage, the possibility of an adherent T cell or null cell is not eliminated.

In summary, we have conducted three major lines of investigation. We have analyzed the culture conditions for rat lymphocytes, and found that the optimal dose of Con A for mitogenesis changes with the development of the rat. We have outlined in detail the development of T-cell mitogen responses in Lewis rat spleen and thymuses, noting that these organs mature at different times, and that the ontogeny of PHA responsive cells differs from the ontogeny of Con A responsive cells. Finally, we have traced the development of a suppressor system present from birth to weaning in rat spleens. We suggest that an adherent, radioresistant suppressor cell present in rat spleens from birth may interact after mitogen stimulation with mature T cells present in rat thymus from birth, but not present in rat spleen until 12-15 days

after birth.

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