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Histocompatibility Recognition in Effector and Helper T Cell Responses of *Xenopus*

by

Roger Louis Lallone

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A Thesis submitted for the degree of
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Histocompatibility Recognition in Effector and Helper T Cell Responses of Xenopus, by Roger L. Lallone.

ABSTRACT

This thesis has attempted to define systems for examining the role played by the amphibian thymus in MHC (major histocompatibility complex) restriction of cytotoxic and helper T cell reactivities. The first part of the investigation examines *in vivo* and *in vitro* reactivity to MHC and to minor H (histocompatibility) antigens. Assays used are skin graft rejection, mixed lymphocyte reactivity (MLR) and cell-mediated lympholysis (CML). Thymus-dependence of MHC and minor H antigen-disparate graft rejection is demonstrated by early thymectomy and thymus reimplantation studies. Unlike MLR, CML reactivity of spleen lymphocytes proves to be a poor *in vitro* correlate of *in vivo* skin graft rejection, since cytotoxic cells could not be demonstrated against minor H antigen-disparate targets, even following fairly rapid second-set minor graft destruction and restimulation *in vitro*. Further CML and MLR studies with *Xenopus* splenocytes revealed weak proliferative and lack of cytotoxic responses to TNP-modified self cells, in contrast to strong *in vitro* reactivities against xenogeneic cells. Overall this 'negative' data precluded the planned investigation on the role of the thymus in self MHC restriction of cytotoxic T cell killing of modified self.

The involvement of helper T cells in *in vivo* and *in vitro* antibody production to foreign erythrocyte antigens is investigated in the second half of the thesis. A primary *in vitro* plaque-forming-cell (PFC) response to rabbit erythrocytes (but not to sheep erythrocytes) is demonstrated with splenocytes taken from control toads, but not from 7-day-thymectomized (Tx) *Xenopus*. In *Xenopus* less than six months old, detection of this cellular antibody production requires homologous complement from young donors, The *in vitro* PFC response of B cells (spleen cells taken from Tx toadlets) to rabbit erythrocytes could be restored to normal only by coculture with T cells (i.e. 3000R irradiated splenocytes) taken from non-operated animals, but *not* by coculture with T cells taken from Tx toadlets bearing adult or larval MHC-compatible thymus implants. This finding precluded the use of allothymus chimeras to investigate the role of the thymus in self-MHC restriction of helper T cell cooperation with B cells.

Possible explanations for the observed immunological differences between mice and *Xenopus* and suggestions for future experiments are discussed.

List of Abbreviations

PFC	Plaque forming cell
CTL	cytotoxic T lymphocyte (cell)
MHC	major histocompatibility complex
SRBC	sheep red blood cell
RRBC	rabbit red blood cell
MØ	macrophage
APC	antigen presenting cell
T cell	thymus derived lymphocyte
B cell	antibody producing lymphocyte
MLR	mixed lymphocyte response
CML	cell mediated lympholysis
XLC'	*Xenopus laevis* complement
GPC'	Guinea pig complement
TNP	trinitro-phenylated
TNBS	trinitro-benzene-sulfonate
TNCB	trinitro-chloro-benzene
CFA	complete Freund's adjuvant
Tx	thymectomized

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Chapter One

General Introduction

Most information regarding the structure and function of genes of the major histocompatibility complex (MHC) and of the antigens for which they code has been derived from experiments involving mice. The use of intra-MHC recombinants is the most common way to examine gene structure and antigen function (see Klein, 1979). It is generally agreed that cell surface antigens of the MHC (i.e. the H-2 in mice) are involved in antigen recognition and cell interactions leading to activation of T cells and possibly of B cells (Katz et al., 1978; Singer and Hodes, 1982). Two classes of highly polymorphic MHC antigens are particularly important in the function of certain distinct sub-populations of T lymphocytes (Bach et al., 1973; Dennert, 1974a; Cantor and Boyse, 1975; Bach et al., 1976). Class I MHC antigens (coded for by H-2, K/D loci) elicit strong cytotoxic T cell (CTL) responses by providing target antigens for allogeneic-MHC-reactive CTL and interaction antigens for non-MHC-(i.e. minor antigens, viral antigens and haptens) reactive CTL (Alter et al., 1973; Nabholz et al., 1974; Forman, 1975; Shearer et al., 1975; Blanden et al., 1975; Burakoff et al., 1976; Dennert and Hyman, 1977). Class II MHC antigens (coded for by the H-2, I locus) elicit strong mixed lymphocyte reactions (MLR; as do allogeneic MIs antigens) by providing target antigens for allogeneic-MHC-reactive proliferative T cells and interaction antigens for non-MHC-reactive proliferative T cells. (Abbasi et al., 1973; Meo et al., 1973; Rollinghoff et al., 1975; Schmitt-Verhulst et al., 1977). The same class II antigens also appear to provide interaction structures for helper T cells in T cell - B cell collaboration (Armerding et al., 1974; Katz and Benacerraf, 1975) and in T cell - T cell collaboration (Schendel

and Bach, 1974; Kreeb and Zinkernagel, 1980; Lutz et al., 1981; Weinberger et al., 1981; Guimezanes andSchmitt-Verhulst, 1981).

Physical self linkages (inhibitable by cytocholasin B) appear to be required for cellular and humoral immune responses to most conventional antigens (Zinkernagel and Doherty, 1974). Cytotoxic T cell activation requires simultaneous recognition of infecting or modifying antigen and Class I MHC antigens, and antibody production requires simultaneous recognition (by a helper T cell) of foreign antigen and Class II MHC antigens at the stimulator (antigen presenting cell) and target (von Boehmer and Haas, 1976; Miller et al., 1976) or B cell (Kutz et al., 1973; Kappler and Marrack, 1976; Sprent, 1978) surfaces. Without simultaneous self MHC antigen recognition signals, cytotoxic T cells and B cells are rendered unresponsive (Kindred, 1975; Bennick and Doherty, 1980).

Cytotoxic T cells preferentially lyse antigenically modified histocompatible target cells and helper T cells preferentially cooperate with histocompatible B cells. The histocompatibility determinants involved in these interactions are limited to antigens coded for by genes of the MHC. Non-MHC (minor antigens) coded for by genes outside of the MHC, including the MIs locus (Berumen et al., 1983), are recognized as modifiers by cytotoxic T cells (in vivo as well as in vitro; see Silvers et al., 1982) and probably play no part in helper T cell - B cell interactions (Gordon et al., 1975; Bevan, 1975b, 1975c).

Two independent processes are thought to contribute to the development of such a final T cell population. During evolution the T cell must have developed a receptor with the capacity to recognize conventional antigen in the context of MHC antigens and

during ontogeny the T cell receptor repertoire must have been expanded and educated to discriminate self from non-self. Together these processes produce functional populations of self-MHC restricted or at least biased (Blanden and Andrew, 1979; Wagner et al., 1980; Good and Nossal, 1983) cytotoxic and helper T cells, which recognise foreign antigen, together with an appropriate MHC antigen, on a target or B cell surface.

Even after receiving a great deal of attention, the precise mechanism of MHC restricted antigen recognition (i.e. the structure of the T cell receptor, see Jensenius and Williams, 1982) and the mechanism for establishing self-MHC preferences (i.e. the process of T cell education, see Howard, 1978) both remain conjectural. Cell surface and MHC antigen haptenation (Forman et al., 1977; Forman, 1977a, 1977b; Henkart et al., 1977), competitive cold target, free hapten and anti-hapten antibody inhibition (Dennert, 1975; Forman, 1975; Zinkernagel, 1976a; Shevach et al., 1982), radiation chimeras (Zinkernagel and Doherty, 1974; Zinkernagel, 1976b; Pfizenmaier et al., 1976), negative selection (Wilson et al., 1977; von Boehmer et al., 1978a), clonal selection or deletion (Thomas and Shevach, 1977; Ching and Marbrook, 1979), cross reactive modified H-2 incompatible target lysis (Pfizenmaier et al., 1976; Burakoff et al., 1976) and neonatal tolerance induction (Zinkernagel et al., 1977) techniques are the more common methods used to examine the mechanism of dual specificity. Two hypotheses (with modifications) have been proposed to explain this phenomenon. The first hypothesis involves a T cell receptor composed of two independent, but functionally linked, antigen binding sites. One site recognizes self-MHC (R) and another recognizes foreign or modifying antigen (X). The second

hypothesis involves a T cell receptor composed of a single binding site. The single site recognises self-MHC and foreign modifying antigen (RX) as a neo-antigenic determinant (NAD). To date, most experimental evidence seems to be consistent with the "altered-self" hypothesis (Matzinger, 1981; Hunig and Bevan, 1982).

Fully allogeneic thymus-host, single F1 thymus into parental host, double F1 thymus into parental host, and parental thymus into F1 host chimeras created by implanting thymus tissue into either adult thymectomized, irradiated, or bone marrow reconstituted mice (Bevan, 1977; Fink and Bevan, 1978; Zinkernagel et al., 1978c; Waldmann et al., 1979) or into athymic nude mice (Zinkernagel et al., 1979; Lake et al., 1980; Kindred, 1980) are the most common methods used to examine the mechanism of establishing self (host) MHC preferences (compared in Zinkernagel et al., 1978a; Kindred, 1981). Two hypotheses have been put forward to explain the mechanism by which self (thymic) T cell tolerance is established and restriction specificity conferred. Any acceptible hypothesis must not only explain how T cell populations become self tolerant and self restricted, but must at the same time account for the fact that these two processes may be unrelated (Kindred et al., 1975; Sprent and von Boehmer, 1976; Zinkernagel et al., 1977). The first hypothesis has been derived from experiments involving semi-allogeneic thymus chimeras such as as an F_1 (AXB) host bearing a parental (AXA) thymus graft. Such experiments have shown that cytotoxic and helper T cells are able to recognise conventional antigens only in association with thymic and not with non-thymic MHC antigens. This result would tend to imply that the restriction specificity (i.e. the response phenotype) of a T cell population (possibly excluding T helper cells involved in a cytotoxic response, see Bennink and Doherty, 1978) is learned and will

correspond to the genotype of the epithelial cells (Zinkernagel, 1982) or antigen presenting cells (Longo and Schwartz, 1980) of the parental thymus in which they develop and not necessarily to the F_1 genotype of the bone marrow from which they originate. Such a T cell population might develop if pre-thymic or intra-thymic T cells (Kruisbeek et al., 1981; Morrissey et al., 1982) with a set of germ line receptors for self-MHC were positively selected, mutated, and clonally expanded in the thymus (Blanden and Ada, 1978) and if only those T cells whose receptors were not potentially autoreactive left the thymus (von Boehmer et al., 1978b; Teh et al., 1982). Once in the periphery, extra-thymic antigen presenting cells could (but would not necessarily be required to) drive their maturation and make further selections on the T cell repertoire (Zinkernagel et al., 1978b; Erb et al., 1979, Stockinger et al., 1980). A second hypothesis has been derived from experiments involving fully allogeneic thymus chimeras such as a parental (AXA) host bearing another parental (BXB) thymus. Such experiments have shown that cytotoxic T cells (but not helper T cells, see Singer et al., 1981; Singer et al., 1982) are able to recognize conventional antigens in association with either thymic or non-thymic (i.e. bone marrow) MHC antigens (Wagner et al., 1980). This result would imply that the restriction specificity may not simply be the result of thymic T cell education, but rather the result of some other process (Zinkernagel et al., 1980). Such a T cell population might develop if pre-thymic or intrathymic T cells with sets of germ line receptors for self and allogeneic MHC were negatively selected (i.e. not expanded) by the thymus (or by some other yet unidentified environmental influence;

see Miller et al., 1983) and if only those T cells whose receptors were not potentially auto-reactive left the thymus. Such a T cell population might also result if cytotoxic T cells (but not helper T cells) could develop and mature along either an intra-thymic or extra-thymic differentiation pathway (Kruisbeek et al., 1983). Once mature and present in the periphery, antigen presenting cells could (and would be required to) select out, expand, and mature self MHC restricted T cells (a step which may, see Stutman 1978, or may not, see Scollay et al., 1984, occur), while allogeneic MHC restricted T cells would have to be suppressed (Billings et al., 1978). To date, few experiments have data which suggest that allorestricted T cells may be suppressed (Pierce et al., 1976; Smith and Miller, 1980) and many others suggest that they are not (Fink and Bevan, 1978; Swierkosz et al., 1978; Zinkernagel and Althage, 1979; Zinkernagel et al., 1979).

High alloreactivity of T cells (in comparison to that of B cells) is another unexplained aspect of T cell antigen recognition, and its physiologicalrole (for example in relation to malignancy; see Burnet, 1968; Burnet, 1970) is often disputed. Structurally, most allogeneic MHC antigens differ relatively little. Limiting dilution (Waldmann et al., 1975; Ching and Marbrook, 1979) and polyclonal cytotoxic T cell activation (Bevan and Cohn, 1975; Bevan et al., 1976) techniques have revealed an unexpectedly high frequency of T cells with receptors for foreign MHC (mouse H-2) alleles (see Dutton et al., 1978). For instance, while the frequency of T cells responsive to various soluble protein or erythrocyte antigens is approximately 1 in 100,000 and to modified self (TNP)

antigens is approximately 1 in 15,000, the frequency of T cells responsive to allogeneic MHC antigens can be from 1 in 1,000 to 1 in 100 (Marrack et al., 1974; Skinner and Marbrook, 1976; Lindahl and Wilson, 1977). Monitoring the sequential development (Schmitt-Verhulst et al., 1981) or re-development of T cell subpopulations Buszello et al., 1983), selectively activating T cell subpopulations by antigen stimulation (Bevan, 1977; Lemmonier et al., 1977a; 1977b; Burakoff et al., 1978), selectively deleting T cell subpopulations by filtration through irradiated hosts (Wilson et al., 1977) or by BUdR-plus-light-induced cell suicide (Schmidt-Verhulst and Shearer, 1977; Janeway et al., 1978) or by binding to cell monolayers (Schnagl and Boyle, 1981), and using T cell receptor immunosorbents and purified MHC antigens (Binz et al., 1979) are the most common methods used to examine the relationship between modified self-reactive and allogeneic-MHC-reactive T cells. Two hypotheses have been made to explain the mechanism of alloreactive T cell responses. The first hypothesis involves a single population of T cells which bind allogeneic MHC antigens either by way of a highly cross reactive conventional antigen T cell receptor or a unique (and separate) allo antigen T cell receptor (Teh et al., 1978; Finberg et al., 1978; Lutz et al., 1980). The second hypothesis involves separate populations of T cells which bind only conventional antigens or allogeneic MHC antigens and which represent either fundamentally different T cell populations or separate parts of a continuous T cell spectrum (Cunningham, 1975; Doherty et al., 1976; Cunningham and Lafferty, 1977; Droege, 1979). To date, most experimental evidence suggests that T cell receptors are generated from a random repertoire of V genes (as suggested

by Janeway et al., 1976). However, this does not exclude the possibility that they are generated from an alloreactive repertoire (as suggested by Jerne, 1971).

It is becoming increasingly common to examine some of the remaining questions in immunology (such as the role of MHC antigens in T cell function and the role of the thymus in T cell education) in phylogenetically less advanced and embryonically more easily manipulated animals such as Xenopus. This is often because experiments using the mouse, and other more accepted mammalian (and avian) models, reach impasses which may be due to technical difficulties (related especially to difficulties in producing non-contaminated irradiation bone marrow chimeras and to difficulties in the use of nude mice (Zinkernagel et al., 1978; Wagner et al., 1980).

The amphibians, especially the anuran amphibians (frogs and toads), have received a great deal of attention from developmental biologists and, more recently, from immunobiologists. This is partially for ontogenetic reasons, since they develop free from maternal interactions through interesting and unique stages, which may be useful for examining aspects of immune development. There are also phylogenetic reasons since anurans occupy a transitional point in the development of terrestrial life, and possibly in the development of a modern integrated immune system.

The organization of the anuran immune system is in many ways homologous to that found in the mouse. In Xenopus, for example, lymphoid organs are relatively few in number and not very complex histologically; the two major organs of the immune system are the thymus and spleen (Manning and Horton, 1969;

Turner, 1973; Manning and Horton, 1982). Xenopus lymphocytes are comprised of a heterogeneous collection of B cells (antibody producing) and T cells (thymus derived) which have been tentatively identified either by differential expression of surface immunoglobulin, or by differential adherence to nylon wool (Blomberg et al., 1980; Bleicher and Cohen, 1981; Hsu and Du Pasquier, 1983). The Xenopus T cell population appears to be at least functionally heterogeneous, in that thymectomy at different stages of larval life has different effects on graft rejection and antibody responses: thus thymectomy only at early stages of development impairs the alloimmune response, while quite late larval thymectomy (Horton and Sherif, 1977) continues to interfere with help er T cell populations involved in antibody production. Xenopus T cell responses, which include acute skin graft rejection (Horton and Manning, 1972; Chardonnens, 1975), mixed lymphocyte responses (Du Pasquier and Miggiano, 1973), cell mediated lympholysis (Bernard et al., 1979) and T-dependent B cell responses (Bernard et al., 1981) appear to be controlled by antigens coded for by genes which segregate together (with some erythrocyte antigens) as a single (Du Pasquier et al., 1977) linked MHC homologue (Du Pasquier et al., 1975), termed XLA. An MHC may be a general feature of anurans, however this has only been superficially examined in relation to acute skin graft rejection in one species other than Xenopus (see Roux and Volpe, 1975). The Xenopus MHC codes for both class I and class II cell surface antigens (Flajnik et al., 1983b), while minor histocompatibility antigens (including some erythrocyte antigens) and immunoglobulin (heavy chain) genes are coded for by independently segregated genes (Du Pasquier and Kobel, 1977a; 1977b; Obara et al., 1983). The Xenopus thymus

appears to be the site to which pre-T cells migrate (Volpe et al., 1979) to be matured into both helper and cytotoxic T cells (Tochinai et al., 1976; Kawahara et al., 1980) and to be conferred with MHC restriction specificity (Flajnik et al., 1983a).

One important (theoretically and practically) and as yet unanswered question about antigen recognition and function of helper and cytotoxic T cells concerns the role played by the thymus in educating T cells as to what should be recognized as self and non-self. The experiments reported in this thesis are an attempt to define a system, using Xenopus, with which this question could be addressed. The experiments in Chapter One describe a series of attempts to adapt the in vitro chromium-51 release assay to measure the response of spleen cells from thymus-reimplanted Tx animals to MHC and non-MHC (minor) antigens and thereby examine restriction specificities of 'chimeric' cytotoxic T cells. These experiments are extended and their results confirmed in Chapter Two, in an attempt to make a preliminary survey of what types of pertinent questions concerning T cell function in Xenopus might be examined using the conventional mixed lymphocyte culture and cell mediated Chromium-51 release assays. A slightly modified amphibian culture technique is described in Chapter Three, which supports a primary immune response to foreign erythrocytes (as measured by a plaque forming cell assay). It was hoped that this culture technique might more readily support either a primary CTL response to MHC alloantigens, or even a secondary CTL response to minor alloantigens, as measured by a Chromium-51 release assay. Finally, the experiments in Chapter Four describe a series of

attempts to use the in vitro PFC assay to measure the T dependent antibody response of spleen cells from thymectomized animals placed in co-culture with spleen cells from thymus-reimplanted Tx animals, and thereby examine restriction specificities of 'chimeric' helper T cells.

Chapter Two

Effector T cells in *in vitro* proliferative and cytotoxic responses to MHC and non-MHC antigens. I. Responses to MHC but not to minor alloantigens in normal and thymus re-implanted, thymectomized animals.

Introduction

The primary purpose for the work presented in this thesis was to use the amphibian, Xenopus, to examine the role of the thymus in conferring restriction specificity on T cells. Experiments began by investigating the role of the thymus and thymic MHC antigens in generating T cells responsive to foreign alloantigens and also the role of cytotoxic T lymphocytes (CTL) in the response to MHC and non-MHC alloantigens.

It has been known for some time that early larval thymectomy adversely affects the cellular immune system of Xenopus (Tochinai, 1975; Donnelly et al., 1976; Donnelly and Cohen, 1979), although its effect on histological development may be limited (Horton and Manning, 1974a; Tochinai, 1976). Thymectomy, if performed early enough (at 7 days or earlier), impairs in vivo skin graft rejection (Horton and Manning, 1972; Tochinai and Katagiri, 1975; Kaye and Tompkins, 1983), in vitro T cell mitogen induced lymphocyte proliferation, and the in vitro mixed lymphocyte response (Du Pasquier and Horton, 1976; Horton and Sherif, 1977). Evidence that thymectomy impairs the immune response by selective deletion of thymus derived lymphocytes comes from experiments examining B cell populations and T-independent B cell responses, which tend to remain intact (Collie et al., 1975; Tochinai, 1976b; Bleicher and Cohen, 1981), and also from experiments investigating the effect of thymus reimplantation or adoptive transfer of normal thymocytes, which tend to restore immune reactivity (Horton and Horton, 1975; Tochinai et al., 1976; Kawahara et al., 1980).

The thymus is generally considered to be the sole source of T cells in Xenopus and there is as yet no evidence for extrathymic T cell maturation pathways. Recently, however, questions have been raised concerning the involvement of non-thymus derived lymphocytes in typical T cell responses such as allogeneic skin graft rejection and the allogeneic mixed lymphocyte reaction (Nagata and Cohen, 1983). For this reason some initial experiments were carried out to examine the effect of thymectomy and thymus reimplantation on in vivo allograft rejection, on in vitro proliferative responses and on in vitro allogeneic target cell lysis. Incorporated into these experiments is a preliminary comparison of the in vivo and in vitro responses to such MHC and non-MHC alloantigens, which extends previous work (Bernard et al., 1979).

