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Studies on the Effect of Sequential Thymectomy on *in vitro* Responses of Lymphocytes in the Amphibian, *Xenopus laevis*

by Nour El-Din H.S. Sherif, B.Sc.

being a Thesis submitted for the Degree of

Master of Science

in the University of Durham (Department of Zoology)

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CONTENTS

ABSTRACT i

CHAPTER 1 GENERAL INTRODUCTION 1

CHAPTER 2 *IN VITRO* RESPONSES TO THYMUS-DEPENDENT MITOGENS 6

Introduction 6

Materials and Methods 7

Results 11

A) Response of thymus and spleen of control toadlets to PHA and Con A. 11

B) Effect of sequential thymectomy on PHA responsiveness of spleen. 13

Discussion 14

Tables 18

Figures 21

CHAPTER 3 MIXED LEUCOCYTE CULTURE REACTIVITY 24

Introduction 24

Materials and Methods 25

Results 26

A) Splenic MLR in control toadlets 26

B) Effect of sequential thymectomy on splenic MLR 27

Discussion 28

Tables 30

Figure 42

CHAPTER 4 GENERAL DISCUSSION 43

ACKNOWLEDGEMENTS 47

REFERENCES 48

DECLARATION

The work presented in Chapters 2 and 3 forms the basis of one publication:

HORTON, J.D. and SHERIF, N.E.H.S. (1977): "Sequential thymectomy in the clawed toad: effect on mixed leucocyte reactivity and phytohaemagglutinin responsiveness." in: Developmental Immunobiology, Eds. J.B. Solomon and J.D. Horton. North Holland Publishing Co., Amsterdam (in press).

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ABSTRACT

Spleen and thymus leucocytes from control *Xenopus* toadlets react similarly *in vitro* to classical thymus-dependent (T) mitogens (phytohaemagglutinin (PHA) and Concanavalin A) as measured by enhanced DNA synthesis. The effect of different concentrations of foetal calf serum on the dose response curves to those two mitogens is examined.

The effect of sequential thymectomy on PHA responsiveness of spleen leucocytes reveals that thymectomy until stage 55 of development (31 days) abrogates PHA reactivity. In contrast, thymectomy at stage 56/7 (35-40 days - just prior to metamorphosis) and in early adult life has no apparent debilitating effect on the response to this mitogen.

Optimal conditions for splenic mixed leucocyte reactivity (MLR) in control *Xenopus* have been investigated. The use of allogeneic serum enhances MLR stimulation indices.

The effect of sequential thymectomy on splenic MLR shows that thymus removal only until stage 51 (15-16 days) abrogates this allogeneic response. Thymectomy from stage 52-57 (21-40 days), and in adulthood, failed to abolish MLR.

It appears that lymphocytes that can participate in MLR are established in the periphery (e.g. spleen) early in development, in contrast to the slower "emergence" of PHA-reactive cells. These studies support a concept of T cell heterogeneity in amphibians.

CHAPTER 1

GENERAL INTRODUCTION

Two broad categories of lymphocytes are found in birds and mammals. Thus, lymphocytes that are spawned in the thymus, and which participate in cell-mediated immunity, are termed thymus-derived (T) cells, whereas those that develop within the bursa of Fabricius (birds) or mammalian bursa equivalent (e.g. foetal liver and spleen) (Owen, Raff and Cooper, 1975) are the precursors of antibody-forming cells (humoral immunity) and are called bursa or "bursal-equivalent"-derived (B) lymphocytes (see Greaves, Owen and Raff, 1973 and Nossal, 1976 for reviews).

Functional and physical differences have proved useful in recent years to characterise T and B cells. These include: 1) Differential reactivity to mitogens (see Oppenheim and Rosenstreich, 1976) and to the carrier and haptenic portions of antigenic molecules (Miller et al., 1971), 2) Differences in migratory pathways (de Sousa, 1976), 3) Presence or absence of certain surface antigens and immunoglobulins (Ig's) (Greaves et al., 1973), and 4) Ultrastructural differences (Lebouteiller et al., 1976). Moreover, it is now realized that these major populations themselves consist of a heterogeneous collection of lymphoid cells which are, in various ways, intimately involved with the initiation and regulation of immune responses (Greaves et al., 1973; Möller, 1975 and Loor and Roelants, 1977).

The question of whether T and B cell equivalents exist in poikilotherms is now being examined. At the functional level,

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evidence is beginning to accumulate that T-like and B-like cells are found in fishes, amphibians and reptiles (see Cooper, 1976a and 1976b; Manning and Turner, 1976 and Wright and Cooper, 1976). Thus in the leopard frog, different culture conditions are required to obtain maximal proliferation of lymphocytes to classical T cell mitogens (phytohaemagglutinin (PHA) and Concanavalin A (Con A)) on the one hand and B cell mitogens (bacterial lipopolysaccharide (LPS) and purified protein derivative of tuberculin (PPD)) on the other (Goldstine et al., 1975). Cell fractionation studies on goldfish (Warr et al., 1977) and alligator (Cuchens et al., 1976) lymphocytes are also indicative of functional lymphocyte heterogeneity in poikilotherms. Moreover, hapten-carrier effects have been observed in a variety of fishes (Stolen and Mäkelä, 1975, 1976; Yocum, Cuchens and Clem, 1975) and amphibians (Ruben, 1975, 1976; Ruben, Van der Hoven and Dutton, 1973). By analogy with mammalian experimentation, these hapten-carrier experiments provide suggestive evidence for cell cooperation in poikilotherm humoral immunity.

Physical differences amongst poikilotherm lymphocytes are less well established. Indeed the majority of lymphocytes in the thymuses of fishes and larval amphibians contain readily demonstrable surface Ig (see Ellis and Parkhouse, 1975 (for fishes); Du Pasquier, Weiss and Loor, 1972; Jurd and Stevenson, 1976 (for amphibians)) and also papers presented in the symposium on Phylogeny of Thymus and Bone Marrow-Bursa Cells (see Wright and Cooper, 1976)). This contrasts with the situation in mammals, where the presence of readily detectable surface Ig has been

used as a marker for B cells. Ig is believed to act as the receptor for antigen on these lymphocytes (Greaves, Owen and Raff, 1973). The nature of the T cell antigen-receptor is still unknown and remains an important open question in immunology. The finding of surface Ig, by sensitive techniques, on endotherm T cells and the presence of (endogenous) surface Ig positive thymus cells in poikilotherms lends weight to the concept that the T cell receptor is also Ig (see discussion in Marchalonis, 1976) and indicates that absence of Ig may not be a hallmark of these cells.

The origin of lymphocytes in poikilotherms is currently being examined. Whether or not the developmental history of cells functionally equivalent to T and B lymphocytes in amphibians is comparable to the situation in birds and mammals is still unclear (Turpen and Cohen, 1976a; Turpen, Volpe and Cohen, 1973, 1975). Volpe and Turpen (1976) have suggested that virtually all the lymphocytes of amphibians emanate from the thymus. In this respect, proliferative responses to T but not to B cell mitogens by thymic lymphocytes have been reported in certain fishes (e.g. rainbow trout (Etlinger et al., 1976)) and in the clawed toad (Donnelly et al., 1976). These experiments imply that the thymus is the source of T- but not B-like lymphocytes. However, other studies on fishes (see Cuchens et al., 1976 and review by Etlinger et al., 1976) and experiments on <u>Xenopus</u> in this laboratory (Smith and Horton, unpublished observations) suggest that lymphocytes responding to <u>both</u> T and B mitogens exist in the thymus. Further experimentation is required to determine whether such B-like lymphocytes are actually spawned in the thymus of these lower

vertebrates, or are just temporarily residing in this organ.

Thymectomy (tx) experiments with amphibian larvae are proving to be useful in tackling this question (Manning and Collie, 1975). For example, in the clawed toad, Xenopus laevis, (an excellent model for observing the long-term effects of thymectomy, since it is one of the rare species that can be successfully thymectomized very early in life and does not runt after this operation (Manning, 1971; Horton and Manning, 1974; see also Du Pasquier, 1976)) thymic ablation in the first week of life impairs allograft rejection (Horton and Manning, 1972) and abolishes antibody production to thymus-dependent antigens (Turner and Manning, 1974 and Horton et al., 1977). However, antibody production to thymus-independent antigens remains unaffected (Collie et al., 1975), even when the thymus is removed at only 4 days (Tochinai, 1976). Moreover, lymphocytes with surface Ig are still present in good numbers in early tx Xenopus (Weiss, Horton and Du Pasquier, 1973). In vitro studies on these animals also demonstrate (see Ch.2) thymus-dependent and thymus-independent lymphocyte populations. Despite the possibility that thymectomy could, to a certain extent, effect an alternative pathway of lymphocyte differentiation (Turpen and Cohen, 1976b) these thymectomy studies on Xenopus provide compelling evidence for a separate maturation of T-like and B-like cells in amphibians. It is now of central importance to elucidate the origin of B cells in these animals, and Turpen (1977) is currently examining this issue.

The major emphasis of this thesis is to investigate the role the thymus plays in establishing immune development later in

ontogeny. The effect of sequential thymectomy on PHA responsiveness and mixed leucocyte reactivity of *Xenopus* spleen leucocytes is examined. The studies suggest that the amphibian thymus contains a functionally heterogeneous population of lymphocytes.

CHAPTER 2

IN VITRO RESPONSES TO THYMUS-DEPENDENT MITOGENS

INTRODUCTION

In vitro studies on Xenopus thymectomized at 7-8 days of age (stage 48) illustrate that thymus-dependent and thymus-independent lymphocyte populations exist in amphibians (Donnelly et al., 1976; DuPasquier and Horton, 1976 and Manning et al., 1976). Thus early thymic ablation abrogates or severely impairs PHA and Con A responsiveness of spleen and peripheral blood leucocytes, but has no apparent effect on LPS and PPD reactivity. These experiments did not, however, investigate the length of time for which the thymus must be present in vivo to establish (T-dependent) mitogen-reactive cells. The work in this chapter therefore concentrated on this issue, by examining the effect of thymectomy performed at several different stages of larval development and also in adulthood. However, first it was necessary to develop in this laboratory the techniques for culturing Xenopus lymphocytes and to define the optimal conditions for obtaining maximal stimulation of control cells by T-dependent mitogens. The effect of mitogen and foetal calf serum concentrations has been examined, and moreover a comparison of mitogen responses in spleen and thymus has been made to see if any functional heterogeneity exists (in terms of mitogen reactivity) between peripheral T cells and thymocytes.

MATERIALS & METHODS

Rearing and care of animals

The South African clawed toad, Xenopus laevis was used. All animals used in the studies presented in this thesis were bred and reared in the laboratory under the general conditions described by Henriques (1964) and Horton and Manning (1972). Spawning was induced by the injection of chorionic gonadotrophin into the dorsal lymph sac of adult male and female Xenopus laevis (Daudin). Stock larvae were reared in 8-litre tanks (three larvae/litre) containing aerated, standing tap water, and were fed nettle powder. Developmental stages were determined using the Normal Table of Nieuwkoop and Faber (1967) and larvae were selected for thymectomy if they had reached the appropriate stage at the age given in the Table. Metamorphosed animals (toadlets) were kept in similar numbers per litre. They were fed Tubifex worms twice weekly. All animals were maintained at 23 $\pm 1^{\circ}$C.

