Reconstitution of a T cell-dependent antibody response in the thymectomized clawed toad Xenopus laevis

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Reconstitution of a T Cell-Dependent Antibody Response in the Thymectomized Clawed Toad *Xenopus laevis*

by

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DECLARATION

Preliminary accounts of the experiments reported in Chapters 2* and 6+ have been communicated to the International Society for Developmental and Comparative Immunology and are to be published in abstract form. The assistance of collaborators J.D. Horton and A.J.H. Gearing in Chapter 2 is gratefully acknowledged. Some of the work presented in Chapter 3 has been published++.

* Gearing, A.J.H., Horton, J.D. and Cribbin, F.A. (1984) A survey of the immune system of thymectomized Xenopus implanted with allogeneic thymus in larval life. Abstract in Proceedings 2nd Congr. Dev. comp. Immun. Los Angeles, 1983. Pergamon Press, New York. In press.

+ Cribbin, F.A. and Horton, J.D. (1984) Restoration of cellular antibody production to sheep erythrocytes in thymectomized Xenopus by reconstitution with allogeneic and MHC-compatible splenocytes and thymocytes. Abstract in Proceedings 2nd Congr. Dev. comp. Immun. Los Angeles, 1983. Pergamon Press, New York. In press.

++ Williams, N.H., Cribbin, F.A., Zettergren, L.D. and Horton, J.D. (1983) Ontogeny and characterization of mitogen-reactive lymphocytes in the thymus and spleen of the amphibian, Xenopus laevis. Immunology, 49, 301-309.

Reconstitution of a T Cell-Dependent Antibody Response in the Thymectomized Clawed Toad Xenopus laevis by Frances A. Cribbin.

Abstract - Larvally thymectomized Xenopus laevis are unable to mount an antibody response to thymus-dependent antigens. In Chapter 2, larval thymuses incompatible at the major histocompatibility complex (MHC) (either irradiated or non-irradiated), and both MHC-compatible and incompatible "adult" thymuses implanted to thymectomized animals early in larval life, were found to restore cellular and serum antibody production to the thymus-dependent antigen sheep erythrocytes.

Functional studies on thymocytes and splenocytes were carried out in Chapter 3 to determine whether thymocytes were relatively free from contaminating B cells and could be used as a "pure" source of T cells for cellular reconstitution experiments. It was shown that both thymocytes and splenocytes could be stimulated in vitro by the B cell mitogen Escherichia coli lipopolysaccharide to differentiate into cytoplasmic immunoglobulin M-producing cells. Thus thymocytes were found to include a population of B lymphocytes.

The experiments reported in Chapter 4 were designed to find a suitable method for the depletion of B cells from lymphocyte populations to be used for cellular reconstitution. Nylon wool filtration proved to be inefficient at depleting B cell numbers. However γ-irradiation was able to effectively remove B cell activity from unprimed lymphocyte populations.

In Chapter 5, the ability of injected lymphocytes to migrate to the spleen was investigated. It was shown using ^{51}Cr labelling and ploidy-marked cells that lymphocytes could reach the spleen within 6 hours after injection and remain there for at least 6 days.

Finally, in Chapter 6, cellular reconstitution experiments showed that injection of thymocytes or splenocytes was not very effective in restoring the cellular antibody production of thymectomized animals to sheep erythrocytes. However splenocyte/peripheral blood lymphocyte mixtures, even after irradiation to remove functional B cells, were able to fully restore the cellular antibody response, irrespective of whether the injected cells were MHC-compatible or incompatible to the host. Thus it appears that the short-term primary in vivo antibody response to sheep erythrocytes does not require MHC identity of the T and B lymphocyte populations.

CHAPTER ONE

GENERAL INTRODUCTION

Cellular Interactions in Mammalian Immune Responses

Two types of immune responses are distinguished, namely cell-mediated immunity (which protects against intracellular viral and bacterial infections, and is centrally involved in allograft rejection) and humoral immunity (effected by release of antibodies, which bind extracellular antigens, enhancing their phagocytosis, and which can neutralize toxins). Two populations of lymphocytes are involved with mediating these immune responses. Thus T lymphocytes (processed by, or in some way dependent on, the thymus) are responsible for cell-mediated immunity, while B lymphocytes (bursa-dependent in birds, bursa-equivalent-dependent in mammals) synthesize circulating antibody. There are three major classes of T lymphocytes: cytotoxic T cells, which are involved in the lysis of specific antigenic target cells; suppressor T cells, which bring about the suppression of immune responses; and helper T cells, which can stimulate both T and B cell function (see Hood, Weissman and Wood, 1978 for review).

Two distinct groups of antigens are involved in humoral responses. Thymus-independent antigens (e.g. lipopolysaccharide, ficoll and polyvinylpyrrolidone) trigger antibody production by B cells, without the involvement of helper T cells. These antigens tend to be high molecular weight polymers with a simple repeating structure and are poorly metabolised (Mosier and Subbarao, 1982). In contrast, thymus-dependent antigens (e.g. sheep erythrocytes, rabbit erythrocytes and human immunoglobin G) require the co-operation of helper T cells to induce specific antibody production in B cells. This Thesis will be

mainly concerned with the response to the thymus-dependent antigen, sheep erythrocytes (SRBC).

The first experiments demonstrating that both T and B cells were required for antibody production to certain antigens came in 1966, when Claman, Chaperon and Triplett discovered that irradiated mice injected with thymocytes and bone marrow (a mammalian bursa-equivalent) cells together, were able to mount a good antibody response to SRBC, whereas a poor response was obtained with either thymocytes or bone marrow cells alone. This study was later confirmed and expanded by Mitchell and Miller and co-workers. They were able to restore the cellular antibody response of neonatally thymectomized mice (which respond relatively poorly to SRBC) using thoracic duct lymphocytes or thymocytes (Miller and Mitchell, 1968; Miller, Mitchell and Weiss, 1967). Most of the antibody-producing cells were found to be derived from the host, not from the injected lymphocytes, by using cells which could be identified by the H-2 antigen (a surface marker) or by chromosome markers (Miller and Mitchell, 1968; Nossal, Cunningham, Mitchell and Miller, 1968). Another series of experiments employed adult-thymectomized animals that were lethally irradiated and protected with an injection of bone marrow, before being reconstituted with thoracic duct lymphocytes or thymocytes. Once more it was found that most of the antibody-producing cells were derived from host cells, presumably bone marrow-derived (Mitchell and Miller, 1968). It was also shown that in lethally irradiated mice restored with mixtures of thoracic duct lymphocytes and bone marrow cells, the antibody-forming cells produced were of bone marrow origin (Nossal, Cunningham, Mitchell and Miller, 1968). These experiments showed that two types of lymphocytes were required for production of a good antibody response to

SRBC - thymus-derived T cells and bone marrow-derived B cells.

Further support for the T-B cell collaboration hypothesis was obtained from hapten-carrier experiments. A primary antibody response to a hapten (a small non-immunogenic molecule) was found to take place only if the hapten was coupled to a larger carrier molecule. In order to produce a secondary response, both hapten-primed and carrier-primed cell populations were required (Mitchison, 1971; Rajewsky, Shirrmacher, Nase and Jerne, 1969). It was also shown by using cells which carried different immunoglobulin allotype genes, that all the anti-hapten antibody produced was of the allotype of the hapten-primed cells, and that the carrier-primed cells did not themselves produce antibody - rather they were functioning in a helper capacity (Mitchison, 1971).

The mouse mutant known as nude, which has a congenitally hypoplastic thymus (Pantelouris, 1968), is deficient in the ability to mount an antibody response to SRBC (Kindred, 1971a; Reed and Jutila, 1972). In experiments designed to determine whether the antibody-producing capacity of these mice could be restored with an injection of thymocytes, it was shown that restoration would only take place if the injected cells were related to the nude recipients (Kindred, 1971b). The required relationship was found to be compatibility at the major histocompatibility complex (MHC), as a lasting restoration of antibody production was only produced with MHC-compatible cells (Kindred and Shreffler, 1972).

The MHC is a cluster of closely-linked genes carried on a single chromosome. The products of these genes are important in a variety of immune responses. There are three main classes of loci within the MHC. Class I loci control the major transplantation antigens (K and D in the mouse): these molecules are found on all mammalian cells, excluding some in early embryonic and final stages of development,

and are found in high concentrations on B and T lymphocytes and macrophages. Class II regions control immune responsiveness (I in the mouse). Included within the class II regions are the IA loci. The Ia molecules are found mainly on B cells, and also on T cells, epidermal cells and macrophages to a lesser extent. Finally, the class III regions control the components of the complement system (see Hobart and McConnell, 1975; Hood, Weissman and Wood, 1978).

Many experiments have been performed in recent years, both in vivo (e.g. Sprent, 1978a; Sprent, 1978b) and in vitro (e.g. Jones and Janeway, 1981; Yamashita and Shevach, 1978) that confirm the existence of the phenomenon of MHC restriction between helper T cells, B cells and macrophages in the T cell-dependent antibody response (see also reviews by Julius, 1982 and Sprent, 1978c). Cytotoxic T cells also show an MHC-based restriction specificity. Thus cytotoxic T cells were found to kill only virus-infected cells of the same K and D MHC type as themselves, and not virus-infected cells of a different MHC type (Zinkernagel and Doherty, 1974).

The precise mechanisms for the cellular interactions involved in the production of antibody in a T cell-dependent response are still under debate. However, a number of steps can be identified (reviewed in Howie and McBride, 1982). The foreign antigen is first of all taken up by macrophages, and processed into a form capable of stimulating helper T cell activity (Unanue, 1981). These macrophages then present the processed foreign antigen, now associated with the macrophage membrane, to specific helper T cells, in some form of association with their own Ia antigens (Yano, Schwartz and Paul, 1977). This step initiates the differentiation and proliferation of helper T cells, a process which also appears to require certain lymphokine factors,

such as interleukin 1, produced by antigen-stimulated macrophages, and interleukin 2, produced by a T cell subset (Oppenheim and Gery, 1982; Smith, 1981; Smith and Ruscetti, 1981; Unanue, 1981). The activated helper T cells then stimulate B lymphocytes to divide and differentiate, to produce antibody-secreting cells. A variety of lymphokines may also be important in the interactions between T and B cells, and in the clonal expansion of B lymphocytes (Farrar and Hilfiker, 1982; Martinez and Coutinho, 1981; Schimpl and Wecker, 1975; Taussig, 1980).

There is some controversy as to the level at which MHC restriction takes place during T-B-macrophage interactions. Some workers believe that only the activation of helper T cells by macrophages is MHC restricted, and that the interaction between helper T and B cells is unrestricted (Erb, Meier, Matsunaga and Feldmann, 1979; Ertl, 1981; McDougal and Cort, 1978; Singer, Hathcock and Hodes, 1979; Singer, Hathcock and Hodes, 1980; Vogt, Erb, Keller and Feldmann, 1981). Others, however, consider that both the helper T cell-macrophage and the helper T-B cell interactions are MHC restricted (Hünig and Schimpl, 1979; Jones and Janeway, 1981; Sprent, 1978a; Sprent, 1978b; Yamashita and Shevach, 1978). This apparent discrepancy may relate to the state of differentiation of the B cell. It has been shown that polyclonally activated B cell blasts can be stimulated to secrete antibody by antigen-activated helper T cells, irrespective of their MHC type. On the other hand, small resting B cells require histocompatible helper T cells to enable them to respond to specific antigen (Andersson, Schreier and Melchers, 1980; Schreier, Andersson, Lernhardt and Melchers, 1980). Another possible explanation for the discrepancy is that different B cell subsets may have different

requirements for activation by helper T cells. The expression of the B cell surface differentiation antigen Lyb-5 appears to be important in this respect. Thus the activation of Lyb-5^- B cells by helper T cells is MHC restricted, whereas Lyb-5^+ B cells are activated by helper T cells without the requirement for identity at the Ia locus (Asano, Singer and Hodes, 1981; Singer, Morrissey, Hathcock, Ahmed, Scher and Hodes, 1981).

Recognition of and restriction to self-MHC antigens (K and D) by cytotoxic T cells, was originally thought to be "learnt" in the thymus during development. Zinkernagel et al. performed experiments with bone marrow radiation chimeras (lethally irradiated mice, reconstituted with bone marrow depleted of functional T cells), in which the thymus of the host was of type A and the differentiating T cells (from the bone marrow) of type A x B. They were able to demonstrate that the restriction specificity of cytotoxic T cells was determined by the MHC type of the thymus, not by the MHC type of the lymphocytes themselves. The role of the thymus was confirmed using thymus grafts. Thus irradiated thymuses of type A were grafted into adult-thymectomized, lethally irradiated animals (type A x B), protected with an injection of bone marrow depleted of functional T cells (type A x B). Again, cytotoxic T cells were generated that were restricted to killing virally-transformed type A target cells, but not type B. Hence it was concluded that the radioresistant portion of the thymus determines the K and D MHC restriction specificity of cytotoxic T cells during maturation of T cells in the thymus (Zinkernagel, Callahan, Althage, Cooper, Klein and Klein, 1978). Similar results have been shown for helper T cells using bone marrow radiation chimeras (Singer, Hathcock and Hodes, 1981), i.e. helper T cells from allogeneic chimeras would

only provide help for B cells with the same MHC (Ia) type as found in the host thymus.

However, conflicting data has come from experiments using thymus-grafted nude mice. The early work with these animals suggested that thymic selection alone is not sufficient for the expression of the restriction specificity repertoire of the T cells. Thus nude mice grafted with a fully allogeneic thymus were found to possess cytotoxic T cells (Zinkernagel, Althage, Waterfield, Kindred, Welsh, Callahan and Pincetl, 1980) and helper T cells (Kindred, 1978) restricted to the MHC type of the nude host, rather than to the donor thymus. In contrast, other workers have more recently shown that helper T cells and cytotoxic T cells can be restricted to the donor thymus MHC type, when nude mice are grafted with allogeneic thymuses (Singer, Hathcock and Hodes, 1982 and Kruisbeek, Sharrow, Mathieson and Singer, 1981, respectively). However, Kruisbeek et al. found that the spleens of thymus-engrafted nude mice had precursor cytotoxic T cells restricted to the MHC of the nude host, unlike the precursor cytotoxic T cells in the thymuses which were restricted to the MHC of the thymus. This suggested that there are two pathways for the restriction of cytotoxic T cells, one being intrathymic and restricted to the MHC of the thymus, and the other extrathymic, restricted to the nude host MHC type. Non-operated nude mice have also been shown to produce MHC-restricted cytotoxic T cell responses in the absence of a thymus (Hünig, 1983).

To summarise, there is some confusion concerning the site at which restriction to self-MHC is learnt by developing cytotoxic and helper T cells (see also Howard, 1980); however, the concensus of opinion seems to be that MHC restriction can take place in the thymus, although other sites for restriction also seem to exist.

Cellular Interactions in Amphibian Immune Responses

Primordial cell-mediated immunity with short-term memory is first shown in advanced invertebrates, e.g. annelids and echinoderms. However, only vertebrates possess an integrated cell-mediated and humoral immunity, with both functional T and B cells (Hildemann, 1974; Tam, Reddy, Karp and Hildemann, 1976). Even the most primitive of vertebrates, the agnathans, have T and B cells, although these animals do not possess a thymus and their lymphoid tissue lacks organization, with no clear separation of primary and secondary lymphoid organs, and stem cell sources. On the other hand, the jawed vertebrates all have a thymus, in which the differentiation of T cells takes place, as well as peripheral (secondary) lymphoid organs, such as the spleen (Manning, 1981).

As vertebrates become more advanced, their cell-mediated immunity also improves, with the development of the MHC (Cohen, 1980). The primitive agnathans show a chronic allograft rejection with short-term memory - i.e. accelerated rejection of second set grafts. This graft rejection is still chronic in the primitive bony fish, but the teleosts show acute rejection of allografts [(one indication of the possession of an MHC complex (Cohen, 1971)] with memory (Hildemann, 1974; Tam, Reddy, Karp and Hildemann, 1976). The more primitive amphibians - the apodans and urodeles - have only chronic graft rejection, but the anuran amphibians reject skin allografts in an acute fashion (Cohen, 1971; Cohen, 1980; Tam, Reddy, Karp and Hildemann, 1976). Xenopus laevis, a primitive anuran amphibian rejects skin allografts in a sub-acute fashion, perhaps indicating that Xenopus has fewer "strong" histocompatibility antigens than the more advanced anurans, such as Rana pipiens or Rana catesbeiana (Cohen, 1971). The reptiles again

show a chronic graft rejection, but the birds and mammals have a prompt and vigorous transplantation immunity, and a well-developed MHC (Cohen, 1980; Tam, Reddy, Karp and Hildemann, 1976).