Allogeneic histocompatibility antigens in Xenopus are divided into different classes according to their relative antigenic strength. Different classes of antigen elicit either acute, sub-acute, or chronic skin graft rejection and either weak, or very weak, mixed lymphocyte reactions (Du Pasquier et al., 1975). Based in part on this notion of relative antigen strength and in part on the concept of cumulative gene dose, the rate of in vivo graft rejection, together with the magnitude of the mixed lymphocyte reaction, have been used to arbitrarily assign MHC haplotypes to the various Xenopus laevis-gilli clones (Kobel and Du Pasquier, 1975) and to determine whether MHC compatible clones differ by non-MHC (minor) antigens. Clones such as LG-17 (ac) and LG-5 (ad)

are said to differ by a single MHC haplotype, whereas clones such as LG-17 (ac) and LG-15 (ac) differ only by minor antigens.

Methods

Animals: Cloned Xenopus laevis-gilli hybrids were used throughout the experiments reported in this chapter (these animals are described in Kobel et al., 1981). The animals used either for breeding or for experimentation were healthy, feeding, and free from obvious infection (Emerson and Norris, 1905; McClure, 1925; Rose, 1946; Modzelewski and Culley, 1974). First and second generation animals were obtained through artificial breedings induced by repeatedly injecting both male and female animals with human chorionic gonadotrophin (Griffin and George, Sussex, England) (Rugh, 1934a; Zwarenstein, 1937; Heilbrunn, 1939; Ryan and Grant, 1940; Gitlin, 1942). Animals were reared at constant temperature (23°C) and immunized at elevated temperature (26°C) in fresh dechlorinated and aerated water, on constant 12 hour dark-light cycles (Rugh, 1934b; Rugh, 1935). Prior to metamorphosis animals were fed hydrated nettle powder, for 3-4 months following metamorphosis toadlets were fed live Tubifex worms, and thereafter adult animals were fed a combination of live Tubifex worms and minced ox liver (Morrill, 1923; Briggs and Davidson, 1942). Xenopus laevis-gilli hybrids (clonotypes LG-17, LG-15, LG-5 or LG-3) which appear to be MHC heterozygous (haplotypes ac, ac, ad, and bd respectively) were donated by L. Du Pasquier(Basel Institute for Immunology, Basel, Switzerland) and their offspring were obtained by in vitro

activation of diploid eggs using U.V. light-inactivated sperm (Kobel and Du Pasquier, 1975; Kobel and Du Pasquier, 1970). Animals were larvally thymectomized by the electrocautery technique (Horton and Manning, 1972) at developmental stages 47-48 (Nieuwkoop and Faber, 1956) - approximately 7 days post-hatching. Some thymectomized animals were implanted subcutaneously with a single syngeneic or allogeneic thymus (from a 4 week old donor) at approximately 3 weeks post-thymectomy (Du Pasquier and Horton, 1982). All dissections and surgical procedures were carried out under light general anesthesia using MS 222 (Sandoz Labs, Basel, Switzerland) solutions of varying strengths. All thymectomy and thymus reimplantation operations were performed by Dr. John Horton (University of Durham).

Preparation of cell suspensions: Xenopus leukocyte suspensions were prepared from the spleen. Spleens were dissected out intact and manually dissociated into amphibian strength phosphate buffered saline (Flow Labs, Irvine, Scotland). Single cell suspensions were prepared by repeated pipetting. Tissue clumps were allowed to settle from suspension and were removed. Cells were pelleted by 15 minutes centrifugation (at 360 x g) and the supernatants aspirated and discarded. Prior to use all cells were washed through 2-3 cycles of centrifugation (15 minutes at 360 x g at 5°C) and gentle vortexing in fresh medium containing 1% fetal calf serum. Cells were counted, unstained, under high mangnification using a hemacytometer. Cells were resuspended in appropriate culture medium at appropriate concentrations following the final wash.

Preparation of antigens: Cells to be used as stimulators in one way mixed lymphocyte culture were inactivated in suspension (at 5×10^6 leukocytes per ml.) by 5 minutes exposure to a cobalt-60 source at a dose rate of 1200 rad/minute (6000R total dose) (Elves, 1969; Bach and Bach, 1972; Lafferty et al., 1974). The irradiation dose rates are shown in Appendix 1 and were calculated using a Fricke ferrous ion chemical dosimeter and U.V. spectrometer (Unicam SP 1805)(Weiss et al., 1956; O'Donnell and Sangslier, 1970). The irradiation doses necessary for inactivation of Xenopus spleen lymphocytes are shown in Appendix 3. Prior to use, irradiated cells were transferred to fresh medium, washed, and re-counted as previously described.

Preparation of serum: Whole blood was collected from rabbits by ear vein puncture. Undiluted blood was allowed to clot for two hours at room temperature, centrifuged (30 minutes at 360 x g), and the serum removed. Rabbit serum to be used as a source of anti-Xenopus antibody was heat-inactivated (one hour at 56°C) and stored frozen (at -20°C) until use.

In vivo immunization: Immunization of rabbits to Xenopus spleen cells was performed by injection of intact Xenopus spleen leukocytes. Cell suspensions were injected via an ear vein. Rabbits received two injections of 2×10^7 cells, each two weeks apart. Blood was collected two weeks after the final injection and rabbit anti-Xenopus serum prepared.

Immunization of Xenopus to allogeneic histocompatibility antigens was performed by applying an appropriate dorsal skin

allograft and animals bearing grafts were kept at an elevated temperature of 26-27°C (Barlow and Cohen, 1981). The host animal was left overnight in shallow water while the graft was allowed to heal in place unsutured. Animals were examined periodically for signs of rejection. Four weeks after the grafts were rejected, animals were either re-grafted or sacrificed and their spleens removed for use in vitro.

In vitro conditions: The complete tissue culture medium consisted of a 60% Leibovitz-15 (L-15, Flowlabs, Irvine, Scotland) base, diluted with double glass distilled water, and supplemented with 1% or 10% heat-inactivated (56°C for 1 hour) fetal calf serum (FCS; lot no. 29072126, Flow Labs), with 10 mM HEPES buffer, 20 mM sodium bicarbonate, 2mM **1**-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, 2.5 mg/ml Fungizone (required in our culture facility) (all from Flow Labs) and 0.05 mM 2-mercapto-ethanol (British Drug Houses, Poole, England). All tissue culture glassware was sterilized by autoclaving and all tissue culture solutions were sterilized by filtration through 0.22 μm millipore filters (Millipore S.A., Molsheim, France) (see Sykes, 1969). All cultures were incubated at 26-27°C in a water saturated atmosphere of 5% carbon dioxide in air.

Assays: In vitro proliferative responses were measured in one way mixed lymphocyte culture (Weiss and Du Pasquier, 1973) (see Appendix 4). Some in vitro proliferative responses were augmented by preculturing both responder and stimulator spleen cells for at least 72 hours without stimulating antigen (MLC responses assayed by either protocol are impaired by thymectomy). Cells were pre-

cultured in 24 well flat-bottomed plates (Linbro type 76-033-05-Flow), with 5×10^6 cells per well, at a concentration of 5×10^6 cells per ml (see Appendix 5). Pre-cultured cells were harvested by pipetting and gentle scraping of the culture well. The contents were transferred to 12 x 75 mm test tubes (Polystyrene Falcon Tubes; Beveridge Co., Newcastle, England) and washed twice before being assayed for activity. Non-primed or primed and non-precultured or precultured spleen cells to be assayed for proliferative activity were suspended in complete L-15 medium containing 1% FCS (as above). Mixed lymphocyte cultures were set up in triplicate in 96 well, conical bottom, tissue culture plates (Product number M25ARTL, Sterilin Ltd., Middlesex, England). Each culture contained 1×10^5 non-irradiated responder spleen leukocytes combined with 1×10^5 irradiated (6000R) stimulator spleen leukocytes (since the use of mitomycin-C-treated stimulator cells had adverse effects on the responder cell population) in a total volume of 0.2 ml. After a further 72 hours each culture was pulse labelled with 2μCi of tritiated thymidine (3H-TdR, TRA120, 5 Ci/mole; Amersham International). After 24 hours cultures were harvested onto glass fibre filters using a semi-automatic Skatron cell harvester (Flow Labs). Each filter was dried and placed directly into 5 ml of a toluene based PPO/POPOP liquid scintillator (Packard) for beta counting.

In vitro cytolytic responses were measured by the short term chromium-51 release assay technique (Burton et al., 1975; Bernard et al., 1979). In vitro immunization or restimulation of Xenopus spleen cells to allogeneic MHC and minor antigen disparate spleen cells was performed by culturing 5×10^6 non-primed or primed,

non-irradiated responder spleen leukocytes at a final concentration of 5×10^6 cells per ml in complete culture medium containing 10% heat-inactivated FCS. In antigen stimulated cultures, equal numbers (5×10^6) or half numbers (2.5×10^6) of irradiated (6000R) allogeneic spleen leukocytes were added. In PHA stimulated cultures, mitogenic doses (20 mg/ml of PHA-M (Difco)) were added (Rollinghoff et al., 1975). In "3 cell" experiments, 5×10^6 or 2.5×10^6 irradiated (6000R) third party allogeneic spleen leukocytes were added (Schendel et al., 1974). In amplifier T cell experiments, 10×10^6 or 5×10^6 irradiated (6000R) syngeneic (to the responder) thymocytes from 6 month old animals were added (Wagner, 1973; Cohen and Howe, 1973). Cell suspensions were cultured in 24 well flat-bottomed tissue culture plates. In vitro stimulated spleen cells to be assayed for lympholytic activity were harvested (in the same fashion as precultured cells used in MLC) on day 5 of in vitro culture and resuspended in complete L-15, containing 10% FCS. Cytotoxic test cultures were set up in 12 x 75 mm round bottomed tissue culture tubes (Polystyrene Falcon Tubes; A.J. Beveridge, Newcastle, England). Each culture contained 5×10^6 originally cultured effector spleen leukocytes combined with 5×10^4 target spleen cells (E:T ratio of 100/1) in a total volume of 1 ml. All cells used as targets were 5-day PHA-M-(20mg/ml)-induced lymphoblasts. The lymphoblasts were radioactively labelled by pre-incubation for 18 hours with 100 µCi/ml of chromium-51 (^{51}Cr, CJS.4, 350-600 mCi/ml; Amersham International). The labelled lymphoblasts were purified (to remove dead cells, small lymphocytes, and erythrocytes) by centrifugation (10 minutes at 360 x g) onto Ficoll-Isopaque density gradients (density of 1.070 g/ml). After six to eight hours, each tube was vortexed,

centrifuged (10 minutes at 360 x g) and 0.5 ml of the supernatant removed for gamma counting.

Data Collection: The criterion for skin graft rejection was taken to be complete pigment cell breakdown. Data is expressed as the number of days required for an animal to show signs of complete graft rejection. Data is presented as mean and standard error of triplicate animals.

Scintillation counting for tritium was performed by an automatic liquid scintillation counter (Packard Tricarb 300C) with programable quench curves and automatic CPM to DPM conversions. Data is expressed as a stimulation index, which represents the increased rate of thymidine uptake due to antigenic stimulation in one way mixed lymphocyte combinations. Data is presented as mean and standard error of the stimulation index for triplicate cultures (using non-stimulated cultures, containing 2×10^5 viable non-irradiated leukocytes, as a base-line). This data was calculated by dividing both the mean DPM and SEM of test cultures by the mean DPM of control cultures.

Scintillation counting for Cr-51 was performed by an automatic solid crystal gamma well scintillation counter with preset energy windows (Phillips PW4580; Medical Physics Department, Dryburn Hospital, Durham, England). Data is expressed as the percent specific chromium release, which represents the CPM ratio of test release minus spontaneous release, divided by maximum release minus spontaneous release (x100). Spontaneous release values represent the amount of chromium release by target cells alone

under identical culture conditions. Spontaneous release values were always between 25% and 30% maximum release values. Maximum release values represent the amount of chromium released by target cells cultured in water and put through three cycles of freezing and thawing. Maximum release values were always between 80% and 95% of total uptake values. The data is presented as single culture determinations.

Results

The role of the thymus in allograft rejection: Xenopus rejects first-set skin grafts from allogeneic donors usually in acute fashion and second-set skin grafts in accelerated fashion. This rejection appears to be at least partially thymus dependent. Graft rejection times for normal, thymectomized, and thymus reimplanted animals are shown in Table 2.1. Thymectomy of an LG-17 host impairs, but does not prevent rejection of skin grafts from LG-5 donors and also delays (possibly prevents) rejection of skin grafts from LG-15 donors. Larval reimplantation of a syngeneic (3 week) thymus restores rejection times for skin grafts from both donors to normal.

While the thymus may not be absolutely required for all forms of skin graft rejection, its own MHC antigens can affect in vivo reactivity to allografts. The influence of the thymus (and its genotype) on the fate of certain allografts is also shown in Table 2.1. Depending on the alleles of the donor-host combination, allogeneic thymus reimplantation can either restore reactivity toward or induce tolerance to skin grafts from LG-15 and LG-5 donors.

The role of the thymus in MLR: Xenopus also mounts a proliferative response to allogeneic cells in primary mixed lymphocyte culture.

This response has been shown elsewhere to be thymus dependent (Du Pasquier and Horton, 1976). The proliferative response of spleen cells from normal and Tx animals implanted with a larval thymus is shown in Table 2.2. Normal and syngeneic thymus reimplanted LG-17 animals mount comparable responses to LG-5 stimulators, which are stronger than those to LG-15. The response of thymectomized LG-17 animals was not tested. However, preliminary experiments showed that mixed lymphocyte responses (both with and without pre-culturing) are thymus dependent (data not shown). LG-17 Tx animals reimplanted with an allogeneic thymus also mount responses to LG-5 (and possibly LG-15) stimulators. A response to LG-5 stimulators is displayed by Tx LG-17 animals reimplanted with either an LG-5 or LG-3 thymus, even though such allogeneic thymus chimeras are unable to reject LG-5 skin grafts.

The role of the thymus in CML: Antigen specific cytotoxic cells arise in the spleen following allograft rejection and subsequent mixed lymphocyte culture. Preliminary work showed that primed cytotoxic cells from normal LG-17 animals lyse LG-5 targets. In contrast, no *in vitro* cytotoxicity was induced to LG-15 following *in vivo* rejection and *in vitro* restimulation with these cells. Levels of LG-5 and LG-15 target cell lysis by primed and *in vitro* restimulated spleen cells from normal, thymectomized, and thymus reimplanted LG-17 animals are shown in Table 2.3. Thymectomy abrogates the cytotoxic response and implantation of a syngeneic larval thymus restores the response to normal. The cytotoxic responses of animals reimplanted with an allogeneic thymus and

which did not reject skin grafts (especially from LG-5 donors) were not examined. Although the in vivo rejection (by LG-17) of both LG-5 and LG-15 allografts is thymus dependent, only the lysis of LG-5 target cells can be readily detected in a short term in vitro chromium-51 release assay.

The effect of stronger in vivo and in vitro immunization on CML: Generating an in vitro cell mediated lympholysis response to strong MHC alloantigens (which elicit acute graft rejection) requires both in vivo and in vitro pre-stimulation. In order to obtain a response to weak minor alloantigens (which elicit sub-acute graft rejection and extremely weak proliferative responses) stronger in vivo immunization and/or stronger in vitro restimulation may be required. First- and second-set skin graft rejection times for MHC and minor alloantigen disparate skin grafts are shown in Table 2.4. LG-17 hosts reject second-set grafts from both LG-15 and LG-5 donors in accelerated fashion and second-set rejection of LG-15 grafts occurs as rapidly as first-set rejection of LG-5 grafts. However, even though strong in vivo immunization (first- plus second-set allograft challenge) boosted the cytotoxic response to MHC alloantigens, it had no effect on the response to minor alloantigens. Levels of LG-15 and LG-5 target cell lysis by cytotoxic cells from LG-17 animals which rejected second-set grafts are shown in Table 2.5. Even after second-set grafting and in vitro restimulation, primed cytotoxic cells from LG-17 specifically lysed only target cells from LG-5 and not from LG-15. Also shown in Table 2.5 is the effect of adding mitogenic doses ofPHA and/or equal numbers of allogeneic third party stimulator

cells to increase proliferation during restimulation. Addition of PHA during restimulation depressed the level of lysis of both targets and addition of irradiated (6000R) third party stimulators (reciprocally either LG-5 or LG-15) seemed to increase only the level of background lysis (i.e. the level which occurs without restimulation by the specific target cell) or both targets. Addition of large numbers (5×10^6 or 10×10^6) of irradiated or non-irradiated syngeneic thymocytes had no effect on the response of LG-17 to either target (data not shown). No cytotoxic (guinea pig complement-fixing) antibody could be detected in the serum of LG-17 animals which had rejected first- plus second-set skin grafts (see Appendix 6).

Discussion

In mice, primary challenge with either MHC or minor alloantigens results in a strong immune response and secondary challenge results in a specific amnestic response (Billingham et al., 1954a; Billingham et al., 1954b; Wagner and Rollinghoff, 1976). These are associated with a strong cytotoxic T cell mediated component (Oppenheim et al., 1965; Dutton, 1966; Cerottini et al., 1970; Andersson and Häyry, 1973; Cerottini et al., 1974; Cerottini and Brunner, 1974; Alter et al., 1976) and a weak cytotoxic antibody and complement-mediated component (Biesecker, 1973). Cytotoxic responses can readily be generated in primary mixed lymphocyte culture (Häyry and Defendi, 1970; Hodes and Suedmyr, 1970) to most antigens (although the response to some minor antigens may require pre-in vivo-priming). Cellular immunity is highly dependent

on the presence of a thymus, since thymus deficient animals (athymic nude or neonatally thymectomized mice) accept allografts and xenografts (Miller, 1961; Wortis, 1971), are highly susceptible to infection, tumor induction, and wasting disease (Wilson et al., 1964; McIntire et al., 1964) and demonstrate impaired (possibly abrogated) cell-mediated cytotoxic reactions (Feldmann et al., 1972). The deficiency is directly due to a lack of T cells since it can be restored (at least partially, see Kindred and Loor, 1974) not only by thymus reimplantation, but also by thymocyte and T cell infusion later in life (Miller, 1961; Miller, 1962; Pennycuik, 1971; Pantelouris, 1971; Feldmann et al., 1972; Kindred, 1974).

The preliminary results in this chapter (together with the results of many previous experiments) suggest that most of these findings also apply to Xenopus. However, these results also point out two important features (which have already been noted by others, (see Flajnik Ph.D. Thesis, 1983) and may make the model interesting, but difficult to use, for examining the role of the thymus in cytotoxic T cell education. First, it must be determined why allogeneic thymus reimplantation restores responsiveness to third party MHC alloantigens but not necessarily to third party minor alloantigens when presented in the context of non-thymic (host) MHC antigens. Second, it must be determined why thymus reimplantation inhibits the rejection of skin grafts from the thymus donor but restores the in vitro mixed lymphocyte response to cells of the thymus donor.

Thymus reimplantation may induce tolerance to class I MHC antigens but not to class II MHC antigens and developing cytotoxic T cells may learn to recognize minor alloantigens only in the context of class I thymic MHC antigens. Cytotoxic T cells may become tolerant to and restricted to class I MHC antigens of the thymus (especially the epithelium) but proliferative (and possibly helper T cells) may not become either tolerant or restricted to class II MHC antigens of the thymus (van Ewijk et al., 1980; Rouse and Weissman, 1981).

T cells derived from the thymus implant may induce suppressor T cell mediated tolerance to thymic MHC and minor alloantigens (Chen and Splitter, 1983). Active suppression of the response to MHC and/or minor alloantigens shared between the thymus and skin graft donors used in these experiments may prevent the in vivo graft rejection and be reflected in the in vitro mixed lymphocyte response (Kindred, 1976; Kindred and Sordat, 1977).

There are two other features of the present study that also warrant further comment. First, in Xenopus, it has been shown that in vitro cytotoxicity requires both in vivo and in vitro restimulation to reach detectable levels. Second, it has been shown that little, if any, cytotoxic T cell response to minor alloantigens can be detected (in the short term release assay), even after 'rigorous' in vivo and in vitro immunization schedules.

One explanation for these findings is that the culture conditions used to generate CML responses to MHC alloantigens (Kindred, 1980) and non-MHC alloantigens (MacPhail et al., 1982)

or the PHA lymphoblast targets used to measure CML responses to minor alloantigens (Bevan and Cohn, 1975; Hale and Paulus, 1979) may limit the sensitivity of the assay. The use of poor or inappropriate experimental techniques may make in vitro CML a poor correlate of skin graft rejection and may make it impossible to either generate or detect a primary in vitro response to MHC antigens and even a secondary response to minor antigens. Another explanation might be that all alloantigens (i.e. both MHC and minor) in Xenopus may be considerably weaker than their mammalian counterparts. If antigen strength is proportional to the frequency of antigen reactive lymphocytes present, in vivo priming plus in vitro restimulation may be required to elevate the numbers of MHC alloantigen reactive cytotoxic and/or helper T cells (Bevan, 1976; Finberg et al., 1979) to detectable levels. Even with priming, however, the numbers of MHC reactive T cells in thymectomized animals, or of non-MHC reactive T cells in normal animals, may never reach levels detectable at E:T ratios of 100:1.

A third explanation is that cytotoxic T cells in Xenopus may require activation signals from specialized antigen presenting cells, (Pettinelli et al., 1979; Czitrom et al., 1982; Sunshine et al., 1982; Silvers et al., 1982; Czitrom and Liddell, 1983) such as MHC antigen-bearing macrophages or dendritic cells (Steinman and Witmer, 1978), which may be in limited numbers in the spleen. Spleen cells capable of presenting class II MHC alloantigens in vitro may require prior in vivo antigen contact, while cells capable of presenting minor alloantigens (plus self class II MHC)(Bevan 1976) may be in too low numbers (Lu et al., 1979) to generate a response in vitro even after in vivo priming.