Thymectomy

Larvae were thymectomized (tx) or sham thymectomized (sh) by microcautery as described by Horton and Manning (1972)(the procedure is summarised below) at the following stages of development: st. 48 (7-8 days), st. 51 (15-16 days), st. 52/3 (21-23 days), st. 54/5 (28-31 days), and st. 56/7 (35-40 days). A Martin-Elektrotom 60 high frequency (1.75 MHz) cautery apparatus was used. Larvae are positionod ventral side down in a small petri-dish containing a layer of charcoal-agar. Under a stereomicroscope the thymus is exposed by lifting

away a flap of dorsal skin using fine tungsten needles. The tip of the neutral electrode is pushed into the agar and the tip of the positive electrode (a fine tungsten needle) is placed on the thymus. Current is applied and the localised generation of heat which ensues at the tip of the positive electrode destroys the thymus. Adult thymuses were removed surgically (Horton, Rimmer and Horton, 1977) when toadlets were 6 months old. Details of thymus histogenesis at these various stages have been given elsewhere (Horton, Rimmer and Horton, 1977). The effect of thymectomy at any particular stage of development was generally studied in at least two separate batches of animals. Thymic absence was always confirmed at autopsy.

Preparation of lymphocyte suspensions

Control *Xenopus* and those thymectomized as larvae were used in the *in vitro* experiments when 20-30 weeks old, whereas adult thymectomized animals were assayed when 12-18 months. Animals were anaesthetized in MS 222 (Sandoz), placed in a laminar flow clean air bench and swabbed (on the ventral surface) with alcohol. They were then exsanguinated by cutting the heart ventricle, which reduces the number of erythrocytes (and other circulating blood cells) in the spleen. Spleens and/or thymuses were surgically removed under sterile conditions and transferred intact to a watch glass containing a few drops of Leibovitz (L15) culture medium (modified as below), washed and excess tissue cut off. Organs were then transferred to another watch glass containing approximately 3 mls of fresh medium and, under a stereomicroscope, teased with fine forceps

so as to release the cells into the medium. The suspension was then transferred to a sterile 10 ml centrifuge tube, and the cell clumps and debris allowed to settle to the bottom for one minute. The supernatant spleen or thymus cell suspension was then transferred to another centrifuge tube and made up to 10 ml with fresh medium. The cells were washed twice by centrifugation (at 300 g for 10 minutes) and finally adjusted to a cell concentration of $5x10^6$ leucocytes/ml, using a Neubauer American Optical hemacytometer. Leucocyte suspensions were prepared at room temperature.

The medium for cell culture was modified from the one used by Du Pasquier and Miggiano (1973). L15 (Flow laboratories) + 2mM L. glutamine was diluted in the ratio 58 parts L15 : 24 parts double-distilled water, with the following additives: 0.8 parts Hepes buffer 1.0M (Welcome) (corresponds approximately to 10^{-2}M final concentration), 0.8 parts penicillin/streptomycin (Flow) (final concentration 50 i.u/ml penicillin, 50 µg/ml streptomycin), 3.3 parts 2mM mercaptoethanol ($8x10^{-5}$M). All additives were sterilized by filtration using "Millex" filters (Millipore, pore size 0.22 µm).

Mitogens

The mitogens used were:

a) PHA (M) used at concentrations (of the commercial Difco material reconstituted to 5 ml with double-distilled water) of 1:50 (corresponds approximately to 200 µg PHA/ml final concentration), 1:250 (40 µg/ml), 1: 1250 (8µg/ml), and 1:2500 (4 µg/ml).

b) Con A (Difco) used at 50, 10, 2.5, 0.5 and 0.1 µg Con A/ml final concentration.

Assay for mitogen-reactive cells

The technique was modified from the method of Weiss and Du Pasquier (1973). Forty microlitres of spleen or thymus cells ($2x10^5$ leucocytes) were distributed in individual wells of sterile Cooke microtitration plates (M 220 25 ARTL-GIBCO) with V-shaped wells. Ten microlitres of mitogen (freshly diluted with medium) were then added, while control cultures received 10 µl of culture medium without mitogen. Finally, 10 µl of heat-inactivated foetal calf serum (FCS- lot 420076 Flow) was added at 10% or 1% final concentrations. All cultures were set up at least in triplicate, using calibrated microlitre pipettes with sterile disposable tips. The cultures were incubated in humidified atmosphere at 28°C for 48 hours. They were then pulsed with 10 µl of 1:10 tritiated thymidine (i.e 1 µCi ^{3}HTdR, specific activity 5 Ci/mmol, Radiochemical Centre, Amersham) 24 hours before harvesting. This supplies the cells with a radioactive ingredient for DNA synthesis. The nuclei of those cells in which DNA is being replicated (s phase of cell cycle) become labelled over the pulse period. The cells were harvested with the aid of a Skatron cell harvester (Flow). Each row of wells was flushed for one minute with double distilled water, this transferred the cells from the culture wells to individual glass fibre filter discs, and washed away any non-incorporated ^{3}HTdR. The filters were placed in glass scintillation counting vials and the cellular material digested for one hour with 0.5 ml Protosol (New England Nuclear) at 56°C. Five ml of scintillation fluid (42 ml Liquifluor (NEN) + 1 litre of toluene) were finally added to each vial. The amount of incorporated ^{3}HTdR was counted in a Nuclear Enterprise liquid scintillation β counter.

Calculation of stimulation indices

The stimulation index (S.I.) was calculated by dividing the average counts per minute (CPM) for mitogen stimulated cultures by the average CPM of the corresponding control culture, which had not received mitogen.

S.I.'s were considered positive only when they reached 1.4 (mean (± S.E.) coefficient of variation (=S.D./mean) in cpm for triplicate cultures in preliminary experiments on the control toadlets was 19% ± 1.7 for PHA studies on spleen and thymus and 23% ± 2.0 for Con A studies on spleen and thymus. The figures are a little higher (27% ± 1.7) in the sequential tx experiments employing PHA).

RESULTS

A) Response of thymus and spleen of control toadlets to PHA and Con A

One pool of 6 toadlets was used for the 10% FCS supplementation experiments (A & C in Figures 2.1 and 2.2), while all the data for the 1% FCS experiments (B & D in Figures 2.1 and 2.2) came from organs removed from a different pool of 8 animals.

PHA stimulation (Figure 2.1 & Table 2.1)

With 10% FCS supplementation, peak S.I.'s were recorded in the spleen with 1:250 PHA (mean S.I. ± S.E. = 6.6 ± 0.6). The use of 1% FCS achieved significantly higher S.I.'s at this mitogen concentration (40 ug/ml) (15.5 ± 1.8. $P = < 0.001$ (Student's t test)) and also when 1:1250 PHA was used (mean

S.I. ± S.E. = 13.4 ± 1.9. P = <(0.01).

Results with thymic leucocytes from the same animals were comparable to the extent that 1:250 PHA proved optimal for 10% FCS supplementation (mean S.I. ± S.E. = 17.1 ± 4.3). Moreover, 1:250 and 1:1250 PHA were optimal for 1% FCS (9.9 ± 2.3; 9.2 ± 2.7 respectively). However, maximal stimulation of thymus cells with 10% and 1% FCS were not significantly different (Student's t test).

Enhancement of PHA responsiveness of spleen cells, with low conentration of FCS appeared to be related to the low background CPM (mean ± S.E. = 3,184 ± 810 with 1%FCS compared with 9,829 ± 1,123 with 10%) rather than to elevated CPM in PHA stimulated cultures under these conditions (see Fig. 2.1). In contrast, the failure of low FCS supplementation to enhance mitogen reactivity of thymus cells reflected the similarity in background CPM with 1% FCS (mean ± S.E. = 651 ± 130) and 10% FCS (1096 ± 414).

<u>Con A stimulation</u> (Figure 2.2 & Table 2.2)

With 10% FCS supplementation, peak S.I.'s were recorded in the spleen with 2.5 µg/ml Con A (mean S.I. ± S.E. = 4.6 ± 0.4). In contrast to PHA stimulation, the use of 1% FCS did not result in significantly enhanced maximal S.I.'s. Con A concentrations at 0.5 and 1 µg/ml were now necessary to produce maximal stimulation (mean S.I's ± S.E. = 11.8 ± 4.3 and 7.5 ± 1.6 respectively).

Thymocytes were also optimally stimulated by Con A at 2.5 µg/ml when 10% FCS was used (mean S.I. ± S.E. = 8.4 ± 0.5). Maximal stimulation with 1% FCS again occurred at 0.5 and 1.0 µg/ml Con A (mean S.I.'s ± S.E. = 14.5 ± 6.2 and 10.6

±5.8). The lower concentration of FCS failed to significantly enhance maximal stimulation of thymus cells by Con A.

Summary

In view of the consistency of the PHA stimulation experiments with spleen cells and because of the higher maximal S.I.'s generally recorded with this mitogen, PHA was chosen for use in the sequential thymectomy experiments, along with 1% FCS supplementation.

B) Effect of sequential thymectomy on PHA responsiveness of spleen

Figure 2.3 shows the dose response curves of spleen leucocytes from 23 sh and 29 tx toadlets to PHA using 1% FCS supplementation. Table 2.3 gives details of S.I.'s recorded. All cultures from sh toadlets displayed enhanced uptake of ^{3}HTdR following addition of mitogen at the three lower dilutions. Peak S.I.'s were recorded with 1:250 and 1:1250 dilutions (means ± S.E. were 13.9 ± 2.9 and 13.8 ± 2.7 respectively). The highest S.I. recorded at these dilutions was 73. PHA at 1:50 occasionally failed to stimulate splenocytes.

The reduced numbers of lymphocytes in spleens of tx toadlets together with the use of these spleens in other mitogen studies, not presented here, generally precluded testing lymphocytes from any tx individual with more than one PHA dose. PHA at 1:250 or 1:1250 were therefore used and S.I.'s discussed below refer to data at these optimal dilutions. Lymphocytes from all Xenopus tx at stages 48, 51, 52/3 and 54/5 failed to display PHA reactivity. Indeed S.I.'s were

nearly always (18/20 cases) < 1.0, i.e. PHA had resulted in inhibition of ^{3}HTdR incorporation (see Du Pasquier and Horton, 1976 for discussion on this point).

In contrast, thymectomy just prior to metamorphosis (st. 56/7) or in adulthood failed to interfere with PHA responsiveness. In these animals S.I.'s $\pm$ S.E. were 5.8 ± 1.6 and 9.4 ± 3.0 respectively, which did not differ significantly from S.I.'s in toadlets sh at these stages (8.9 ± 2.1 and 5.7 ± 0.5). However, it should be noted that 2/6 S.I.'s in the st. 56/7 tx animals (1.8 and 2.9) fell below the lowest S.I. recorded (3.6) with any control.