Immunoglobulins are universally found in vertebrates. Even the agnathans can produce IgM-type antibodies and this class of immunoglobulin is present in all vertebrates. As the vertebrates become more advanced, the number of immunoglobulin classes increases. The anuran amphibians are the first group to display a second class of immunoglobulin - IgRAA. Reptiles also have two classes of immunoglobulin (IgM and IgRAA), while birds and eutherian mammals have four (IgM, IgRAA, IgA and IgE) and five classes (IgM, IgG, IgA, IgE and IgD) respectively (Jurd, 1984).

The anuran amphibian Xenopus laevis (the South African clawed toad) is a good animal model to use for the examination of both ontogenetic and phylogenetic aspects of immunity. This species can be reared and maintained easily in the laboratory, genetic "strains" are becoming available and a good knowledge of its immune system has already been provided (see Cohen and Turpen, 1980). Xenopus, like many other ectotherms, has a free-living larval stage so that the development of its immune system is essentially free from any continuous maternal influence. Xenopus possesses a variety of lymphoid organs and tissues - the thymus, spleen, bone marrow, kidney, liver and gut-associated lymphoid tissue - as well as the basic cell types of the immune system, such as macrophages and functionally distinct T and B lymphocytes (Manning and Horton, 1982).

Thymus-dependent and thymus-independent lymphocytes have been demonstrated in Xenopus by using the mitogens phytohaemagglutinin (PHA) and Concanavalin A (Con A) which selectively stimulate T cells in mice, and lipopolysaccharide (LPS) from Escherichia coli and

purified protein derivative from Mycobacterium tuberculosis which are mitogenic for mammalian B cells (Greaves and Janossy, 1972). Lymphocytes from the spleens of control Xenopus are able to respond to Con A and PHA (Donnelly, Manning and Cohen, 1976; Green and Cohen, 1979; Horton, Smith, Williams, Smith and Sherif, 1980; Williams, Cribbin, Zettergren and Horton, 1983), and to LPS (Horton, Smith, Williams, Smith and Sherif, 1980; Williams, Cribbin, Zettergren and Horton, 1983) and purified protein derivative (Donnelly, Manning and Cohen, 1976; Green and Cohen, 1979). In contrast, in animals thymectomized early in larval life the mitogen response to PHA is virtually abolished (Du Pasquier and Horton, 1976; Horton and Sherif, 1977; Manning, Donnelly and Cohen, 1976), as is the response to Con A (Manning, Donnelly and Cohen, 1976). The B cell mitogen responses to LPS and purified protein derivative however, remain normal following early thymectomy (Manning, Donnelly and Cohen, 1976).

The thymus-dependent and thymus-independent immune responses shown by Xenopus can also be demonstrated using antigens that are thymus-dependent and thymus-independent in mammals. Thymectomized animals are unable to mount an antibody response to the thymus-dependent antigens SRBC (Horton, Rimmer and Horton, 1976; Turner and Manning, 1974), rabbit erythrocytes (Tochinai and Katagiri, 1975), human immunoglobulin G (IgG; Turner and Manning, 1974) and dinitrophenylated-keyhole limpet haemocyanin (Du Pasquier and Wabl, 1976), whereas non-thymectomized animals respond well. On the other hand, thymectomized animals can respond normally to the thymus-independent antigens LPS (Collie, Turner and Manning, 1975) and polyvinylpyrrolidone (Tochinai, 1976).

Xenopus displays a sub-acute skin allograft rejection response (see Cohen, 1971) which is impaired following early thymectomy (Horton

and Manning, 1972; Kaye and Tompkins, 1983; Rimmer and Horton, 1977). Interestingly, a very chronic allograft rejection can still be mediated following early thymectomy if the thymectomized host and skin donor are sufficiently disparate (Nagata and Cohen, 1983), whereas alloimmunity appears to be abrogated in certain donor and host combinations (Tochinai and Katagiri, 1975). The mechanism of the "thymus-independent" chronic graft rejection remains to be elucidated (see Nagata and Cohen, 1983).

The mixed lymphocyte response (MLR), another manifestation of an MHC (see Cohen, 1980) is also abrogated in early-thymectomized animals (Du Pasquier and Horton, 1976; Horton and Sherif, 1977). The genetics of the Xenopus MHC has been described by Du Pasquier and co-workers. They found that animals from one family could be divided into four groups of MLR-identical siblings, whereas only a small number of "unrelated" animals were MLR-identical. This led to the suggestion that MLR reactivity depends upon one genetic region (Du Pasquier and Chardonnens, 1975; Du Pasquier, Chardonnens and Miggiano, 1975; Du Pasquier and Miggiano, 1973; Du Pasquier and Weiss, 1973). Studies on tolerance induction to allografts at metamorphosis showed that thiourea-blocked siblings given a skin graft at this time could also be segregated into four classes of mutually tolerant animals. On the other hand, "unrelated" animals rejected between 90 and 100% of grafts. Hence it was suggested that graft rejection at metamorphosis was controlled by one genetic region (Chardonnens, 1975; Chardonnens and Du Pasquier, 1973; Du Pasquier and Chardonnens, 1975).

Further experiments demonstrated that MLR and acute graft rejection in adult Xenopus seemed to be controlled by the same genetic region. Grafts exchanged between siblings differing at two MLR

haplotypes appeared to be rejected more rapidly than when animals differed at one MLR haplotype, and both of these groups rejected their grafts significantly faster than grafts between MLR-identical siblings (Du Pasquier, Chardonnens and Miggiano, 1975). These latter authors were also able to produce agglutinating antisera specific for certain red blood cell antigens. These antigens were found to be coded by the same genetic region that controls MLR and acute graft rejection. Using similar antisera, graft rejection at metamorphosis was also shown to be controlled by this same genetic region (Du Pasquier and Chardonnens, 1975). The genetic relationship between MLR, graft rejection and some red blood cell antigens suggests the existence of a genetic region in Xenopus equivalent to the MHC of mammals and birds.

Some products of the MHC are expressed in Xenopus larvae, as an MLR reaction can be detected between tadpoles of the same stage (Du Pasquier and Weiss, 1973). However, two MHC antigens at least are known to appear at metamorphosis. Immunofluorescence analysis has been used to show that one antigen, present on lymphocytes, appears 10-15 days before the end of metamorphosis, and a second antigen present on erythrocytes (and presumably lymphocytes) appears 1.5 months post-metamorphosis (Du Pasquier, Blomberg and Bernard, 1979).

Xenopus is able to produce two classes of antibody - IgM and an IgG-like molecule, sometimes called IgRAA (Jurd, 1984). Primary immunization of this frog with foreign antigen stimulates the production of IgM early in the response, with the appearance of Ig"G" about 3-6 weeks later (Hadji-Azimi, 1971; Lykakis, 1969; Wabl and Du Pasquier, 1976). Booster antigen stimulations cause a secondary response, with an increase in the levels of both IgM and

Ig"G". However, there is no shift from IgM to Ig"G" activity, as is found in the mammalian response (Hadji-Azimi, 1971). [In mammals, primary immunization causes early secretion of IgM antibody, followed by the appearance of IgG several days later. With the increase in IgG levels, the production of IgM declines, until about 2 weeks after the injection of antigen, the IgM is completely replaced by IgG. Secondary immunization brings about secretion of greater amounts of IgG, but only relatively low levels of IgM are produced (Uhr, 1964).]

A variety of techniques (immunofluorescence, immunoferritin and immunoperoxidase microscopy) have been used to show that Xenopus possesses two populations of peripheral lymphocytes, one surface Ig positive and one surface Ig negative (Du Pasquier, Weiss and Loor, 1972; Hadji-Azimi, 1977; Jurd and Stevenson, 1976; Nagata and Katagiri, 1978). Some early experiments suggested that a large percentage of thymocytes were surface Ig positive in both larval and young adult Xenopus. This level of surface Ig-positive cells was found to be much lower in the thymocytes of older adults (animals > 1 year old; approximately 10% surface Ig-positive cells) (Du Pasquier, Weiss and Loor, 1972; Jurd and Stevenson, 1976; Nagata and Katagiri, 1978).

However, more recently it has been demonstrated in experiments performed on Rana catesbeina, that antisera against IgM may contain contaminating antibodies that cross-react with carboyhydrate on the thymocyte surface (Mattes and Steiner, 1978a; Mattes and Steiner, 1978b). Similar experiments performed with antisera to mouse and trout immunoglobulins have also shown the anti-carbohydrate activity of such antisera (Layton, 1980; Yamaga, Kubo and Etlinger, 1978a; Yamaga, Kubo and Etlinger, 1978b). It has also been shown, by using antisera to deglycosylated immunoglobulins, that Xenopus larval

thymocytes do not possess detectable surface Ig, whereas a large percentage of larval splenocytes are surface Ig positive (Hadji-Azimi and Schwager, 1980). Both surface IgM and Ig"G" have been detected on Xenopus splenocytes but the majority of surface Ig-positive cells carry IgM, with only a small percentage of cells being Ig"G" positive (Hadji-Azimi and Schwager, 1982; Jurd and Stevenson, 1976).

Xenopus also possesses cells capable of producing lymphokines. Thus adherent peritoneal exudate cells (a macrophage-enriched population), when stimulated with a non-mitogenic dose of LPS, produce a supernatant which is mitogenic for thymocytes, suggesting the existence of an amphibian interleukin 1, a lymphokine which is produced by macrophages in mammals (A.J.H. Gearing, personal communication). Xenopus spleen cells stimulated with either a mitogenic dose of PHA or Con A, or allogeneic splenocytes, produce a supernatant which potentiates the response of thymocytes to sub-optimal doses of PHA or Con A, in a manner analogous to mammalian interleukin 2 (a T cell product) (Gearing, 1984).

Some evidence has been obtained from in vitro experiments for the requirement for T and B cell collaboration in the elicitation of T cell-dependent antibody responses in Xenopus. The secondary immune response to dinitrophenylated-fowl gamma-globulin, in vitro, was found to require both hapten (dinitrophenyl-keyhole limpet haemocyanin)-primed and carrier (fowl gamma-globulin)-primed lymphocytes. The carrier-specific cells were found to be non-nylon wool adherent, X-ray resistant and surface Ig negative - i.e. they possessed the characteristics of T lymphocytes. The hapten-reactive, antibody-secreting cells had the characteristics of B lymphocytes - they were nylon wool adherent, X-ray sensitive and surface Ig positive (Blomberg, Bernard and Du Pasquier, 1980).

Further experiments, again using carrier-primed and hapten-primed lymphocytes, were performed with cells of various genotypes taken from isogenic Xenopus hybrids - Xenopus laevis x Xenopus gilli clones. [The animals within a single clone are MHC-identical (Kobel and Du Pasquier, 1975).] It was found that the primed T and B cells would co-operate to produce low molecular weight (Ig"G") anti-dinitrophenyl antibody if they were taken from clones of animals having either one or two MHC haplotypes in common. If the carrier-primed T cells were histoincompatible with the dinitrophenyl-primed B cells, then no Ig"G" antibody response was observed, although occasional IgM antibody responses took place. These experiments strongly suggest that T-B collaboration to produce Ig"G" antibody in in vitro secondary antibody responses is MHC restricted in Xenopus, in a way similar to that described in mammals (Bernard, Bordmann, Blomberg and Du Pasquier, 1981).

The demonstration of MHC restriction by in vivo experimentation has not yet been forthcoming. In this respect, thymectomized Xenopus should be suitable, since one can attempt to reconstitute their T cell-dependent antibody responses with thymus implants or thymus-derived cells taken from MHC-compatible or MHC-incompatible donors. As already mentioned, Xenopus can be thymectomized as early as 5-7 days after fertilization (or stages 46-48 of Nieuwkoop and Faber, 1967) using the method of Horton and Manning (1972) or even as early as 4 days after fertilization (stage 45) according to Tochinai (1975). At these early stages, the thymus contains only small numbers of cells ($< 2{,}000$ at 8 days; Horton and Horton, 1975) and the differentiation of small lymphocytes does not occur until stage 49 (Nagata, 1977). Hence there is little chance of any prolonged lymphocyte seeding to the periphery from the thymus before thymectomy takes place.

Thymectomy has not been achieved at such an early stage of thymus development in the mouse, because of the period of gestation within the mother. Neonatally thymectomized mice are still able to respond to SRBC, albeit poorly (Miller, Mitchell and Weiss, 1967) whereas thymectomy at 5-7 days in Xenopus causes complete abrogation of the response to SRBC (Horton, Rimmer and Horton, 1976; Turner and Manning, 1974). Even nude mice are able to mount a low antibody response to SRBC (Kindred, 1971a; Reed and Jutila, 1972). These mice possess both precursor cytotoxic T lymphocytes and helper T cells in low numbers. Immunization of nude mice with SRBC, together with administration of lymphokine preparations containing interleukin 2, results in the appearance of SRBC-specific helper T cells in the spleen, and antibody production to SRBC can be demonstrated (see Hünig, 1983). Thus the thymectomized Xenopus is in some respects a superior T cell-depleted animal model, compared to neonatally thymectomized and nude mice.

The experiments reported in this Thesis were carried out in an attempt to determine whether the T cell-dependent antibody response to SRBC in the thymectomized Xenopus could be restored with implanted allogeneic thymus implants or with injected allogeneic lymphocytes. The Thesis is subdivided into chapters as follows. Chapter two is concerned with the restoration of anti-SRBC reactivity in thymectomized larvae reconstituted with whole thymus implants. In Chapter three, the differentiation of cytoplasmic immunoglobulin M-positive lymphocytes is examined in cultured thymocytes and splenocytes. This was performed to characterise the lymphocyte populations used in later cellular reconstitution experiments and to check the extent to which B cells were found in the thymus. Chapter four investigates the suitability of nylon wool treatment and irradiation for producing

cell populations depleted of B lymphocytes. Chapter five details experiments designed to show whether or not lymphocytes injected into an animal reach the spleen. In Chapter six, the ability of lymphocytes, from different organs and after various treatments, to restore the T cell-dependent antibody response to SRBC in the spleens of thymectomized animals was examined. In the final Chapter (seven) the major findings of this Thesis are discussed and some suggestions for future experiments are given.

CHAPTER TWO

RESTORATION OF THE ANTIBODY RESPONSE TO SHEEP ERYTHROCYTES IN THYMECTOMIZED XENOPUS IMPLANTED WITH A LARVAL (MHC-INCOMPATIBLE) OR ADULT (MHC-COMPATIBLE OR MHC-INCOMPATIBLE) THYMUS

Introduction

Thymectomized Xenopus laevis are deficient in a variety of immune responses, including humoral antibody production to a range of T cell-dependent antigens, as discussed in Chapter 1. Reconstitution experiments, involving the implantation of MHC-compatible or MHC-incompatible thymuses into such animals are now beginning to be performed. Larval MHC-incompatible thymuses implanted into larval, thymectomized animals have been shown to be repopulated with lymphocytes of host origin (Gearing, Horton and Cribbin, 1984) and a similar immigration of lymphoid cells occurs in "adult" MHC-incompatible thymuses implanted into adult, thymectomized hosts (Nagata and Cohen, 1984). The skin allograft response of thymectomized Xenopus reconstituted with genetically-undefined foreign larval thymus is fully restored, when skin grafts are MHC-disparate to both host and thymus donor (Horton and Horton, 1975). Thymectomized Xenopus laevis/gilli and Xenopus laevis/muelleri hybrids implanted with genetically-defined MHC-compatible or MHC-incompatible larval thymuses have recently been shown to respond normally to dinitrophenylated-keyhole limpet haemocyanin. The IgM antibody produced was similar in quantity, affinity and specificity to that of non-operated controls; and Ig"G" antibody was produced which had an antibody spectrotype of the host type, showing that any B cells coming from

the donor thymus were not involved in antibody synthesis in the reconstituted animals. Similar restoration of antibody levels is found with irradiated (1,000 rad) thymus implants (Du Pasquier and Horton, 1982).