Finally, *in vitro* cytotoxic T cell (especially spleen T cell) responses in *Xenopus* may be highly suppressed (Rich and Rich, 1974). High numbers of suppressor cells, activated either in mixed lymphocyte culture (Hirano and Nordin, 1976a; Hirano and Nordin, 1976b; Fitch et al., 1976; Crosier and Broom, 1981) or by mitogen stimulation (Peavy and Pierce, 1974), may dampen down *in vitro* responses; these may be overridden by *in vivo* priming with MHC alloantigens, but are not overridden and may be enhanced by *in vivo* priming with minor alloantigens (in contrast to the effect of *in vivo* priming in mice (Starzinski-Powitz et al., 1976; MacPhail and Stutman, 1982).

Table 2.1 Primary allogeneic skin graft rejection times of normal, early larval thymectomized, and syngeneic or allogeneic thymus reimplanted animals at 26°C.

Skin graft Recipient	Skin Graft Donor	
	LG-15(ac)	LG-5(ad)
normal LG-17(ac)	36.0±0.8	27.3±0.7
Thymectomized LG-17	>100 (NR)	70 ±20.2
TxLG-17 plus LG-17 Thymus	27.3±1.7	21.3±0.7
TxLG-17 plus LG-5 Thymus	57.3±6.5	>100 (NR)
TxLG-17 plus LG-3 Thymus	>100 (NR)	>100 (NR)

number of days required to complete skin graft rejection

NR = not rejected

mean number of days ± SEM

Responder animals are 6 month old LG-17

3 animals per group

Table 2.2 Levels of proliferation in one way MLR by normal and various thymus reimplanted animals.

MLR Responder	6000R MLR Stimulators		
	LG-17(ac)	LG-15(ac)	LG-5(ad)
normal LG-17 (ac)	1.5±0.1	1.9±0.3	4.1±0.3
thymectomized LG-17	ND*	ND*	ND*
TxLG-17 plus LG-17 thymus	1.7±0.1	2.0±0.1	5.0±0.4
TxLG-17 plus LG-5 thymus	1.5±0.1	1.7±0.1	3.2±0.1
TxLG-17 plus LG-3 thymus	1.3±0.1	1.2±0.1	2.7±0.2

Levels of stimulation in one way MLC

Test MLC was set up using responder and stimulator cells which had been pre-cultured for 96 hours prior to assay.

ND = not done using LG-17; however previous experiments using outbred animals have shown that thymectomy abrogates the MLR as measured by this protocol.

Mean stimulation index ± SEM

Responder cells are from 6 month old LG-17

Triplicate cultures from pools of 3 animals.

Table 2.3 Levels of specific target cell lysis in Cr-51 release by normal and thymus reimplanted animals.

Cytotoxic Effector	Cr-51 labelled target cell	
	LG-15(ac)	LG-5(ad)
normal LG-17 (ac)	-1%	35.5%
thymectomized LG-17	0%	2.1%
TxLG-17 plus LG-17 thymus	9.1%	50.5%
TxLG-17 plus LG-5 thymus	ND	ND
TxLG-17 plus LG-3 thymus	ND	ND

Percent specific Cr-51 release from labelled lymphoblast targets

Target cell lysis was measured following first set skin graft rejection and one way restimulation

ND = not done because skin grafts were not rejected

% specific Cr-51 release of single cultures

Effector cells are from 6 month old LG-17

R/s ratio = 2/1 E/T ratio = 100/1

Individual cultures from pools of 3 animals

Table 2.4 Primary and secondary allogeneic skin graft rejection times of normal animals (LG-17) at 26°C.

LG-17 Host	Skin Graft Donor		
	LG-17	LG-15	LG-5
FIRST SET	NR	42.5±1.1	25.6±0.3
SECOND SET	NR	20.6±0.9	16.3±1.4
$\bar{d}$	-	21.0±0.6	9.3±1.7

Number of days required to complete skin graft rejection

NR = not rejected

mean number of days ± SEM

Responder animals are 6 month old LG-17

3 animals per group

Table 2.5 Levels of specific target cell lysis in Cr-51 release by animals which were *in vivo* primed by skin grafting and whose spleen cells were *in vitro* restimulated by a variety of protocols.

In vitro restimulator	Cr-51 labelled target cell	
	LG-15(ac)	LG-5(ad)
no stimulators	0%	2.5%
LG-15 stimulators	9.1%	22.5%
LG-5 stimulators	23.0%	79.3%
LG-15 plus LG-5 stimulators	22.7%	65.7%
PHA only stimulator	-1%	-3%
PHA plus LG-15 stimulators	-4%	ND
PHA plus LG-5 stimulators	ND	-1%
PHA plus LG-15 plus LG-5	-2%	-1%

Percent specific Cr-51 release from labelled lymphoblast targets

Target cell lysis was measured following first plus second set skin graft rejection and one way restimulation

ND = not done

% specific Cr-51 release of single cultures

Effector cells from 9 month old LG-17

R/s ratio = 2/1 PHA conc = 20μg/ml E/T ratio = 100/1

Individual cultures pooled from 4 animals

Chapter Three

Effector T cells in *in vitro* proliferative and cytotoxic responses to MHC and non-MHC antigens. II. Responses to allogeneic and xenogeneic MHC antigens, but not to allogeneic minor or hapten-modified-self antigens.

Introduction

In the previous chapter it was confirmed that with *in vivo* priming (by skin grafting) and *in vitro* restimulation (by MLC) *Xenopus* spleen cells could be induced to lyse allo MHC antigen disparate target cells. However, even using strong (antigen specific and non-antigen-specific) *in vitro* stimulation protocols (i.e. involving the use of mitogens and third party allogeneic cells) non-*in vivo* primed CTL could not be induced to lyse MHC disparate target cells, nor could *in vivo* primed CTL be induced to lyse minor antigen disparate target cells. In light of these apparent differences between cytotoxic responses of *Xenopus* and of mice and also between major and minor histocompatibility antigen responses of *Xenopus*, it was decided to carry out a more comprehensive (though admittedly limited due to limited access to suitable *Xenopus* strains) survey of the types of responses which could be examined using the Chromium-5^1 release assay.

Experiments are currently underway in a variety of laboratories designed to examine immune responses to various types of cell surface antigens in *Xenopus*. Some of these experiments are concerned with the role of the thymus in the development of tolerance and reactivity to minor alloantigens (DiMarzo and Cohen, 1982; Flajnik et al., 1983a), others are concerned with MHC restricted recognition of virally infected or hapten modified cells (Watkins and Cohen, 1983; Lallone and Horton, 1983), and still others are concerned with the thymus and T cell dependence of xenoantigen recognition (Knowles et al., 1981; Clothier et al.,

1983). The experiments reported in this chapter were carried out to enable comparisons to be made between *in vitro* proliferative and cytotoxic T cell responses to major and minor alloantigens with responses to hapten modified (TNP) self MHC and xenogeneic MHC antigens.

In vitro proliferative responses in MLC to allo MHC antigens do not require *in vivo* priming in *Xenopus*. However, allo-immunization (by skin grafting) will specifically increase stimulation indices (Barlow and Cohen, 1981). *In vitro* cytotoxic responses in short term CML to allo MHC antigens strictly require *in vivo* priming followed by *in vitro* restimulation to reach detectable levels (Bernard et al., 1979). Therefore, incorporated into these experiments is an examination of the effect of *in vivo* and *in vitro* priming on proliferative and cytotoxic responses. The effect of using pre-cultured spleen cells rather than fresh spleen cells in the MLR assay is also examined.

Methods

Animals: Cloned *Xenopus laevis-gilli*, outbred *Xenopus laevis* and outbred *Xenopus tropicalis* were used in these experiments. Outbred animals were purchased commercially (Xenopus Ltd., Surrey, England). LG animals were bred and reared as previously described (Chapter 2).

Preparation of cell suspensions: *Xenopus* leukocyte suspensions were prepared from the spleen and from peripheral blood. Spleen cell suspensions were prepared as previously described (Chapter 2).

Peripheral blood was collected either by cardiac puncture or femoral vein puncture, and diluted into heparinized (5 units heparin/ml; Flow Labs, Irvine, Scotland) amphibian strength (0.77 x mammalian strength) phosphate buffered saline (Flow Labs, Irvine, Scotland). Erythrocytes were removed by 20 minutes centrifugation (at 100 x g) through Ficoll-410- (Pharmacia Labs, Uppsala, Sweden) Isopaque (Nyegaard Co., Uppsala, Sweden) density gradients (density of 1.100g/ml) (Boyum, 1968; Harris and Ukaejiofo, 1970). Peripheral blood leukocytes were aspirated from the density interface. Prior to use, the cells were washed and counted as previously described (Chapter 2).

Preparation of antigens: Cells to be used as antigen for in vivo immunization or as stimulators in one way mixed lymphocyte culture were inactivated by irradiation (6000R) as previously described (Chapter 2).

Irradiated leukocytes to be hapten modified were resuspended in amphibian strength phosphate buffered saline containing 1mM trinitrobenzene sulfonic acid (at pH 7.3) (TNBS; Sigma Chemical Co., Poole, England) (Arrottini and Garvin, 1972; Forman, 1977; Clement and Shevach, 1979; Shearer et al., 1979; Polisson et al., 1980). After 10 minutes incubation (at 29°C) the hapten coupling reaction was stopped by addition of excess fetal calf serum and the cells were washed. The presence of TNP groups on the surface of hapten treated cells was verified by F. Cribbin (University of Durham), by indirect fluorescence, using rabbit anti-DNP-BSA antibody and FITC-labelled goat anti-rabbit Ig antibody (both

from Miles-Yeda). Prior to use, cells were washed and recounted as previously described (Chapter 2).

In vivo immunization: Immunization of Xenopus to allogeneic or xenogeneic spleen cells was performed either by application of an appropriate skin graft (as previously described in Chapter 2), or by dorsal lymph sac injection of an appropriate suspension of 5×10^6 fresh, irradiated (6000 R) spleen and blood leukocytes. Animals were sacrificed either four weeks following the final stages of graft rejection or four weeks following the final injection.

Immunization of Xenopus to TNP-modified self spleen cells was performed either by subcutaneous injection of 0.1 mg of trinitrochlorobenzene (TNCB; British Drug Houses, Poole, England) emulsified in 0.2 ml of complete Freund's adjuvant (CFA; Grand Island Biological, New York, U.S.A.) or by dorsal lymph sac injection of a suspension of 5×10^6 fresh TNP-modified (10 minutes in 1mM TNBS at 29°C), irradiated (6000 R) blood leukocytes (Friedlander et al., 1973; Thomas et al., 1977; Fugiwara et al., 1979; Finberg et al., 1979). Animals were sacrificed either 7 or 21 days following the injection of TNCB in CFA, or 4 weeks following the final injection of TNP-modified leukocytes.

In vitro immunization: Immunization of Xenopus spleen cells to allogeneic (MHC and minor disparate), xenogeneic, or hapten (TNP) modified syngeneic spleen cells was performed as previously described (Chapter 2). All cultures were incubated for 5 days, harvested, and prepared for assay as previously described (Chapter 2).

Assay: In vitro proliferative and lympholytic responses to allogeneic (MHC and minor disparate), xenogeneic, or hapten (TNP)

modified syngeneic spleen cells were measured as previously described (Chapter 2).

Data collection: Scintillation counting for tritium and Chromium-51 was performed and data is expressed and presented as previously described (Chapter 2).

Results

In vitro proliferative and cytotoxic T cell responses: The experiments reported here confirm that non-immune spleen cells proliferate in response to alloantigens in one or two way mixed lymphocyte culture and that stimulation indices can be non-specifically augmented by preincubation of responder and stimulator cells for 3-5 days prior to the assay (see Appendix 5). Other experiments have shown that even using culture conditions which support an *in vitro* primary antibody response, the *in vitro* cytotoxic response to alloantigens can be detected only after *in vivo* pre-immunization (see Appendix 8).

The role of T cells in the response to MHC and minor alloantigens: MHC antigen disparities elicit a stronger *in vitro* proliferative response than do minor antigen disparities. The levels of stimulation which occur between non-primed or primed LG-17 responder cells and irradiated (6000 R) LG-5 or LG-15 stimulator cells is shown in Table 3.1. In direct one way mixed lymphocyte culture, the response to both LG-5 and LG-15 is low. Skin graft rejection has little priming influence on either response. Incubating both responder cells and stimulator cells (separately) for 5 days, without antigen (except those present in FCS), prior to MLC

augments both responses. Together in vivo priming and pre-culturing act synergistically, and using such a procedure LG-5 stimulator cells still elicit a stronger proliferative response by LG-17 responder cells than do LG-15 stimulator cells.

MHC antigen disparities, but not minor antigen disparities, elicit in vitro cytotoxic responses detectable in the short term assay. The levels of cell mediated lympholysis which occur after 7 hours between in vivo primed and/or in vitro primed LG-17 effector cells and either LG-5 or LG-15 target cells is shown in Figure 3.1. Non-in vivo primed and/or non-in vitro restimulated LG-17 effector cells do not lyse either LG-5 or LG-15 target cells. After in vivo first- or first- plus second-set skin graft rejection and in vitro one way restimulation, appropriately primed LG-17 effector cells lyse LG-5 target cells but not LG-15 targets. The reciprocal combination was not examined, due to a limited number of LG-17 and LG-15 animals, and no other combinations were examined since suitable clones were not available for study.

The role of T cells in the response to modified self MHC antigens: Allogeneic cells elicit stronger in vitro proliferative responses than do TNP-modified self cells. The levels of proliferation which occur between non-primed or primed (TNCB sensitized) responder cells and irradiated (6000 R) allogeneic or TNP-modified MHC compatible stimulator cells is shown in Table 3.2. In direct one-way mixed lymphocyte culture, the response to both allogeneic and TNP-self cells is low. TNCB-CFA sensitization for 7 days has little effect on either response and TNCB-CFA sensitization for 21 days has a detrimental effect on both responses. Incubating

responder cells with or without equal numbers of irradiated (6000 R), TNP coupled, self cells and incubating stimulator cells (separately) without irradiation or haptenation for 3 days prior to assay augments both responses. In all cases, the proliferative response elicited by allogeneic cells is higher than the response elicited by TNP-self cells. The detrimental effect seen as a result of TNCB/CFA priming may be due to non-specific effects of CFA alone, since such effects were not observed when immunization was carried out using haptenated, Ficoll-Isopaque separated, peripheral blood leukocytes, or thioglycollate-induced peritoneal exudate cells.

Allogeneic, but not TNP-modified, syngeneic cells elicit in vitro cytotoxic responses in the short term assay. The level of cell mediated lympholysis which occurs between in vivo primed (allogeneic or TNP-self PBL injected) and/or in vitro primed effector cells and allogeneic or TNP-self target cells is shown in Figure 3.2. Non-in vivo primed and/or non-in vitro primed effector cells do not lyse either allogeneic or TNP-self target cells. After one or two in vivo immunizing injections and in vitro one way restimulation, effector cells lyse allogeneic target cells but not TNP-self target cells.

Similar proliferative and lympholytic results were found using a variety of different haptens with different chemical natures (including FITC, arsanilic acid and sulfanilic acid) and using a variety of different Xenopus responder strains.

The role of T cells in the response to xenogeneic MHC antigens: Allogeneic and xenogeneic cells elicit *in vitro* responses which are comparable in strength. The levels of proliferation which occur between non-primed or primed *Xenopus laevis* responder cells and irradiated (6000 R) *Xenopus laevis* (allogeneic) or *Xenopus tropicalis* (xenogeneic) stimulator cells is shown in Table 3.3. In direct one way mixed lymphocyte culture, the response to both allogeneic and xenogeneic cells is low. Skin graft rejection has little priming influence on either response. Incubating both responder cells and stimulator cells (separately) for 3 days, augments both responses. Together both *in vivo* priming and pre-culturing act synergistically and using such a procedure the levels of proliferation elicited by allogeneic cells and by xenogeneic cells are similar.

Both allogeneic and xenogeneic cells elicit *in vitro* cytotoxic responses in the short term assay. The levels of cell mediated lympholysis which occur between *in vivo* primed and/or *in vitro* primed *Xenopus laevis* effector cells and either *Xenopus laevis* or *Xenopus tropicalis* target cells is shown in Figure 3.3. Non-*in vivo* primed and/or non-*in vitro* primed effector cells do not lyse either allogeneic or xenogeneic target cells. After first- or first- plus second-set graft rejection and *in vitro* one way restimulation, effector cells lyse both allogeneic and xenogeneic target cells. The levels of lysis elicited by allogeneic and by xenogeneic cells are similar.

The reciprocal combination (i.e. *X. tropicalis* versus *X. laevis*) was not examined due to a limited number of *X. tropicalis*

available commercially and no other xenogeneic combinations were examined since other Xenopus species were not available.

Discussion

In mice, cytotoxic (presumably T) cells appear to be responsible for immune responses to cells carrying foreign MHC antigens (Cerottini et al., 1970), foreign minor antigens (Bevan, 1975b), hapten-modified self MHC antigens (Shearer, 1974), and xenogeneic MHC antigens (Carnaud et al., 1977). Target cell destruction is purely cell mediated and responses can be measured in vitro using either the tritiated-thymidine uptake assay (this technique is used in both one- and two-way mixed lymphocyte culture), or the short-term Chromium-51 release assay (this technique is used at effector to target cell ratios between 10:1 and 100:1. The allo-antigen (and possibly the xenoantigen) responses are directed toward foreign MHC antigens recognized as individual determinants (or as interaction determinants, see Fathman and Nabholz, 1977) expressed on the target cell surface, whereas both minor alloantigen and modified self antigen responses are directed towards antigens that are recognized as interaction determinants (Bevan, 1975) associated with self MHC antigens.

The results in this chapter demonstrate that using identical immunization and assay techniques, Xenopus spleen cells can be induced to proliferate (although stimulation indices are low) in response to allogeneic (MHC and minor), hapten-modified self, and xenogeneic antigens, but cannot be induced to lyse (at effector to target ratios not exceeding 100:1 and assay times not exceeding

8 hours) target cells carrying either hapten-modified self or minor allogeneic antigens. Although this study is limited in its overall scope, in that it has not examined how Xenopus responds to all minor alloantigens (such as those in normal outbred and inbred populations) or to all modified self antigens (such as virally-infected or tumour-transformed cells), or to all xenogeneic antigens (such as those in other Xenopus and non-Xenopus species), these initial in vitro comparisons might tend to suggest that care should be taken when interpreting the results of in vivo experiments examining how the thymus is involved in establishing the immune response to such antigens, and especially how it is involved in educating self MHC restricted cytotoxic cells. First, it must be determined why in vivo responses to some xenogeneic and allogeneic MHC antigens appear to be thymus independent and yet the in vitro responses to these antigens appear to involve cytotoxic T cells. Second, it must be determined why modified self and minor alloantigens appear to elicit thymus dependent in vivo immune responses (which seem even to be restricted by thymic MHC antigens in chimeric animals) and yet appear to elicit no in vitro cytotoxic T cell response.

While all foreign histocompatibility antigens in Xenopus may elicit cytotoxic T cell responses, only the strongest of these antigens may be able to elicit responses which can be detected either in vivo (in thymectomized animals) or in vitro (in normal animals). The strongest antigens may tend to be MHC alloantigens; MHC xenoantigens of animals such as Xenopus tropicalis may closely resemble alloantigens of Xenopus laevis, so that the two are

responded to in similar fashion (Dennert, 1974b; Dennert and Hyman, 1977; Lindahl and Bach, 1975). Cytotoxic T cells may be responsible for mediating *in vivo* responses, such as skin graft rejection (in all animals to all antigens), but in some cases (such as modified self and minor alloantigens) the response may be too weak to be detected using conventional assays such as MLR and CML.

Cytotoxic T cells in normal *Xenopus* may mediate responses to foreign MHC antigens (including xeno-MHC) but not to foreign non-MHC antigens (including hapten-modifying self MHC). The T cell receptor may not readily accommodate non-MHC antigens, or non-MHC antigens may not readily form appropriate cell surface associations on appropriate antigen presenting cells required for activation of helper and/or cytotoxic T cells (Schmitt-Verhulst and Shearer, 1975; Matsunaga and Simpson, 1978; Pettinelli et al., 1979; Gualde et al., 1982; Heber-Katz and Shevach, 1980). Encountering such antigens may alter normal immuno-regulatory circuits (Scott and Long, 1976; Long and Scott, 1977; Tagart, 1977; Ptak and Rozycka, 1977; Butler et al., 1981; Tagart et al., 1978) and result in activation of non-T cell mediated immune mechanisms, and even suppression of the cytotoxic T cell.

Many individual (possibly cross-reacting) non-MHC antigen (i.e. modified-self) reactive cytotoxic (Starzinski-Powitz, 1976) and/or helper (Levy et al., 1980) T cell clones may be deleted or suppressed during development in *Xenopus*, leaving the T cell repertoire deficient in non-MHC antigen- (Chardonnens and Du-Pasquier) and even MHC antigen- (DuPasquier et al., 1979) reactive cells. During metamorphosis, a variety of new minor histocompatability

antigens arise and tolerance to these must be readily established in order to prevent autoimmune reactions (DuPasquier and Bernard, 1980; DiMarzo and Cohen, 1982; see also Triplett, 1962). In order to accommodate such changes cytotoxic T cells with specificity for new adult minor antigens may be deleted, and at the same time possibly because of cross-reactivity between modified-self and foreign MHC antigens) allogeneic and xenogeneic MHC specific clones may also be deleted. This may help to account both for the lack of response to many randomly chosen modified self antigens and the comparative weakness of the response to MHC alloantigens.