DISCUSSION

The experiments on control toadlets indicated that thymus and spleen behaved similarly in terms of mitogenic reactivity to PHA and Con A. The same concentrations of these mitogens induced maximal proliferation of splenocytes and thymocytes, and stimulation indices in the two organs were comparable. These studies therefore suggest that functionally mature lymphocytes exist in the thymus and that these cells behave similarly (in terms of mitogen reactivity) to the T cells existing in the spleen. The demonstration of good levels of reactivity to PHA and Con A in thymus cells contrasts with recent work by Donnelly et al. (1976) since they reported only minimal stimulation of *Xenopus* thymocytes with these mitogens. However, the single dilution of mitogen used in their experiments may not have been optimal for obtaining maximal stimulation, since they were working with low concentrations of cells in culture. Thus studies in this laboratory

(Alan Smith, unpublished) reveal that cultures with few cells require less mitogen for maximal stimulation. Donnelly et al. (1976) did, however, record good PHA and Con A stimulation in thymus with one fraction (low density) of bovine serum albumin -separated cells.

With respect to background CPM, the experiments reported here reveal two interesting differences between thymus and spleen. The first is that thymus cells in 10% and 1% FCS behaved similarly, unlike spleen lymphocytes which displayed higher background CPM with 10% FCS. These results suggest that splenocytes are either preferentially stimulated by the high concentration of xenogeneic protein, or are more viable under these conditions. The other feature is that background CPM of thymus cells were generally lower than spleen cells, even when 1% FCS was used. This feature has been commented on before by Weiss and Du Pasquier (1973) who suggested that either the proportion of proliferating lymphocytes may be higher in the spleen than in the thymus, or that the generation time is shorter for splenic lymphocytes. Since autoradiography shows that thymus cells (particularly cortical lymphocytes) are heavily labelled 4 hours following <u>in vivo</u> injection of ^{3}HTdR (Horton & Horton, 1975), this argues against the idea that the thymus contains a high percentage of long-lived cells. It is also possible that the culture conditions have not been ideally established for thymocytes.

The experiments on tx animals confirm previous reports that PHA and Con A responsiveness (Donnelly et al., 1976; Du Pasquier and Horton, 1976; and Manning et al., 1976) are thymic-dependent functions in <u>Xenopus</u>. Good levels of stimulation were recorded in spleen leucocytes from sh toadlets,

whereas animals thymectomized at 7-8 days failed to respond. No residual response to PHA by early-tx individuals was detected here, in contrast to that occasionally recorded by others (Donnelly et al., 1976; and Manning et al., 1976). Several explanations of residual T-cell responses in thymectomized _Xenopus_ have been put forward by Manning and Collie (1977). They suggest that these may be: a) effected by genuinely thymus-independent mechanisms; b) brought about by alternative pathways for T cell differentiation from extra-thymic sources; c) the result of incomplete functional distinction between T and B cells in amphibians; d) due to early seeding of cells from the thymus (prior to its removal) to the periphery. Since thymectomy as late as 23 days does not enhance the level of residual responses to PHA and Con A (Manning and Collie, 1977), this argues against the last explanation.

The present work has concentrated however, on the effect of thymectomy later in life, and has shown that thymectomy until stage 55 of development (31 days) abrogates PHA reactivity. In contrast, tx at 35-40 days and after metamorphosis had no apparent debilitating effect on response to this mitogen. Preliminary experiments with Con A are producing similar results, since spleen lymphocytes from two _Xenopus_ tx at stage 48 and two tx at stage 53 failed to display enhanced ^{3}HTdR uptake following Con A stimulation, in contrast to animals sh at these stages (mean S.I. $\pm$ S.E. = 9.5 $\pm$ 3.0). Tx at stage 56 (35 days) had no apparent effect on the one animal tested with Con A (S.I. = 8.5). It appears, therefore, that a permanent, self-replicating population of mitogen-reactive T cells is not established until just prior to metamorphosis.

Further discussion of this issue is left until Chapter 4. The fact that the adult *Xenopus* thymus no longer plays an important regulatory role with respect to PHA reactivity contrasts with experiments recently performed on mice, at a similar period post-adult thymectomy (Jacobs and Byrd, 1975).

TABLE 2.1 EFFECT OF FCS CONCENTRATION ON RESPONSE OF SPLEEN AND THYMUS LEUCOCYTES TO PHA

	Animals	SPLEEN S.I.'s at PHA concentrations			THYMUS S.I.'s at PHA concentrations		
		1:1250	1:250	1:50	1:1250	1:250	1:50
10% FCS	1	5.8	7.4	4.4	6.6	18.0	9.6
	2	3.7	4.3	3.0	3.6	9.3	3.9
	3	4.1	5.7	2.3	14.0	23.9	9.7
	4	5.1	8.0	6.8	-	-	-
	5	4.3	7.6	7.1	-	-	-
	6	3.6	6.8	2.8	-	-	-
1% FCS	7	10.8	15.4	8.2	5.6	13.5	11.4
	8	6.8	9.4	5.2	6.3	13.6	12.6
	9	18.2	22.5	5.3	19.1	17.4	3.5
	10	16.1	16.0	2.5	16.1	6.6	1.2
	11	10.4	12.8	5.1	2.3	4.1	2.2
	12	18.1	16.7	0.7	-	-	-

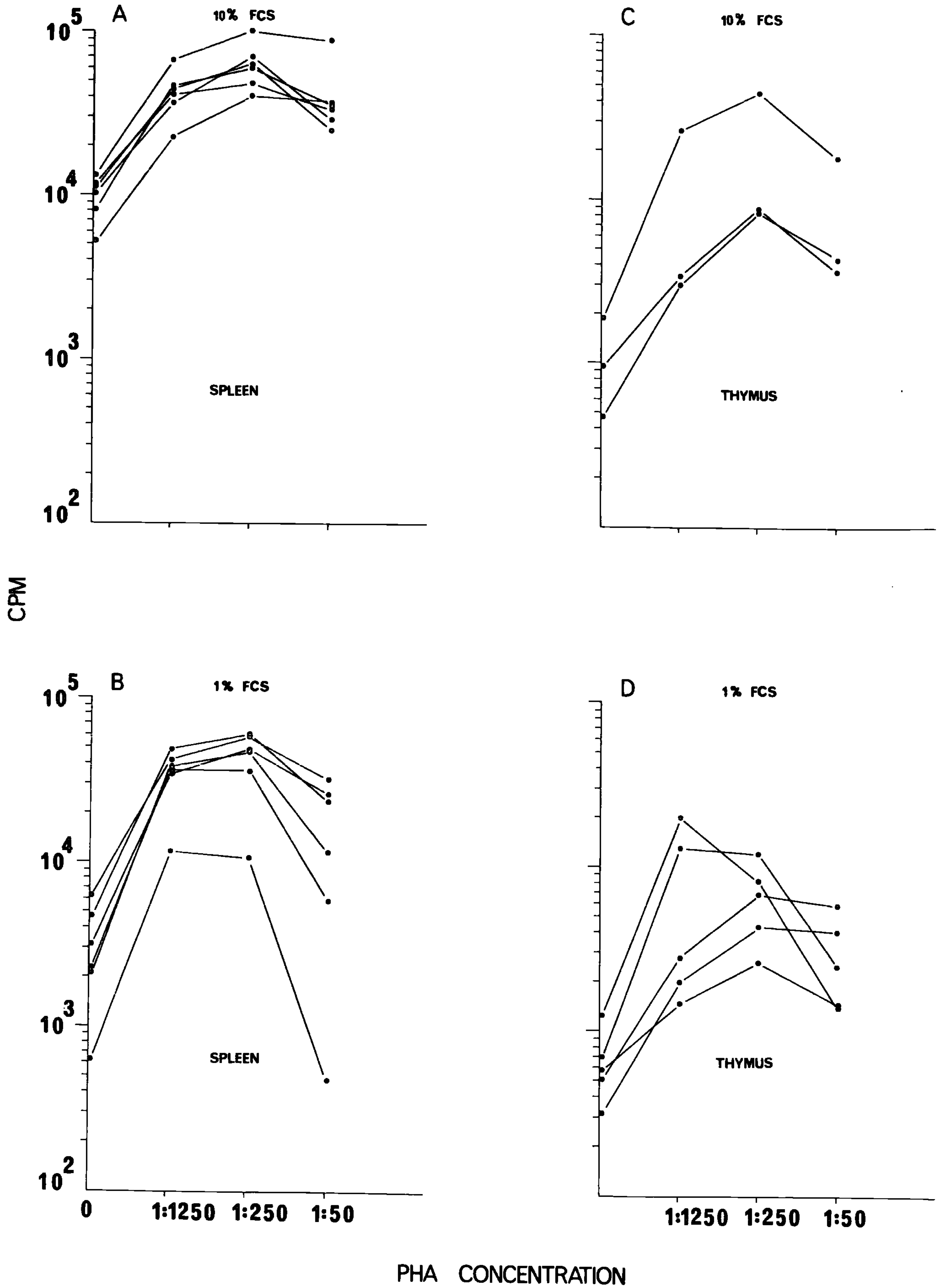
TABLE 2.2 EFFECT OF FCS CONCENTRATION ON RESPONSE OF SPLEEN AND THYMUS LEUCOCYTES TO CON A

		SPLEEN S.I.'s at Con A concentrations (µg/ml)						THYMUS S.I.'S at Con A concentrations (µg/ml)					
	Animals	0.1	0.5	1.0	2.5	10	50	0.1	0.5	1.0	2.5	10	50
10% FCS	1	-	-	2.6	5.2	1.8	0.1	-	-	1.4	3.1	0.9	0.1
	2	-	-	2.0	3.0	1.5	0.04	-	-	2.6	3.7	0.8	0.3
	3	-	-	2.6	3.8	1.6	0.02	-	-	7.4	18.3	1.0	0.1
	4	-	-	3.5	5.2	2.8	0.6	-	-	-	-	-	-
	5	-	-	-	5.4	1.6	0.2	-	-	-	-	-	-
	6	-	-	-	4.8	3.4	0.6	-	-	-	-	-	-
1% FCS	9	-	15.9	14.6	4.5	-	-	-	32.8	27.9	3.6	-	-
	10	-	5.1	5.1	1.4	-	-	-	11.2	5.9	1.1	-	-
	11	0.7	8.6	8.7	3.5	-	-	1.2	5.4	3.4	0.6	-	-
	12	2.3	31.3	6.8	0.8	-	-	1.5	8.7	5.3	1.2	-	-
	13	0.8	3.5	3.3	-	-	-	-	-	-	-	-	-
	14	0.6	6.6	6.3	-	-	-	-	-	-	-	-	-