In this Chapter, experiments were performed (in collaboration with A.J.H. Gearing and J.D. Horton) to examine the ability of MHC-incompatible larval thymus (irradiated or non-irradiated) and "adult" thymus implants (MHC-compatible or MHC-incompatible) to restore the cellular and serum antibody response of Xenopus laevis to SRBC. "Adult" thymuses were used because it is only after metamorphosis that some MHC antigens are found on Xenopus cells (Du Pasquier, Blomberg and Bernard, 1979; Du Pasquier, Flajnik, Hsu and Kaufman, 1984).

Materials and Methods

Animals

The majority of animals used in this Thesis were bred and reared in the laboratory. Wild adult Xenopus laevis were purchased commercially (Xenopus Ltd.). Inbred (G-line) Xenopus laevis were a gift from C. Katagiri (Hokkaido University, Sapporo, Japan). These latter animals are MHC-identical (Katagiri, 1978), but have minor histocompatability disparities (J.C. Arnall, unpublished observations; DiMarzo and Cohen, 1982). Spawning was induced by the injection of chorionic gonadotrophin (Griffin and George) into the dorsal lymph sac of adult males and females. The animals were allowed to mate overnight, and the eggs laid were then transferred to aerated, standing water. Tadpoles were fed with nettle powder and kept at $23 \pm 2^{\circ}C$. After metamorphosis, toadlets were fed on Tubifex worms and older animals (> 4 months old) were fed on ground

ox liver. Some experiments in this Thesis were performed using 8-12 month old outbred Xenopus laevis purchased commercially (laboratory reared by Xenopus Ltd.).

A small number of the animals used in this Chapter as thymus donors, were a strain of isogenic Xenopus hybrid-- Xenopus laevis x Xenopus gilli clone LG5 (Kobel and Du Pasquier, 1975). These animals are MHC-identical.

Thymectomy

Thymectomy was carried out using the method of Horton and Manning (1972). The thymuses were removed by microcautery when the larvae were approximately 7 days old (or stage 47 according to the normal table of Nieuwkoop and Faber, 1967). Thymectomies were performed by J.D. Horton. The larvae were checked for absence of thymus regeneration before metamorphosis, and also at post-mortem of experimental animals.

Thymus implantation

Thymus implantation was performed when the thymectomized larvae were 4-6 weeks old (stages 54-57), by implanting a single thymus under the skin behind one eye. The donor thymuses used were larval thymuses of similar stage to the host, or suitably sized fragments of thymuses of 4-5 month old "adult" toadlets. All reconstitutions with larval thymuses were performed using MHC-incompatible thymuses, some of which were irradiated (1,000 or 5,000 rads) before use. Adult thymuses were either MHC-compatible or MHC-incompatible. Thymus implantations were performed by J.D. Horton and A.J.H. Gearing. Larvae were checked for the presence of an implant before metamorphosis, and also at post-mortem.

Irradiation of thymuses

Thymuses were dissected out of the donor larvae, and placed in test-tubes containing amphibian strength Leibovitz L-15 culture medium (Flow Laboratories) for irradiation. The thymuses were given a dose of either 1,000 rads, at a dose rate of 200 rads/minute, or 5,000 rads, at a dose rate of 1,000 rads/minute, by exposure to a cobalt-60 source.

Antigen preparation and immunization

Sterile sheep red blood cells (SRBC) in Alsevers solution (Tissue Culture Services) were washed three times in saline, and resuspended in a 10% vol/vol suspension in saline for injection.

Animals aged 6-12 months were immunized by injection of 0.05 ml of 10% SRBC per gramme body weight, via the intraperitoneal route. Toadlets received either a single injection of SRBC and were tested for plaque-forming cells (PFC) 6 days later; or three injections given 3 days apart (the "multiple-injection" schedule: see Results), and tested for splenic PFC and serum antibody production 2 weeks after the final injection. Immunized animals were kept at the elevated temperature of 26°C.

Preparation of lymphocyte cell suspensions

Spleens were removed aseptically in a laminar flow hood, after animals had been anaesthetised with MS222 (Sandoz). They were then transferred to small petri dishes containing L-15 culture medium, modified for use with amphibians, by diluting 5:3 with double-distilled water. This amphibian strength medium also contained 1% heat-inactivated foetal calf serum (FCS; Flow) to protect the cells

during centrifugation, and was sterilized by filtration through a 0.22 μm filter (Millipore). Spleens were teased apart using watch makers forceps, and transferred to polystyrene tubes. Tissue clumps were further broken up by gentle pipetting, and cell clumps and debris were allowed to settle. The supernatants, now single cell suspensions, were then transferred to fresh test-tubes, and washed three times by centrifugation at 350 x g for 10 minutes, at 4°C. The lymphocyte concentration was then determined using a Neubauer American Optical counting chamber and adjusted to an appropriate concentration.

All spleen and thymus cell suspensions used in this Thesis were prepared in this way.

Plaque-forming cell assay

A modification of the slide method of the haemolytic plaque assay originally described by Cunningham and Szenberg (1968) was used. Spleen cell suspensions were prepared, chilled on ice. The cell suspensions were adjusted to a maximum concentration of 5×10^6 lymphocytes/ml in a medium containing 5 parts L-15: 3 parts double-distilled water: 1 part FCS. Aliquots (160 μl) of each spleen cell suspension were mixed with 12 μl 25% SRBC and 40 μl 1:10 guinea-pig serum (Wellcome) as a source of complement. The guinea-pig serum was first absorbed with SRBC before use. The assay mixtures were prepared on ice, and allowed to warm up to room temperature before insertion into the double microscope slide chambers, to prevent the formation of air bubbles during the incubation period. The slide plaque chambers were prepared using 3 M double-sided Scotch tape (No. 400) between acetone/alcohol-cleaned slides, giving two 90 μl chambers per slide. The assay mixtures were then gently pipetted

into the plaque chambers (two to four chambers per sample) and the edges of the chambers sealed with a 2:1 mixture of molten parafin wax and petroleum jelly. After 2 hours incubation at 30°C, the PFC were counted under low power magnification. Only when a central Xenopus lymphocyte could be seen in the plaque was a PFC scored. Plaque-forming cells were expressed as the number per 10^6 leucocytes.

Serology

Blood was collected by cardiac puncture, using drawn pasteur pipettes, coated with a silicone/chloroform mixture (1:1). The blood was allowed to clot at 4°C, and then centrifuged at 350 x g for 10 minutes to pellet any loose cells, prior to removal of the serum.

Haemolyzing antibody levels were determined using a microhaemolysis test performed in V-well microtitration plates (Titertek). Twenty-five microlitres of serum was serially diluted in mammalian strength phosphate-buffered saline (PBS; Flow) or 0.1 M 2-mercaptoethanol (BDH), diluted in PBS. 2-Mercaptoethanol-treated plates were incubated at 37°C for 1 hour - this removes IgM antibody activity, but leaves Ig"G" antibody intact (Hadji-Azimi, 1971; Turner and Manning, 1974; Uhr, 1964). Five microlitres of 5% SRBC and 20 µl 1:10 guinea-pig complement (absorbed with SRBC) were then added. The contents of the wells were mixed by gentle agitation, and the plates then incubated for 2 hours at room temperature. The maximum dilution of serum which caused lysis of the SRBC was taken as the titre of the lysing antibody, and was expressed as $-\log_2$ titre.

Experimental design

Splenic PFC responses and serum antibody production, in thymectomized animals reconstituted with a thymus implant - MHC-incompatible larval

thymus (irradiated or non-irradiated) and adult thymus (MHC-compatible or MHC-incompatible) - were compared with the responses of control (non-operated) and thymectomized animals. Splenic PFC and serum antibody assays were also performed on non-injected animals, to determine background antibody levels. A small number of thymectomized larvae were reconstituted with implants of larval MHC-incompatible spleen or liver fragments. This was to see whether tissues other than the thymus could restore the PFC and serum antibody response of thymectomized animals, to SRBC.

The animals used in the MHC-incompatible reconstitution experiments included: (i) outbred thymectomized hosts reconstituted with outbred non-sibling thymuses; (ii) outbred thymectomized hosts reconstituted with inbred G-line thymuses; and (iii) inbred G-line thymectomized hosts reconstituted with cloned LG5 thymuses. (See Tables 2.1-2.4 for animals used in each experiment). Where outbred: outbred combinations were used, there was a good chance that the donor and host differed from each other by two MHC alleles, as outbred non-siblings were shown always to reject each others skin grafts in a normal sub-acute fashion (approx. 3 weeks at 24-26^{o}C). The same is true for the outbred: G-line and G-line: LG5 combinations (J.D. Horton, personal communication). For the MHC-compatible reconstitution experiments, both thymectomized hosts and thymus donors were inbred G-line animals, which are MHC-compatible (see "Animals" section).

Results

(a) Background cellular and serum antibody production

The levels of cellular and serum antibody present in animals that were not primed with SRBC are shown in Table 2.1.

Controls. Control (non-operated) animals did not show a background level of spleen cells producing antibody against SRBC. However, some animals (three out of eight tested) did give a positive background total serum antibody titre. After treatment of the serum with 2-mercaptoethanol to destroy IgM antibody, no detectable serum antibody remained, i.e. there was no background level of Ig"G".

Thymectomized. Thymectomized animals behaved in a similar fashion to controls. There was no background level of splenic PFC in the one animal tested, but two out of three animals possessed background levels of total serum antibody. This was not significantly different from the background levels of total serum antibody in the control animals ($P > 0.1$). There was no background serum Ig"G" antibody, as shown by the titration of 2-mercaptoethanol-treated serum.

Thymectomized animals reconstituted with a larval MHC-incompatible thymus. Two such animals were tested for their background levels of cellular and serum antibody. Neither animal responded to SRBC in the PFC assay, but both gave a background total serum antibody titre, which was similar to the background total serum antibody titre of thymectomized animals ($P > 0.1$). Treatment of the serum with 2-mercaptoethanol wiped out this response, hence there was no detectable background Ig"G" antibody.

(b) Cellular antibody production in animals immunized with a single injection of antigen

The splenic PFC numbers given by control, thymectomized and thymectomized animals reconstituted with MHC-incompatible larval thymus are shown in Table 2.2. Control animals gave a response of 215 ± 180 PFC/10^6 leucocytes (mean $\pm$ S.D.) whereas thymectomized

animals did not respond to SRBC. However, implantation of an MHC-incompatible larval thymus restored the ability of thymectomized animals to produce plaques against SRBC. The number of PFC obtained was $108 \pm 98/10^6$ leucocytes (mean $\pm$ S.D.) which was not significantly different from the control response, as shown by the Students t-test ($P > 0.1$).

(c) Cellular and serum antibody production in animals given a multiple antigen injection schedule

The levels of cellular and serum antibody produced by animals given three injections of antigen are shown in Tables 2.3 and 2.4. The responses of control, thymectomized and thymectomized animals reconstituted with larval or irradiated larval MHC-incompatible thymus are shown in Table 2.3, and the responses of thymectomized animals reconstituted with MHC-compatible or MHC-incompatible adult thymus are shown in Table 2.4.

Controls. Control animals all responded to SRBC with a cellular antibody level of 85 ± 43 PFC/10^6 leucocytes and a total serum antibody titre of 7.4 ± 1.2 (mean $\pm$ S.D.). After treatment of the immune sera with 2-mercaptoethanol, the Ig"G" antibody titre was 4.0 ± 2.1 (mean $\pm$ S.D.).

Thymectomized. Thymectomized animals were unable to respond to SRBC. None of the nine animals tested gave any anti-SRBC PFC. Six out of nine animals did show a positive total serum antibody titre, but the mean titre of 3.1 ± 2.5 was similar ($P > 0.1$) to the titre of 2.7 ± 2.3 (mean $\pm$ S.D.) given by non-immunized thymectomized animals. This value of 3.1 ± 2.5 was also significantly lower than the titre of 7.4 ± 1.2 (mean $\pm$ S.D.) given by immunized controls ($P < 0.001$ as measured by a Students t-test). The immunized

thymectomized animals did not produce any 2-mercaptoethanol-resistant antibody.

Thymectomized animals reconstituted with a larval MHC-incompatible thymus. The implantation of an MHC-incompatible larval thymus restored the ability of thymectomized animals to respond to SRBC. The cellular antibody response was 38 ± 36 (mean $\pm$ S.D.) PFC/10^6 leucocytes, which was significantly different from the control level ($P < 0.02$). The total serum antibody titre obtained was 7.4 ± 1.8 and the 2-mercaptoethanol-resistant antibody titre was 3.5 ± 1.2. These values however were not significantly different from the control levels ($P > 0.1$). Hence the serum antibody titres have been restored to control levels, but the cellular antibody response is significantly lower.

Thymectomized animals reconstituted with an irradiated larval MHC-incompatible thymus. Irradiated MHC-incompatible larval thymuses were also able to restore the ability of thymectomized animals to respond to SRBC. Thymuses given 1,000 and 5,000 rads were equally effective in restoring the response ($P > 0.1$ for the PFC response, total serum antibody titre and 2-mercaptoethanol-resistant antibody titre, as compared between thymectomized animals reconstituted with thymuses given 1,000 or 5,000 rads). Animals given 1,000 rad irradiated thymus produced a cellular antibody response of 87 ± 19/10^6 leucocytes (mean $\pm$ S.D.). The total serum antibody titre was 7 ± 1 and the 2-mercaptoethanol-resistant antibody titre was 1.3 ± 1.2 (mean $\pm$ S.D.; only two out of three animals gave 2-mercaptoethanol-resistant antibody). The 5,000 rad irradiated thymus was able to restore the cellular antibody response of thymectomized animals to 118 ± 92 PFC/10^6 leucocytes (mean $\pm$ S.D.). The total serum antibody titre was 6.7 ± 1.5, and the 2-mercaptoethanol-resistant antibody titre was 1.3 ± 1.2 (mean $\pm$ S.D.; again with only two out of three

animals responding). The cellular antibody response and total serum antibody titres are comparable to control levels, in both 1,000 and 5,000 rad irradiated thymus reconstituted animals ($P > 0.1$, as measured using the Students t-test). The 2-mercaptoethanol-resistant antibody titres for both 1,000 and 5,000 rad thymus reconstituted animals appeared to be lower than the control levels, but this difference was not significant ($P > 0.5$, but < 0.1 as measured by the Students t-test). However, only six animals were used in the irradiated thymus experiments (three with 1,000 rad thymuses, and three given 5,000 rad thymuses) so more experiments are needed to confirm that the 2-mercaptoethanol-resistant antibody titres are restored to control levels.

Thymectomized animals reconstituted with an adult thymus. Both MHC-compatible and MHC-incompatible adult thymuses were able to restore the response of thymectomized animals to SRBC, but to different extents. Animals reconstituted with an MHC-compatible thymus gave a cellular antibody response of 137 ± 102 PFC/10^6 leucocytes (mean $\pm$ S.D.), and a total serum antibody titre of 7 ± 3. Two out of three animals produced 2-mercaptoethanol-resistant antibody, with a mean level of 2.3 ± 2.1. Reconstitution with an MHC-incompatible thymus enabled thymectomized animals to give a cellular antibody response of 25 ± 18 PFC/10^6 leucocytes, and a mean total serum antibody titre of 5.3 ± 1.8. Only two out of eight animals were able to produce 2-mercaptoethanol-resistant antibody, with a mean level of 0.8 ± 1.4. The responses given by animals reconstituted with an MHC-compatible thymus were comparable to control responses ($P > 0.1$ for cellular antibody response and both total serum and 2-mercaptoethanol-resistant antibody titres). However, animals reconstituted with MHC-incompatible adult thymuses did not respond as well to SRBC

as control animals. The cellular antibody response, total serum antibody titre and 2-mercaptoethanol-resistant antibody titres were all significantly lower in the animals given an adult MHC-incompatible thymus ($P < 0.001$, $P < 0.02$ and $P < 0.01$ respectively). Thymectomized animals given an MHC-incompatible adult thymus did not respond as well to SRBC as animals reconstituted with an MHC-compatible adult thymus. Although the total serum and 2-mercaptoethanol-resistant antibody titres were not significantly different ($P > 0.1$), the cellular antibody response was poorer in the animals given an MHC-incompatible thymus ($P < 0.02$).