The presence of a strong cytotoxic T cell-independent component of the immune response may help to account for the ability of early thymectomized Xenopus to survive reductions in lymphocyte numbers, possibly without (see Tochinai, 1975; Tochinai, 1976) chronic wasting disease, and enable them to reject allogeneic (Horton and Manning, 1972) and xenogeneic skin grafts (Clothier et al., 1983). Non-cytotoxic T cell mediated immune mechanisms may mediate the response to allogeneic MHC antigen disparate skin grafts in thymectomized animals (Kindred and Shirrmacher, 1980), and similar mechanisms may mediate the response to allogeneic weak non-MHC antigen disparate skin grafts in normal animals (Hildemann and Mullen, 1973). Many examples of cytotoxic T cell independent cell mediated immune mechanisms can be found in animals which are either phylogenetically more advanced or more primitive than Xenopus (Keissling et al., 1976; Cohen and Horan, 1977; Herberman and Holden, 1978). Such immune mechanisms include cytotoxic antibody (Winn et al., 1973; Jooste and Winn, 1975;

Jooste et al., 1981), cytotoxic and phagocytic macrophages (Dennert and Lennox, 1973; Haskill et al., 1976; Cabilly and Gallily, 1981a; 1981b), natural killer (NK) cells (Keissling et al., 1975; 1976; Nemlander et al., 1983), and antibody-dependent cytotoxic cells (ADCC) (Dennert and Lennox, 1972; Goldstein et al., 1973; Haskill et al., 1977).

Future experiments could be designed to determine whether antibody-dependent cell mediated immune responses (Jurd and Doritis, 1977) are involved in the cytotoxic response to hapten (TNP) modified syngeneic cells in either thymectomized or normal Xenopus.

Table 3.1 In vitro proliferative responses in allogeneic one way mixed lymphocyte culture.

LG-17 Responders	6000R irradiated stimulators		
	LG-17	LG-5	LG-15
Non-precultured			
non-grafted	0.8±0.1	1.5±0.1	1.2±0.2
LG-5 grafted	0.7±0.1	2.7±0.1	1.3±0.1
LG-15 grafted	0.9±0.1	1.8±0.1	1.2±0.1
72 hr precultured			
non-grafted	1.5±0.1	4.1±0.4	2.5±0.1
LG-5 grafted	1.7±0.3	12.9±0.6	2.1±0.2
LG-15 grafted	1.4±0.1	6.0±0.2	4.1±0.4

Levels of stimulation in one way MLC

Test MLC was set up using responder and stimulator cells removed directly from the spleen or which had been pre-cultured for 72 hours prior to the assay

MLR was measured using non-primed animals or following first set skin graft rejection

Mean stimulation index ± SEM

Responder cells are from 9 month old LG-17 and stimulator cells from LG-5 and LG-15 animals

Triplicate cultures from individual animals

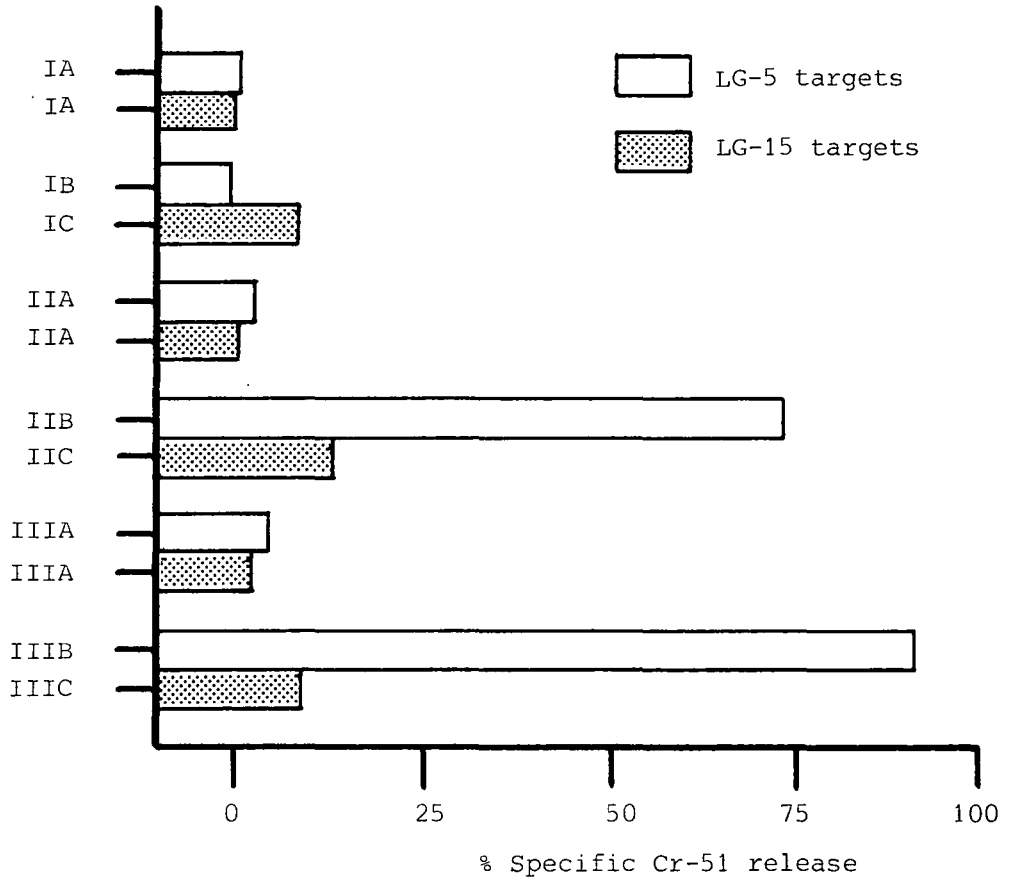


Figure 3.1 Levels of *in vitro* six hour antigen specific target lysis by spleen cells taken from normal 9 month old *Xenopus* LG-17 which were either;

(I) non primed
(II) primed by single set grafting of skin from either LG-5 or LG-15 animals
(III) primed by double set grafting of skin from either LG-5 or LG-15 animals

Prior to the assay, spleen cells were put into mixed lymphocyte culture for 5 days, with either;

(A) no stimulator cells
(B) LG-5 stimulator cells
(C) LG-15 stimulator cells

R/s ratio = 2/1 E/T ratio = 100/1

Individual cultures from pools of 4 animals

Table 3.2 In vitro proliferative responses in hapten modified syngeneic one way mixed lymphocyte culture.

X.L. Responders	6000R Irradiated Stimulators		
	SELF	ALLO	TNP-SELF
Non-precultured			
non injected	0.9±0.2	3.4±0.2	0.9±0.1
non injected	1.4±0.2	5.5±0.1	1.3±0.2
7 day CFA injected	1.5±0.1	4.7±0.3	0.9±0.1
7 day TNCB/CFA injected	0.8±0.1	3.8±0.2	1.1±0.1
21 day CFA injected	1.2±0.1	1.4±0.1	0.9±0.1
21 day TNCB/CFA injected	0.9±0.1	0.9±0.1	1.5±0.1
72 hour precultured			
non injected	1.0±0.2	8.1±0.5	3.2±0.3
7 day TNCB/CFA injected	0.9±0.1	12.2±0.7	2.0±0.2
21 day TNCB/CFA injected	1.0±0.1	1.0±0.1	0.8±0.2
72 hour boosted TNP-self			
non injected	0.9±0.1	6.1±0.2	1.4±0.1
7 day TNCB/CFA injected	1.2±0.1	14.8±0.8	1.5±0.1
21 day TNCB/CFA injected	0.8±0.1	1.3±0.1	0.9±0.1

Levels of stimulation in one way MLC

Test MLC was set up using responder and stimulator cells removed directly from the spleen or which had been precultured for 72 hours prior to the assay.
MLR was measured using non-primed animals or following single injection of TNCB emulsified in CFA.
Mean stimulation index ± SEM
Responder cells and stimulator cells from 9 month old outbred animals
Triplicate cultures from individual animals.

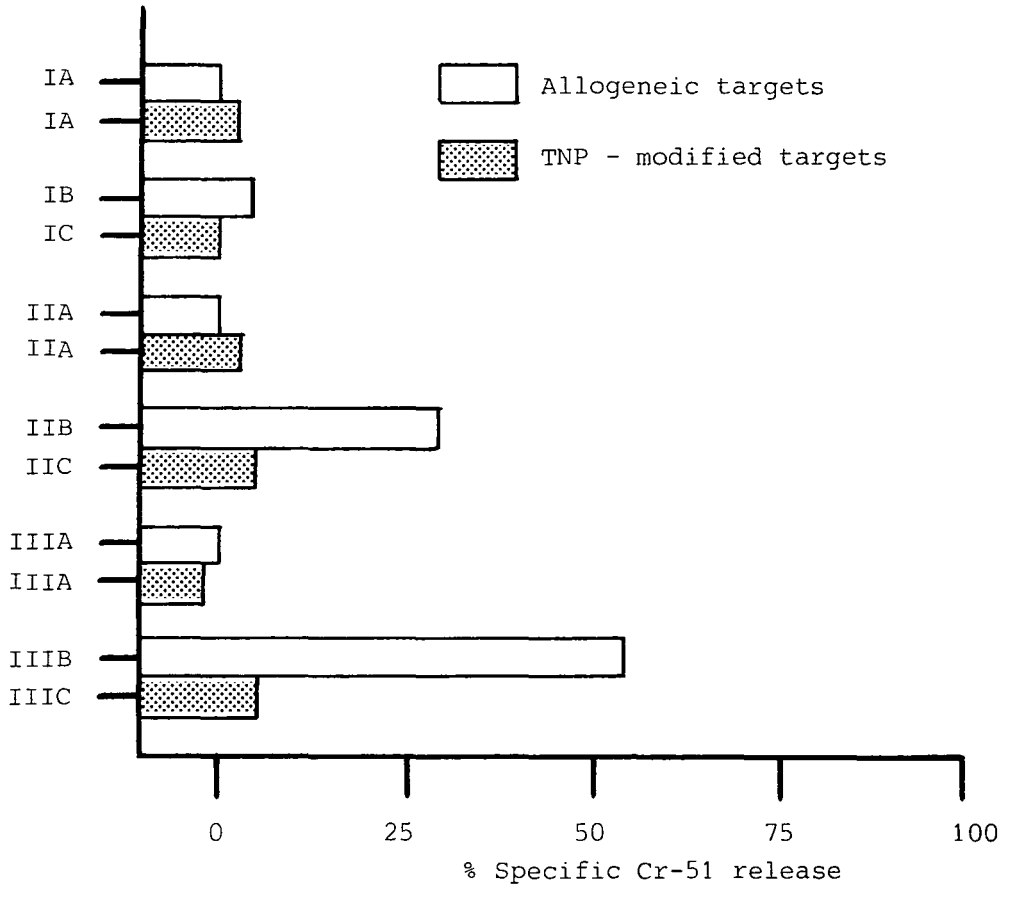


Figure 3.2 Levels of *in vitro* six hour antigen or hapten (TNP) specific target cell lysis by spleen cells taken from normal 9 month old *Xenopus laevis* (G-line) which were either;

(I) non-primed
(II) primed by single injection of 5×10^6 either TNP G-line or LG-17 cells
(III) primed by double injection of 5×10^6 either TNP G-line or LG-17 cells

Prior to the assay, spleen cells were put into mixed lymphocyte culture for 5 days with either;

(A) no stimulator cells
(B) LG-17 stimulator cells
(C) TNP modified G-line stimulator cells

R/s ratio = 1/1 E/T ratio = 100/1

Individual cultures from pools of 4 animals.

Table 3.3 In vitro proliferative responses in Xenogeneic one way mixed lymphocyte culture.

X.L. Responders	6000R Irradiated Stimulators		
	SELF	ALLO	XENO
non-precultured			
non-grafted	0.9±0.02	1.4±0.03	2.2±0.1
allo grafted	1.0±0.05	2.2±0.1	2.0±0.1
xeno grafted	0.9±0.1	1.1±0.05	2.7±0.2
72 hour precultured			
non-grafted	1.1±0.03	2.5±0.2	3.5±0.04
allo grafted	1.2±0.01	4.6±0.2	2.3±0.06
xenografted	1.2±0.02	3.3±0.3	8.4±0.3

Levels of stimulation in one way MLC

Test MLC was set up using responder and stimulator cells removed directly from the spleen or which had been pre-cultured for 72 hours prior to the assay.

MLR was measured using non-primed animals or following first set skin graft rejection.

Mean stimulation index ± SEM

Responder cells are from 9 month old outbred Xenopus laevis and stimulator cells from Xenopus laevis or tropicalis animals.

Triplicate cultures from individual animals.

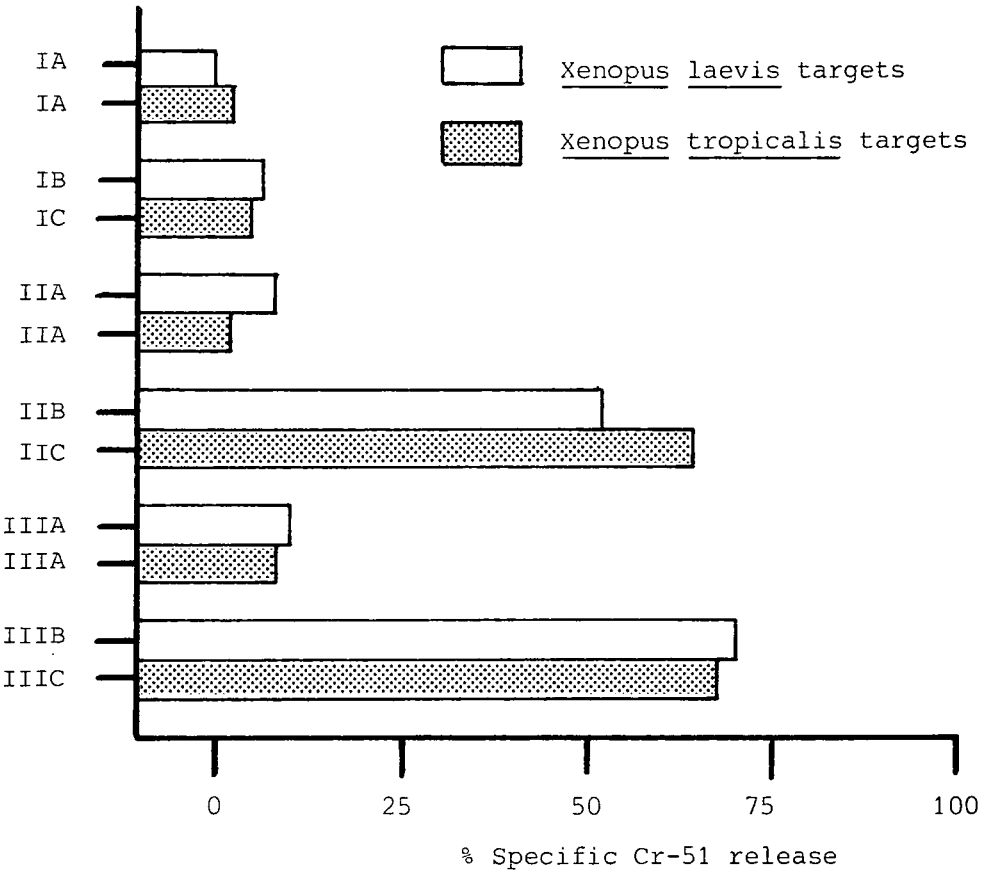


Figure 3.3 Levels of in vitro six hour antigen specific target cell lysis by spleen cells taken from normal 12 month old Xenopus laevis (outbred) which were either:

(I) non primed
(II) primed by single set grafting of skin from either X.L. or X.T. animals
(III) primed by double set grafting of skin from either X.L. or X.T. animals

Prior to the assay, spleen cells were put into mixed lymphocyte culture for 5 days with either;

(A) no stimulator cells
(B) Xenopus laevis stimulator cells
(C) Xenopus tropicalis stimulator cells

R/s ratio = 2/1 E/T ratio = 100/1

Individual cultures from individual animals.

Chapter Four

Helper T cells in *in vitro* plaque forming cell responses to sheep and rabbit erythrocytes. I. Responses to rabbit (but not sheep) erythrocytes detected in young animals using homologous (but not guinea pig) complement.

Introduction

In the previous two chapters it was confirmed that the generation of allogeneic MHC antigen reactive CTL in Xenopus requires a primary in vivo immunization phase and a secondary in vitro restimulation phase (see also Bernard et al., 1979). Both phases are antigen specific and in vitro restimulation requirements could not be circumvented, or overridden, by non-specific antigen or mitogen stimulation. It was also shown that even after primary immunization (either by first- or first-plus second-set skin grafting) and secondary restimulation, neither minor alloantigen nor hapten-modified-self antigen reactive CTL could be detected. This is in sharp contrast to the situation in mice, where MHC alloantigen reactive CTL can be induced either by in vivo or in vitro primary immunization alone, and where minor alloantigen reactive CTL can readily be detected after both immunization and restimulation. In the light of these differences, questions were raised concerning possible deficiencies in the culture conditions used for in vitro immunization and/or for the Cr-51 release assay. It seemed important therfore to examine whether or not similar culture conditions would be able to support a primary Mishell-Dutton (1966; 1967) type antibody response in Xenopus.

Such standard amphibian culture conditions (with minor modifications) have been utilized before to generate in vitro antibody responses in Xenopus (and in other amphibians such as Bufo marinus; see Azzolina, 1975). A good response to soluble

protein antigens has been elicited in culture of dissociated Xenopus spleen cells; however, no response could be detected without prior hapten and carrier priming (Blomberg et al., 1980). A primary response to heterologous (sheep) erythrocytes (either intact or sonicated) has been elicited in cultures of Xenopus spleen fragments (Auerbach and Ruben, 1970); however, this response required at least 14 days to become readily detectable by a direct plaque forming cell assay. The experiments reported in this chapter were carried out to examine whether or not culture conditions (similar to those used in the cytotoxicity assay) could be modified enough to support a primary in vitro PFC response (kinetically resembling a primary in vivo PFC response) to heterologous erythrocytes. Their aim was to use the modified culture condition to generate a primary CTL response to allogeneic targets. By loosely following the mammalian protocol (i.e. for choice of antigen, assay procedure and data collection) a primary response, which appears to be dependent on interactions between multiple cell types, has been generated in vitro using Xenopus spleen cells.

Some additional experiments reported in this chapter were carried out to examine why both in vivo and in vitro PFC responses are often difficult to detect using conventional methods in young Xenopus. Adult frog and toad immunoglobulins are able to fix guinea pig complement (see Romano et al., 1973; Ohnishi, 1980) and this heterologous combination is commonly used in direct plaque forming cell (PFC) assays to study in vivo and in vitro hemolytic antibody responses to foreign erythrocytes. Antibodies

of Xenopus tadpoles (and those of young post-metamorphic animals) appear less able (DuPasquier and Cunningham, unpublished data) or altogether unable (see Williams, PhD Thesis, 1981) to fix guinea pig complement and rosette forming cell (RFC) assays must often be used as an alternative to PFC assays (Kidder et al., 1973; see also Du Pasquier, 1970a,b). Since both larval and adult frogs and toads are know to produce immunoglobulins and complement which are superficially similar to those of mammals (see for example Legler et al., 1969; Weinheimer et al., 1971; Donnelly and Cohen, 1977; Ruben et al., 1977b), attempts were made to use this isologous combination in the PFC assay (a similar protocol has been used successfully in bullfrogs; see Moticka et al., 1973). Unintentionally some interesting results may have emerged: thus following metamorphosis, the proportion of spleen PFC producing antibody capable of fixing guinea pig complement progressively increases; larvae and young post-metamorphic animals can fix only complement from young homologous donors.

Methods

Animals: Inbred (G-line) Xenopus laevis, which appear to be MHC homozygous (JJ; Dimarzo and Cohen, 1982) were used throughout these experiments. Animals were either donated by C. Katagiri (Hokkaido University, Sapporo, Japan) or purchased commercially (Nippon Life Sciences, Sapporo, Japan) and their offspring were produced through controlled matings (Katagiri, 1978). They were reared as previously described (Chapter 2).

Preparation of cell suspensions Xenopus leukocyte suspensions were prepared from the spleen as previously described (Chapter 2). Selected cell populations were removed from some cell suspensions by various separation procedures. Dead cells and other high density cells were removed from suspension by centrifugation (for 10 minutes, at 20°C, at 360 x g) through Ficoll-Isopaque (density of 1.094 g/ml) (Mishell and Shiigi, 1980). Following centrifugation, cells were recovered only from the density interface. Radiosensitive cells (i.e. those cells sensitive to radiation-induced interphase death, (see Anderson and Warner, 1976) which includes immature B cells, (see Anderson et al., 1972; Janeway, 1975) were destroyed in suspension (5×10^6 leukocytes per ml) by 5 minutes exposure to a cobalt-60 source at a dose rate of 600 rad/minute (for 3000 R) (Bernard et al., 1981; Fauci et al., 1978). Following irradiation, cells were transferred to fresh medium. Nylon wool adherent cells were removed from suspension by incubation (at 30°C) on nylon wool (Fenwall, Leukopak, Travenol Labs, Lot IH302R6) columns (0.6g nylon wool per 10 ml syringe) (Julius et al., 1973; Trizio and Cudowicz, 1974; Blomberg et al., 1980). After 1 hour, non-adherent cells (50-60% recovery) were eluted by dropwise addition of fresh (pre-warmed to 30°C) medium. Glass adherent cells were removed from suspension by incubation (at 30°C) on non-siliconized glass bead (type IV, 250-300 mm, Sigma Chemical Co., Poole, England; Lot IIF-0462) columns (18g glass beads per 10 ml syringe) (Shortman et al., 1971; Hudson and Hay, 1976). After 1 hour, non-adherent cells (85-95% recovery) were eluted by dropwise addition of fresh medium. Prior to use, all cells were washed and counted as previously described (Chapter 2).