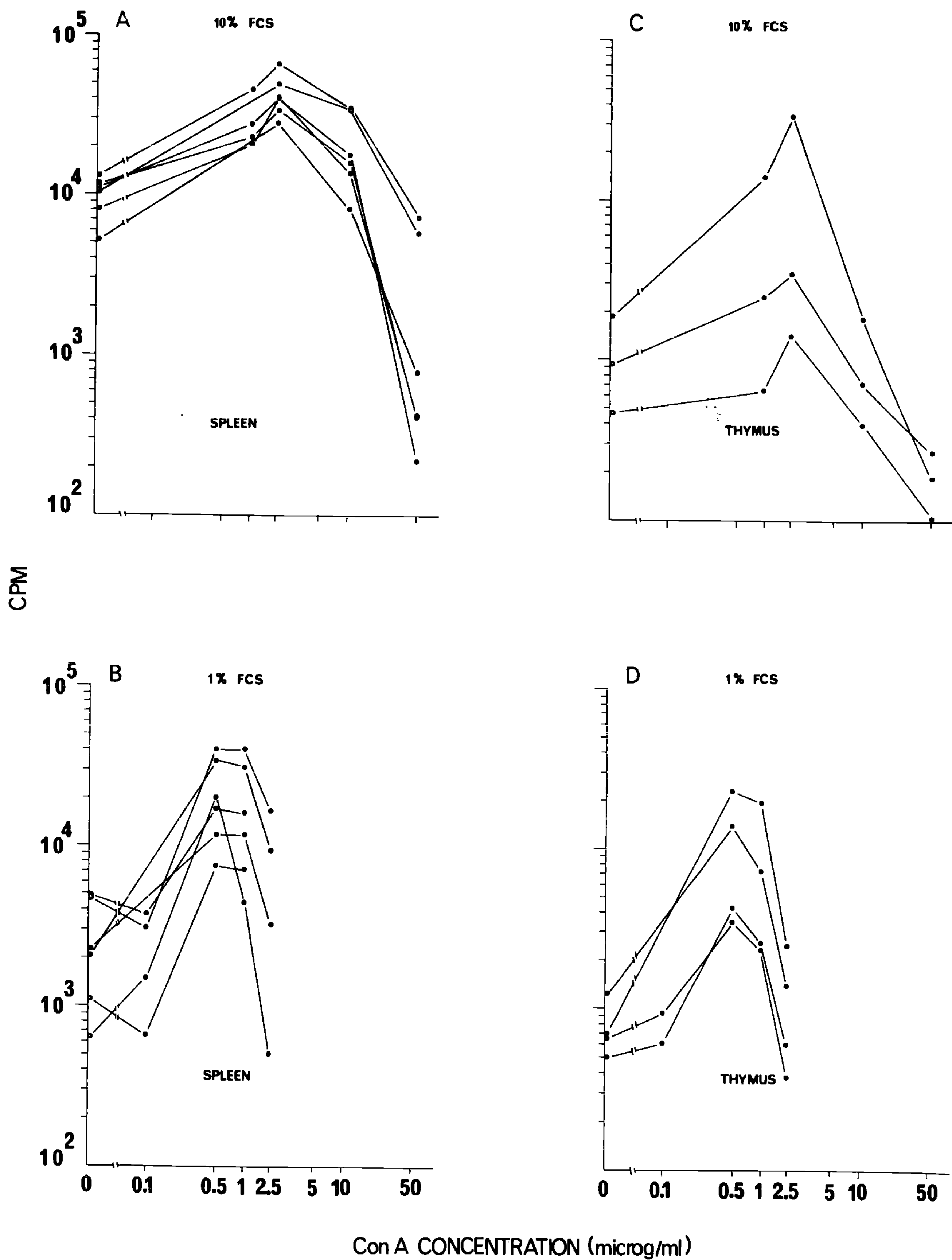
TABLE 2.3 PHA RESPONSES OF SPLEEN LEUCOCYTES:
EFFECT OF THYMECTOMY AT DIFFERENT STAGES OF DEVELOPMENT

Larval age (days) at time of operation	SHAM THYMECTOMIZED S.I.'s at PHA concentrations				THYMECTOMIZED S.I.'s at PHA concentrations			
	1:2500	1:1250	1:250	1:50	1:2500	1:1250	1:250	1:50
7-8 (st. 48)	16.8	29.9	22.4	27.4	-	-	0.52	-
	-	10.9	10.9	10.8	-	-	0.32	-
	22.1	40.7	73.0	14.6	-	0.9	-	-
	-	-	44.1	-	-	0.5	-	-
	-	-	8.0	-	-	0.6	-	-
15-16 (st. 51)	10.1	9.9	9.4	7.0	-	0.4	-	-
	-	13.1	-	-	-	0.5	-	-
	-	-	4.3	-	-	-	1.2	-
	-	-	6.1	-	-	-	0.5	-
					-	-	0.4	-
21-23 (st.52/3)	7.1	7.3	6.1	1.2	1.3	0.94	0.79	0.14
	4.9	8.6	11.3	2.2	-	0.22	1.0	-
	4.7	6.9	6.6	1.0	-	-	0.3	-
					-	-	0.6	-
					-	-	0.3	-
28-31 (st.54/5)	31.6	37.2	20.6	7.9	-	-	0.23	-
	6.9	8.7	7.5	2.5	-	-	0.21	-
	7.0	9.7	-	2.7	-	-	0.19	-
	-	11.6	-	-	-	0.7	-	-
	-	4.3	-	-	-	0.9	-	-
35-40 (st.56/7)	10.5	14.9	7.0	0.8	-	-	2.9	-
	4.8	8.6	5.4	0.6	-	1.8	4.0	0.8
					-	-	10.4	-
					-	-	11.0	-
					-	-	4.4	-
6 months (adult)	3.6	5.1	6.6	2.2	-	10.8	-	-
	4.9	7.4	6.0	1.8	-	17.4	-	-
	-	-	5.6	-	-	-	5.3	-
	-	-	3.6	-	-	-	4.1	-

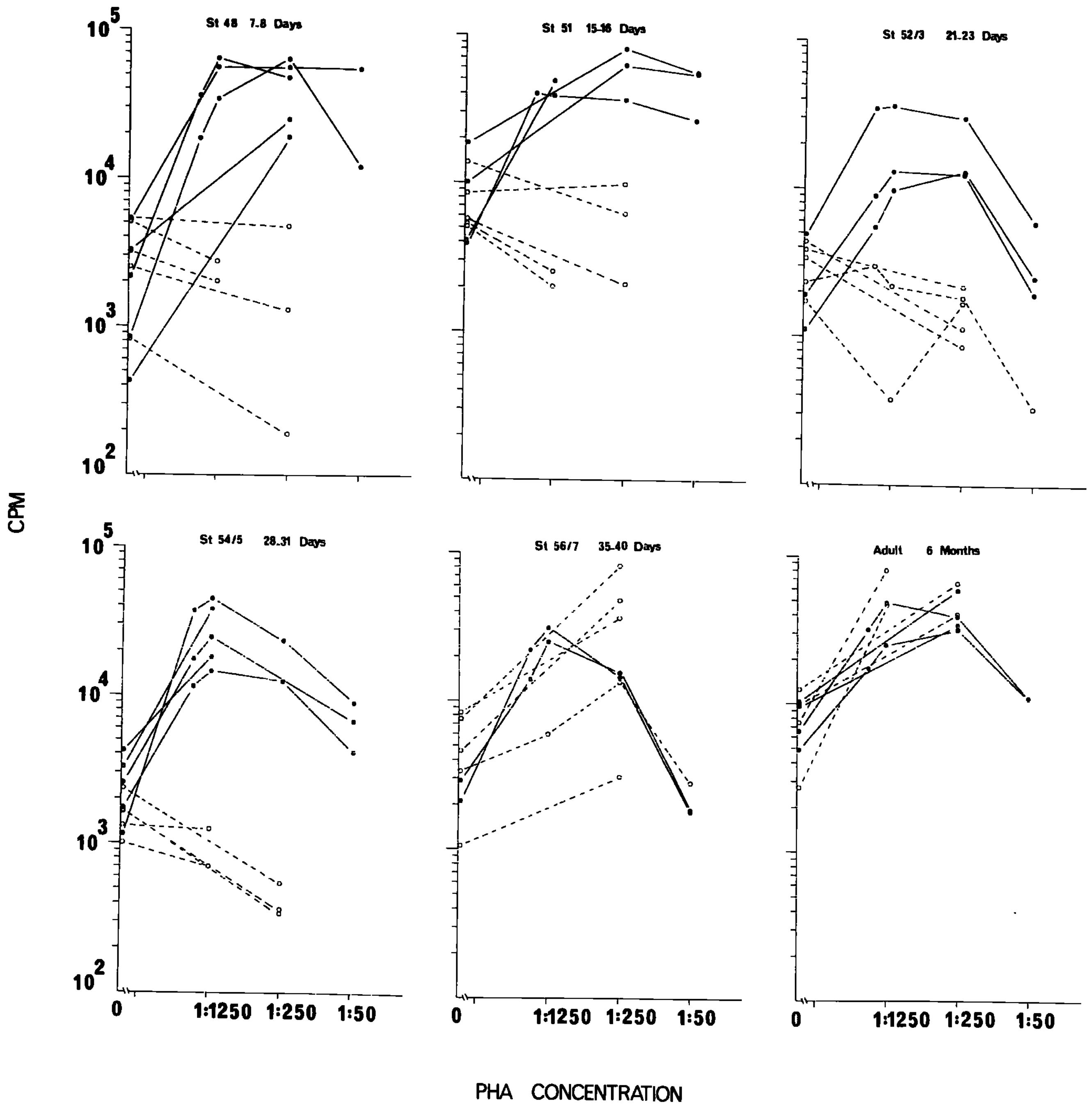
Each horizontal row of data comes from a separate individual.



2.1.
A
10% FCS
B
1% FCS
C
10% FCS
D
1% FCS
SPLEEN
THYMUS
SPLEEN
THYMUS
10^5
10^4
10^3
10^2
CPM
0
1:1250
1:250
1:50
PHA CONCENTRATION


2.2.
A
10% FCS
SPLEEN
C
10% FCS
THYMUS
CPM
B
1% FCS
SPLEEN
D
1% FCS
THYMUS
10^5
10^4
10^3
10^2
0
0.1
0.5
1
2.5
5
10
50
Con A CONCENTRATION (microg/ml)

2.3.



CHAPTER 3

MIXED LEUCOCYTE CULTURE REACTIVITY

INTRODUCTION

Reproducible levels of stimulation occur (as measured by ^{3}HTdR incorporation) when allogeneic lymphocytes are co-cultured from *Xenopus laevis* (Du Pasquier and Miggiano, 1973; Weiss and Du Pasquier, 1973), and from *Bufo marinus* (Goldshein and Cohen, 1972). MLR stimulation and acute skin allograft rejection in anurans appear to be under the control of the same polymorphic genetic region (Du Pasquier, Chardonnens, and Miggiano, 1975). It is suggested that these amphibians therefore possess a major hisotcompatibility complex (MHC) homologous to that existing in birds and mammals (Du Pasquier et al., 1975; Roux and Volpe, 1975).

Normal reaction against foreign histocompatibility antigens in anurans (as in endotherms) is dependent upon an intact thymus early in development. Thus thymectomy in the first week of life in *Xenopus* impairs (Horton and Manning, 1972; Rimmer and Horton, 1977), or possibly even abrogates (Tochinai and Katagiri, 1975) skin allograft rejection and prevents MLR (Du Pasquier and Horton, 1976). To throw more light on the ontogeny of T cells that effect these alloimmune responses, this chapter concentrates on the effect of thymectomy at later stages of development on MLR of *Xenopus* spleen cells, and compares these findings with similar *in vivo* experiments performed elsewhere. First, however, optimal conditions for MLR in control *Xenopus* were investigated.

MATERIALS AND METHODS

General

Thymectomy, breeding and rearing procedures and preparation of lymphocyte suspensions were similar to those described in detail in Chapter 2.