The MHC-incompatible adult thymuses were also not as efficient as MHC-incompatible larval thymuses at restoring the response to SRBC. Although the cellular antibody response was not significantly different ($P > 0.1$), both the total serum and 2-mercaptoethanol-resistant antibody titres were significantly lower in animals given an MHC-incompatible adult thymus ($P < 0.05$ and $P < 0.01$ respectively).

Thymectomized animals reconstituted with larval spleen or liver. One thymectomized animal reconstituted with MHC-incompatible spleen failed to produce a cellular antibody response to SRBC. The total serum antibody titre of 3 was similar to that of a thymectomized animal, and no titre was seen with 2-mercaptoethanol-treated serum. Two thymectomized animals reconstituted with MHC-incompatible liver also failed to produce a cellular antibody response to SRBC. One animal gave a total serum antibody titre of 3, and a 2-mercaptoethanol-resistant antibody titre of zero. (Antibody titres were not determined for the second animal.) It would seem that in contrast to thymus, spleen and liver are unable to restore the response of thymectomized animals to SRBC, as thymectomized animals given these lymphoid organ implants behaved in the same way as non-implanted

thymectomized animals.

(d) Morphological and histological appearance of the implanted thymuses

Histological studies were made on non-irradiated and irradiated MHC-incompatible larval thymuses that were implanted to opposite sides of the head. This allowed a direct comparison of the effect of irradiation on the implanted thymuses in the same thymectomized host.

Non-irradiated larval thymuses. Non-irradiated thymus implants remain visible throughout the life of the animal. These thymus implants increase in size during the first few post-metamorphic months, and can still be detected under the skin, medial to the eye, at post-mortem. The appearance of a typical thymus implant 30 days after implantation, can be seen in Fig. 2.1, on the left hand side of the animal. There is a clearly defined cortex and medulla.

Irradiated larval thymuses. Thymuses given 1,000 or 5,000 rads irradiation remain visible for a varying length of time after implantation. Some thymuses given 1,000 rads can still be detected at post-mortem, whereas other seem to have disappeared. The appearance of a thymus given 1,000 rads can be seen *in situ*, 30 days after implantation, in Fig. 2.1, on the right hand side of the animal. As with the non-irradiated thymus there is a clearly defined cortex and medulla, but the irradiated thymus is much smaller than the non-irradiated thymus. (Fig. 2.1 is a section through the centre of both thymuses.)

Thymuses given a dose of 5,000 rads cannot be detected at post-mortem. However, their appearance is similar to that of thymuses given 1,000 rads, within the first few weeks after implantation.

Adult thymuses. Both MHC-compatible and MHC-incompatible adult thymus implants remain readily visible and are detectable adjacent to the eye at post-mortem.

Discussion

The results presented in this Chapter show that all the types of thymus implant used are able to restore the antibody response of thymectomized Xenopus, at least to some extent. Non-irradiated, MHC-incompatible larval thymus implants display a normal histology and, as demonstrated by ploidy-marker experiments (Gearing, Horton and Cribbin, 1984), are repopulated by host cells. The splenic cellular antibody response in thymectomized animals given such a thymus is restored, but is significantly lower than the response of non-operated, control animals. However, the levels of serum antibody (both total antibody and 2-mercaptoethanol-resistant antibody) are restored to control levels. This restoration of the response to SRBC was not simply due to a non-specific "allogeneic effect", since non-SRBC-injected animals reconstituted with an MHC-incompatible thymus displayed only background anti-SRBC reactivity.

Experiments performed with T cell-depleted nude mice (aged 2-8 weeks) have shown that an implanted neonatal thymus, syngeneic or allogeneic, also becomes repopulated with host cells (Kindred, 1978; Kindred and Loor, 1975; Pritchard and Micklem, 1973). However, the ability of such reconstituted mice to mount an antibody response to SRBC and to reject a third party skin graft is only partially restored. Spleen cells from these reconstituted mice can also now respond to the T cell mitogens Con A and PHA to some extent (Kindred, 1978). The response to PHA is also restored in thymectomized Xenopus reconstituted with larval MHC-incompatible thymus (Gearing, Horton

and Cribbin, 1984).

Irradiated, MHC-incompatible larval thymus implants initially contain lymphocytes (although their origin - host or donor - was not examined here), but these implants remain very much smaller than non-irradiated implants and tend to disappear after metamorphosis. Thus long-term repopulation of irradiated Xenopus thymus does not seem to occur. This is, however, a little better than the situation in nude mice reconstituted with irradiated, allogeneic perinatal thymus. These thymuses remain small and largely epithelial, with only a few lymphocytes, and do not attain normal structure (Loor and Hägg, 1977). In contrast to the nude mouse, in which the irradiated thymus implants usually fail to restore the response to T cell-dependent antigens (Kindred, 1978), in Xenopus, irradiated (both 1,000 and 5,000 rads), MHC-incompatible larval thymus implants restored the antibody response to control levels. It would seem quite possible that restoration with the irradiated larval implants (where lymphocyte numbers are low) is achieved in large measure through release of thymic hormone from radioresistant thymic epithelial cells. Thymic factors have been described in amphibians (Dardenne, Tournefier, Charlemagne and Bach, 1973) and are well characterized in mammals (see Bach, 1980).

As in the nude mouse (Radov, Sussdorf and McCann, 1975), adult MHC-compatible thymus implants restore the antibody response to SRBC in Xenopus to control levels. On the other hand, in nude mice, adult MHC-incompatible thymus implants fail to restore humoral responsiveness to SRBC above background levels, whereas in Xenopus such implants partially restore both the cellular antibody response in the spleen and 2-mercaptoethanol-sensitive (presumably IgM: see Materials and Methods) serum antibody titres. However, the levels

of 2-mercaptoethanol-resistant antibody (Ig"G") are very much lower than in the control toadlets. Future thymus reconstitution experiments with Xenopus should concentrate on the use of "adult" thymus implants and determine whether there are real differences in Ig"G" antibody production between MHC-compatible and MHC-incompatible implanted animals. [This could be done by using monoclonal antibodies against Xenopus Ig"G", that are becoming available, in an enzyme-linked immunosorbent assay (see Johnstone and Thorpe, 1982).]

Apart from the adult MHC-incompatible thymus implantation work reported here, the restoration experiments in this Thesis and those performed by others, using antigens that are known to require T-B MHC-restricted collaboration (Du Pasquier and Horton, 1982), would tend to lead to the conclusion that host-derived T cells, that have developed in the foreign thymus, can repopulate the periphery, and there collaborate perfectly well with host-derived B lymphocytes in primary in vivo antibody responses. The amphibian thymus may, then, not be centrally involved in self-restriction of helper T cells. On the other hand, the involvement of (MHC-incompatible) donor-derived T and B lymphocytes in effecting the antibody responses observed cannot be ruled out in these experiments where non-irradiated thymus implants are used to reconstitute. Thus both T and B-like lymphocytes exist in the Xenopus thymus (see Chapter 3 and also Hsu, Julius and Du Pasquier, 1983; Williams, Cribbin, Zettergren and Horton, 1983) even prior to metamorphosis (Williams, Cribbin, Zettergren and Horton, 1983). However, it is relevant to report here that Nagata and Cohen (1984) have very recently found either no donor-derived cells or up to 32% of donor-derived cells in the spleen (depending on the host/donor combination used) 6-13 months after thymectomized Xenopus were implanted (as toadlets) with "adult"

MHC-compatible or MHC-incompatible thymuses. (Interestingly, these authors reveal that "adult" MHC-incompatible thymuses fully restore the splenic cellular PFC response to SRBC.)

Experiments with irradiated thymus reported here, and elsewhere (Du Pasquier and Horton, 1982), were set up to remove the possibility of donor B cell contamination. B cell activity of Xenopus is eliminated by a dose of 500 rads in vitro (Blomberg, Bernard and Du Pasquier, 1980). Horton and Du Pasquier (1982) have also shown that the antibody produced in MHC-incompatible thymus-implanted animals is of the host spectrotype which tends to rule out the involvement of donor B cells in the restoration of the response to SRBC. The possibility that donor (radiation-resistant) T cells (rather than host-derived T cells) might be involved in the immune response of restored Xenopus still cannot be excluded, although a dose of 5,000 rads destroys the ability of almost 100% of thymus cells to survive in vitro (R.L. Lallone, personal communication). Whether T cells of one genotype injected into a thymectomized host of another genotype can co-operate with the B cells of that host in primary anti-SRBC reactivity is the central issue of the remainder of this Thesis. A long-lasting co-operation does not occur in anti-SRBC responses of nude mice injected with MHC-incompatible thymocytes or splenocytes (Kindred, 1971b; Kindred, 1975; and Kindred and Weiler, 1972) . The outcome of such experiments in Xenopus should allow a clearer interpretation of the red cell studies with MHC-incompatible thymus-reconstituted animals reported in this Chapter.

The MHC-restriction of T cells by the foreign Xenopus thymus may also be obscured in these thymectomy-reimplantation studies, by early seeding of immature, but already host MHC-restricted, T-axis lymphocytes

prior to removal of the thymus at 7 days of age (stage 48 of Nieuwkoop and Faber, 1967). These lymphocytes could then mature into functional T cells under the hormonal influence of the thymus implant. This seems unlikely, as the differentiation of small lymphocytes in the thymus does not take place until stage 49 (Nagata, 1977). However, experiments have recently been performed by Cohen, Flajnik and Du Pasquier (1984) which address this issue. They used chimeric Xenopus in which the anterior "half" of an embryo is joined to a posterior "half" of an MHC-dispartate embryo. The anterior portion contained the thymic anlage of one MHC haplotype and the posterior portion contained the haemopoetic stem cell source of a different MHC haplotype. Hence during ontogeny of these chimeras, lymphocyte precursors exclusively differentiate in an MHC-incompatible thymus epithelial environment. It was found that two out of three animals could produce a cellular antibody response to SRBC, whereas the third animal could not. Other animals were still able to produce both IgM and Ig"G" antibodies to dinitrophenylated-keyhole limpet haemocyanin, but the Ig"G" response showed delayed kinetics and lower antibody titres. Thus it appears that the thymus may play some part in the education of Xenopus helper T cells, but some extrathymic component is also centrally involved.

It will be important in the future to consider more closely the actual MHC-restricting elements within the thymus. It has been suggested that self-MHC restriction is learnt in the mammalian thymus by T cells upon interaction with MHC products on thymic antigen-presenting cells, which migrate into the thymus from the bone marrow (Longo and Schwartz, 1980). Thus host-derived MHC-restricting antigen-presenting cells may well migrate into the MHC-incompatible thymus, along with lymphoid stem cells, and so

partially obscure the organ's vital role in T cell education.

In conclusion, one can say relatively little about the role of the Xenopus thymus in MHC restriction experiments reported to date, since in none of these experiments is it known whether the host T cells developing are restricted to the thymus donor type or to the genotype of the thymectomized host. This aspect could be examined by studying the potential of T cells from thymus-implanted animals to co-operate with B cells of various genotypes in a primary in vitro antibody assay. Such an assay has recently been established for anti-rabbit erythrocyte responses (Lallone, 1984).

Table 2.1 Background cellular and serum antibody production to SRBC in non-immunized control, thymectomized and reconstituted animals

	Control	TX	TX + larval thymus*
PFC/10^6 spleen leucocytes	0 0 0 0 0 $\bar{x} = 0$	0	0 0 $\bar{x} = 0$
Total serum antibody titre ($-\log_2$)	0 4 3 0 0 0 0 3 $\bar{x} = 1.8 \pm 1.3$	0 4 4 $\bar{x} = 2.7 \pm 2.3$	2 3 $\bar{x} = 2.5 \pm 0.7$
2-Mercaptoethanol-resistant antibody titre ($-\log_2$)	0 0 0 0 0 $\bar{x} = 0$	0 0 $\bar{x} = 0$	0 0 $\bar{x} = 0$

Results are expressed as mean $\pm$ S.D. where appropriate.

* Animals used were thymectomized (TX) outbred X. laevis reconstituted with inbred X. laevis G-line thymuses.

All animals were aged 6-8 months.

Table 2.2 Cellular antibody production to SRBC in control, thymectomized and reconstituted animals, after a single injection of antigen

	Control	TX	TX + larval thymus*
PFC/10^6 spleen leucocytes	26 210 165 457	0 0 0 0	40 30 241 121
	$\bar{x} = 215 \pm 180$	$\bar{x} = 0$	$\bar{x} = 108 \pm 98$

Results are expressed as mean ± S.D. where appropriate.

The assay was performed 6 days after the injection of antigen.

* Animals used were thymectomized (TX) outbred X. laevis, reconstituted with outbred X. laevis thymuses.

All animals were aged 7 months.

Table 2.3 Cellular and serum antibody production to SRBC in control, thymectomized and thymectomized animals reconstituted with a larval MHC-incompatible thymus, after multiple antigen injections

	Control	TX	TX + larval thymus*	TX + irradiated larval thymus+	
				1000 rads	5000 rads
PFC/10^6 spleen leucocytes	105 26 107 114 88 133 105 136 109 32 14 54 $\bar{x}$ = 85 ± 4.3	0 0 0 0 0 0 0 0 0 $\bar{x}$ = 0	83 71 11+ 6 2 17+ 25 85 $\bar{x}$ = 38 ± 26	106 85 69 $\bar{x}$ = 87 ± 19	198 139 18 $\bar{x}$ = 118 ± 92
Total serum antibody titre ($-\log_2$)	10 7 8 6 6 7 7 7 8 8 $\bar{x}$ = 7.4 ± 1.2	3 4 4 5 0 0 0 6 6 $\bar{x}$ = 3.1 ± 2.5	10 7 7+ 6 6 5+ 10 8 $\bar{x}$ = 7.4 ± 1.8	8 6 7 $\bar{x}$ = 7.0 ± 1.0	7 5 8 $\bar{x}$ = 6.7 ± 1.5
2-Mercaptoethanol-resistant antibody titre ($-\log_2$)	7 6 0 4 3 4 4 4 $\bar{x}$ = 4.0 ± 2.1	0 0 0 0 0 0 0 $\bar{x}$ = 0	5 4 4+ 2 4 2+ $\bar{x}$ = 3.5 ± 1.2	0 2 2 $\bar{x}$ = 1.3 ± 1.2	2 2 0 $\bar{x}$ = 1.3 ± 1.2

Table 2.4 Cellular and serum antibody production to SRBC in thymectomized animals reconstituted with MHC-compatible or MHC-incompatible "adult" thymus, after multiple antigen injections

	MHC-compatible	MHC-incompatible
PFC/10^6 spleen leucocytes	176 197 25 313 75 35 141 $\bar{x}$ = 137 ± 102	20* 12* 58* 39* 27+ 1+ 32+ 28+ 4* $\bar{x}$ = 25 ± 18
Total serum antibody titre ($-\log_2$)	4 10 7 $\bar{x}$ = 7.0 ± 3.0	6* 4* 6* 9* 4+ 5+ 4+ 4+ $\bar{x}$ = 5.3 ± 1.8
2-Mercaptoethanol-resistant antibody titre ($-\log_2$)	0 4 3 $\bar{x}$ = 2.3 ± 2.1	3* 0* 0* 3* 0+ 0+ 0+ 0+ $\bar{x}$ = 0.8 ± 1.4

Results are expressed as mean ± S.D. where appropriate.

Animals were given three injections of antigen, 3 days apart and the assay was performed 2 wks after the final injection.