Preparation of antigens: Sheep red blood cells from a single sheep were purchased commercially (Tissue Culture Services, Slough, England) and rabbit red blood cells from a single rabbit were collected via an ear vein (McCarthy and Dutton, 1975). Prior to storage, the red blood cells were centrifuged and the buffy coats were aspirated and discarded. Red blood cells were stored in Alsever's solution (Flow Labs, Irvine, Scotland) for at least one week, and up to 4 weeks prior to use. Prior to use, cells were washed as previously described (Chapter 2).

Preparation of serum: Whole blood was collected from non-immunized Xenopus of varying age either by cardiac or leg vein puncture. Undiluted blood was allowed to clot for two hours at room temperature, centrifuged (30 minutes at 5°C at 360 x g) and the serum was removed. Xenopus serum used as a source of hemolytic complement was maintained thereafter at 0°C on ice.

In vivo immunization: Immunization of Xenopus to sheep or rabbit erythrocytes was performed by injection of washed and intact red blood cells suspended in amphibian strength saline. Cell suspensions were injected via the dorsal lymph sac at appropriate times prior to sacrifice. Post-metamorphic Xenopus received standard 0.05 ml per gram body weight injections of a 0.0025% cell suspension for low dose priming, or of a 10% cell suspension for high dose priming. Xenopus tadpoles received standard 0.005 ml intraperitoneal injections of a 50% cell suspension for high dose priming only. Animals were sacrificed either after six days or after various numbers of days as indicated in the text.

In vitro immunization: Immunization of Xenopus spleen cells to sheep or rabbit erythrocytes was performed by culturing 3×10^6 cells per ml in complete culture medium containing 10% FCS. In antigen stimulated cultures, varying numbers, or equal numbers (3×10^6) of intact sheep or rabbit red blood cells were also added.

Responder spleen cell suspensions were cultured in 24 well tissue culture plates, with or without antigen, in complete tissue culture medium as previously described (Chapter 2). Cultures were fed on days 1, 3 and 5 with one drop (approximately 30 µl) of nutritive mixture. The nutritive mixture consisted of a 60% L-15 base (see recipe in Chapter 2) supplemented with 20% FCS, 20mM HEPES buffer, 40mM sodium bicarbonate, 10mM **1**-glutamine, 5 x Eagles non-essential amino acids (from 100x stock; Flow Labs, Irvine, Scotland), 5 x Eagles essential amino acids (from 50x stock, Flow Labs, Irvine, Scotland) and 1% (w/v) d-glucose (Sigma Chemical Co., Poole, England). All cultures were incubated for an appropriate length of time, harvested, and prepared for assay as previously described (Chapter 2).

Assay: In vivo and in vitro antibody responses to sheep or rabbit erythrocytes were measured by the thin layer direct plaque forming cell assay technique (Cunningham and Szenberg, 1968). Spleen cells to be assayed for PFC activity were suspended at varying dilutions in 60% L-15 supplemented with 10% FCS. A 150 µl aliquot of each cell suspension was combined with 15 µl of a 25% suspension of indicator erythrocytes (either sheep or rabbit) and 40 µl of 1:10 guinea pig or Xenopus serum (both of which were antigen-absorbed, as in Mishell and Shiigi, 1980). Guinea

pig serum used as a source of complement was purchased commercially in lyophilized form (Wellcome Reagents Ltd., Beckenham, England; best of two lots from Wellcome Labs and two lots from Flow Labs) and reconstituted immediately prior to use. Xenopus serum used as a source of complement was collected from non-immune G-line animals and used fresh (Ruben et al., 1977b).

The final assay mixtures (approximately 0.2 ml) were pipetted into double microscope slide chambers and the edges of the chambers were sealed with a 2:1 mixture of molten paraffin wax and petroleum jelly. The sealed chambers were incubated at 30°C for 1-2 hours and PFC were counted under low power of a dissecting microscope. Only plaques with a clear central lymphocyte were scored as PFC. Plaques without a central cell were assumed to be false PFC and were therefore not counted.

Data collection: Plaque forming cells were counted on 2 separate slides at spleen cell dilutions which gave approximately 100 plaques per slide. Data is expressed either as in vivo PFC per 10^6 originally-recovered spleen leukocytes, or as in vitro PFC per 10^6 originally-cultured leukocytes (in order to avoid obtaining artificially high counts due to high cell recovery in vivo or low viable cell recovery in vitro). Data is presented as single measurements made using spleen cells pooled from several inbred (G-line) animals.

Results

The in vivo PFC response: Both rabbit and sheep erythrocytes elicit strong and specific antibody responses in vivo in G-line Xenopus. Requirements for optimal in vivo spleen PFC responses

are shown in Tables 4.1 and 4.2. The strongest response is elicited by high doses (10% suspensions) of RBC, although a weak response can be elicited by low doses (0.0025% suspensions). The largest numbers of direct anti-RRBC or anti-SRBC PFC are found approximately 7 days post-injection. Using Xenopus (isologous) serum rather than guinea pig (heterologous) serum as a source of complement increases the sensitivity of the in vivo 7 day, but not necessarily the 11 or 15 day, direct PFC assay. Both types of complement must be absorbed, and Xenopus serum must be used fresh and not frozen (data not shown). Mature PFC (presumably differentiated plasma cells) generated in vivo are not immediately destroyed by 3000 R irradiation, they do not readily adhere to nylon wool columns, and they do not adhere to glass bead columns (see Appendix 7).

The in vitro PFC response: Rabbit, but not sheep, erythrocytes elicit strong antibody responses in vitro in cultures of spleen cells from G-line Xenopus. Requirements for optimal in vitro PFC responses are shown in Tables 4.3 and 4.4. In the absence of antigen, low levels of direct PFC arise spontaneously with specificity for both types of RBC. At all RBC to leukocyte ratios tested, the presence of SRBC decreased the formation of anti-SRBC PFC while the presence of RRBC increased the formation of anti-RRBC PFC. The largest numbers of direct anti-RRBC PFC are found approximately 7 days post culture initiation. Higher numbers of direct PFC are detected on days 7, 11 and 15 when young Xenopus serum, rather than guinea pig serum, is used as a source of complement.

The negative effect of SRBC is antigen specific and the positive effect of RRBC is also antigen specific. The differential effects of SRBC and RRBC may be a unique feature of the in vitro PFC response of inbred (G-line) animals reared in our laboratory. In vitro differentiation of immature B cells into mature antibody producing plasma cells is prevented by destroying irradiation-(3000 R)-sensitive cells, by removing nylon wool adherent cells, or by removing glass bead adherent cells prior to culture (again see Appendix 7).

The role of complement in the 6 day PFC response: Parallel changes occur in antibody and complement during development which can be detected in an in vivo PFC response. Some suggestion of a relationship between PFC donor age and Xenopus complement donor age is shown in Table 4.5. Young animals (and tadpoles) generate PFC in their spleens following SRBC challenge, but their antibodies are unable to fix either guinea pig or adult Xenopus complement. Responses can be detected only using complement from similarly aged Xenopus donors. Adult animals also generate PFC in their spleens following SRBC challenge and their antibodies are able to fix complement from any source tested. Responses can be detected equally well using complement from guinea pig, young Xenopus, or adult Xenopus serum donors. The development of PFC able to fix guinea pig complement occurs gradually. The ratio of PFC detected using young Xenopus complement to PFC detected using guinea pig complement appears inversely proportional to the age of the spleen cell donor, and this ratio remains constant even when spleen cells are primed in either younger or older irradiated (3000 R), adoptive transfer hosts (data not shown).

Parallel changes occurring in antibody and complement can even more readily be detected in the *in vitro* PFC response. The relationship between spleen cell donor age and complement donor age is shown in Table 4.6. PFC generated *in vitro* display a spleen cell donor, age-dependent, complement requirement which is comparable to that displayed by PFC generated *in vivo*. The ratio of PFC detected using *Xenopus* complement to PFC detected using guinea pig complement appears inversely proportional to the age of the original spleen cell donor, and this ratio remains constant when spleen cells are primed in co-culture with irradiated (3000 R) spleen cells from either younger or older donors (data not shown).

Discussion

In mice, antibody responses can be stimulated *in vivo* in the spleens of normal animals and/or *in vitro* in cultures of dissociated, non-fractionated spleen cells (Marbrook, 1967; Mishell and Dutton, 1967; Kettman and Dutton, 1970; Click et al., 1972; Marbrook and Haskill, 1974; Schreier, 1978). Responses can be elicited by a wide variety of antigens including soluble proteins and intact cells. Primary stimulation results largely in the production of high molecular weight immunoglobulin (IgM) (Mishell and Dutton, 1967) and secondary stimulation results largely in the production of low molecular immunoglobulins (IgG) (North and Maizels, 1977; Eipert et al., 1978). Antibody production can be measured on a cellular basis using direct and/or indirect plaque forming cell (PFC) assays. Mouse immunoglobulins readily fix commercially prepared (lyophilized) guinea pig complement and this combination is commonly used to measure anti-hapten and anti-RBC responses.

The results in this chapter demonstrate that Xenopus of any age are able to mount a strong in vitro PFC response which is comparable to an in vivo response. The in vitro culture techniques which have been used to generate this response are modified slightly from those commonly used for Xenopus (i.e. using higher nutrient and cell concentrations) but closely resemble those used for mice. The assay techniques are also slightly modified from those commonly used for Xenopus (i.e. using a selected RBC species and isologous complement) but again resemble those used for other animals. However, some remaining questions must be resolved before this technique will be useful as a way to examine primary antibody responses of Xenopus. First, it must be determined why PFC responses can be stimulated by rabbit but not by sheep RBC (and also whether responses can be stimulated by soluble as well as cellular antigens). Second, it must be determined why removal or destruction of selected spleen cell subpopulations (which do not appear to remove or destroy mature PFC) prevents the response.

All antibody responses in Xenopus may be dependent on and controlled by a variety of antigen specific regulatory cells (including antigen specific helper and suppressor cells) which are activated differentially depending on the antigen. In our G-line Xenopus, in vitro stimulation with RRBC may activate large numbers of helper cells and thereby specifically generate a RRBC PFC response, while in vitro stimulation with SRBC may stimulate large numbers of suppressor cells (possible generated as a result of feeding cross-reactive antigens) and thereby specifically inhibit a SRBC PFC response.

In vitro antibody responses to all RBC antigens in *Xenopus* may be dependent on accessory cells (which readily adhere to nylon wool or to glass) and/or soluble factors which they elaborate. The *in vitro* response to some antigens may be prevented by removal of, or by sub-threshold numbers of, appropriate (antigen specific) spleen accessory cells. The presence of low numbers of accessory cells in the spleen with specificity for RRBC may make PFC responses dependent on high cell density (at least 1×10^7 per ml) and the presence of even lower numbers of accessory cells with specificity for SRBC may make PFC responses impossible, even at high cell density.

The results in this chapter further reveal that following metamorphosis, the proportion of spleen PFC producing a class of antibody capable of fixing guinea pig complement progressively increases, and that this may be accompanied by changes in the antibody binding capacity of some serum complement components. However, some remaining questions must again be resolved before conclusions can be drawn concerning the ontogeny of antibody and complement systems in *Xenopus*. First, it must be determined why direct PFC responses of young *Xenopus* can be more readily detected, or only detected, using complement from sibling *Xenopus*. Second, it must be determined why the requirement for isologous complement is not displayed in direct PFC responses of adult *Xenopus*.

During the primary anti-RBC response of young animals, one immunoglobulin (either a high or low molecular weight Ig class (Du Pasquier and Haimovich, 1974; 1976; Hadji-Azimi, 1979) or B cell class (such as an intrathymic or thymus derived B cell

class) (DuPasquier et al., 1972; DuPasquier and Weiss, 1973; Williams et al., 1983; Hsu et al., 1983) may predominate. The predominant production of a high molecular weight immunoglobulin class in young animals may result in a requirement for isologous complement, and as the predominance changes toward the production of a low molecular weight class (either as a result of antigen choice, priming schedules, or age), the requirement for isologous complement may disappear.

Larval Xenopus may produce a unique larval immunoglobulin class (which has not been verified using molecular weight criteria and therefore may be an IgM subclass): following metamorphosis the production of this immunoglobulin may slow and stop. A uniquely larval immunoglobulin subclass may merely have an altered complement receptor not found in adults and which is able to bind complement components found in isologous, but not heterologous, serum. Following metamorphosis in Xenopus (and other amphibians), a variety of new adult proteins appear and replace larval ones which subsequently disappear (studied most extensively in the larger bullfrog tadpole, see Wald, 1958; Manwell, 1966; Wise,1970). Not unexpectedly, changes occur in the nature and relative amounts of various serum proteins, and this may include certain high (or low) molecular weight immunoglobulins and certain complement components (Richmond, 1968; DuPasquier et al., 1979).

In most cases an obvious advantage can be associated with making a larval to adult protein switch. For example, tadpole and adult hemoglobulin have markedly different oxygen binding

affinities (Hamada et al., 1976). However, in the case of immunoglobulins, such an advantage is not so obvious. Larval high and low molecular weight antibodies appear able to bind antigen specifically, to bind complement specifically, and appear adequate with respect to the size of their repertoire. Nevertheless, it is possible that the larval system is less efficient than the adult system. Amphibian larvae have far fewer lymphocytes than do their adult counterparts and yet their antigen repertoires (though possibly smaller than mammals; DuPasquier and Wabl, 1978) do not appear limited. This could imply that some larval antibodies (such as high molecular weight anti-RBC antibodies, but possibly not anti-TNP and anti-DNP antibodies - see Haimovich and DuPasquier, 1973) are more highly cross-reactive than adult antibodies, and therefore bind antigen with a lower average affinity than adult antibodies (DuPasquier and Haimovich, 1974). Despite lower binding affinity, complement activation would be necessary for target destruction and this may call for a specialized complement receptor (present either on high or low molecular weight Ig, or only on a unique larval Ig). Later in life, antigenic challenge may place a severe enough stress on the immune system so as to cause this predominantly larval immunoglobulin class to be produced in early post-metamorphic Xenopus.

There are examples of other protein changes which have been studied primarily in amphibians, other than Xenopus, that take place during metamorphosis, which may set a precedent. For example, during metamorphosis individual tadpole erythrocytes begin production

of adult hemoglobin, eventually these cells are replaced by adult erythrocytes, and yet under severe anemic stress (induced by phenylhydrazine), adult cells can be induced to reactivate production of tadpole hemoglobin (Moss and Ingram, 1965; Hamada and Sukuya, 1966; Maniatis et al., 1969; Maniatis and Ingram, 1972).

Future experiments could be designed to examine whether more rigorous immunization schedules, involving either repeated antigen injection, or the use of different antigens (including TNP-KLH), or the use of CFA, will produce PFC capable of fixing guinea pig complement in tadpoles and young froglets. Experiments could also be designed to determine whether or not the level of cross reactivity between SRBC and RRBC (or between different antigens) detected in the PFC response of young animals, with small numbers of lymphocytes, is higher than that of older animals, with higher numbers of lymphocytes.

Table 4.1 Antigen and complement dependence of the in vivo Xenopus anti-SRBC and anti-RRBC PFC response.

In vivo Immunization	RBC DOSE	PFC/10^6 originally recovered spleen leukocytes			
		ANTI-SRBC-PFC		ANTI-RRBC-PFC	
	%	GPC'	XLC'	GPC'	XLC'
SRBC injected	0	0	0	0	0
	0.0025	1	110	0	0
	10	30	380	0	0
RRBC injected	0	0	0	0	0
	0.0025	0	0	0	180
	10	0	0	45	431

PFC were assayed using 1/50 diluted Xenopus serum from 6 month old G-line animals or guinea pig serum as a source of complement.

PFC were measured six days following immunization at 26°C.

Responder animals are 6 month old G-line.

Individual measurements from pools of 3 animals.

Table 4.2 Time course of the *in vivo* *Xenopus* anti-SRBC and anti-RRBC PFC response

In vivo Immunization	RBC Dose %	Target RBC	C' Source	PFC/10^6 originally recovered spleen leukocytes			
				Day 3	Day 7	Day 11	Day 15
Non injected	0	R	XL				0
			GP				0
	0	S	XL				0
			GP				0
SRBC injected	10	R	XL	0	0	0	0
			GP	0	0	0	0
	10	S	XL	0	588	39	5
			GP	0	117	173	89
RRBC injected	10	R	XL	0	874	45	18
			GP	0	189	157	15
	10	S	XL	0	184	0	0
			GP	0	0	0	0

PFC were assayed using 1/50 diluted *Xenopus* serum from 6 month old G-line animals or guinea pig serum as a source of complement.

Responder animals are 9 month old G-line

Individual measurements made from pools of 3 animals

Table 4.3 Antigen and complement dependence of the *in vitro* *Xenopus* anti-SRBC and anti-RRBC PFC response.

In vitro Immunization	RBC/leuko ratio	PFC/10^6 originally cultured spleen leukocytes			
		ANTI-SRBC PFC		ANTI-RRBC PFC	
		GPC'	XLC'	GPC'	XLC'
SRBC stimulated	0	0	35	0	60
	0.001	0	15	0	61
	0.01	0	1	0	55
	0.1	0	0	0	63
	1.0	0	0	0	58
	10	0	0	0	49
RRBC stimulated	0	0	25	0	57
	0.001	0	20	0	61
	0.01	0	18	0	155
	0.1	0	20	0	270
	1.0	0	21	0	510
	10	0	19	0	103

PFC were assayed using 1/50 diluted *Xenopus* serum from 6 month old G-line animals or guinea pig serum as a source of complement.

PFC were measured on day six of culture

Responder cells are from 6 month old G-line animals

Individual cultures from pool of six animals.

Table 4.4 Time course of the in vitro Xenopus anti-SRBC and anti-RRBC PFC response

In vitro Immunization	RBC/ leuko ratio	Target RBC	C' source	PFC/10^6 originally cultured spleen leukocytes			
				Day 3	Day 7	Day 11	Day 15
Non-stimulated	0	R	XL	28	63	12	2
			GP	0	0	0	0
	0	S	XL	10	2	0	0
			GP	0	0	0	0
SRBC stimulated	1.0	R	XL	18	43	20	3
			GP	0	0	0	0
	1.0	S	XL	1	0	0	0
			GP	0	0	0	0
RRBC stimulated	1.0	R	XL	241	594	268	55
			GP	3	3	0	0
	1.0	S	XL	15	0	0	0
			GP	0	0	0	0

PFC were assays using 1/50 diluted Xenopus serum from 6 month old G-line animals or guinea pig serum as a source of complement.

Responder cells are from 6 month old G-line animals

Individual cultures from pool of 10 animals.

Table 4.5 Parallel changes in the antibody and complement systems during aging in Xenopus, demonstrated in a primary in vivo anti-SRBC PFC response.

Responder Age	SRBC Dose %	PFC/10^6originally recovered spleen leukocytes and PFC/ spleen					
		GPC'		6moXLC'		Adult XLC'	
		10^{-6}	$spleen^{-1}$	10^{-6}	$spleen^{-1}$	10^{-6}	$spleen^{-1}$
2 mos Xenopus	0	0	0	0	0	ND	ND
(tadpole - stage 56/7)	50	0	0	322	16	ND	ND
4 mos Xenopus	0	0	0	4	9	0	0
	10	0	0	894	2056	0	0
12 mos Xenopus	0	0	0	0	0	0	0
	10	51	331	431	2801	0	0
18 mos Xenopus	0	0	0	0	0	0	0
	10	282	3384	535	6420	0	0
Adult Xenopus	0	0	0	0	0	0	0
(Full grown, unknown age)	10	59	2832	72	3456	109	5232
Adult Mouse	0	0	0	0	0	0	0
	10	556	10564	0	0	0	0

PFC were assayed using 1/50 diluted Xenopus serum from 6 month old or adult G-line animals or guinea pig serum as a source of complement.

PFC were measured six days following immunization.

Responder animals are G-line Xenopus or Swiss Albino Mouse.

Individual measurements from pools of 20 tadpoles or 1-4 frogs.

Table 4.6 Parallel changes in the antibody and complement systems during aging in Xenopus, demonstrated in a primary in vitro anti-RRBC PFC response.

Donor Age	RRBC/ Leuko Ratio	PFC/10^6 originally cultured spleen leukocytes		
		GPC'	6moXLC'	AdXLC'
4 mos Xenopus	0	0	53	0
	1.0	0	525	0
12 mos Xenopus	0	17	33	0
	1.0	120	412	0
18 mos Xenopus	0	58	56	ND
	1.0	593	605	ND
Adult Xenopus (Full grown, unknown age)	0	77	ND	ND
	1.0	573	ND	ND

PFC were assayed using 1/50 diluted Xenopus serum from 6 month old or adult G-line animals or guinea pig serum as a source of complement.

PFC were measured on day six of culture.

Responder cells are from G-line animals

Individual cultures from pools of 1-4 animals.

Chapter Five

Helper T cells in *in vitro* plaque forming cell responses to sheep and rabbit erythrocytes. II. Responses in co-culture of spleen cells from MHC compatible normal and thymectomized animals.