MLR Assay

Forty µl of the spleen cell suspensions (20 µl from each animal) were plated in individual wells of V-shaped culture plates. In mixed culture (i.e. lymphocytes from two siblings) and in the control culture (lymphocytes from one animal), the total number of lymphocytes was identical ($2x10^5$). Cultures were generally pulsed after 72 hours incubation (see Results, section A) with 1 µCi ^{3}HTdR and harvested at 96 hours. They were prepared for scintillation counting as before.

Calculation of stimulation indices

The stimulation index (S.I.) was calculated by dividing the average counts per minute (CPM) from the mixed culture by the average of the mean CPM's from the two corresponding control culture values. S.I.'s were considered positive only when they reached 1.4 (mean ($\pm$ S.E.) coefficient of variation in CPM for triplicate cultures in the sequential tx experiments was 33% $\pm$ 1.6).

RESULTS

A) Splenic MLR in control toadlets

Experiment 1 (summarised in Table 3.1.)

The optimal length of culture period for obtaining maximal MLR was examined first. L15 medium was supplemented with 10% FCS, since this was used by Weiss and Du Pasquier (1973) in successful MLR studies with *Xenopus*. MLR of spleen cells from individual animals was assayed on days 3,4 and 5 of culture. The highest MLR generally appeared to be in 4 and 5 day cultures, i.e. those pulsed at 3 and 4 days. From this experiment it was decided to adopt 72 hours as the time for pulsing in further experiments.

Experiment 2 (summarised in Table 3.2(a))

The effect of addition of allogeneic serum was examined here. Ten µl heat-inactivated serum from one adult *Xenopus* was added to each well, diluted so as to give a final concentration of 0.5%. This improved the results (i.e. higher S.I.'s), (see also Du Pasquier and Horton, 1976). However, we noticed (unpublished) that serum from some *Xenopus* do not result in better MLR S.I.'s. Ten per cent FCS was added to the medium in this experiment.

Experiment 3 (summarised in Table 3.2(b))

The effect of using less (1%) FCS was tested here. Allogeneic serum at 0.5% was again added. This improved MLR S.I.'s considerably - compare Table 3.2(b) with Table 3.2(a).

Summary

The use of Xenopus serum (from individual "A") gave higher S.I.'s with both 10% and 1% FCS. Furthermore, less (1%) FCS resulted in the best S.I.'s. The optimal culture conditions of experiment 3 were therefore used in the sequential tx experiments.

B) Effect of sequential thymectomy on splenic MLR

MLR experiments (on st. 48, 51, 52/3, 54/5, 56/7 and adult operated animals) are given in Tables 3.3 - 3.11. Where more than one Table is presented for animals tx at a particular stage, this reflects that more than one batch of toadlets was tested. S.I.'s for all experiments, which involved 32 sh and 35 tx toadlets are outlined in Fig. 3.1. All combinations between shams resulted in significant stimulation, the mean (± S.E.) S.I. being 5.1 (±0.5) (range 1.4-14.0). S.I.'s in the animals sh as adults were the highest, undoubtedly related to the particularly low levels of ^{3}HTdR incorporation in unstimulated spleen cells of these older toadlets (see Table 3.11).

Thymectomy at 7-8 days (Tables 3.3 and 3.4) abrogates this MLR. Combinations amongst 7 early-tx animals were uniformly non-stimulatory (S.I.'s ranged from 0.6-1.3). Lymphocytes from these tx animals could, however, still stimulate control lymphocytes to proliferate, since 6/8 tx X sh combinations displayed positive MLR, although S.I.'s were generally lower than in sh X sh combinations, where twice as many responder cells can be expected. Thymectomy at 15-

16 days (Tables 3.5-3.7) still had a considerable effect on MLR, since 5/8 combinations (involving 13 animals) were non-stimulatory.

In contrast to the impairment achieved by thymectomy at stages 48 and 51, ablation at all later stages of development failed to prevent splenic MLR (Tables 3.8 - 3.11). Mixtures of spleen cells from such tx animals always resulted in positive S.I.'s, although when compared with combinations involving control cells, these were often of a lower order of magnitude. Further studies with greater numbers of animals are required to test the significance of this observation. The occasional (5/41) non-stimulating sh X tx combination is probably due to matching of histocompatibility antigens in these particular siblings (see Du Pasquier and Miggiano, 1973).

DISCUSSION

The preliminary experiments on MLR in control *Xenopus* revealed good levels of stimulation, comparable to those obtained by Du Pasquier, Chardonnens and Miggiano (1975). One per cent FCS proved to be quite adequate for the present *in vitro* studies, and so the high cost of using 10% FCS supplementation (Du Pasquier et al., 1975) is avoided. As noted by Du Pasquier and Horton (1976) addition of 0.5% *Xenopus* serum potentiates MLR.

The experiments on 7-8 day tx animals reported here confirm previous studies (Du Pasquier and Horton, 1976) which showed that MLR is abolished by early thymic ablation. Moreover, thymectomy at stage 51 (15-16 days) still has a consider-

able effect on MLR. In contrast, thymectomy performed later in life has little, if any, effect. This *in vitro* finding correlates precisely with *in vivo* graft rejection studies on *Xenopus*. Thus thymectomy as late as 16 days (Horton and Rimmer, unpublished) impairs first-set allograft destruction, whereas later thymus ablation fails to debilitate (Horton and Manning, 1972). The ability to mount a normal allograft rejection in this species is therefore probably related to the emergence of MLR-positive lymphocytes in the periphery. In this respect, Du Pasquier et al.(1975) have noted that residual (chronic) allograft rejection can occur between MLR identical siblings, whereas acute graft rejection is correlated with positive MLR. The residual graft rejection capacity seen in *Xenopus* thymectomized by microcautery early in development (Horton and Horton, 1975; Horton and Manning, 1972 and Rimmer and Horton, 1977), would seem to depend on either a thymic-independent source of lymphocytes, or on very early seeding (see Tochinai and Katagiri, 1975), prior to thymic ablation of MLR-negative T cells. Whatever their origin, these lymphocytes may be responding only to minor histocompatibility antigens.

TABLE 3.1

MLR BETWEEN CONTROL XENOPUS SPLEEN LEUCOCYTES: EFFECT OF LENGTH OF CULTURE PERIOD

Animals	Pulse at (hr.)	C_a	C_b	C_c
C_a	48	7,436 ± 1159	15,166 ± 543(1.9)	15,693 ± 4,015(2.1)
	72	5,585 ± 3894	23,985 ± 959(3.2)	25,256 ± 3,302(2.4)
	96	8,858 ± 891	20,126 ± 504(2.3)	16,245 ± 5,158(2.8)
C_b	48	-	8,499 ± 1,409	13,132 ± 180(1.8)
	72	-	9,332 ± 708	20,078 ± 1,749(1.6)
	96	-	6,650 ± 713	13,843 ± 750(3.0)
C_c	48	-	-	7,194 ± 1,464
	72	-	-	15,601 ± 2,020
	96	-	-	2,682 ± 820

Results are given in (^{3}H) Counts per minute ± Standard deviation. Stimulation indices in parentheses. 10% FCS final concentration in medium. C_a to C_c are siblings. Autogeneic combinations (e.g. C_a x C_a, etc.) correspond to control values (i.e. background).

TABLE 3.2

MLR BETWEEN CONTROL *XENOPUS* SPLEEN LEUCOCYTES:

EFFECT OF VARIATION IN CONCENTRATION FCS AND ADDITION OF *XENOPUS* SERUM

a) 10% FCS supplementation

Animals	*Xenopus* serum added				medium only added			
	C_d	C_e	C_f	C_g	C_d	C_e	C_f	C_g
C_d	7,941 ± 1,726	22,696 ± 2,102 (3.2)	23,045 ± 2,954 (2.9)	21,345 ± 3,758 (2.6)	8,876 ± 1,868	19,182 ± 3,946 (2.3)	21,937 ± 1,291 (2.0)	15,894 ± 4,915 (1.8)
C_e	-	6,351 ± 1,051	29,433 ± 2,673 (4.1)	21,935 ± 1,502 (2.9)	-	7,884 ± 1,512	25,725 ± 2,432 (2.5)	5,596 ± 1,520 (0.7)
C_f	-	-	8,149 ± 2,440	16,624 ± 1,686 (2.0)	-	-	12,887 ± 2,142	19,452 ± 1,941 (1.8)
C_g	-	-	-	8,563 ± 2,196	-	-	-	8,786 ± 1,033

Results are given in (^3H) CPM ± S.D. S.I.'s in parentheses. 0.5% *Xenopus* serum final concentration added. Pulse at 72 hours. C_d - C_g are siblings.

TABLE 3.2 (contd.)

b) 1% FCS supplementation

Animals	*Xenopus* serum added					medium only added				
	C_h	C_i	C_j	C_k	C_l	C_h	C_i	C_j	C_k	C_l
C_h	2,155 + 883	28,888 ± 3,842 (4.2)	21,426 ± 5,253 (8.4)	22,717 ± 2,314 (7.0)	26,168 ± 2,761 (5.1)	2,711 ± 509	13,557 ± 793 (2.4)	10,612 ± 223 (2.2)	16,170 ± 1,898 (3.5)	11,897 ± 1,587 (2.9)
C_i	-	11,558 ± 2,947	24,807 ± 8,504 (3.4)	24,103 ± 6,220 (3.0)	30,632 ± 1,816 (3.1)	-	8,362 ± 1,724	16,736 ± 2,125 (2.2)	21,409 ± 752 (2.9)	19,777 ± 3,183 (2.8)
C_j	-	-	2,932 ± 748	30,332 ± 9,376 (8.3)	23,775 ± 10,388 (4.3)	-	-	7,024 ± 2,479	18,181 ± 1,060 (2.7)	10,986 ± 5,556 (1.7)
C_k	-	-	-	4,349 ± 2,164	32,670 ± 2,698 (5.3)	-	-	-	6,540 ± 227	n.d.
C_l	-	-	-	-	8,042 ± 1,379	-	-	-	-	5,570 ± 560

Results are given in (^3H) CPM ± S.D. S.I.'s in parentheses. 0.5% *Xenopus* serum final concentration added. Pulse at 72 hours. n.d. = not done. C_h - C_l are siblings.

TABLE 3.3

MLR BETWEEN SIBLING XENOPUS SPLEEN LEUCOCYTES:

EFFECT OF THYMECTOMY AT ST.48 (7-8 DAYS)

Animals	Sh_1	Sh_2	Sh_3	Tx_1	Tx_2	Tx_3
Sh_1	2,594 ± 506	16,628 ± 2,529 (3.4)	13,786 ± 2,764 (5.2)	9,521 ± 8,307 (1.9)	3,247 ± 147 (1.6)	6,824 ± 2,282 (1.1)
Sh_2	-	7,094 ± 609	21,477 ± 1,794 (4.4)	5,276 ± 634 (0.7)	6,700 ± 1,546 (1.6)	15,938 ± 577 (1.9)
Sh_3	-	-	2,686 ± 1,284	7,145 ± 0 (1.4)	16,395 ± 7,576 (8.1)	n.d.
Tx_1	-	-	-	7,174 ± 2,961	2,492 ± 595 (0.6)	4,778 ± 1,036 (0.6)
Tx_2	-	-	-	-	1,344 ± 0	7,221 ± 4,531 (1.3)
Tx_3	-	-	-	-	-	9,479 ± 1,953

Results are given in (^3H) CPM ± S.D. S.I.'s in parentheses.