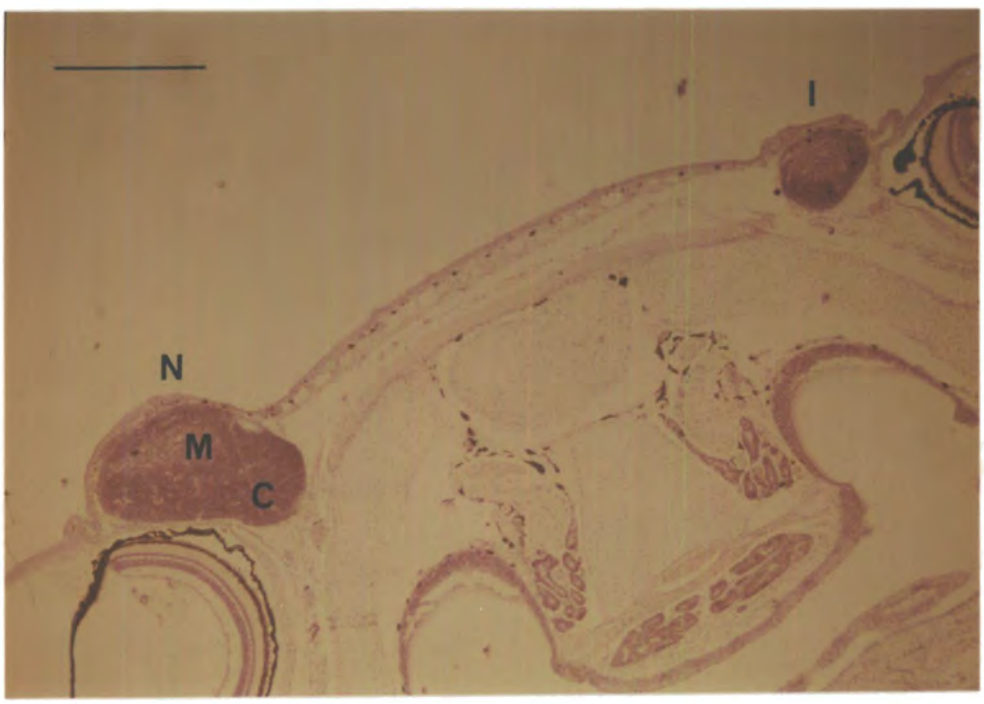
Both thymectomized host and donor thymus were inbred *X. laevis* G-line in the MHC-compatible combinations.

* Animals used were thymectomized outbred *X. laevis* reconstituted with inbred *X. laevis* G-line thymuses.

\+ Animals used were thymectomized inbred *X. laevis* G-line, reconstituted with *X. laevis/gilli* clone LG5.

All animals were aged 9-12 months.

Fig. 2.1 Histology of a non-irradiated and an irradiated MHC-incompatible larval thymus implanted to the same thymectomized host



This section was made 30 days post-implantation. C, cortex; I, irradiated thymus; M, medulla; N, non-irradiated thymus. Scale bar = 1 mm.

CHAPTER THREE

USE OF A B CELL MITOGEN TO GENERATE CYTOPLASMIC IMMUNOGLOBULIN M-POSITIVE CELLS IN BOTH SPLENOCYTE AND THYMOCYTE CULTURES

Introduction

At the outset of the planned experiments to effect cellular restoration of the anti-erythrocyte response in thymectomized Xenopus, it was unclear which of the two major lymphoid organs - the thymus or spleen - would be the most suitable source of (helper) T lymphocytes. It could not be assumed that the thymus would contain a purer population of T cells (albeit in various stages of differentiation), free from B cell "contamination", than the spleen since it has been shown that the mammalian B cell mitogen Escherichia coli lipopolysaccharide (LPS) is equally efficient at inducing blastogenesis in both splenocyte and thymocyte cultures of 6 month old Xenopus (Williams, Cribbin, Zettergren and Horton, 1983; Williams and Horton, 1980). Whether or not LPS was actually stimulating B cells in the thymus in these experiments remains controversial: thus Bleicher and Cohen (1981) have recently demonstrated, using monoclonal antibodies to deplete surface immunoglobulin (Ig) M-positive cells from Xenopus splenocytes, that surface Ig-negative lymphocytes can still display a significant (albeit reduced) mitogenic response to LPS. They therefore suggested that LPS may well induce both frog B and T cells to proliferate.

In view of both this controversy and the need to procure a T cell-enriched lymphocyte suspension for reconstitution work, it was decided that a closer examination of the question of the existence of thymic B cells was necessary. This Chapter therefore examines

the ability of LPS to induce the differentiation of cytoplasmic IgM-positive lymphocytes in Xenopus thymocyte and splenocyte cultures. In experiments performed with mice, LPS can be shown to induce the formation of Ig-secreting plaque-forming cells in vitro (Andersson, Sjöberg and Möller, 1972; Melchers, 1977). These Ig-secreting cells can be visualized using cytoplasmic immunofluorescence methods (Kearney and Lawton, 1975).

Materials and Methods

Lymphocyte culture

Spleen and thymus cell suspensions were prepared as described in the previous Chapter, but using supplemented amphibian strength L-15 culture medium. The L-15 medium was diluted 5:3 with double-distilled water, and contained 0.08 mM 2-mercaptoethanol (BDH), 10 mM Hepes buffer (Flow), 50 IU/ml penicillin (Flow, 50 µg/ml streptomycin (Flow), 2.5 µg/ml fungizone (Flow), and 1.25 mM L-glutamine (Flow). One per cent foetal calf serum (FCS) was added to the medium used for washing the cells, but the cells were resuspended in serum-free medium for counting. The cell suspensions were adjusted to a lymphocyte concentration of 5×10^6 total cells/ml.

Lymphocytes were cultured in Cooke V-well microtest plates (M25-ARTL, Sterilin). Forty microlitres of lymphocyte suspension were distributed to individual wells, then 10 µl mitogen and 10 µl FCS were added. Spleen cultures contained a final concentration of 1% FCS, and thymus cultures contained 10% FCS to try and increase viability of thymocytes. Cultures were incubated at 28°C in humidified air for 5, 6, 7 or 8 days. Cultures were fed with 30 µl L-15 containing 1% or 10% FCS after 3 days.

Mitogens

PHA 'M' (Difco) at 20 μg/ml and LPS (055:B5, Difco) at 2.0 mg/ml were used. Mitogens were freshly diluted from stock solutions just before use.

Dye-exclusion

Nigrosine in PBS (0.2%) was mixed with an equal volume of a cell suspension in a small glass tube. After thoroughly mixing, the contents of the tube were left to incubate at room temperature for 5 minutes, after which time the cells were counted in a haemocytometer to determine the percentage viable cells.

Staining for cytoplasmic immunoglobulin

Lymphocyte cultures were harvested into tubes, and washed twice in amphibian strength phosphate-buffered saline (PBS, Flow). The cell concentration was then adjusted to 1 x 10^6/ml in PBS containing 50% FCS and a cell smear prepared using a cytocentrifuge (Shandon). Cell suspension samples (100 μl) were centrifuged at 600 rpm for 5 minutes. The slides were then air-dried and fixed for 30 minutes in absolute alcohol containing 5% glacial acetic acid, at -20°C. The slides were washed three times in PBS containing 0.1% sodium azide (NaN_3), and stored at 4°C in the final wash until used.

The slides were blotted dry, leaving a film of PBS over the cell smear. Five microlitres of rabbit IgG anti-*Xenopus* μ chain antiserum (0.2 or 0.08 mg/ml, a gift from L.D. Zettergren, Carroll College, Waukesha, Wisconsin, U.S.A.; see Williams *et al.*, 1983 for details of preparation) was added to the cell smear, and the slides were incubated for 30 minutes in a moist chamber. The slides were washed three times in PBS containing 1% NaN_3, and the smears were

incubated with 10 µl 1:10 goat IgG anti-rabbit IgG, fluorescein-isothiocyonate (FITC)-conjugated (Miles-Yeda; molar ratio F/P ≒ 3.7) for 30 minutes in a moist chamber. The slides were again washed three times in PBS containing NaN_3, and finally mounted in polyvinyl alcohol, containing 1% NaN_3. The slides were either examined immediately, or stored at 4°C in the dark, until viewed under incident light illumination with a Zeiss Ultraphot microscope.

Cytoplasmic immunoglobulin M-positive ($cIgM^+$) cells and cytoplasmic immunoglobulin M-negative ($cIgM^-$) lymphoblasts were counted, using a x100 objective oil immersion lens, in fifty fields from the centre of the smear.

Experimental design

Splenocytes (taken from 5-11 month old outbred animals) and thymocytes (from 5-7 month old outbred animals) were cultured with or without LPS or PHA and then stained for cytoplasmic IgM. The purpose of these experiments was to determine whether or not LPS could stimulate B-lineage lymphocytes in the spleen and thymus to produce cytoplasmic IgM, detectable by immunofluorescence. Phytohaemagglutinin was used to compare the effects obtained with a T cell mitogen.

Specificity of antisera. The following experiments were carried out to test the specificity of the rabbit IgG anti-*Xenopus* µ chain and the FITC-conjugated goat IgG anti-rabbit IgG antiserum.

Cytocentrifuge preparations of splenocytes and thymocytes were treated with normal rabbit IgG at 0.2 mg/ml, prior to addition of the FITC-labelled goat IgG anti-rabbit IgG. No fluorescent cells were obtained, showing that rabbit IgG does not bind non-specifically to *Xenopus* lymphocytes.

Preparations were treated with 1:10 FITC-labelled goat IgG anti-rabbit IgG alone, which also did not produce any $cIgM^+$ cells. This demonstrates that the FITC-labelled antibody does not stick non-specifically to the cells.

As a final control, preparations were treated with a blocking IgG antiserum (non-FITC-labelled goat IgG anti-rabbit IgG; Miles-Yeda) between application of the rabbit anti-Xenopus μ chain and the FITC-labelled goat anti-rabbit IgG antisera. This treatment also prevented fluorescence, showing that the FITC-labelled antibody does not stick non-specifically to the rabbit anti-Xenopus μ chain antiserum.

Results

(a) Lipopolysaccharide-induced differentiation of $cIgM^+$ cells and $cIgM^-$ lymphoblasts in cultured splenocytes

The results obtained when splenocytes, cultured with or without LPS, were stained for cytoplasmic immunoglobulin M are shown in Table 3.1.

$cIgM^+$ cells. The numbers of $cIgM^+$ cells increased from background levels obtained in unstimulated cultures, to the levels shown in LPS-treated cultures. After 5 days in culture, the numbers of $cIgM^+$ cells increased from zero in the absence of LPS to 2.9% with LPS; and after 6 days in culture from $0.6 \pm 0\%$ without LPS to $2.5 \pm 0.8\%$ with LPS. A similar increase was found after 7 days, from a background of $0.6 \pm 1.0\%$ to $5.7 \pm 2.4\%$ in the presence of LPS (this increase was found to be highly significant, as determined using a Students t-test, with $P < 0.001$), and after 8 days in culture from 0.25% without LPS to 8.8% with LPS. There was a trend from 5 through 8 days of culture, for the number of $cIgM^+$ cells produced in the LPS-treated cultures to increase.

cIgM$^-$ lymphoblasts. The lymphoblasts counted were large cells (> 10 μm) with a greater proportion of cytoplasm surrounding the nucleus than the small lymphocytes. Counts were made of cIgM$^-$ lymphoblasts, as there appeared to be a difference in the numbers of these cells present in unstimulated cultures, as compared with LPS-treated cultures.

The numbers of unstained lymphoblasts (cIgM$^-$ blasts) increased from the background levels in unstimulated cultures to higher levels shown in LPS-treated cultures. After 6 days in culture, the numbers of cIgM$^-$ blasts increased from a background of 2.7 ± 0.4% to 11.2 ± 2.5% with LPS added, and after 7 days in culture from 1.5 ± 1.0% without LPS to 10.3 ± 4.8% with LPS. (This latter increase was shown to be highly significant, using a Students t-test, with $P < 0.001$.) A similar increase was seen after 8 days in culture, from 2.8% in the absence of LPS to 17.1% with LPS.

Morphology of cell types. The morphology of the cIgM$^+$ cells varied from lymphoblasts with cytoplasmic fluorescence restricted to Golgi and associated cisternae, to lymphoblasts with cytoplasmic fluorescence scattered throughout the cell, to plasmablasts that displayed intense cytoplasmic fluorescence. These cell types are illustrated in Fig. 3.1. A cytospin of a 7-day unstimulated culture is shown in Fig. 3.1 (a), whereas a cytospin of an LPS-treated culture after 7 days is shown in Fig. 3.1 (b). Unstained lymphoblasts and small lymphocytes can also be seen in Fig. 3.1. A high power magnification of a cIgM$^+$ cell with most of its fluorescence restricted to the Golgi area is shown in Fig. 3.2 (a), together with a cIgM$^+$ cell displaying a more general cytoplasmic fluorescence and a cIgM$^-$ small lymphocyte.

That the small, brightly staining "cells" visible in Fig. 3.1 are probably dead cells/fragments of dead cells that have non-specifically

absorbed the stain is suggested by dye-exclusion data. Thus, one culture examined was recorded by dye exclusion as 88% viable when unstimulated, and 75% viable when treated with LPS. Similar values of 93% and 85% viability were obtained by subtracting the number of very small, brightly staining "cells" from the total cell number in the fluorescence counts for the same culture. Another culture, which was discarded because of its poor viability, was 34% viable when unstimulated and 28% viable when treated with LPS, as determined by dye-exclusion. When stained for fluorescence microscopy, most of the cells in this culture, unstimulated or LPS-treated, appeared as very small, brightly stained "cells".

(b) Phytohaemagglutinin-treated splenocyte cultures

A number of experiments were performed using the T cell mitogen PHA to stimulate the spleen cells. Unfortunately, the PHA caused the cells to agglutinate, so that it was impossible to make accurate quantitative observations from the stained cytocentrifuge preparations. However, qualitative observations of eight cultures (performed on 5, 6 and 7 days) showed there was no visible difference between the numbers of $cIgM^+$ cells and $cIgM^-$ blasts in PHA-treated and unstimulated cultures. One 7 day culture was sufficiently good for a cell count to be performed: the unstimulated culture contained 1.5% $cIgM^+$ cells and 1.1% $cIgM^-$ blasts, whereas the PHA-treated culture contained 0.3% $cIgM^+$ cells and 2.5% $cIgM^-$ blasts.

(c) Lipopolysaccharide-induced differentiation of $cIgM^+$ cells and $cIgM^-$ lymphoblasts in cultured thymocytes

The results obtained when thymocytes, cultured with LPS, were stained for cytoplasmic IgM are shown in Table 3.2. After 5 days

of culture, the numbers of $cIgM^+$ cells and $cIgM^-$ blasts in LPS-stimulated cultures were $8.7 \pm 4.1\%$ and $2.1 \pm 0.7\%$ respectively. Similar numbers were found after 7 days, with $6.6 \pm 3.5\%$ $cIgM^+$ cells and 2.9 ± 2.9 $cIgM^-$ blasts. The morphology of the cell types obtained was similar to that of the spleen cell types. (A high power magnification of a $cIgM^+$ thymocyte with cytoplasmic fluorescence scattered throughout the cell is shown in Fig. 3.2b.) It was possible to perform cell counts on only one 5 day and one 7 day unstimulated culture. After 5 days, the numbers of $cIgM^+$ cells and $cIgM^-$ blasts in the unstimulated cultures were 1.1% and 0.6% respectively. Similar numbers were obtained after 7 days with 0.7% $cIgM^+$ cells and zero $cIgM^-$ blasts.

(d) Phytohaemagglutinin-treated thymocyte cultures

Cultures from animal T6 treated with PHA showed 0.2% $cIgM^+$ cells and 0.2% $cIgM^-$ blasts after 5 days, and animal T5 showed zero $cIgM^+$ cells but 0.4% $cIgM^-$ blasts after 7 days. Thus the numbers of $cIgM^+$ cells and $cIgM^-$ blasts were considerably smaller in the unstimulated and PHA-treated cultures, when compared to the LPS-stimulated cultures.

(e) Viability of thymocyte cultures

The reason for the lack of quantitative data concerning the unstimulated and PHA-treated thymocyte cultures was the poor viability of these thymocyte cultures, even when using 10% FCS supplementation of the culture medium. Most of the thymocytes in the cytospin preparations appeared as small, brightly staining cells, possibly through non-specific absorption of the stain by dead cells, as already discussed. Treatment with LPS increased the viability of

the cultures, and induced the differentiation of $cIgM^+$ cells. An indication of the increased viability of thymocyte cultures after LPS treatment is also given by counts of broken, damaged cells and intact small lymphocytes and lymphoblasts visible in cytospin preparations stained with Leishmans. One 3 day unstimulated culture contained 75.2% damaged cells, 24.6% intact small lymphocytes and 0.2% intact lymphoblasts. After treatment with LPS, this culture contained 30.2% damaged cells, 65.8% intact lymphocytes and 4.0% intact lymphoblasts. Another unstimulated culture, harvested at 6 days, contained mainly damaged cells, but after LPS treatment this culture contained 65.7% damaged cells, 26.9% intact small lymphocytes and 7.4% intact lymphoblasts.