Introduction

The primary purpose behind the current projects in this laboratory has been to examine the role of the thymus in conferring restriction specificity on T cells. Since even using improved *in vitro* culture techniques (i.e. involving higher spleen cell density and nutrient concentration, see Appendix 8), a cytotoxic T cell assay proved to be unsuited for this purpose, attempts were made to adapt the primary *in vitro* PFC assay (refer to Chapter 3) for use in a helper T cell assay, which might have provided an alternative way to examine T cell restriction. A primary T lymphocyte dependent antibody assay (rather than a secondary) seemed particularly appropriate, since restriction specificity of a helper T cell population could be examined without the possibility of interference from an *in vivo* antigen presenting cell induced bias. It seemed that it might be feasible to proceed by combining *in vitro*, unfractionated B cells from thymectomized animals (since PFC do not appear to adhere to nylon wool columns in this laboratory; see Appendix 7) with unfractionated, irradiated, T cells, either from syngeneic and allogeneic normal animals or from syngeneic and allogeneic thymus chimera animals.

A large proportion of comparative experiments performed to date have been concerned with thymus and T cell function in *Xenopus* and in particular with the part they play in B cell responses. It has already been shown that larval thymectomy (and possibly hydrocortisone treatment - see Ruben and Vaughan, 1974) or adult thymectomy (see Gruenwald and Ruben, 1979) impairs most (but not

all, see Collie et al., 1975; Tochinai, 1976b) *in vivo* and *in vitro* antibody responses (Turner et al., 1974; Tochinai and Katagiri, 1975; Ruben et al., 1977a). Following larval thymectomy, the immune response can be restored by syngeneic or allogeneic thymus reimplantation (Tochinai et al., 1976; DuPasquier and Horton, 1982), or by thymocyte reconstitution, either in adoptive transfer (Kawahara et al., 1980) or in co-culture (Ruben et al., 1977a). It has also been shown that normal (T dependent) antibody responses require T and B lymphocyte collaboration (Blomberg et al., 1980) and that helper T cell function is genetically (MHC) restricted (Bernard et al., 1981).

It is not the purpose of the experiments reported in this chapter to reinvestigate or to challenge the results of previous reports. Rather it is intended that these experiments should examine the behaviour of spleen lymphocyte populations from normal, thymectomized, and thymus reimplanted Tx animals. *In vitro* restoration of B cell populations (from the spleen) from thymectomized animals is the assay which we wished to use to examine the restriction specificity of helper T cells developing in animals bearing a foreign thymus graft. Unfortunately the results point out peculiarities in the *in vitro* behaviour of spleen cells from Tx animals given a syngeneic thymus.

Methods

Animals: Inbred (G-line) *Xenopus laevis*, cloned hybrid *Xenopus laevis gilli* (LG) or *laevis muelleri* (LM), or random outbred

Xenopus laevis were used throughout these experiments. They were bred and reared as previously described (Chapter 2). Immunologically deficient animals were produced by irradiation and/or thymectomy. Animals were irradiated by whole body exposure to a cobalt-60 source (Williams and Chase, 1976). The lethal irradiation doses for Xenopus are shown (see Appendix 2) and the LD_{50} was taken to be approximately 3000 R. Animals were thymectomized early in larval life as previously described (Chapter 2). Prior to immunization some irradiated and/or thymectomized animals were reconstituted with MHC compatible thymocytes or splenocytes by a single dorsal lymph sac injection of 20×10^6 thymus or 10×10^6 spleen leukocytes. Some thymectomized animals were reimplanted with a single thymus (either adult or larval thymus, see Loor and Haag, 1977) as previously described (Chapter 2).

Preparation of cell suspensions: Xenopus leukocyte suspensions were prepared from the spleen and from the thymus. Spleen and thymus cell suspensions were prepared as previously described (Chapter 2). Spleen cells to be used as a source of primed or non-primed helper T cells (Katz et al., 1970; Janeway, 1975) which were devoid of B cell activity were inactivated by irradiation (3000 R) as previously described (Chapter 4). Prior to use, the cells were washed and counted as previously described (Chapter 2).

Preparation of antigens: Sheep and rabbit red blood cells were obtained and prepared for use as previously described (Chapter 4).

Preparation of serum: Xenopus serum used as a source of complement was collected and prepared for use as previously described (Chapter 4).

In vivo immunization: Immunization of Xenopus to sheep or rabbit red blood cells was performed as previously described (Chapter 4) using 10% SRBC or RRBC suspensions. Animals were sacrificed after two days (for short term priming) and after six days (for long term priming).

In vitro immunization: Immunization of Xenopus spleen cells to rabbit red blood cells was performed as previously described (Chapter 4) using RRBC/leukocyte ratios of approximately 1/1. In mixed (Kettman and Dutton, 1971 or in reconstituted (Hirst and Dutton, 1970; Munro and Hunter, 1970) cultures, 3×10^6 non-irradiated responder cells were co-cultured with equal numbers (3×10^6) of irradiated (3000 R) syngeneic or allogeneic helper cells. All cultures were incubated for an appropriate length of time, harvested, and prepared for assay as previously described (Chapter 4).

Assay: In vivo and in vitro PFC responses to sheep or rabbit erythrocytes were measured as previously described (Chapter 4) using serum from six month old (C-line) Xenopus laevis as a source of complement throughout.

Data collection: Plaque forming cells were counted and data is expressed and presented as previously described (Chapter 4). (Note: The approach taken in designing these experiments was to examine as many technical variations as time and resources would allow and therefore individual experiments were not exhaustively repeated. All the experiments which are reported as single data points were obtained using cells collected and pooled from multiple MHC-compatible sibling animals. In vivo and in vitro experiments were carried out using pools of cells, which were redivided into

identical aliquots, and then either incubated under varying culture conditions, or assayed under varying test conditions. Whenever possible, attempts were made to provide internal controls by establishing base-line values which could be compared to previous experiments and to separate parts of the same experiment).

Results

The effect of thymectomy and irradiation on the in vivo PFC response: The six day in vivo primary direct PFC response in Xenopus is sensitive to both irradiation and thymectomy. The effect of whole body irradiation (3000 R) and/or early larval thymectomy (7 day) is shown in Table 5.1. Both irradiation and thymectomy prevent a primary direct PFC response from developing on day 6. The effect of adoptively transferring unfractionated spleen or thymus cells into these animals is also shown. Non-irradiated (but not 3000 R irradiated) spleen or thymus cells produce a strong PFC response on day 6 when transferred into irradiated hosts. However, when the same cells are transferred into thymectomized hosts, no response occurs. The low number of PFC found in the spleens of thymectomized animals injected with normal spleen cells appear to originate from donor (not host) B cells (since they are not affected by irradiation of the host, but are destroyed by irradiation of the transferred donor cells).

The effect of thymectomy and irradiation on the in vitro PFC response: The in vitro primary direct PFC response of Xenopus spleen cells is also sensitive to irradiation and to thymectomy. The effect of irradiation and/or thymectomy is shown in Figure 5.1. Irradiation

prevents PFC from ever developing *in vitro*, but thymectomy does not. Although some anti-RRBC PFC arise spontaneously (i.e. in the absence of antigen) in cultures of normal spleen cells, this background level is especially high in cultures of spleen cells from thymectomized animals. This is particularly true when using young animals with low numbers (2×10^6 per spleen) of spleen leukocytes. In the presence of antigen, spleen cells from normal animals develop a response (the kinetics of the response is highly dependent on cell concentrations) but spleen cells from thymectomized animals do not. In the case of spleen cells from thymectomized animals far fewer PFC arise when antigen is added to the cultures than when it is not.

The effect of in vivo priming on the in vitro PFC response:

In normal, but not thymectomized animals, a primary PFC response initiated *in vivo* continues *in vitro* even in the absence of antigen. The effect of 0, 3, 5 and 7 day *in vivo* priming on six day *in vitro* spontaneous (i.e. non-*in vitro* antigen-induced) PFC formation is shown in Figure 5.2. In normal animals, the number of PFC recovered on day 6 of culture is proportional to the number of PFC/10^6 cells cultured on day 0. In thymectomized animals, antigen sensitization *in vivo* has no effect on either the number of PFC/10^6 on day 0 or the number of PFC recovered on day 6 of culture.

Irradiated (3000 R), *in vivo* primed (2 day short-term primed) spleen cells from normal animals, but not thymectomized animals, are able to induce an *in vitro* primary PFC response by normal, non-irradiated, spleen cells in cultures without antigen.

The effect of 2 day *in vivo* primed stimulator cells on 6 day spontaneous *in vitro* PFC formation is shown in Table 5.2. Primed cells from normal animals (possibly only MHC compatible normal animals) are slightly stimulatory, but primed cells from thymectomized animals are not.

Syngeneic in vitro cellular restoration: Normal MHC compatible spleen cells restore the *in vitro* PFC response of post-thymectomy spleen cells to normal levels with normal kinetics. The result of combining irradiated (3000 R), non-primed or primed spleen cells from normal animals with responder spleen cells from normal or thymectomized animals is shown in Figure 5.3. Non-primed helper cells have a feeder cell (increased concentration increases response kinetics) effect on the *in vitro* PFC response of normal responder cells and may have a restorative effect on the response of post-thymectomy responder cells. Primed helper cells further accelerate the kinetics of the response of both normal and post-thymectomy responder cells.

Allogeneic in vitro cellular restoration: Normal, fully allogeneic, spleen cells do not restore the *in vitro* PFC response of spleen cells from thymectomized animals. The result of combining allogeneic, irradiated (3000 R), non-primed or primed, spleen cells with responder spleen cells from normal or thymectomized animals is shown in Figure 5.4. Non-primed and primed allogeneic helper cells slightly augment the *in vitro* PFC response of normal responder cells but do not restore the response of post-thymectomy responder cells. The augmented response of normal spleen cells may be due to positive allogeneic bystander effects, but the inability of

normal allogeneic cells to restore post-thymectomy spleen cells is not due to negative allogeneic effects. In this experiment, non-primed and primed helper spleen cells were obtained from Xenopus LM hybrids (LM-3) which themselves respond normally in vitro to RRBC. Similar results were obtained using randomly-chosen, outbred, allogeneic Xenopus laevis combinations. However, in one experiment involving hybrid LG combinations, the 6 day anti-RRBC response of spleen cells from thymectomized LG-17 (ac) animals was restored (from 91 PFC/10^6) not only by irradiated (3000 R) syngeneic spleen cells (567 PFC/10^6), but also by semi-allogeneic LG-5 (ad) spleen cells (700 PFC/10^6) and by fully-allogeneic LG-3 (bd) spleen cells (686 PFC/10^6).

Lack of restoration of in vitro helper cell function with cells from thymus-implanted, thymectomized toads: Reimplantation of a MHC compatible or syngeneic thymus into larvally thymectomized hosts restores their in vivo PFC response, but does not restore their ability to reconstitute the in vitro PFC response of spleen cells taken from thymectomized animals. The result of combining MHC compatible irradiated (3000 R), non-primed helper spleen cells from 6 month old thymectomized animals, which were reimplanted with a single adult thymus (taken from 6 month old donors) during larval life (3 weeks post-thymectomy), with responder spleen cells from normal or thymectomized animals, is shown in Figure 5.5. Helper cells from such thymus-restored animals have no effect on the in vitro PFC response of either normal or spleen cells from thymectomized animals. The result of combining syngeneic, irradiated (3000 R), non-primed, helper spleen cells from animals

which were reimplanted with a larval thymus (taken from 3 week old donors) during larval life with responder spleen cells from normal or thymectomized animals is shown as part of Appendix 8. Helper cells from such thymus restored animals do not display either a feeder effect when cultured with normal spleen cells nor a restoring effect when cultured with post-thymectomy spleen cells.

Discussion

In mice, foreign erythrocyte challenge results in a primary PFC response followed by a secondary response which is higher in magnitude and quicker to peak (Dutton and Mishell, 1967; Kettman and Dutton, 1971). In vivo and in vitro antibody responses are dependent on anti-theta plus complement sensitive cells (T cells; Gorczynski et al., 1972), on radio sensitive cells (B cells) and on adherent cells (accessory cells; Hartmann et al., 1970; Gorczynski et al., 1971). Humoral immunity is highly dependent on thymus derived T cells (Aden et al., 1972) since thymus deficient animals (athymic nude or neonatally thymectomized mice) respond poorly to (and may even be temporarily tolerized by; see Kindred, 1975) T dependent antigens, are lymphopenic and gammaglobulinemic (Pantelouris, 1968; 1971; Wortis, 1971) and demonstrate impaired, possibly abrogated, PFC reactions (Friedman, 1964; Takeya et al., 1965; Kindred, 1971; Feldmann et al., 1972; Lefkovitz, 1973). Thymus deficiencies are directly due to a lack of T cells, since they can readily be restored, not only by whole syngeneic or allogeneic neonatal thymus reimplantation (Radov et al., 1975;

Kindred and Loor, 1975; Loor and Haag, 1977), but also by infusion of syngeneic or allogeneic thymocytes (Miller and Mitchell, 1968; Kindred, 1974), thoracic duct lymphocytes (Miller et al., 1967), hydrocortisone resistant thymocytes (Blomgren and Anderson, 1971) or educated T cells (Hartmann, 1970), and by co-culture with syngeneic or allogeneic non-adherent, radio resistant (Munro and Hunter, 1970; Mosier et al., 1970) spleen cells (Lefkovitz, 1973; Hirst and Dutton, 1970; Schimpl and Wecker, 1971) or thymus cells (Feldmann et al., 1972; Adams, 1972; Aden et al., 1972). The collaborative interactions which take place between primed and non-primed helper cells and either antigen presenting cells (McDougal and Cort, 1978; Shih et al., 1980; Singer et al., 1980a; 1980b) and/or B cells (Jones and Janeway, 1981) is MHC restricted (although MHC restriction of the latter depends to some extent on the state of the B cell prior to challenge - see Schreier et al., 1980; Anderson et al., 1980; Ratcliffe and Julius, 1982; Julius et al., 1982; Speck and Pierce, 1982). However, under certain conditions (particularly in vitro) interactions between MHC disparate cells can result in positive allogeneic and allogeneic bystander effects (Ekpha-Mansa and Kennedy, 1971; Vann and Galloway, 1973; Kettman and Skarvall, 1974).

The results in this chapter support a suggestion that most of these findings also apply to Xenopus. However, the results also point out some interesting features of thymectomized Xenopus which must be resolved before such a model system will prove suitable for studying the role of the thymus in T cell function.

First, it must be determined what cells are affected by thymectomy and why short-term cellular restoration of the PFC response is difficult *in vivo* and is MHC restricted *in vitro*. Second, it must be determined why *in vitro* helper cell activity (but not *in vivo* helper cell activity, see Du Pasquier and Horton, 1982) is limited in animals which were larvally thymectomized and later thymus reimplanted.

Thymectomy in *Xenopus* appears to non-specifically increase the background level of spleen PFC and this is apparent both *in vivo* and *in vitro*. This may represent a real increase in B cell activity, possibly due to a lack of regulatory T cells, or merely a proportional increase in B cell numbers, due to a non-specific enrichment of non-T cells. The spleens of thymectomized animals are enriched for surface IgM positive lymphocytes (Bleicher and Cohen, 1981). Antigen contact in these animals appears to specifically depress the level of spleen PFC *in vitro*. Because this does not occur *in vivo*, it is unlikely to be due to the activation of suppressor T cells migrating from the thymus prior to thymectomy at 7 days. It is more likely to be due to antigen induced B cell paralysis, due to the absence of helper T cells (Kindred, 1975).

Cellular restoration of the PFC response of thymectomized *Xenopus* appears to be possible using spleen cells from normal animals and readily occurs *in vitro* but not *in vivo* (at least in the short term). Because an *in vivo* response following whole body irradiation can be restored in the short term by cell transfer,

it is unlikely that the inability to restore thymectomized animals in this manner is due to poor homing. It is more likely that PFC responses require the presence of a third cell (possibly an antigen presenting macrophage or B cell; see Chesnut and Grey, 1981), that is either thymus derived (Lu et al., 1980; Robinson, 1983; Hosono et al., 1984) or developmentally dependent on T cells (Schrader, 1973; Tzehoval et al., 1981), and which is present in insufficient numbers in spleen or thymus. If such a cell was required to participate in the PFC response, then it would not be difficult to explain why full in vivo restoration using spleen (Cribbin, in preparation) or thymus (Nagata, 1980) cells can occur over several weeks (this may be long enough for the expansion or maturation of such a cell to take place) and why full short-term (6 day) restoration can occur only by transferring an irradiated mixture of spleen and blood (Cribbin, in preparation) leukocytes (peripheral blood may contain significant numbers of such a cell).

Thymic restoration of helper T cell function in thymectomized Xenopus is possible using MHC compatible or incompatible thymus implants and the restored function can be readily detected in vivo (in the reconstituted host) but not in vitro (when irradiated spleen cells from a thymus reimplanted animal are co-cultured with spleen cells from a thymectomized animal). The developmental stage at which our thymectomized hosts receive thymus implants, or the developmental age of our thymus donors, may make it impossible to effect full restoration. The proper development of helper T

cells and/or antigen presenting cells may be impaired by such a transient early absence of the thymus. Because similar results were found using either adult or larval implants, it is unlikely that such an incomplete restoration is due to changes within the donor thymus (Loor and Haag, 1977). It is more likely that incomplete retoration is due to early changes (occurring before 4 weeks) in the thymectomized host which make it difficult or impossible for any thymus implant to completely restore full function.

A system involving late thymus implants and an *in vitro* primary PFC response may be altogether unsuitable for examining the MHC restriction and thymic education of helper T cells. Only a system involving embryonic chimeras (Volpe et al., 1979) and/or a secondary anti-hapten response may be suitable. However, the use of different and more sensitive assays may detect more subtle differences in the primary reconstitution system. Future experiments could be designed to determine whether or not changes in immunoglobulin isotype can be detected in this system using ELISA and RIA techniques with monoclonal anti-*Xenopus* IgM and IgG preparations.

Table 5.1 Cellular restoration of the *in vivo* PFC response following early larval thymectomy and/or whole body lethal irradiation.

Responder Animal Treatment	no SRBC	10% SRBC	SRBC and adoptive transfer			
			10×10^6 spleen		20×10^6 thymus	
			OR	3000R	OR	3000R
Normal	0	1990	ND	ND	ND	ND
3000R Irradiated	0	1	638	0	1027	2
Thymectomized	0	0	32	0	0	0
Thymec. and Irrad.	ND	ND	56	0	4	0

Anti-SRBC-PFC per 10^6 originally recovered spleen leukocytes

PFC were assayed using 1/50 diluted *Xenopus* serum from 6 month old G-line animals as a source of complement.

PFC were measured six days following simultaneous immunization and adoptive transfer

Responder animals are six month old G-line

Individual measurements from pools of 2 animals.