0.5% Xenopus serum and 1% FCS added. Pulse at 72 hours.

n.d. = not done, Sh = Sham Thymectomized, Tx = Thymectomized.

one sibship used.

TABLE 3.4

MLR BETWEEN SIBLING *XENOPUS* SPLEEN LEUCOCYTES:

EFFECT OF THYMECTOMY AT ST.48 (7-8 DAYS)

Animals	Sh_4	Sh_5	Sh_6	Sh_7	Tx_4	Tx_5	Tx_6	Tx_7
Sh_4	4,273 ± 633	36,395 ± 14,113 (8.3)	11,001 ± 5,397 (3.4)	38,490 ± 9,022 (4.7)	n.d.	n.d.	n.d.	n.d.
Sh_5	-	4,467 ± 669	9,399 ± 3,307 (2.8)	n.d.	n.d.	n.d.	n.d.	n.d.
Sh_6	-	-	2,131 ± 855	n.d.	n.d.	n.d.	n.d.	n.d.
Sh_7	-	-	-	12,068 ± 7,169	n.d.	n.d.	n.d.	n.d.
Tx_4	-	-	-	-	5,362 ± 2,535	6,906 ± 3,020 (1.0)	5,895 ± 682 (1.3)	n.d.
Tx_5	-	-	-	-	-	8,738 ± 1,428	5,770 ± 2,703 (0.9)	6,903 ± 2,854 (1.3)
Tx_6	-	-	-	-	-	-	3,926 ± 2,128	n.d.
Tx_7	-	-	-	-	-	-	-	1,800 ± 657

See Table 3.3 for legend

TABLE 3.5

MLR BETWEEN SIBLING *XENOPUS* SPLEEN LEUCOCYTES:

EFFECT OF THYMECTOMY AT ST.51 (15-16 DAYS)

Animals	Sh_8	Sh_9	Sh_{10}	Tx_8	Tx_9	Tx_{10}	Tx_{11}
Sh_8	5,595 ± 3,700	13,251 ± 2,126 (2.3)	10,260 ± 918 (2.2)	n.d.	n.d.	n.d.	n.d.
Sh_9	-	6,064 ± 2,686	9,376 ± 4,949 (2.0)	n.d.	n.d.	n.d.	n.d.
Sh_{10}	-	-	3,586 ± 2,326	n.d.	n.d.	n.d.	n.d.
Tx_8	-	-	-	3,302 ± 1,139	2,290 ± 1,288 (0.9)	n.d.	n.d.
Tx_9	-	-	-	-	1,811 ± 367	n.d.	n.d.
Tx_{10}	-	-	-	-	-	7,284 ± 1,663	9,254 ± 209 (1.7)
Tx_{11}	-	-	-	-	-	-	3,878 ± 654

See Table 3.3 for legend

TABLE 3.6

MLR BETWEEN SIBLING <u>XENOPUS</u> SPLEEN LEUCOCYTES:

EFFECT OF THYMECTOMY AT ST.51 (15-16 DAYS)

Animals	Sh_{11}	Sh_{12}	Sh_{13}	Tx_{12}	Tx_{13}	Tx_{14}	Tx_{15}	Tx_{16}
Sh_{11}	4,216 ± 2,479	13,304 ± 6,411 (3.8)	8,692 ± 5,687 (2.8)	n.d.	n.d.	n.d.	n.d.	n.d.
Sh_{12}	-	2,697 ± 661	3,167 ± 1,091 (1.4)	6,735 ± 129 (3.6)	n.d.	23,642 ± 12,227 (5.8)	25,156 ± 4,066 (8.8)	n.d.
Sh_{13}	-	-	1,944 ± 671	2,679 ± 713 (1.8)	n.d.	n.d.	16,570 ± 3,846 (6.7)	n.d.
Tx_{12}	-	-	-	1,018 ± 474	3,951 ± 2,833 (2.0)	n.d.	n.d.	n.d.
Tx_{13}	-	-	-	-	2,945 ± 948	n.d.	n.d.	n.d.
Tx_{14}	-	-	-	-	-	5,485 ± 1,925	7,838 ± 3,295 (1.8)	3,054 ± 579 (0.5)
Tx_{15}	-	-	-	-	-	-	3,001 ± 1,082	3,379 ± 1,611 (0.7)
Tx_{16}	-	-	-	-	-	-	-	6,931 ± 2,696

See Table 3.3 for legend

TABLE 3.7

MLR BETWEEN SIBLING *XENOPUS* SPLEEN LEUCOCYTES:

EFFECT OF THYMECTOMY AT ST.51 (15-16 DAYS)

Animals	Sh_{14}	Sh_{15}	Sh_{16}	Tx_{17}	Tx_{18}	Tx_{19}	Tx_{20}
Sh_{14}	267 ± 103	31,325 ± 2,675 (9.8)	33,405 ± 16,359 (10.2)	n.d.	n.d.	n.d.	n.d.
Sh_{15}	-	6,145 ± 4,317	26,232 ± 5,800 (4.2)	n.d.	n.d.	n.d.	n.d.
Sh_{16}	-	-	6,303 ± 3,438	n.d.	n.d.	n.d.	n.d.
Tx_{17}	-	-	-	4,600 ± 2,568	4,753 ± 803 (1.2)	n.d.	n.d.
Tx_{18}	-	-	-	-	3,173 ± 676	n.d.	n.d.
Tx_{19}	-	-	-	-	-	1,610 ± 1,575	1,012 ± 989 (0.5)
Tx_{20}	-	-	-	-	-	-	2,249 ± 339

See Table 3.3 for legend

TABLE 3.8

MLR BETWEEN SIBLING *XENOPUS* SPLEEN LEUCOCYTES:

EFFECT OF THYMECTOMY AT ST.52/3 (21-23 DAYS)

Animals	Sh_{17}	Sh_{18}	Sh_{19}	Sh_{20}	Tx_{21}	Tx_{22}	Tx_{23}	Tx_{24}
Sh_{17}	6,281 ± 3,591	25,978 ± 4,779 (2.7)	26,190 ± 2,240 (2.5)	32,452 ± 10,643 (4.3)	20,591 ± 6,805 (4.1)	4,925 ± 751 (0.9)	22,378 ± 4,289 (3.4)	n.d.
Sh_{18}	-	12,811 ± 2,963	n.d.	41,091 ± 1,433 (3.8)	28,141 ± 8,163 (3.4)	15,810 ± 6,444 (1.8)	17,443 ± 1,450 (1.8)	n.d.
Sh_{19}	-	-	15,080 ± 182	35,873 ± 3,080 (3.0)	29,466 ± 3,733 (3.1)	n.d.	10,937 ± 1,111 (1.0)	n.d.
Sh_{20}	-	-	-	8,982 ± 3,225	9,906 ± 2,978 (1.6)	n.d.	n.d.	n.d.
Tx_{21}	-	-	-	-	3,706 ± 244	5,984 ± 1,969 (1.4)	10,586 ± 3,727 (2.0)	10,102 ± 3,106 (3.2)
Tx_{22}	-	-	-	-	-	4,763 ± 759	12,624 ± 2,273 (2.2)	8,892 ± 1,795 (2.4)
Tx_{23}	-	-	-	-	-	-	6,974 ± 3,087	8,875 ± 2,589 (1.9)
Tx_{24}	-	-	-	-	-	-	-	2,552 ± 588

See Table 3.3 for legend

TABLE 3.9

MLR BETWEEN SIBLING *XENOPUS* SPLEEN LEUCOCYTES:

EFFECT OF THYMECTOMY AT ST.54/5 (28-31 DAYS)

Animals	Sh_{21}	Sh_{22}	Sh_{23}	Sh_{24}	Tx_{25}	Tx_{26}	Tx_{27}	Tx_{28}
Sh_{21}	6,182 ± 1,408	32,888 ± 2,181 (6.6)	25,880 ± 3,891 (5.7)	26,177 ± 10,934 (4.9)	n.d.	n.d.	n.d.	n.d.
Sh_{22}	-	3,810 ± 1,898	20,412 ± 4,107 (6.0)	32,964 ± 0 (7.8)	n.d.	n.d.	n.d.	n.d.
Sh_{23}	-	-	2,946 ± 1,098	n.d.	n.d.	n.d.	n.d.	n.d.
Sh_{24}	-	-	-	4,611 ± 2,431	n.d.	n.d.	n.d.	n.d.
Tx_{25}	-	-	-	-	1,697 ± 456	5,314 ± 0 (1.9)	12,372 ± 1,516 (2.1)	30,041 ± 16,875 (5.3)
Tx_{26}	-	-	-	-	-	3,806 ± 2,866	n.d.	n.d.
Tx_{27}	-	-	-	-	-	-	10,061 ± 2,936	14,508 ± 0 (1.5)
Tx_{28}	-	-	-	-	-	-	-	9,567 ± 562

See Table 3.3 for legend

TABLE 3.10

MLR BETWEEN SIBLING XENOPUS SPLEEN LEUCOCYTES: EFFECT OF THYMECTOMY AT ST. 56/7 (35-40 DAYS)

Animals	Sh_{25}	Sh_{26}	Sh_{27}	Sh_{28}	Tx_{29}	Tx_{30}	Tx_{31}
Sh_{25}	4,081 ± 1,895	13,414 ± 2,011 (3.3)	41,069 ± 3,848 (5.6)	n.d.	n.d.	31,662 ± 8,153 (5.0)	35,527 ± 4,272 (7.2)
Sh_{26}	-	4,005 ± 2,654	21,296 ± 6,475 (2.9)	n.d.	n.d.	32,915 ± 6,544 (5.3)	14,478 ± 3,670 (3.1)
Sh_{27}	-	-	10,468 ± 2,062	n.d.	n.d.	18,753 ± 8,010 (1.8)	1,944 ± 306 (0.2)
Sh_{28}	-	-	-	8,129 ± 3,672	30,627 ± 4,139 (2.7)	16,246 ± 2,599 (2.0)	n.d.
Tx_{29}	-	-	-	-	14,161 ± 5,339	21,704 ± 5,035 (1.9)	28,435 ± 2,506 (2.9)
Tx_{30}	-	-	-	-	-	8,499 ± 6,968	22,830 ± 6,526 (3.3)
Tx_{31}	-	-	-	-	-	-	5,392 ± 2,356