Discussion

The results described in this Chapter confirm the notion (Horton, Smith, Williams, Smith and Sherif, 1980) that LPS can stimulate thymic B cells in Xenopus. Thus $cIgM^+$ cells are induced to differentiate by LPS in both splenocyte and thymocyte cultures.

There is no significant difference between the numbers of $cIgM^+$ cells found in spleen and thymus cultures after 7 days ($P > 0.1$). However, there was a significant difference in the numbers of $cIgM^-$ blasts after 7 days ($P < 0.01$). The fact that the thymocyte cultures contain less $cIgM^-$ blasts after 7 days than the splenocyte cultures indicates that the spleen of Xenopus has a greater percentage of B lymphocytes capable of responding to LPS than the thymus.

Phytohaemagglutinin-treated splenocyte and thymocyte cultures showed no differentiation of $cIgM^+$ cells. It is perhaps surprising that PHA-treated cultures did not contain increased numbers of $cIgM^-$ lymphoblasts above the control level. However, it is possible

that at the times studied (5-7 days in culture) any PHA-induced lymphoblasts had already degenerated. Maximal tritiated thymidine uptake of PHA-stimulated Bufo marinus lymphocytes is recorded after 3 days of culture and thereafter declines (Goldshein and Cohen, 1972). Furthermore, studies with cultures of trout lymphocytes have shown that cells displaying blast cell morphology appeared 1 day after stimulation of splenocytes with Con A (a T cell mitogen), reached a peak number after 3 days, but by 6 days had degenerated so that reliable morphological identification of specific cell types was impossible (Etlinger, Hodgins and Chiller, 1978). Etlinger et al. also showed that trout peripheral blood lymphocytes stimulated with LPS showed an increase in blast cells after 3 days, which reached a maximal value on day 5. Plasma cells first appeared after 5 days of culture and reached a peak after 7 days.

Studies with mouse splenocytes have also shown that the kinetics of the appearance of T and B cell mitogen-induced blasts differ. Electron microscopy revealed that lymphoblasts appeared in splenocytes cultured with either Con A or LPS after about 1 day in culture. However, whereas the LPS-treated cultures developed to contain lymphoblasts, plasmablasts and plasma cells after $4\frac{1}{2}$ days in culture, the large lymphoblasts in Con A-treated cultures disappeared and these cultures showed mainly intermediate-sized cells after $4\frac{1}{2}$ days (Shohat, Janossy and Dourmashkin, 1973).

Bleicher, Rollins-Smith, Jacobs and Cohen (1983) have shown that commercially purified LPS loses most of its mitogenicity for frog, but not for mouse lymphocytes. In view of the relatively large amounts of LPS required to obtain a good response for frog cells (400-2,000 μg/ml compared to about 50 μg/ml in mice), they suggested that frog cells may be responding to contaminants of LPS.

Taken together with their data that surface IgM-negative cells can respond to LPS to some extent (Bleicher and Cohen, 1981; see Introduction), they further suggested that the response found to LPS in thymocyte cultures may be due to T rather than B cells. The experiments in this Chapter have revealed, however, that whatever the active mitogenic agent is in commercial LPS, and irrespective of the mechanism of induction, that this preparation effects the differentiation of $cIgM^+$ cells - i.e. B equivalent lymphocytes in both spleen and thymus.

The finding of B-lineage cells within the Xenopus thymus is, in fact, not surprising, since a variety of vertebrate species possess intrathymic B lymphocytes, including mice (Micklem, Anderson, Ure and Parry Jones, 1976), chickens (Seto, 1978), bull-frogs (Minagawa, Ohnishi and Murakawa, 1975; Moticka, Brown and Cooper, 1973), snakes (Kawaguchi, Kina and Muramatsu, 1978) and fish (Ortiz-Muniz and Sigel, 1971; Sailendri and Muthukkaruppan, 1975). Very recently, Hsu, Julius and Du Pasquier (1983) have demonstrated that the thymus (as well as spleen) of X. laevis x X. muelleri hybrids produces both IgM and Ig"G" antibody in response to secondary dinitrophenylated-keyhole limpet haemocyanin stimulation in vitro. Interestingly, in their studies, thymic B cells were found to be particularly active in synthesising the low molecular weight Ig. Hsu et al. thus suggest that the frog thymus may be involved in the regulation of memory responses and that such regulation may also occur in the mouse thymus.

In conclusion, the experiments reported in this Chapter reveal that both spleen and thymus of Xenopus contain B cells in addition to T cells. It therefore became necessary to try and remove the B cells before clean T cell reconstitution studies could be performed. Attempts to selectively deplete B cells are described in the following Chapter.

Table 3.1 Cytoplasmic immunoglobulin M-positive cells and unstained lymphoblasts present in unstimulated and LPS-treated splenocyte cultures

Days in culture	Animal	Unstimulated cultures		LPS-treated cultures	
		% $cIgM^+$ cells	% $cIgM^-$ blasts	% $cIgM^+$ cells	% $cIgM^-$ blasts
5	S1	0	12.3	2.9	8.5
6	S2	0.6	2.4	1.9	13.0
	S3	0.6	2.9	3.1	9.4
		$\bar{x} = 0.6 \pm 0$	$\bar{x} = 2.7 \pm 0.4$	$\bar{x} = 2.5 \pm 0.8$	$\bar{x} = 11.2 \pm 2.5$
7	S4	0	2.5	9.2	15.6
	S5	0.7	0.5	3.1	2.5
	S6	0*	0	6.8	13.7
	S7	0.2	1.2	6.7	6.4
	S8	2.8*+	1.6	6.7	14.9
	S9	0+	1.4	2.4	8.9
	S10	0.7+	3.0	4.7	10.2
		$\bar{x} = 0.6 \pm 1.0$	$\bar{x} = 1.5 \pm 1.0$	$\bar{x} = 5.7 \pm 2.4$	$\bar{x} = 10.3 \pm 4.8$
8	S4	0.25	2.8	8.8	17.1

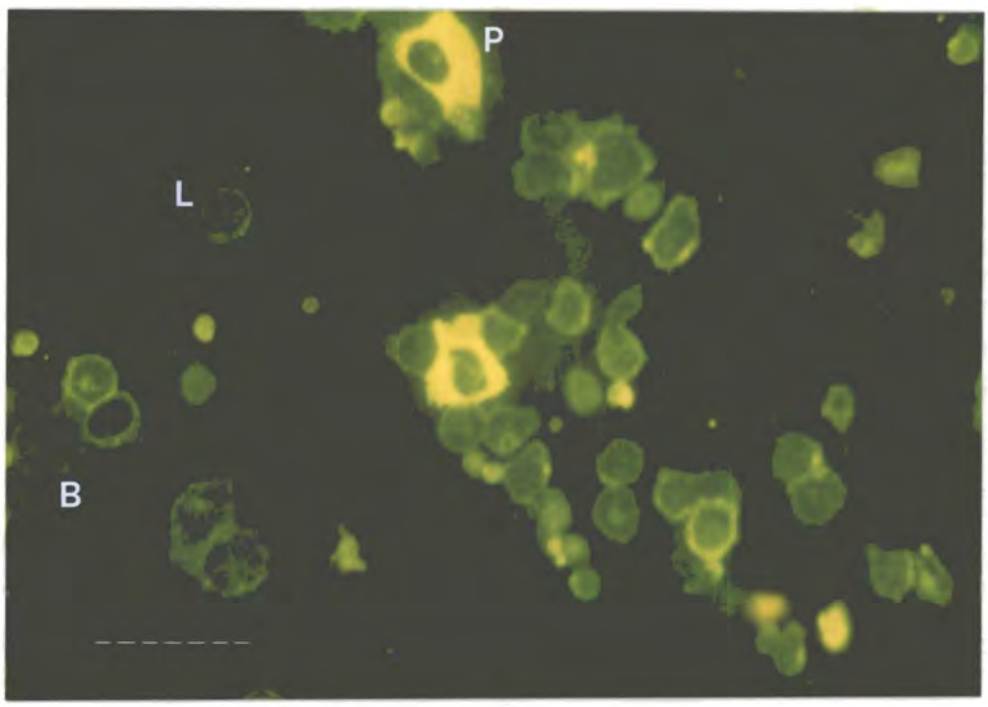
Table 3.2 Cytoplasmic immunoglobulin M-positive cells and unstained lymphoblasts present in unstimulated and LPS-treated thymocyte cultures

Days in culture	Animal	Unstimulated cultures		LPS-treated cultures	
		% $cIgM^+$ cells	% $cIgM^-$ blasts	% $cIgM^+$ cells	% $cIgM^-$ blasts
5	T1	-	-	5.1	1.2
	T2	-	-	6.0	1.5
	T3	-	-	10.8	3.1
	T4	-	-	6.7	2.3
	T6	1.1	0.6	14.9	2.2
				$\bar{x} = 8.7 \pm 4.1$	$\bar{x} = 2.1 \pm 0.7$
7	T1	-	-	4.6	0.8
	T2	-	-	3.1	0.6
	T3	-	-	9.9	4.8
	T4	-	-	4.5	1.2
	T5	0.7	0	10.9	7.0
				$\bar{x} = 6.6 \pm 3.5$	$\bar{x} = 2.9 \pm 2.9$

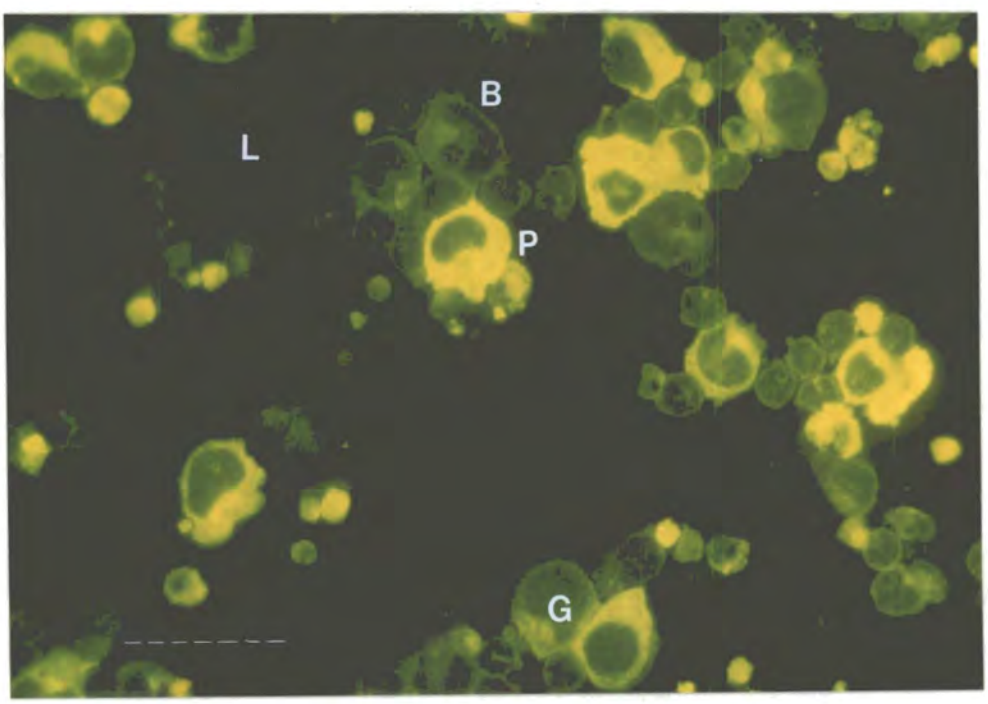
Results are expressed as mean ± S.D. where appropriate.
The LPS concentration was 2.0 mg/ml.

Fig. 3.1 Cytocentrifuge preparations of splenocytes cultured with and without LPS, stained for cytoplasmic IgM

(a)



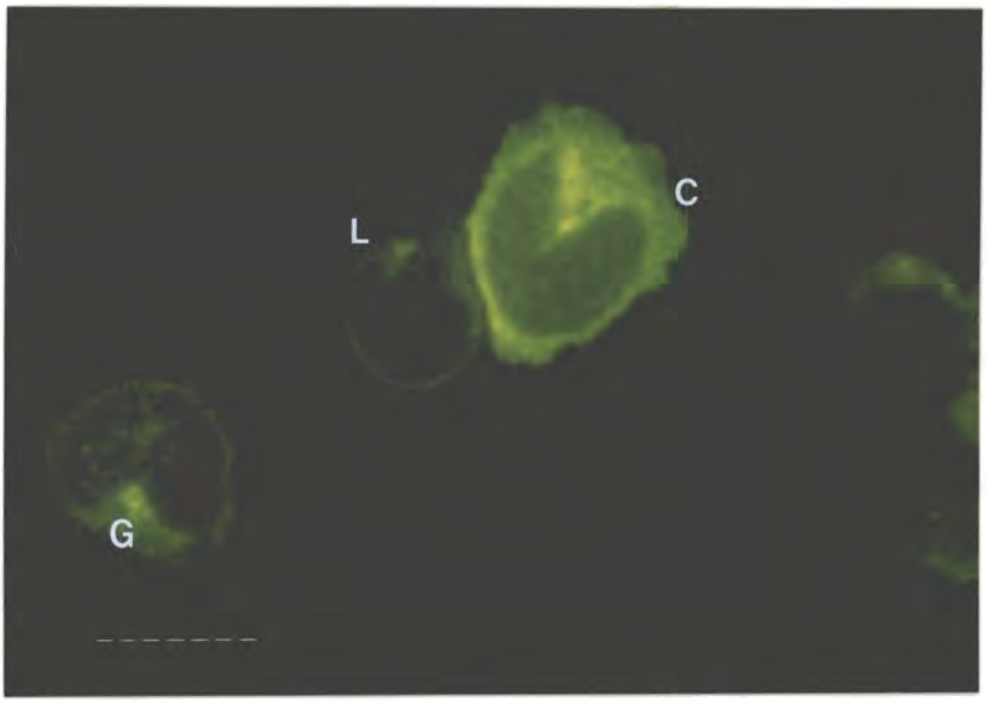
(b)



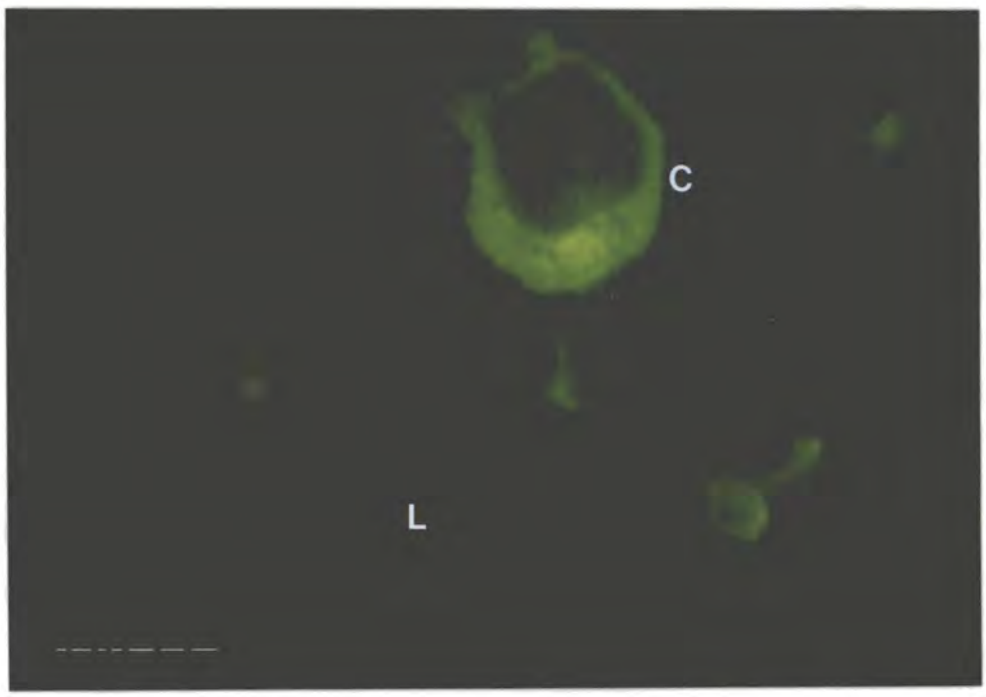
Cytocentrifuge preparations of 7 day splenocyte cultures stained using rabbit IgG anti-*Xenopus* μ chain antiserum, followed by goat IgG anti-rabbit IgG, FITC conjugated. (a) Unstimulated culture; (b) LPS-treated culture; 2.0 mg/ml. B, $cIgM^-$ lymphoblast; G, lymphoblast with $cIgM^+$ golgi zone; L, $cIgM^-$ lymphocyte; P, heavily stained $cIgM^+$ plasmablast. Scale bars = 30 μm.