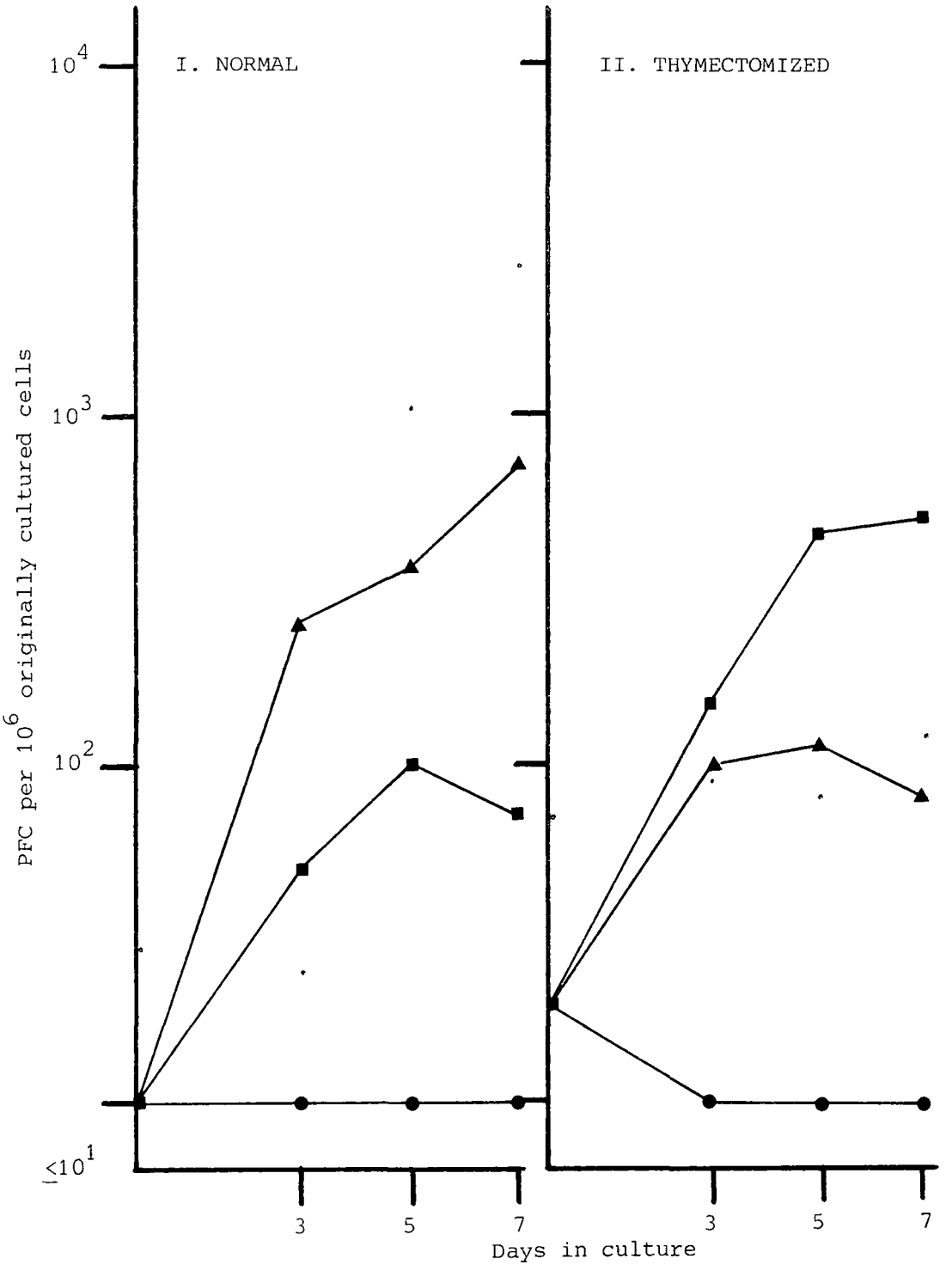


Figure 5.1 kinetics of the in vitro anti-RRBC PFC response by;

(I) normal spleen cells
(II) spleen cells from Tx animals

Cultures contained;

(■) non-irradiated spleen cells without antigen
(▲) non-irradiated spleen cells with antigen
(●) irradiated spleen cells with antigen

Responder cells are from 6 month old G-line animals

Individual cultures from pools of 9 animals each

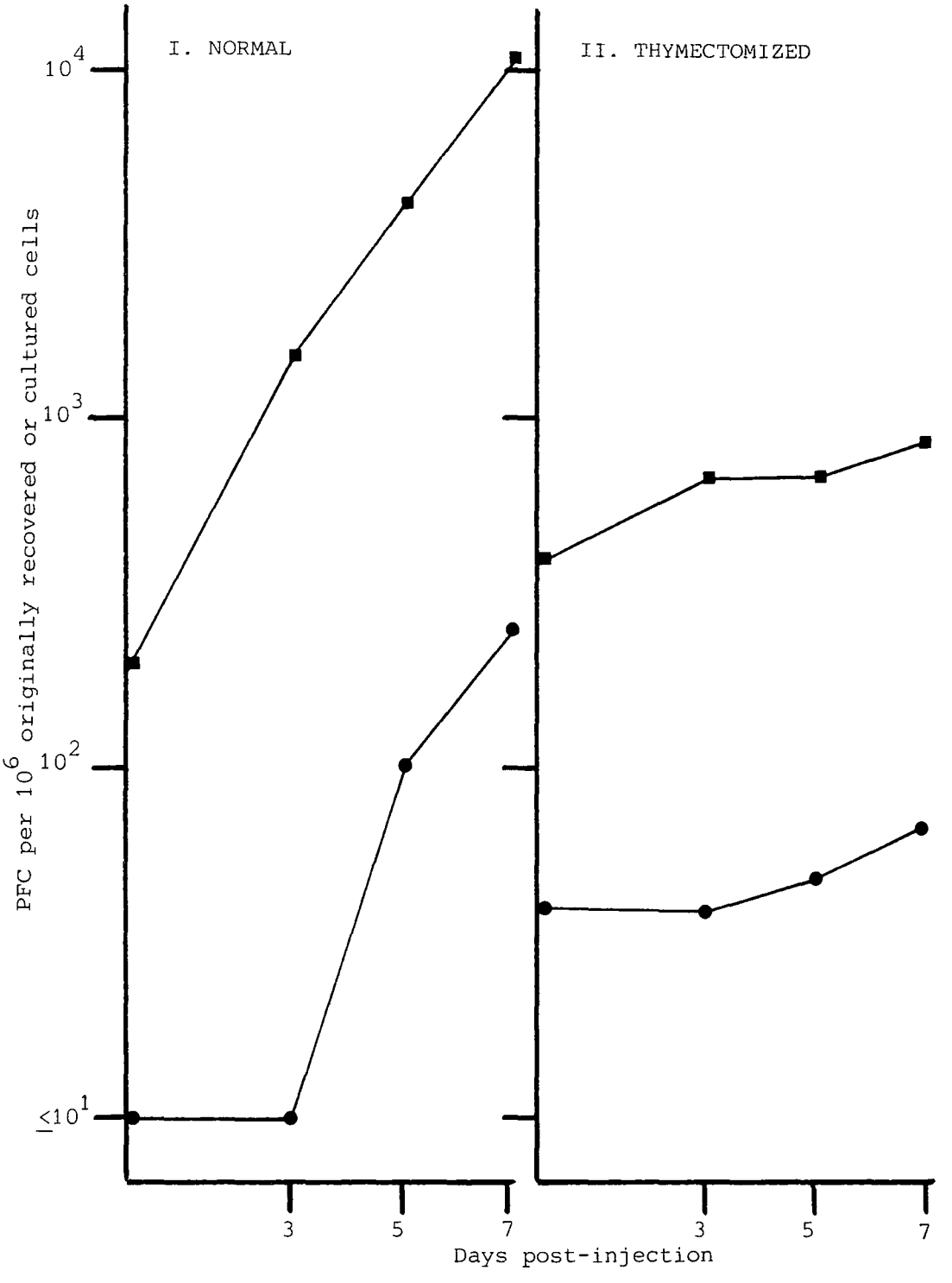


Figure 5.2 kinetics of the in vivo anti-RRBC PFC response by;

(I) normal animals and normal spleen cells
(II) thymectomized animals and spleen cells from Tx animals

PFC were measured;

(●) when recovered from the animal
(■) following six days culture without antigen

Responder cells are from 9 month old G-line animals

Measurements and cultures from pools of 2 animals each

Table 5.2 Induction of an *in vitro* PFC response by normal spleen cells using *in vivo* antigen pulsed stimulator cells from normal or thymectomized animals.

Source of stimulator cell	3000R Stimulator cells			
	NON-PRIMED		TWO-DAY RRBC PRIMED	
	G-line	LM-3	G-line	LM-3
None	85	-	-	-
Normal	75	90	355	75
Thymectomized	95	110	95	115

Anti-RRBC-PFC per 10^6 originally cultured responder spleen leukocytes

PFC were assayed using 1/50 diluted *Xenopus* serum from 6 month old G-line animals.

PFC were measured on day six of culture without antigen

Responder cells from normal 6 month old G-line and stimulator cells from either G-line (MHC compatible) or LM-3 (allogeneic) animals.

Individual cultures from pools of 2-6 animals.

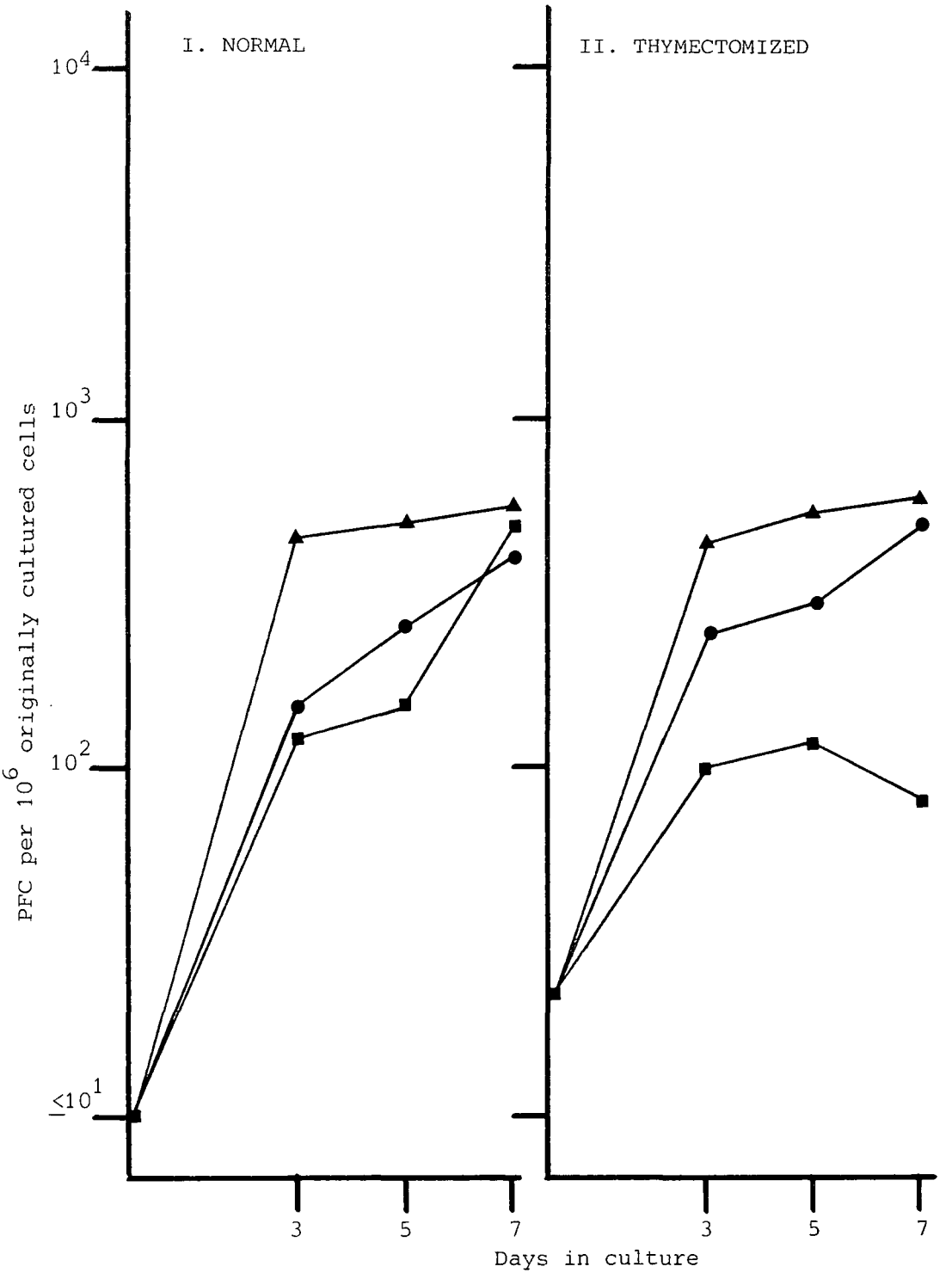


Figure 5.3 kinetics of the in vitro anti-RRBC PFC response by;

(I) normal spleen cells
(II) spleen cells from Tx animals

Cultures contained non-irradiated spleen cells supplemented with;

(■) no irradiated spleen cells
(●) normal MHC compatible irradiated spleen cells
(▲) normal MHC compatible two day in vivo primed irradiated spleen cells

Responder cells are from 6 month old G-line animals

Individual cultures from pools of 6-9 animals each

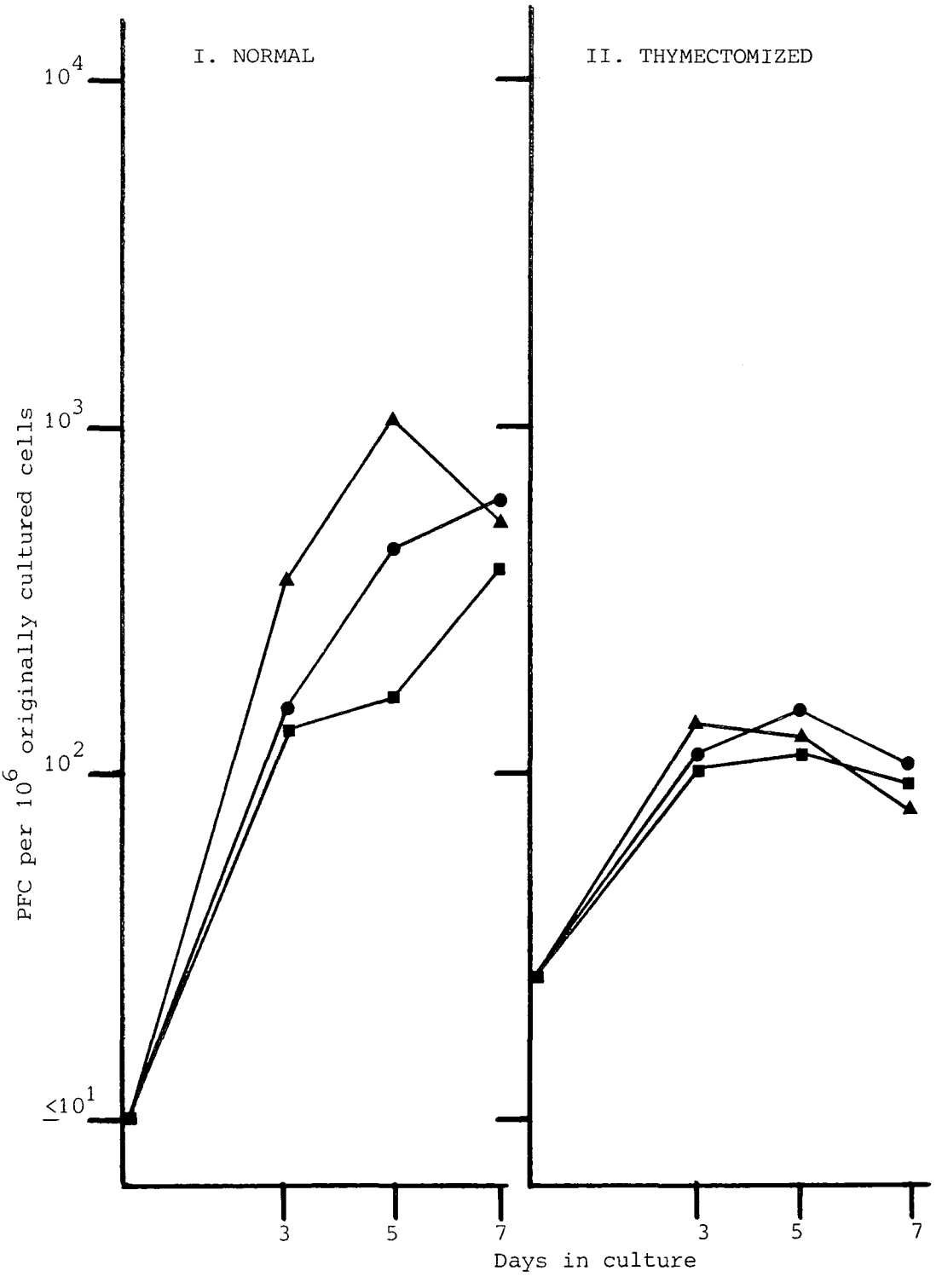


Figure 5.4 kinetics of the in vitro anti-RRBC PFC response by;

(I) normal spleen cells
(II) spleen cells from Tx animals

Cultures contained non-irradiated spleen cells supplemented with;

(■) no irradiated spleen cells
(●) normal allogeneic irradiated spleen cells
(▲) normal allogeneic two day in vivo primed irradiated spleen cells

Responder cells are from 6 month old G-line or LM-3 animals

Individual cultures from pools of 6-9 animals each.

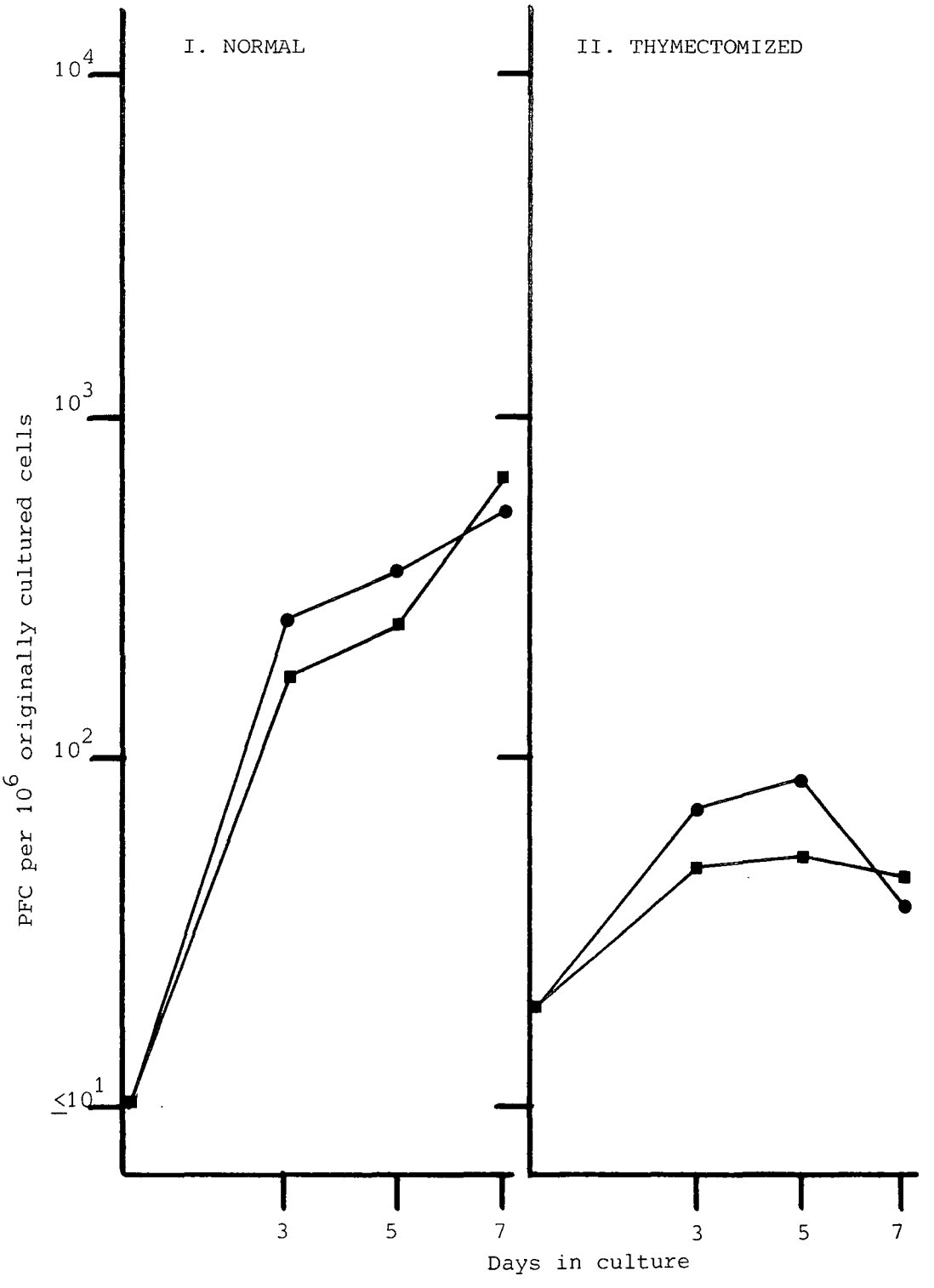


Figure 5.5 kinetics of the in vitro anti-RRBC PFC response by;

(I) normal spleen cells
(II) spleen cells from Tx animals.

Cultures contained non-irradiated spleen cells supplemented with;

(■) no irradiated spleen cells
(●) MHC compatible irradiated spleen cells from larvally thymectomized animals reimplanted with an adult thymus

Responder cells are from 6 month old G-line animals

Individual cultures from pools of 3-6 animals each.

Chapter Six

Concluding Remarks

The experiments described in this thesis have been an attempt to design a system which could be used to examine thymic and T cell function from a comparative point of view, using Xenopus as a phylogenetically-primitive model system. One advantage of using such an approach is that it sometimes provides insights into how the immune system of higher animals, such as mammals, evolved.

Invertebrates have no thymus or true T cells and yet their immune systems appear adequate to ensure survival. Vertebrates (jawed) on the other hand do have a thymus and some, or all, known classes of T cells. What little early thymectomy information is available for the various species indicates a heavy reliance on the thymus. A gradual and progressive loss of non-T cell regulated on non-T cell mediated responses during evolution may have resulted in a progressive increase in thymic dependence. The level of damage done by early thymectomy to an animal's immune response and to its chance of survival may be directly proportional to the phylogenetic position which it occupies. Although proving such a hypothesis will require a great deal more phylogenetically-oriented early thymectomy data, some evidence can be derived from Xenopus. For example, while early thymectomy (or abnormal thymus development) in mammals may abolish normal humoral and cellular immunity, interfere with histological development of lymphoid tissues, and be lethal, in amphibians it may only partially impair humoral and cellular immunity, have relatively little effect on histological development of lymphoid tissues, and is not lethal.

Such a pattern of evolution would tend to indicate a selective advantage of possessing a lymphocyte population with some characteristics of T cells. One characteristic which distinguishes T cells of all classes from other lymphoid cells is the way in which self MHC antigen recognition is integrated into their function (see Langman, 1978; see Zinkernagel, 1979). Dual specificity for foreign hapten-carrier type antigens assures that humoral responses are elicited only by intact and B cell-bound foreign antigens, while dual specificity for foreign modified-self type antigens ensures that cellular responses are elicited only by intact and infected target cells. Once intracellular antigen is released and once extra cellular antigen is broken down, both become accessible to phagocytosis.