See Table 3.3 for legend

TABLE 3.11

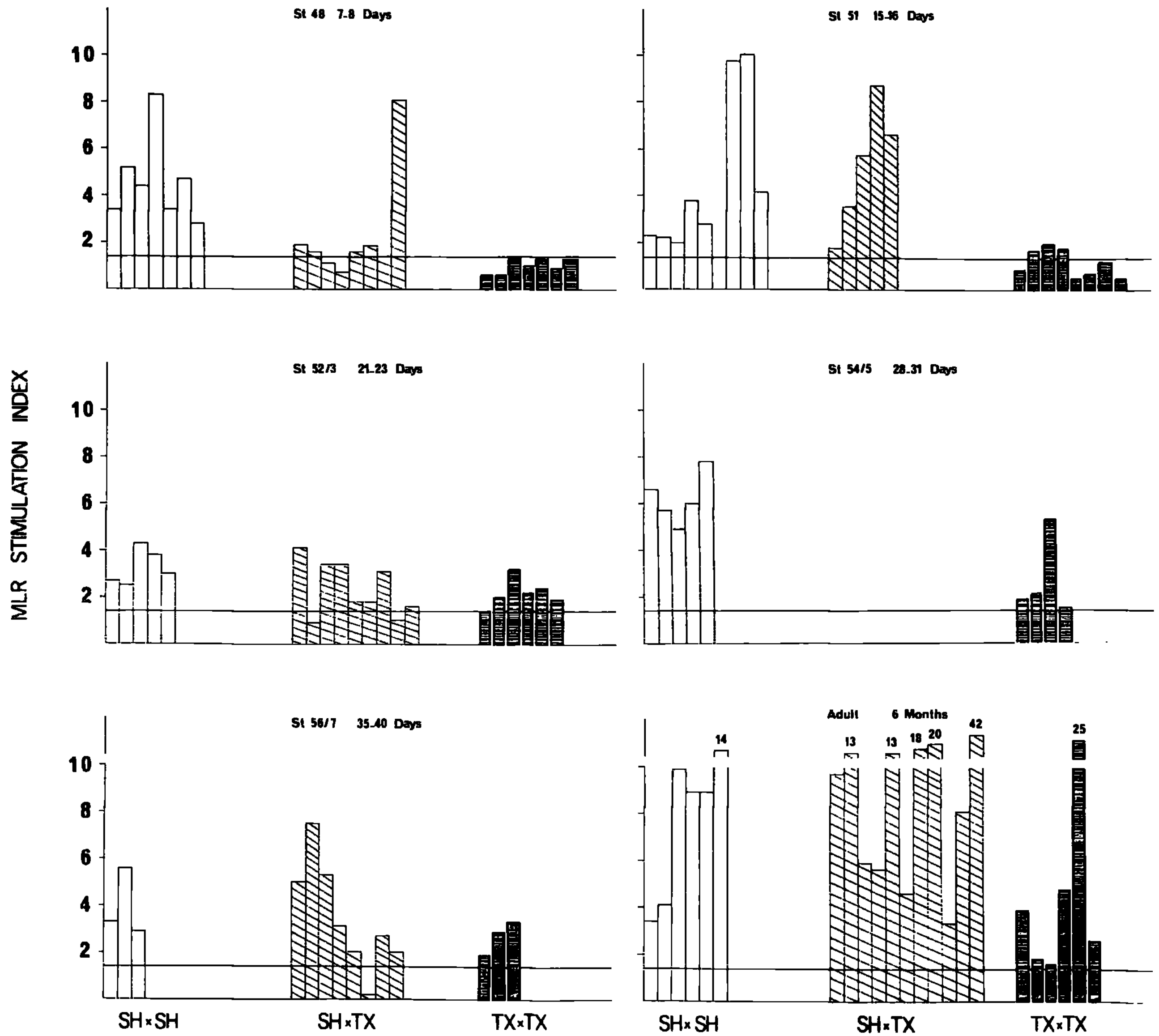
MLR BETWEEN SIBLING XENOPUS SPLEEN LEUCOCYTES:

EFFECT OF THYMECTOMY IN ADULTHOOD (6 MONTHS)

Animals	Sh_{29}	Sh_{30}	Sh_{31}	Sh_{32}	Tx_{32}	Tx_{33}	Tx_{34}	Tx_{35}
Sh_{29}	2,062 ± 1,037	5,414 ± 2,397 (3.4)	6,092 ± 5,353 (4.1)	12,130 ± 2,728 (9.9)	17,764 ± 7,488 (9.7)	14,450 ± 5,895 (13.4)	14,057 ± 4,193 (5.9)	n.d.
Sh_{30}	-	1,161 ± 567	9,073 ± 2,976 (8.9)	6,926 ± 4,763 (8.9)	7,706 ± 6,252 (5.6)	8,353 ± 3,392 (13.4)	8,961 ± 3,151 (4.6)	n.d.
Sh_{31}	-	-	876 ± 595	9,327 ± 3,322 (14.7)	22,916 ± 4,984 (18.4)	9,555 ± 3,390 (19.8)	6,010 ± 7,225 (3.3)	n.d.
Sh_{32}	-	-	-	390 ± 298	8,073 ± 4,209 (8.1)	9,983 ± 10,460 (41.8)	n.d.	n.d.
Tx_{32}	-	-	-	-	1,612 ± 884	3,290 ± 1,390 (3.9)	3,920 ± 390 (1.8)	1,642 ± 371 (1.6)
Tx_{33}	-	-	-	-	-	88 ± 30	6,772 ± 1,857 (4.8)	6,449 ± 3,319 (24.5)
Tx_{34}	-	-	-	-	-	-	2,737 ± 83	4,143 ± 3,204 (2.6)
Tx_{35}	-	-	-	-	-	-	-	437 ± 41

See Table 3.3 for legend

3.1.



CHAPTER 4

GENERAL DISCUSSION

Studies in this thesis confirm previous reports that PHA responsiveness (Donnelly et al., 1976; Du Pasquier and Horton, 1976 and Manning et al., 1976) and MLR (Du Pasquier and Horton, 1976) are thymic-dependent functions in *Xenopus*. Good levels of stimulation to both mitogen and allogeneic cells were recorded in spleen leucocytes from control toadlets, whereas animals thymectomized at 7-8 days failed to respond *in vitro*. The present work concentrated on the effect of thymectomy later in life, and has shown the thymic dependence for PHA responsiveness and MLR to be of different longevity.

MLR is generally abrogated by thymectomy performed up to 16 days of age, whereas later thymectomy has little, if any, effect. These *in vitro* findings agree with *in vivo* graft rejection studies (see Chapter 3 for discussion). Responsiveness of *Xenopus* spleen cells to PHA requires a prolonged thymic influence when compared with establishment of allo-reactive T cells. Thus thymectomy as late as 31 days abrogates PHA reactivity. Such late larval tx also abolishes antibody production to sheep erythrocytes (SRBC's)(Horton et al., 1977 and Collie, 1976). These effects of sequential tx are tabulated in Table 4.1. It should be noted that rosette-forming cell assays performed before metamorphosis (Horton et al., 1977) reveal that some seeding from the thymus of anti-SRBC reactive lymphocytes has already occurred prior to thymectomy at 21-23 days. It therefore appears that until

5/6 weeks of age, there is insufficient seeding to establish a self-replicating population of thymus-dependent cells involved in antibody production to SRBC's and that can participate in mitogenic reactivity. The possibility that sufficient early seeding occurs, but that these T cells require the continued presence of the thymus for humoral maintenance of normal function is, perhaps, unlikely in view of the lack of effect adult tx has on antibody production (Horton et al., 1977) and mitogen responsiveness.

One suggestion that emerges from sequential thymectomy studies performed in this laboratory is that "helper" function appears relatively late in ontogeny (just prior to metamorphosis) when compared with alloimmune reactivity. This ties in well with Du Pasquier and Haimovich's (1976) experiments which show that anuran larvae display normal allograft rejection and MLR, but are deficient in "IgRAA" (the 7S non-μIg of reptiles, aves and amphibians) antibody production. However, sequential tx experiments by others indicate that establishment of "helper" function to different antigens (human gamma globulin and rabbit erythrocytes) may not be a common ontogenetic event (see recent review by Manning and Collie, 1977). Obviously the precise role that the amphibian thymus plays in antibody production requires further experimentation. In this respect Charlemagne and Tournefier (1977) have recently demonstrated <u>enhanced</u> antibody responses to xenogeneic erythrocytes in early-thymectomized urodeles. The intriguing possibility therefore exists that helper, suppressor, and even antibody-forming cells might all develop within this organ in amphibians.

Heterogeneity, with regard to biological function, of amphibian lymphocytes is implicated from a variety of studies (see General Introduction in Chapter 1). That this heterogeneity is also found within the T cell population is indicated by the sequential tx experiments on Xenopus discussed in this Thesis. Furthermore, these experiments indicate that amphibian larvae should prove to be useful models for investigating the distinct possibility (Droege and Zucker, 1975 and Droege, 1976) that T cell subsets are spawned at different stages of development.

TABLE 4.1 IMMUNE RESPONSES IN *XENOPUS* THYMECTOMIZED AT VARIOUS STAGES OF DEVELOPMENT

Age (Stage) at thymectomy	Allograft rejection	MLR	PHA reactivity	Anti-SRBC antibody
7-8 days (48)	-/+	-	-	-
15-16 days (51)	-/+	-/+	-	-
21-23 days (52/3)	+	+	-	-
28-31 days (54/5)	+	+	-	-
35-40 days (56/7)	+	+	+	-/+
6 months (adult)	+	+	+	+

Key: - = No response
-/+ = Impaired
+ = Normal

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REFERENCES

CHARLEMAGNE, J. and TOURNEFIER, A. (1977): "Immunological responses of the axolotl (Ambystoma mexicanum)to erythrocytes and hapton-conjugated erythrocyte antigens. Effects of thymectomy." in Developmental Immunobiology. Eds. J.B. Solomon and J.D. Horton. North Holland Publishing Co., Amsterdam (in press).

COLLIE, M.H. (1976): "Studies on the role of the thymus and the spleen in the immune system of Xenopus laevis (Daudin)." Ph.D. Thesis, University of Hull.

COLLIE, M.H., TURNER, R.J. and MANNING, M.J. (1975): "Antibody production to lipopolysaccharide in thymectomized Xenopus." Europ. J. Immunol., 5: 426-427.

COOPER, E.L. (1976a): "Comparative Immunology." Prentice-Hall Inc. Englewood Cliffs, New Jersey.

COOPER, E.L. (1976b): "Immunity mechanisms." in Physiology of the Amphibia, 3: 164-272. Ed. B. Lofts. Academic Press, New York.

CUCHENS, M., McLEAN, E. and CLEM, W.L. (1976): "Lymphocyte heterogeneity in fish and reptiles." in Phylogeny of Thymus and Bone Marrow-Bursa Cells, pp. 205-213. Eds. R.K. Wright and E.L. Cooper. North Holland Publishing Co., Amsterdam.

de SOUSA, M.A.B. (1976): "Cell traffic." in Receptors and Recognition, Series A, 2: 103-163. Eds. P. Cuatrecasas and M.F. Greaves. Chapman and Hall Publishing Co., London.

DONNELLY, N., MANNING, M.J. and COHEN, N. (1976): "Thymus dependency of lymphocyte subpopulations in Xenopus laevis." in Phylogrhy of Thymus and Bone Marrow-Bursa Cells, pp. 133-141. Eds. R.K. Wright and E.L. Cooper. North Holland Publishing Co., Amsterdam.

DROEGE, W. (1976): "'Early T cells' and 'late T cells', suggestive evidence for two T cell lineages with separate developmental pathways. Europ. J. Immunol., 6: 763-768.

DROEGE, W. and ZUCKER, R. (1975): "Lymphocyte sub-populations in the thymus." Transplant. Rev., 25: 3-26.