Fig. 3.2 Cytocentrifuge preparations of LPS-treated splenocytes and thymocytes, stained for cytoplasmic IgM

(a)



(b)



Cytocentrifuge preparations of 7 day splenocyte (a) and thymocyte (b) cultures, treated with 2.0 mg/ml LPS and stained using rabbit IgG anti-*Xenopus* μ chain antiserum, followed by goat IgG anti-rabbit IgG, FITC conjugated. C, $cIgM^+$ lymphoblast; G, lymphoblast with $cIgM^+$ golgi zone; L, $cIgM^-$ lymphoblast. Scale bars = 10 μm.

CHAPTER FOUR

PRODUCTION OF B CELL-DEPLETED LYMPHOCYTE POPULATIONS: USE OF NYLON WOOL COLUMNS AND GAMMA-IRRADIATION

Introduction

Passage of mammalian lymphocytes through a nylon wool column at 37°C reveals a non-adherent cell population that is enriched for T lymphocytes, as B lymphocytes adhere preferentially to the nylon wool (Greaves and Brown, 1974; Handwerger and Schwartz, 1974; Julius, Simpson and Herzenberg, 1973; Trizio and Cudkowicz, 1974). The B cell-enriched adherent population can be recovered from the column by mechanical agitation (Handwerger and Schwartz, 1974; Trizio and Cudkowicz, 1974). Differential adherence of T and B cells to nylon wool has also been used in attempts to separate lymphocytes of Xenopus (Blomberg, Bernard and Du Pasquier, 1980).

Blomberg et al. (1980) showed that (at 37°C) the nylon wool non-adherent population contained few or no functional B cells, since it was unable to mount a secondary antibody response to dinitrophenylated-keyhole limpet haemocyanin in vitro and possessed low numbers of surface Ig-positive lymphocytes. On the other hand, the non-adherent population maintained its carrier-specific function and had other T cell characteristics such as PHA and MLR reactivity. Since nylon wool separation at 30°C was also shown to be as successful as 37°C incubation at removing surface Ig-positive cells from peripheral blood lymphocytes, the experiments reported here make use of this lower temperature, since amphibian lymphocytes are sensitive to culture temperatures above 30°C (see Blomberg et al., 1980). In this Chapter the ability of nylon wool passage to deplete B cells from thymocyte and splenocyte populations is examined by comparing in vitro

reactivity of non-passaged and non-adherent cells to the T cell mitogen PHA and the B cell mitogen LPS. Since restoration experiments on thymectomized animals using both unprimed and SRBC-primed transfer lymphocytes were planned (see Chapter 6), a brief investigation was carried out on the ability of nylon wool to remove plaque-forming cells from splenocytes taken from SRBC-immunized toadlets.

The use of γ-irradiation for obtaining lymphocyte populations depleted of functional B cells is also examined in this Chapter. Mammalian B lymphocytes have been shown to be more sensitive to doses of X- or γ-irradiation than helper T lymphocytes (Anderson and Warner, 1976; Kataoka and Sado, 1975; Katz, Paul, Goidl and Benacerraf, 1970; Kettman and Dutton, 1971). This relative radiosensitivity of B lymphocytes compared with helper T cells has also been shown in Xenopus. Thus carrier-primed spleen cells given a dose of 3,000 rads are still able to provide help for hapten-primed B cells in an in vitro PFC assay. On the other hand, Xenopus antigen-primed B lymphocytes are X-ray sensitive, as a dose of 500 rads was able to completely eliminate an in vitro secondary PFC response (Blomberg, Bernard and Du Pasquier, 1980). In this Chapter the ability of 3,000 rad irradiated lymphocytes (from unprimed thymus, spleen or spleen/peripheral blood lymphocyte mixtures, and also from spleens taken from SRBC-primed animals) to effect a PFC response when injected into MHC-compatible, lethally irradiated, SRBC-injected Xenopus was examined.

Materials and Methods

Lymphocyte culture and scintillation counting

Lymphocyte culture was as described in Chapter 3 except that: (a) the L-15 culture medium used contained 0.01 M sodium bicarbonate, and

the culture plates were incubated in humidified air containing 5% carbon dioxide. (It was found that Xenopus lymphocytes survived better under these conditions.) (b) The splenocytes were cultured with 1 x 10^5 cells/well rather than 2 x 10^5 cells/well as in Chapter 3. This was because the spleens did not contain enough lymphocytes to compare unpassaged and nylon wool-passaged cells. The thymocyte cultures still used 2 x 10^5 cells/well. Cultures were pulsed with 10 µl (1 µCi) tritiated thymidine ($[^3H]$TdR, specific activity 5 Ci/mmol, Radiochemical Centre, Amersham) after 48 hours, and then harvested using a Skatron cell harvester (Flow) after a total of 72 hours. This resulted in the cells from each well being deposited onto individual glass fibre filter discs, which were then dried overnight at 60°C. The filter discs were transferred to individual polyethylene scintillation vials, and 2.5 ml of a toluene based PPO/POPOP liquid scintillator (Packard) was added to each vial. Tritiated thymidine incorporation was measured using an automatic liquid scintillation counter (Tri-Carb 300C, Packard).

Stimulation indices were calculated for each individual experiment as follows:

$$\text{stimulation index} = \frac{\text{mean dpm in mitogen-stimulated cultures}}{\text{mean dpm in non-stimulated (control) cultures}}.$$

Nylon wool separation

Splenocytes and thymocytes were separated on nylon wool columns as described by Blomberg, Bernard and Du Pasquier (1980). Nylon wool ('Leuko-pak' leukocyte filter, Fenwal Laboratories - purchased from Travenol Laboratories, lot 1H302R6) was washed six times by boiling in double-distilled water for 10 minutes each wash. It was then dried overnight at 37°C, weighed into 1 g lots and teased apart

to remove tangles and knots. The nylon wool was then loosely folded, and packed in a 10 ml syringe to a volume of 7-8 ml, and autoclaved.

The columns were first incubated for 1 hour at 30°C after saturation with 20 ml pre-warmed sterile amphibian PBS containing 5% FCS. Before adding the cell suspension, the columns were rinsed with 10 ml of pre-warmed PBS containing 5% FCS. One to two millilitres of the cell suspension at a concentration of 5-10 x 10^6/ml in L-15 medium containing 5% FCS were then added and washed into the column with 1-2 ml PBS/FCS. The columns were incubated for 1 hour at 30°C, then the non-adherent cells were collected by dropwise addition of 20 ml of pre-warmed FCS/PBS. The recovered cells were washed once by centrifugation, resuspended in L-15 medium, and the lymphocyte concentration adjusted to 5 x 10^6/ml, prior to setting up in culture, as already described.

Plaque-forming cell assay

The PFC assay was performed on spleen cells as described in Chapter 2, except that both guinea-pig and Xenopus serum were used as a source of complement. The Xenopus complement was used because it was found to be more sensitive than the guinea-pig complement, i.e. more PFC were visualized (see Chapter 6 and Lallone, 1984).

Preparation of Xenopus serum for use as complement

Blood was collected by cardiac puncture from animals aged 4-8 months, and allowed to clot at 4°C. After centrifugation at 350 x g for 10 minutes to pellet any loose cells, the serum was removed and kept on ice. The serum was absorbed with SRBC before use in the plaque assay. Prior to absorption, the serum was brought to mammalian tenicity by the addition of NaCl to prevent lysis of the SRBC during

absorption. The Xenopus complement was always prepared freshly on the day of the PFC assay, and was never frozen.

Separation of peripheral blood lymphocytes

Blood was collected from animals by cardiac puncture, and pipetted into 2-3 ml amphibian strength PBS containing 10 units heparin (Flow)/ml. The blood was then layered onto 2-3 ml of a ficoll-isopaque mixture, density 1.1 g/ml, prepared by mixing 9% ficoll 400 (Pharmacia) with 34% isopaque (Nyegaard) in a ratio of 1:0.815. The tubes were centrifuged for 15-20 minutes at 40 x g, which leaves the lymphocytes at the interface of the ficoll-isopaque and PBS, while allowing the more dense erythrocytes to pass through the ficoll-isopaque. The peripheral blood lymphocytes (PBL) were pipetted off, and washed three times in medium, prior to injection.

Irradiation of lymphocytes and animals

Lymphocyte suspensions and whole animals were given a lethal irradiation dose (LD_{50}) of 3,000 rads, at a dose rate of 600 rads/minute by exposure to a cobalt-60 source. For irradiation, the lymphocytes were suspended at a concentration of 5-10 x 10^6 cells/ml, in amphibian strength L-15 containing 10% FCS. The cells were then washed once by centrifugation, prior to injection. The animals were placed in a small container of water for irradiation.

Experimental design

(a) Mitogen studies on nylon wool-passaged and control, non-passaged lymphocytes. These experiments examined the effect of removing nylon wool-adherent cells on in vitro reactivity to T and B cell mitogens. Splenocyte or thymocyte suspensions from control

animals were separated on nylon wool columns and stimulated with PHA or LPS. Mitogen reactivity after nylon wool treatment was compared with mitogen reactivity of the same cell population before nylon wool passaging. The ability of LPS to stimulate the differentiation of cytoplasmic immunoglobulin M-positive ($cIgM^+$) cells in cultures of nylon wool non-adherent splenocytes and thymocytes was also briefly assessed.

(b) Ability of nylon wool to retain cells actively secreting antibody. Toadlets were injected with antigen (SRBC) and the PFC assay performed 6 days later, as described in Chapter 2. Before the assay was carried out, half of the splenocytes were passaged through nylon wool, and the numbers of PFC compared before and after nylon wool treatment. The purpose of this experiment was to determine whether PFC, which are B cells actively secreting antibody, are depleted by the nylon wool treatment.

(c) Studies on the reconstitution of irradiated animals with irradiated or non-irradiated lymphocytes. Toadlets that were to receive lymphocyte injections (i.e. adoptive transfer hosts) were first given a lethal dose of irradiation (3,000 rads). This dose prevents such animals from being able to mount a primary in vivo antibody response to SRBC, a deficiency which is due to the destruction of B cell function, as shown by primary in vitro PFC studies. These studies have shown that 3,000 rad irradiated splenocytes are unable to produce anti-rabbit erythrocyte PFCs, but are still able to provide T cell help for the PFC response of splenocytes from thymectomized (i.e. T cell-deficient) animals (Lallone, 1984). Immediately after irradiation, toadlets were given an injection of 10×10^6 MHC-compatible lymphocytes in a volume of 0.2-0.3 ml amphibian strength L-15, via the dorsal lymph sac. (The lymphocytes

injected were from a variety of sources: thymus; spleen; the spleen of an animal primed with 10% SRBC 6 days previously; or a mixture of splenocytes and PBL at a ratio of 4:1. The injected cells were either non-irradiated or given a dose of 3,000 rads irradiation). The irradiated, adoptive transfer hosts were simultaneously given a single injection of SRBC (i.p.) as described in Chapter 2 and the plaque assay performed 6 days later, using spleen lymphocytes.

In this system, any PFC obtained will originate from functional B cells within the injected population. The experiments therefore test the effectiveness of 3,000 rads irradiation in destroying B cell activity in adoptively transferred unprimed thymocytes, splenocytes and PBL, and primed splenocytes. These experiments were performed using MHC-compatible animals, as irradiated Xenopus injected with MHC-incompatible cells show a rapid deterioration in their state of health, even when the injected cells are first irradiated. One animal given non-irradiated unprimed splenocytes died 4 days after an MHC-incompatible lymphocyte injection, and three others injected with either MHC-incompatible irradiated splenocytes, non-irradiated primed splenocytes or irradiated primed splenocytes, were too sick to be used for a plaque-forming cell assay, after 6 days.

Results

(a) The effect of nylon wool treatment on the proliferative response of splenocytes and thymocytes to T and B cell mitogens

Splenocytes. The results given in Table 4.1 show the effect of nylon wool treatment on the proliferative reactivity of splenocytes to LPS and PHA. Non-nylon wool-passaged cells gave a mean stimulation index of 5.1 $\pm$ 2.1 ($\pm$ S.D.) when cultured with LPS. The S.D.s given

shows the range of stimulation indices of the individual experiments. In many of the experiments described below the stimulation indices vary quite widely from experiment to experiment. However, it should be noted that the S.D.s given for the background counts, stimulated counts and stimulation indices of each individual experiment are relatively small. Nylon wool-passaged non-adherent cells gave a mean stimulation index of 3.5 ± 2.4. Thus the passage of splenocytes through nylon wool brings about a small reduction in the response to LPS, but a substantial response remains in some instances. (This reduction in response was not statistically significant - $P > 0.1$.) In two individual experiments, the response to LPS actually increased after nylon wool passage.

After stimulation with PHA, non-nylon wool-passaged splenocytes gave a mean stimulation index of 32.5 ± 26.9. This mean stimulation index was increased to 45.0 ± 20.7 after nylon wool treatment; however, the increase was not statistically significant ($P > 0.1$). In most of the individual experiments, the PHA reactivity increased after nylon wool treatment, or in one case remained approximately the same. However, in two experiments the response to PHA decreased from 37.4 ± 1.2 and 96.8 ± 24.9 to 19.5 ± 5.5 and 23.3 ± 5.3 respectively, after nylon wool treatment. This decrease in stimulation index was probably due to the fact that the background counts in these two experiments increased after nylon wool treatment, whereas in all of the other experiments, the background counts decreased. This decrease in background counts was not due to an initially poor viability of the passaged cells, as shown in Table 4.2. The viability of fresh cells, measured before culture by nigrosine dye-exclusion, did not change significantly after passage through nylon wool. The same is true for the thymocyte cultures.

(See Discussion for further examination of the decrease in background dpm in the nylon wool non-adherent population as compared to that of unseparated cells.)

Thymocytes. The results given in Table 4.3 show the effect of nylon wool treatment on the LPS and PHA reactivity of thymocytes. Non-nylon wool-passaged cells gave a mean stimulation index of 2.9 ± 0.8 when treated with LPS. After passage through nylon wool, the non-adherent cells gave a mean stimulation index of 2.0 ± 0.8. This small reduction in response to LPS after nylon wool treatment was found not to be statistically significant ($P > 0.05$).

After stimulation with PHA, non-passaged cells gave a stimulation index of 3.9 ± 1.5. After nylon wool treatment, this stimulation index remained more or less constant, at 3.9 ± 1.1. Individual experiments showed either an increase or a decrease in the stimulation index after nylon wool treatment, but the change was small either way and was not statistically significant ($P > 0.1$).

(b) Effect of nylon wool treatment on the ability of LPS to stimulate $cIgM^+$ lymphocytes

Three cultures, previously treated with nylon wool, were examined for their ability to produce $cIgM^+$ cells. The results of this experiment are shown in Table 4.4. Low numbers (in some cases zero) of $cIgM^+$ cells and $cIgM^-$ blasts were observed in the unstimulated and PHA-treated splenocyte and thymocyte cultures. However, good numbers of $cIgM^+$ cells and $cIgM^-$ blasts appeared in the LPS-treated cultures. Thus nylon wool passaging does not appear to significantly affect the number of potential IgM-secreting B cells in a population of splenocytes or thymocytes (cf. data on unseparated cells in Chapter 3 - Tables 3.1 and 3.2). Hence some cells of B lineage are evidently

able to pass through nylon wool.

(c) Effect of nylon wool treatment on the number of plaque-forming cells detectable from an antigen-primed animal

The splenocytes from two SRBC-primed animals gave 210 and 730 PFC/10^6 cells. When these splenocytes were first passaged through nylon wool before performing the plaque assay, the numbers obtained were 131 and 431 PFC/10^6 cells, respectively. Thus some B cells that are actively secreting antibody are retained on the nylon wool column, but the majority of plasma cells (62 and 59% respectively) are not.

(d) "Reconstitution" of irradiated animals

The PFC response obtained after injecting irradiated animals with lymphocytes from a variety of sources, is shown in Table 4.5. The results are similar with guinea-pig and Xenopus complement, but the Xenopus complement is more efficient as the lytic agent, this making the assay more sensitive. (See Chapter 6 for more information on Xenopus complement.)