The data in this report have been obtained mainly either from cell mediated lympholysis or plaque forming cell assays, which, in Xenopus, may or may not address questions concerning the function of separate T cell subsets. The techniques used have been largely adapted from ones originally designed for, and commonly used with, animals other than Xenopus (esp. mice). The results of the experiments have been interpreted primarily in relation to previous work examining antigen recognition, in particular MHC antigen recognition by cytotoxic and helper T cells of animals phylogenetically higher than Xenopus (i.e. again mice). There is as yet no evidence to suggest that the T cell system of Xenopus did not develop according to, and is not governed by, the same set of rules which apply to mice. However, the

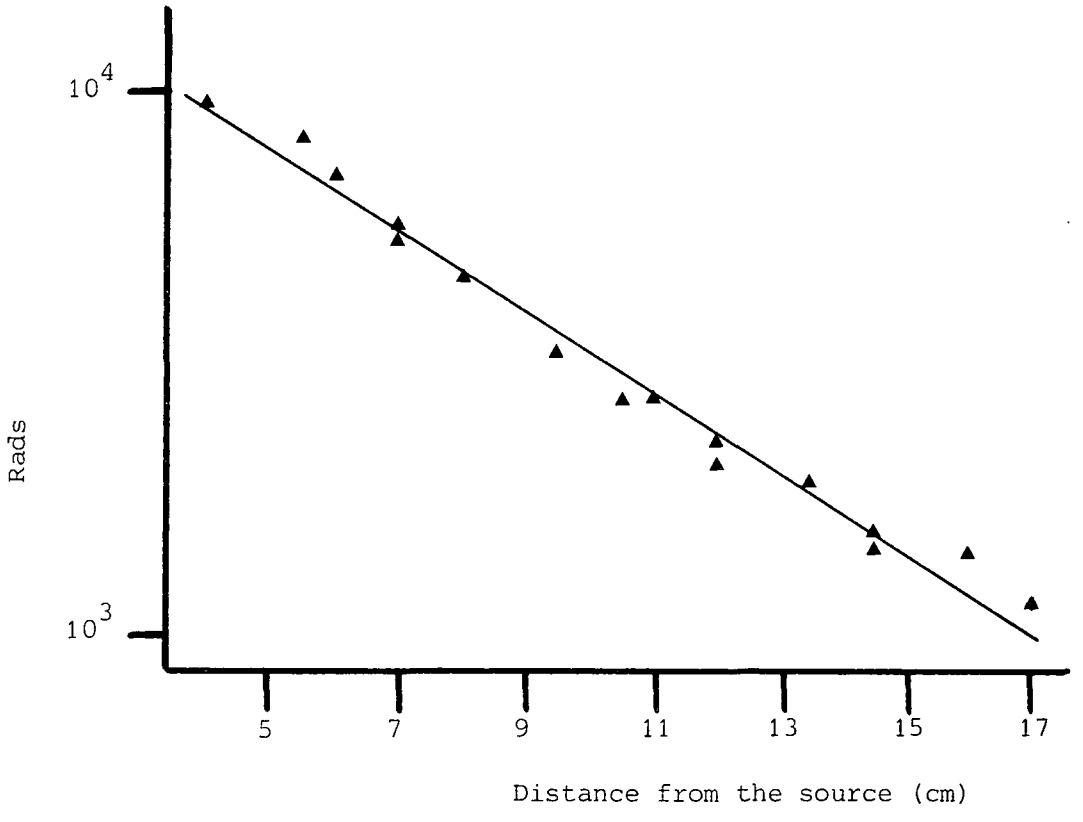
results point out two major discrepancies, primarily concerning the role of MHC antigens and the thymus in these two animals. One discrepancy is that cytotoxic spleen cells from normal and thymus-reimplanted thymectomized animals, which reject both minor and major alloantigen disparate askin grafts, appear able to lyse MHC disparate, but not non-MHC disparate target cells. Furthermore, cytotoxic spleen cells from thymectomized animals, which reject major alloantigen-disparate skin grafts, appear unable to lyse such target cells (in an *in vitro* short-term assay). The other discrepancy is that helper spleen cells from normal animals appear unable to restore *in vitro* antibody responses of allogeneic thymectomized spleen cells. Moreover, helper spleen cells from thymus-reimplanted thymectomized animals, which mount good *in vivo* PFC responses, appear unable to restore *in vitro* antibody responses of spleen cells taken from even MHC compatible thymectomized animals.

Each of these observed discrepancies could certainly be due to technical deficiencies and assay insensitivity, but they could also be due to more inherent differences between *Xenopus* and mice. One major difference may be that the *Xenopus* T cell repertoire is limited a) to cytotoxic T cells capable of reacting with allogeneic (and xenogeneic) MHC antigens but not with allogeneic minor (or modified self) antigens, and b) to helper T cells capable of reacting with antigen plus self MHC (self restricted) B cells, but not with antigen pluse allogeneic MHC (allo restricted) B cells. Another major difference may be

that antigen presenting cells in Xenopus are limited a) to cells capable of presenting allogeneic (and xenogeneic) MHC antigens to cytotoxic T cells in vitro but not allogeneic minor (or modified self) antigens, and b) to cells which develop their capacity for presenting foreign antigen to self MHC-restricted helper T cells exclusively under the influence of the thymus. A final major difference may be that lymphoid cells cultured in vitro are limited a) to target cells (PHA induced lymphoblasts) expressing major but not minor histocompatibility antigens, and b) to B cells (spleen cells from thymectomized animals) capable of receiving help from syngeneic but not allogeneic helper T cells.

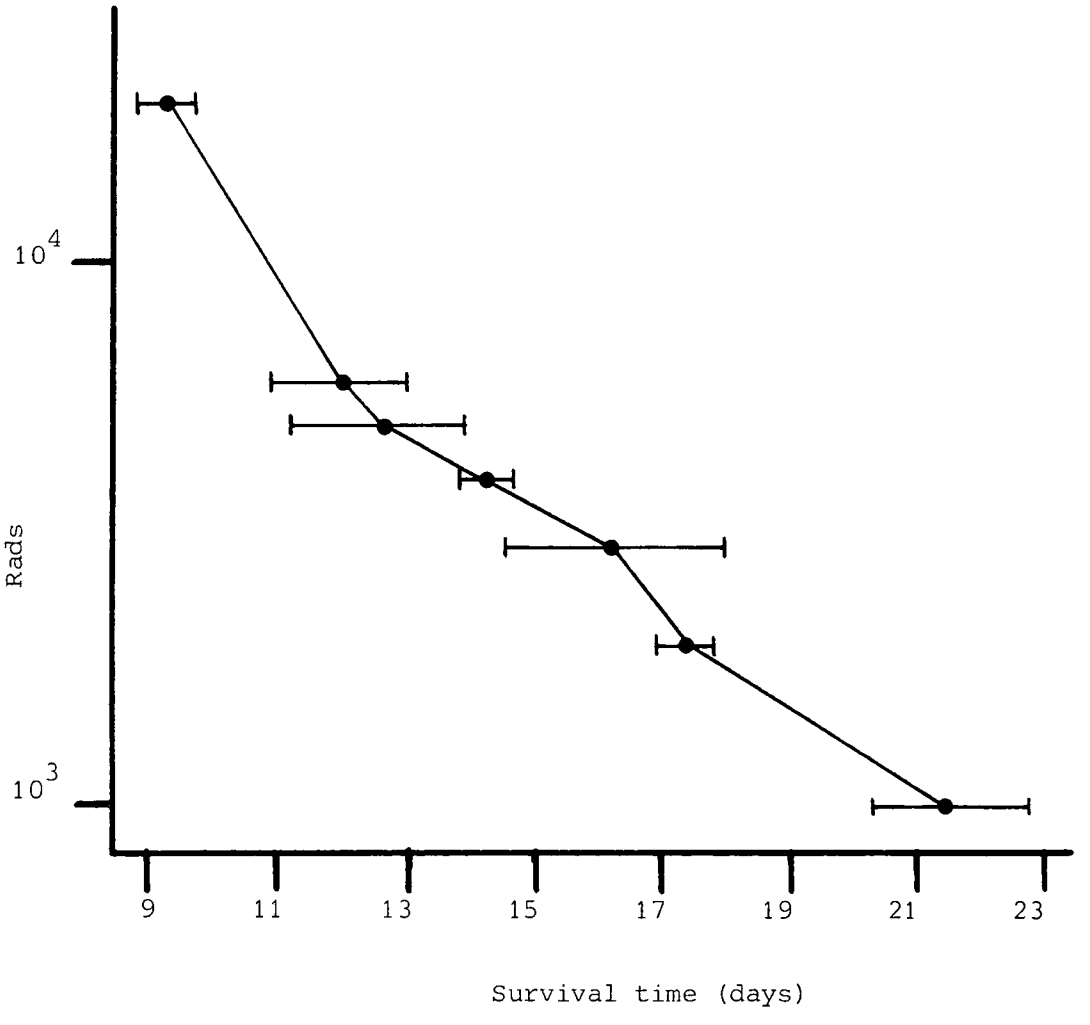
The continued adaptation of new immunological techniques for experimentation in Xenopus may make the model increasingly useful. Using clonal analysis techniques, patterns of T cell repertoire could be examined (for example whether T cells develop from a random self plus X or an allogeneic MHC repertoire). Using better lymphoid cell separation techniques, the development of functional antigen-presenting macrophages and/or B cells could be examined (for example whether such cells arise from the thymus or under the influence of T cells). Using new and well-defined antibody preparations, cell surface antigen expression could be examined (for example whether antigen expression changes during various stages of the lymphocyte cell cycle).

Appendices



Appendix 1 Measurement of the total irradiation dose received after 5 minutes exposure at various distances from the cobalt-60 source.

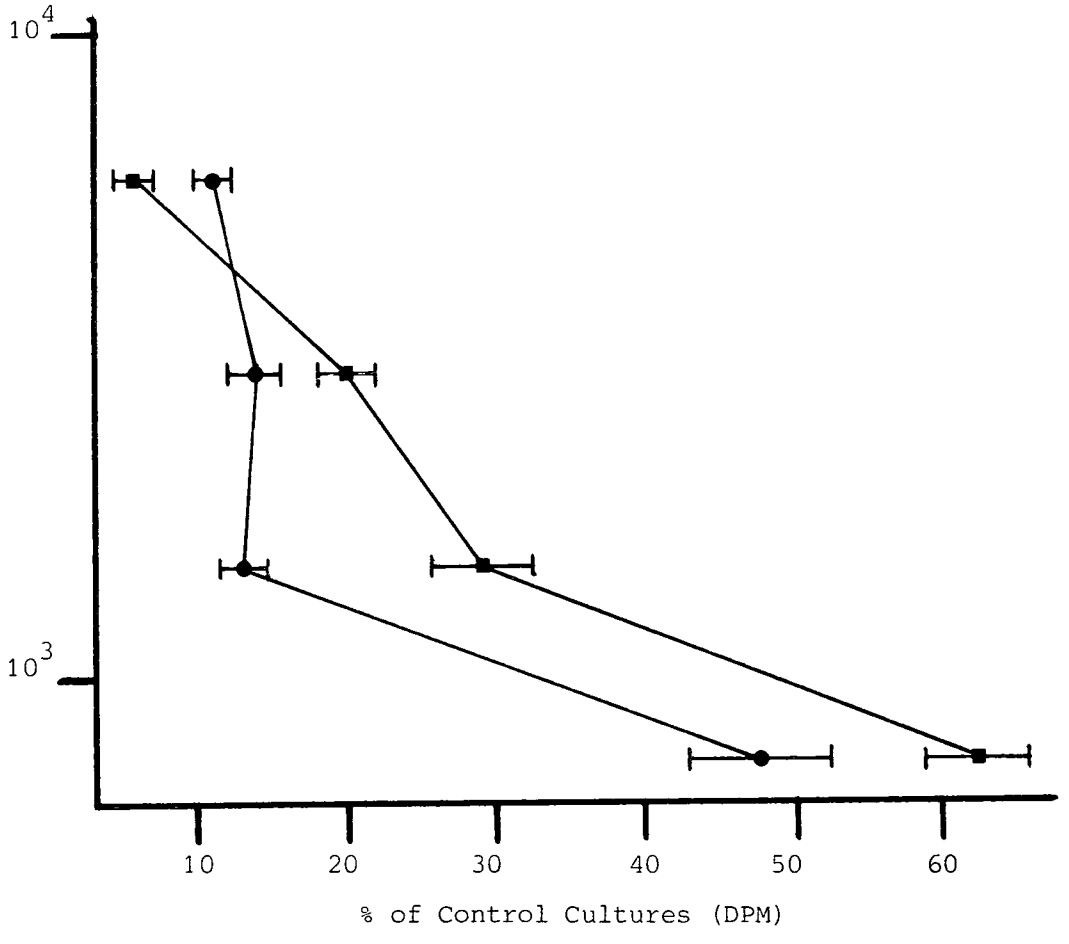
Irradiation doses measured using fervous ion chemical dosimeter.



Appendix 2 Survival times of animals exposed to various irradiation doses from the cobalt-60 source.

Test animals are 6 month old outbred Xenopus.

Each point represents $\bar{x}$ ± SEM of 3 animals per group



Appendix 3. In vitro tritiated thymidine uptake of normal spleen cells exposed to various irradiation doses from a cobalt-60 source

Cultures were pulsed;

(■) at 72 hours and harvested at 96 hours

(●) at 120 hours and harvested at 144 hours

Test cells are from 6 month old outbred animals.

Each point represents $\bar{x}$ ± SEM of triplicate cultures

Appendix 4. Comparisons between one and two way proliferative responses in MLC using normal and irradiation inactivated stimulator cells.

	3H-TdR Uptake in One and Two Way MLC		
Spleen Cells in Culture	Donor 1	Donor 2	Donor 3
S self	10121 ± 351	17316 ± 2805	11613 ± 1120
RS self	126 ± 23	117 ± 13	207 ± 19
S+ RS self	12503 ± 1777	15849 ± 1892	12171 ± 520
S+ Sallo 1	68470 ± 2638	68470 ± 2638	85309 ± 2750
S+ Sallo 2	85309 ± 2750	79853 ± 4338	79853 ± 4338
S+ RSallo 1	29743 ± 772	45947 ± 1826	54849 ± 856
S+ RSallo 2	58808 ± 923	47433 ± 2748	37485 ± 1547

Test MLC was set up using responder and 6000R irradiated stimulator cells removed directly from the spleen.

MLR was measured using non-primed animals.

Mean DPM ± SEM

Responder and stimulator cells from 9 month old outbred animals

Triplicate cultures from 3 individual animals.

Appendix 5. The effect of serum concentration and pre-culturing on the 2 way proliferative response in MLC.

CELLS	FCS SERUM	Mean DPM LG-17	SEM	Mean DPM LG-3	SEM	Mean 2 way S.I.	SEM
Thymus	free	1635	106	1828	56	1.2	0.1
	1%	1730	108	2543	34	2.5	0.1
	10%	2349	97	3118	335	2.4	0.1
Spleen	free	1477	145	1535	104	16.4	0.5
	1%	12026	905	6763	631	3.8	0.1
	10%	29871	1400	17444	873	3.3	0.1

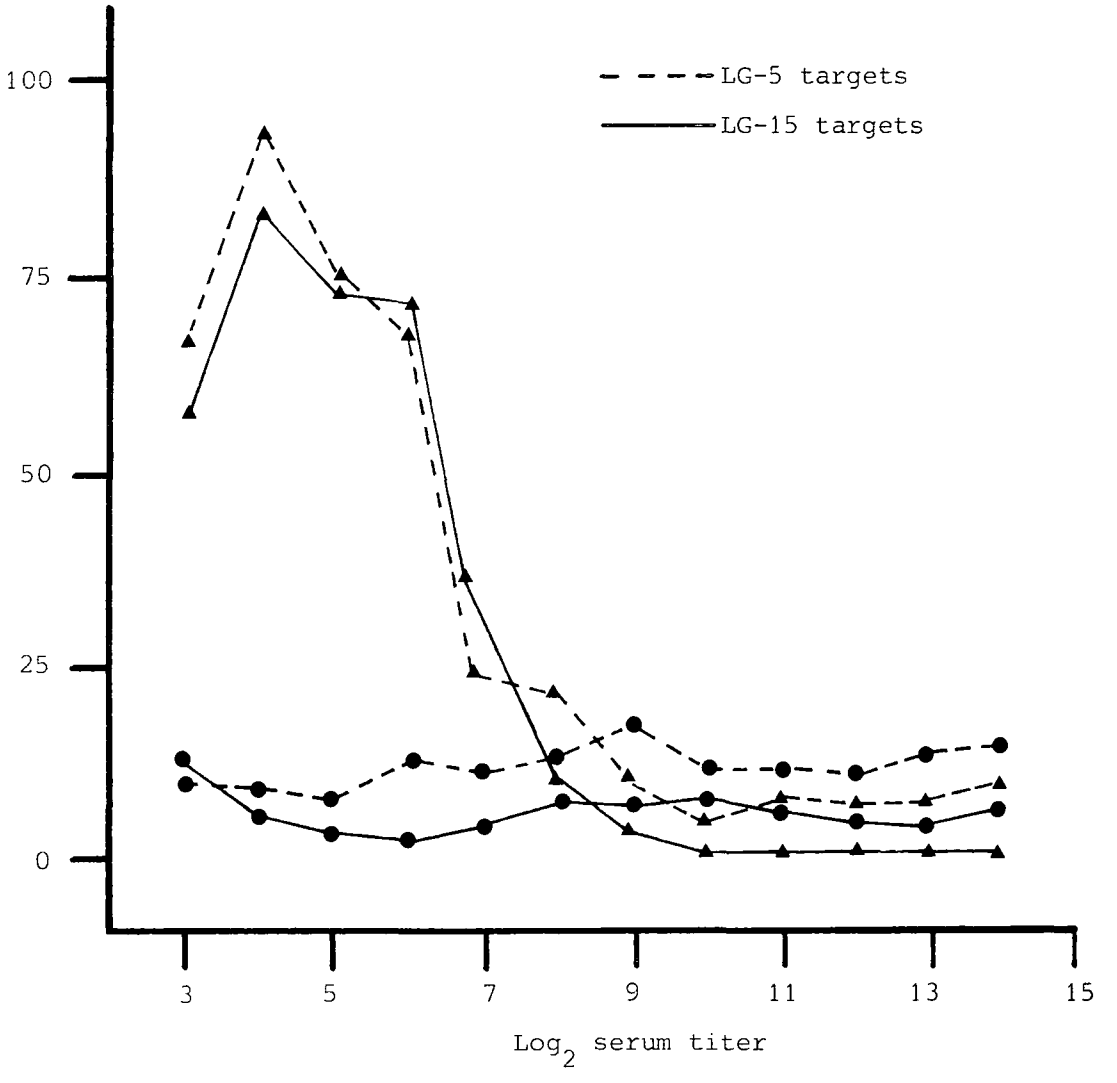
Test MLC was set up using cells removed directly from the animal

CELLS	FCS SERUM	Mean DPM LG-17	SEM	Mean DPM LG-3	SEM	Mean 2 way S.I.	SEM
Thymus	free	384	23	186	24	1.8	0.2
	1%	832	2	307	41	5.8	0.5
	10%	3233	300	1228	108	3.1	0.1
Spleen	free	571	74	498	74	8.7	1.1
	1%	1249	88	1843	239	13.0	0.8
	10%	3372	484	4095	597	7.4	0.3

Test MLC was set up using cells pre-cultured for 72 hours prior to the assay.

Responder and stimulator cells from 9 month old LG-17 or LG-3 animals.

Triplicate cultures from pools of 4 animals.



Appendix 6. Levels of *in vitro* target cell lysis by serum antibody taken either from;

(●) normal *Xenopus* LG-17 primed by double set skin grafting of skin from LG-5 or LG-15.

(▲) normal rabbits primed by double set injection of *Xenopus* leukocytes

Guinea pig serum used as a source of complement

Individual lots of serum pooled from 4 *Xenopus* or 2 rabbits.

Appendix 7. Effect of Nylon wool column separation, glass bead column separation, and irradiation on mature in vivo formed PFC and the primary in vitro PFC response.

RESPONDER	SRBC DOSE %	PFC per 10^6 originally recovered spleen leukocytes					
		N.W. 30°C		G.B. 30°C		3000R	
		Pre-	Post-	Pre-	Post-	Pre-	Post-
Adult	0	0	0	0	0	0	0
	10	305	275	242	250	136	98
Adult	0	0	0	ND	ND	0	0
	10	176	206	ND	ND	144	16

DONOR	RRBC/ LEUKO RATIO	PFC per 10^6 originally cultured spleen leukocytes					
		N.W. 30°C		G.B. 30°C		3000R	
		Pre-	Post-	Pre-	Post-	Pre-	Post-
Adult	0	80	0	58	1	60	0
	1	460	0	593	3	482	0
Adult	0	60	1	77	0	52	0
	1	710	2	573	5	570	0

PFC were assayed using 1/50 diluted guinea pig serum as a source of complement

PFC were measured six days following immunization or on day six of culture

Responder animals are and responder cells are from adult outbred animals

Individual measurements and individual cultures from individual animals

Appendix 8. The effect of altered culture conditions on the in vitro PFC and CTL responses of spleen cells from LG-17 animals.

CULTURE CONDITION	PFC per 10^6 originally cultured spleen leukocytes			
	NORMAL		THYMECTOMIZED	
	$5x10^6$/ml	$10x10^6$/ml	$5x10^6$/ml	$10x10^6$/ml
RRBC to leukocyte ratio	1.0	1.0	1.0	1.0
100% Non-irradiated responder spleen cells	77	710	91	101
50% non-irradiated responder plus 50% irradiated LG-17 normal spleen cells	ND	651	ND	576
50% non-irradiated responder plus 50% irradiated LG-17/17 chimera spleen cells	ND	154	ND	105

PFC were assayed using 1/50 diluted Xenopus serum from 6 month old LG-17 animals

ND = not done

PFC were measured on day six of culture.

CULTURE CONDITION	Percent specific Cr-51 release			
	$5x10^6$/ml (no feed)		$10x10^6$/ml (+ feed)	
Targets	LG-5	LG-15	LG-5	LG-15
Neither primed or stimulated	0%	2%	8%	ND
In vitro stimulated only	7%	5%	10%	ND
In vivo primed only	4%	0%	ND	4%
Primed and stimulated	79%	10%	ND	9%

Target cell lysis was measured without priming or following first plus second set skin graft rejection.

ND = not done

R/s ratio = 2/1 E/T ratio = 100/1

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