DU PASQUIER, L. (1976): "Amphibian models for the study of ontogeny of immunity." in Comparative Immunology, pp. 390-418. Ed. J.J.Marchalonis. Blackwell Scientific Publications, Oxford.

DU PASQUIER, L., CHARDONNENS, X. and MIGGIANO, V.C. (1975): "A major histocompatibility complex in the toad *Xenopus laevis* (Daudin)." Immunogenetics, 1: 482-494.

DU PASQUIER, L. and HAIMOVITCH, J. (1976): "The antibody response during amphibian ontogeny." Immunogenetics, 3: 381-391.

DU PASQUIER, L. and HORTON, J.D. (1976): "The effect of thymectomy on the mixed leucocyte reaction and phytohaemagglutinin responsiveness in the clawed toad, *Xenopus laevis*." Immunogenetics, 3: 105-112.

DU PASQUIER, L. and MIGGIANO, V.C. (1973): "The mixed lymphocyte reaction in the toad *Xenopus laevis*: a family study." Transplant. Proc. 5: 1457-1461.

DU PASQUIER, L., WEISS, N. and LOOR, F. (1972): "Direct evidence for immunoglobulins on the surface of thymus lymphocytes of amphibian larvae." Europ. J. Immunol., 2: 366-370.

ELLIS, A.E. and PARKHOUSE, R.M.E. (1975): "Surface immunoglobulins on the lymphocytes of the skate *Raja naevus*." Europ. J. Immunol. 5: 726-728.

ETLINGER, H.M., HODGINS, H.O. and CHILLER, J.M. (1976): "Characterization of lymphocytes in a primitive teleost, *Salmo gairdneri*." in Phylogeny of Thymus and Bone Marrow-Bursa Cells, pp. 83-91. Eds. R.K. Wright and E.L. Cooper. North Holland Publishing Co., Amsterdam.

GOLDSHEIN, S.J. and COHEN, N. (1972): "Phylogeny of immune competent cells. I. *In vitro* blastogenesis and mitosis of toad (*Bufo marinus*) splenic lymphocytes to phytohaemagglutinin and in mixed lymphocyte cultures." J.Immun. 108:1025-1033.

GOLDSTINE, S.N., COLLINS, N.H. and COHEN, N. (1975): "Mitogens as probes of lymphocyte heterogeneity in anuran amphibians." Adv. exp. Med. Biol., 64: 343-351.

GREAVES, M.F., OWEN, J.J.T. and RAFF, M.C. (1973): "T and B lymphocytes: Origins, Properties and Roles in Immune Responses." Excerpta Medica, North Holland Publishing Co., Amsterdam.

HENRIQUES, U. (1964): "Breeding of Xenopus laevis Daudin." Acta Endocrinol., 90: 89-98.

HORTON, J.D. and HORTON, T.L. (1975): "Development of transplantation immunity and restoration experiments in the thymectomized amphibian." Amer. Zool., 15: 73-84.

HORTON, J.D. and MANNING, M.J. (1972): "Response to skin allografts in Xenopus laevis following thymectomy at early stages of lymphoid organ maturation." Transplantation, 14: 141-154.

HORTON, J.D. and MANNING, M.J. (1974): "Lymphoid organ development in Xenopus thymectomized at eight days of age." J. Morphol., 143: 385-396.

HORTON, J.D., RIMMER, J.J. and HORTON, T.L. (1977): "Critical role of the thymus in establishing humoral immunity in amphibians: studies on Xenopus thymectomized in larval and adult life." Develop. Comp. Immunol., 1: 119-130.

JACOBS, D.M. and BYRD, W. (1975): "Adult thymectomy results in loss of T-dependent mitogens response in mouse spleen cells." Nature, 255: 153-155.

JURD, R.D. and STEVENSON, G.T. (1976): "Surface immunoglobulins on Xenopus laevis lymphocytes." Comp. Biochem. Physiol., 53A: 381-388.

LEBOUTEILLER, P., KINSKY, R.G., VUJANOVIC, N., DUC, H.T. and VOISIN, G.A. (1976): "Morphological differences between thymus derived and bone marrow derived lymphocytes. 2. Electron microscopic and experimental study in unstimulated mice." Differentiation, 6: 125-143.

LOOR, F. and ROELANTS, G.E., Eds. (1977): "B and T cells in immune recognition." Wiley: Chichester (in press).

MANNING, M.J. (1971): "The effect of early thymectomy on histogenesis of the lymphoid organs in Xenopus laevis." J. Embryol. exp. Morph., 26: 219-229.

MANNING, M.J. and COLLIE, M.H. (1975): "Thymic function in amphibians." in Advances in Experimental Medicine and Biology, Vol. 64: Immunologic Phylogeny, pp. 353-362. Eds. W.H. Hildemann and A.A. Benedict, Plenum Press, New York.

MANNING, M.J. and COLLIE, M.H. (1977): "The ontogeny of thymic dependence in the amphibian Xenopus laevis." in Developmental Immunobiology. Eds. J.B. Solomon and J.D. Horton. North Holland Publishing Co., Amsterdam (in press).

MANNING, M.J., DONNELLY, N. and COHEN, N. (1976): "Thymus-dependent and thymus-independent components of the amphibian immune system." in Phylogeny of Thymus and Bone Marrow-Bursa Cells, pp. 123-132. Eds. R.K. Wright and E.L. Cooper. North Holland Publishing Co., Amsterdam.

MANNING, M.J. and TURNER, R.J. (1976): "Comparative Immunobiology." Blackie, Glasgow and London.

MARCHALONIS, J.J., Ed. (1976): "Comparative Immunology." Blackwell Scientific Publications Ltd., Oxford.

MILLER, J.F.A.P., BASTEN, A., SPRENT, J. and CHEERS, C. (1971): "Interaction between lymphocytes in immune responses." Cellular Immunol., 2: 469-495.

MÖLLER, G., Ed. (1975): "Separation of T and B lymphocyte subpopulations." Transplant. Rev., 25:

NIEUWKOOP, P.D. and FABER, J. (1967): "Normal Table of Xenopus laevis (Daudin)." North Holland Publishing Co., Amsterdam.

NOSSAL, G.J.V. (1976): "The nature of immune phenomena in mammals: (a) cellular immunity; (b) antibody formation." in Comparative Immunology, pp. 20-35. Ed. J.J. Marchalonis. Blackwell Scientific Publications Ltd., Oxford.

OPPENHEIM, J.J. and ROSENSTREICH, D.L., Eds. (1976): "Mitogens in Immunobiology." Academic Press, New York.

OWEN, J.J.T., RAFF, M.C. and COOPER, M.D. (1975): "Studies on the generation of B lymphocytes in the mouse embryo." Eur. J. Immunol., 5: 468-473.

RIMMER, J.J. and HORTON, J.D. (1977): "Allograft rejection in larval and adult *Xenopus* following early thymectomy." Transplantation, 23: 142-148.

ROUX, K.H. and VOLPE, E.P. (1975): "Evidence for a major histocompatibility complex in the leopard frog." Immunogenetics, 2: 577-589.

RUBEN, L.N. (1975): "Ontogeny, phylogeny and cellular cooperation." Amer. Zool., 15: 93-106.

RUBEN, L.N. (1976): "The phylogeny of cell-cell cooperation in immunity." in Comparative Immunology, pp. 120-166. Ed. J.J. Marchalonis, Blackwell Scientific Publications Ltd, Oxford.

RUBEN, L.N., VAN DER HOVEN, G. and DUTTON, R.W. (1973): "Cellular cooperation in hapten carrier responses in the newt (*Triturus viridescens*)." Cell. Immunol., 6: 300-314.

STOLEN, J.S. and MÄKELÄ, O. (1975): "Carrier preimmunisation in the anti-hapten response of a marine fish." Nature, 254: 718-719.

STOLEN, J.S. and MÄKELÄ, O. (1976): "Cell collaboration in a marine fish. The effect of carrier preimmunization on the anti-hapten response to nip and nnp." in Phylogeny of Thymus and Bone Marrow-Bursa Cells, pp. 93-97. Eds. R.K. Wright and E.L. Cooper. North Holland Publishing Co., Amsterdam.

TOCHINAI, S. (1976): "Demonstration of thymus-independent immune system in *Xenopus laevis*. Response to polyvinylpyrrolidone." Immunology, 31: 125-128.

TOCHINAI, S. and KATAGIRI, C. (1975): "Complete abrogation of immune response to skin allografts and rabbit erythrocytes in the early thymectomized *Xenopus*." Development, Growth and Differentiation, 17: 383-394.

TURNER, R.J. and MANNING, M.J. (1974): "Thymic dependence of amphibian antibody responses." Europ. J. Immunol., 4: 343-346.

TURPEN, J.B. (1977): "Studies of lymphopoiesis in the developing leopard frog." in Developmental Immunobiology. Eds. J.B. Solomon and J.D Horton. North Holland Publishing Co., Amsterdam (in press).

TURPEN, J.B. and COHEN, N. (1976a): "Localization of thymocyte stem cell precursors in the pharyngeal endoderm of early amphibian embryos." Cell. Immunol., 24: 109-115.

TURPEN, J.B. and COHEN, N. (1976b): "Alternate sites of lymphopoiesis in the amphibian embryo." Ann. Immunol. (Inst. Pasteur), 127C: 841-848.

TURPEN, J.B., VOLPE, E.P. and COHEN, N. (1973): "Ontogeny and peripheralization of thymic lymphocytes." Science, 182:931-933.

TURPEN, J.B., VOLPE, E.P. and COHEN, N. (1975): "On the origin of thymic lymphocytes." Amer. Zool., 15: 51-61.

VOLPE, E.P. and TURPEN, J.B. (1976): "Massive colonization of the spleen by thymic cells." in Phylogeny of Thymus and Bone Marrow-Bursa Cells, pp. 113-122. Eds. R.K. Wright and E.L. Cooper. North Holland Publishing Co., Amsterdam.

WARR, G., DECKER, J.M., DELUCA, D., MARCHALONIS, J.J. and RUBEN, L.N. (1977): "Heterogeneity of lymphocytes in the goldfish, Carrassius auratus: evidence from analysis of surface immunoglobulins and immune response to hapten-carrier conjugates." in Developmental Immunobiology. Eds. J.B. Solomon and J.D. Horton. North Holland Publishing Co., Amsterdam (in press).

WEISS, N. and DU PASQUIER, L. (1973): "Factors affecting the reactivity of amphibian lymphocytes in a miniaturized technique of the mixed lymphocyte culture." J. Immunol. Methods, 3: 273-286.

WEISS, N., HORTON, J.D. and DU PASQUIER, L. (1973): "The effect of thymectomy on cell surface associated and serum immunoglobulins on the toad Xenopus laevis (Daudin): a possible

inhibitory role of the thymus on the expression of immunoglobulins." in Proc. Symp. Phylogenetic and Ontogenetic Study of the Immune Response and its Contribution to the Immunological Theory, pp. 165-174, INSERM, Paris.

WRIGHT, R.K. and COOPER, E.L. (1976): "Phylogeny of thymus and bone marrow-bursa cells." North Holland Publishing Co., Amsterdam.

YOCUM, D., CUCHENS, M. and CLEM, L.W. (1975): "The hapten-carrier effect in teleost fish." J. Immunol., 114: 925-927.

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