When irradiated animals were given an injection of thymocytes (with a simultaneous injection of SRBC), they produced a small number of PFC/spleen. If the thymocytes were irradiated, this response disappeared. Similar results were obtained using splenocytes and a mixture of splenocytes/PBL, although injections of unirradiated unprimed populations gave quite good PFC levels. The PFCs produced by the non-irradiated thymocytes, splenocytes and splenocytes/PBL therefore appear to be due to B cells in the injection. These B cells are destroyed by 3,000 rads of irradiation, so injections of irradiated cells were unable to produce a PFC response to SRBC in the irradiated animals (which themselves do not possess functional B cells).

When irradiated animals were given an injection of primed splenocytes, good numbers of PFCs were found in their spleens. Whether or not SRBC administration to the irradiated recipient was necessary to produce these PFC was not checked here (see Discussion). If the primed splenocytes were irradiated before injection, the number of PFC per spleen was reduced, but a significant level remained. Hence 3,000 rads does not destroy the short-term activity of all primed B cells. The results in Table 4.5 are presented both as PFC/spleen and PFC/10^6 leucocytes. This is because the standard way of expressing PFC data (PFC/10^6 leucocytes) greatly exaggerates the total numbers of PFCs present with respect to the PFC data obtained from the irradiated primed spleen injections. Irradiation of a frog reduced the number of lymphocytes in the spleen from several million to $< 0.3 \times 10^6$. An injection of non-irradiated lymphocytes increased this to $1\text{-}2 \times 10^6$, but an injection of irradiated cells did not increase the spleen lymphocyte numbers to more than 0.5×10^6. Hence the PFC numbers per spleen give a more realistic indication of the total numbers of antibody-forming cells in these particular experiments.

Discussion

The experiments with nylon wool presented here suggest that this technique, if carried out at 30°C, is unsuitable for the preparation of B cell-depleted lymphocyte populations in Xenopus. Nylon wool non-adherent splenocytes and thymocytes were still able to respond to LPS, and with the differentiation of $cIgM^+$ cells. Plaque-forming cells (obtained from SRBC-immunized toadlets) were also shown to be capable of passing through the nylon wool columns. (This also holds true for PFC that were nylon wool passaged at 37°C - data

not shown.) Interestingly, Blomberg, Bernard and Du Pasquier (1980) revealed that, in contrast to nylon wool separation at 37°C (which "eliminates" antibody-producing cells from the non-adherent lymphocyte population), incubation at 27°C resulted in some contaminating B cells (mostly blast cells) in the non-adherent population, as measured by the ability of these lymphocytes to give an *in vitro* secondary antibody response and by the high percentage (22%) of surface Ig-positive cells. Unfortunately, these authors did not examine LPS reactivity, nor did they look at the antibody-producing potential of nylon wool non-adherent lymphocytes passaged at 30°C. However they did show that nylon wool filtration carried out at this latter temperature resulted in the lowest percentage (11%) of surface Ig-positive non-adherent cells. (At 37°C this percentage was 13%.)

Mitogen experiments using murine nylon wool non-adherent lymphocytes reveals them to be poor at LPS reactivity, but enriched in terms of responsiveness to PHA as compared with unseparated cells (Handwerger and Schwartz, 1974). In contrast, no significant enrichment of PHA reactivity could be demonstrated in the nylon wool non-adherent population in the experiments with *Xenopus* reported here nor in those by Blomberg *et al.* (1980). The latter authors suggested that the lack of enrichment may be due to some impairment of the proliferative ability in T cells kept at 37°C for 1 hour, since MLR reactivity of peripheral blood lymphocytes after passage through nylon wool at 27°C was enhanced. (However Blomberg *et al.* did not examine the MLR reactivity of lymphocytes after nylon wool passage at 30 or 37°C.) In the present experiments at 30°C it was noticed that nylon wool-passaged cells generally had lower background counts than the non-passaged lymphocytes. In fact, background counts are

even lower when splenocytes are nylon wool separated at 37°C (data not shown). This decrease in background counts at 30°C was shown not to be due to an immediate decrease in the viability of the passaged cells. However, it is possible that the capacity for survival over the period of the experiment was reduced, resulting in lower background counts. Nylon wool contains a toxic product that decreases the in vitro survival of mammalian cells and requires extensive washing to remove this produce (Julius, Simpson and Herzenberg, 1973). Perhaps Xenopus lymphocytes are more sensitive to this toxic product, such that the thorough washing given to the nylon wool was insufficient. On the other hand, the lowered background counts may be due to the fact that some accessory cells preferentially adhere to nylon wool (Erb, and Feldmann, 1975; Henry, Chen, Stout and Swain, 1980). Such a removal of accessory cells from the non-adherent population may also affect PHA reactivity. Thus macrophages produce interleukin 1 which induces a T cell subset to produce interleukin 2. This latter lymphokine will aid the continued proliferation of mitogen-activated T cells (Howie and McBride, 1982). If the number of macrophages is decreased in nylon wool-passaged populations, the potentiation of the PHA response will also be decreased compared to that in unseparated cells.

In contrast to the lack of success (in terms of B cell depletion) with nylon wool separation, a dose of 3,000 rads of γ-irradiation proved effective in removing the potential of unprimed Xenopus lymphocyte populations to form PFC, when these are transferred to irradiated, MHC-compatible animals. As mentioned in the Introduction and Experimental design section, this dose of irradiation leaves helper T cell function of Xenopus lymphocytes intact (Blomberg, Bernard and Du Pasquier, 1980; Lallone, 1984). On the other hand,

3,000 rads does not destroy all B cell activity in SRBC-primed splenocyte populations - i.e. a substantial PFC response remained 6 days after transfer of the primed irradiated cells into the lethally irradiated host. Others have shown that memory B cells in Xenopus are destroyed by a lower dose of irradiation, since an X-ray dose of 500 rads rendered long-term ($2\frac{1}{2}$ months) in vivo primed lymphocytes unable to produce antibody in an in vitro secondary PFC assay (Blomberg et al., 1980).

It is possible that, in the present experiments, where primed lymphocytes were irradiated within only a few days of antigen administration, radioresistant plasma cells and B lymphoblasts can continue to secrete antibody in the adoptive transfer host. It is known that mature mammalian B cells (plasma cells) are extremely radioresistant (Anderson and Warner, 1976). A time course study, where the time interval between antigen priming and irradiation of the lymphocytes is varied before injection into a lethally irradiated host, is required to determine the longer term potential of the irradiated population. It may be that only the mature B effector cells are radioresistant and in longer term experiments where these cells have died there will be no PFC produced. (The majority of mammalian plasma cells are relatively short-lived and survive for only a few days - Anderson and Warner, 1976.) This could be confirmed by experiments in which the adoptive transfer irradiated host is given irradiated, SRBC-primed splenocytes, but no accompanying injection of antigen. This would reveal whether or not the PFC response seen in the adoptive host spleen is dependent upon interaction of the transferred cells with SRBC within this host.

From the experiments reported in this Chapter, it was decided that irradiated rather than nylon wool-passaged lymphocytes should

be used for the production of B cell-depleted populations for use in the cellular reconstitution experiments on thymectomized animals described in Chapter 6.

Table 4.1 Legend

Cultures were supplemented with 1% FCS.

Number of cells per well = 10^5.

Cultures were pulsed with tritiated thymidine at 48 hours and harvested at 72 hours.

Although mean stimulation indices (S.I.s) can be calculated from the mean counts given, these will not agree precisely with the mean S.I.s given, as the latter figures were calculated from the S.I.s for the individual experiments.

The S.D.s given for the mean counts and mean S.I.s are the S.D.s of those means, rather than the mean of the S.D.s of the counts and S.I.s for the individual experiments.

The animals used were outbred X. laevis aged 9 months.

Table 4.1 The effect of nylon wool treatment on the mitogen response of splenocytes

Mitogen	Animal	Background counts (mean dpm ± S.D.)		Stimulated counts (mean dpm ± S.D.)		Stimulation index ± S.D.	
		Non-passaged	Nylon wool passaged	Non-passaged	Nylon wool passaged	Non-passaged	Nylon wool passaged
LPS	1	1194 ± 432	165 ± 2	3944 ± 586	252 ± 141	3.3 ± 0.5	1.5 ± 0.9
2	2	986 ± 155	965 ± 287	7565 ± 938	3588 ± 507	7.7 ± 1.0	3.7 ± 0.5
	3	2368 ± 483	1075 ± 973	9429 ± 496	1947 ± 624	4.0 ± 0.2	1.8 ± 0.6
	4	1514 ± 570	765 ± 186	6170 ± 160	2068 ± 841	4.1 ± 0.1	2.7 ± 1.1
µg/ml	5	4138 ± 1077	668 ± 182	23980 ± 1478	2213 ± 155	5.8 ± 0.4	3.3 ± 0.2
	6	2897 ± 882	786 ± 713	25892 ± 430	1829 ± 945	8.9 ± 0.2	2.3 ± 1.2
	7	3143 ± 732	1175 ± 465	23771 ± 2901	8660 ± 842	7.6 ± 0.9	7.4 ± 0.7
	8	2012 ± 1092	2566 ± 789	4317 ± 79	2509 ± 411	2.1 ± 0.04	1.0 ± 0.2
	9	668 ± 83	2191 ± 358	2804 ± 180	3253 ± 912	4.2 ± 0.3	1.5 ± 0.4
	10	4885 ± 825	670 ± 89	19052 ± 1600	4420 ± 230	3.9 ± 0.3	6.6 ± 0.3
	11	9959 ± 1905	1615 ± 495	49861 ± 1909	11202 ± 639	5.0 ± 0.2	6.9 ± 0.4
		$\bar{x}$ = 3069 ± 2638	$\bar{x}$ = 1149 ± 711	$\bar{x}$ = 16071 ± 14299	$\bar{x}$ = 3813 ± 3259	$\bar{x}$ = 5.1 ± 2.1	$\bar{x}$ = 3.5 ± 2.4
PHA	1	As above	As above	20244 ± 9038	10818 ± 1344	17.0 ± 7.6	65.6 ± 8.2
20	2			24586 ± 2605	37920 ± 10030	24.9 ± 2.6	39.3 ± 10.4
	3			72940 ± 37237	30100 ± 6723	30.8 ± 15.7	28.0 ± 6.3
µg/ml	4			105902 ± 17419	63675 ± 1133	69.9 ± 11.5	83.2 ± 1.5
	5			72952 ± 7583	24952 ± 3969	17.6 ± 1.8	37.4 ± 5.9
	6			52239 ± 7327	25545 ± 4080	18.0 ± 2.5	32.5 ± 5.2
	7			67603 ± 1299	47519 ± 1989	21.5 ± 0.4	40.4 ± 1.7
	8			75191 ± 2460	49987 ± 14111	37.4 ± 1.2	19.5 ± 5.5
	9			64659 ± 16625	51028 ± 11622	96.8 ± 24.9	23.3 ± 5.3
	10			49387 ± 6922	38817 ± 5160	10.1 ± 1.4	57.9 ± 7.7
	11			138003 ± 18819	110462 ± 17705	13.9 ± 1.9	68.4 ± 11.0
				$\bar{x}$ = 67610 ± 33478	$\bar{x}$ = 44620 ± 26380	$\bar{x}$ = 32.5 ± 26.9	$\bar{x}$ = 45.0 ± 20.7

Table 4.2 Viability of fresh splenocytes and thymocytes before and after passage through nylon wool, as measured by nigrosine dye exclusion

Organ	Viability (%)	
	Before nylon wool passage	After nylon wool passage
Spleen	97.9 98.1	97.3 98.5
Thymus	99.0 99.3	99.3 100.0

The viability of the lymphocytes was measured before culture.

Table 4.3 Legend

Cultures were supplemented with 1% FCS.

Number of cells per well = 2×10^5.

Cultures were pulsed with tritiated thymidine at 48 hours and harvested at 72 hours.

Although mean stimulation indices (S.I.s) can be calculated from the mean counts given, these will not agree precisely with the mean S.I.s given, as the latter figures were calculated from the S.I.s for the individual experiments.

The S.D.s given for the mean counts and mean S.I.s are the S.D.s of those means, rather than the mean of the S.D.s of the counts and S.I.s for the individual experiments.

The animals used were outbred *X. laevis* aged 6-7 months.

Table 4.3 The effect of nylon wool treatment on the mitogen response to thymocytes

Mitogen	Animal	Background counts (mean dpm ± S.D.)		Stimulated counts (mean dpm ± S.D.)		Stimulation index ± S.D.	
		Non-passaged	Nylon wool passaged	Non-passaged	Nylon wool passaged	Non-passaged	Nylon wool passaged
LPS	12	780 ± 44	562 ± 79	2654 ± 376	1096 ± 123	3.4 ± 0.5	2.0 ± 0.2
2	13	822 ± 117	953 ± 282	3141 ± 447	2409 ± 281	3.8 ± 0.5	2.5 ± 0.3
	14	625 ± 72	396 ± 138	1081 ± 301	490 ± 32	1.7 ± 0.5	1.2 ± 0.1
	15	1296 ± 159	696 ± 62	4182 ± 181	1428 ± 178	3.2 ± 0.1	2.1 ± 0.3
mg/ml	16	1352 ± 141	611 ± 248	3685 ± 559	2003 ± 359	2.7 ± 0.4	3.3 ± 0.6
	17	1115 ± 355	592 ± 96	2383 ± 125	847 ± 7	2.1 ± 0.1	1.4 ± 0.01
	18	490 ± 179	350 ± 252	1675 ± 123	450 ± 51	3.4 ± 0.3	1.3 ± 0.2
		$\bar{x}$ = 926 ± 333	$\bar{x}$ = 594 ± 200	$\bar{x}$ = 2686 ± 1090	$\bar{x}$ = 1246 ± 747	$\bar{x}$ = 2.9 ± 0.8	$\bar{x}$ = 2.0 ± 0.8
PHA	12	As above	As above	4611 ± 1060	2635 ± 554	5.9 ± 1.4	4.7 ± 1.0
	13			3543 ± 488	3870 ± 413	4.3 ± 0.6	4.1 ± 0.4
20	14			2082 ± 361	1152 ± 70	3.3 ± 0.6	2.9 ± 0.2
	15			3724 ± 336	2065 ± 93	2.9 ± 0.3	3.0 ± 0.1
µg/ml	16			2301 ± 198	1937 ± 452	1.7 ± 0.2	3.2 ± 0.7
	17			3849 ± 498	2262 ± 366	3.5 ± 0.5	3.8 ± 0.6
	18			2827 ± 278	2070 ± 120	5.8 ± 0.6	5.9 ± 0.3
				$\bar{x}$ = 3277 ± 909	$\bar{x}$ = 2284 ± 830	$\bar{x}$ = 3.9 ± 1.5	$\bar{x}$ = 3.9 ± 1.1

Table 4.4 Cytoplasmic immunoglobulin M-positive cells* and unstained lymphoblasts present in unstimulated, LPS-treated and PHA-treated, nylon wool-passaged splenocytes and thymocytes

Organ and days in culture	Unstimulated cultures		LPS-treated cultures		PHA-treated cultures	
	% $cIgM^+$ cells	% $cIgM^-$ blasts	% $cIgM^+$ cells	% $cIgM^-$ blasts	% $cIgM^+$ cells	% $cIgM^-$ blasts
Spleen 7 day	1.9	3.1	3.9	11.3	-	-
Thymus 5 day	0	0.2	5.7	5.4	0.3	1.1
Thymus 7 day	0	0	8.5	10.4	0	0

* The assay for $cIgM^+$ cells was carried out as described in Chapter 3.

The LPS and PHA concentrations were 2 mg/ml and 20 μg/ml respectively.

All cultures were supplemented with 10% FCS and stained using 0.2 mg/ml rabbit IgG anti-Xenopus μ chain antiserum.

Total number of cells counted per culture = 634 ± 301 (mean ± S.D.).

The animals used were outbred X. laevis aged 5-7 months.

Table 4.5 Legend

Irradiated (3,000 rads) animals were given an injection of SRBC at the same time as the injection of 1×10^7 reconstituting lymphocytes. The PFC assay was performed 6 days later, using both guinea-pig and Xenopus complement on the same spleen cell suspension.

The data presented above was obtained from 15 inbred X. laevis G-line animals (hosts) aged 7-8 months. The donor lymphocytes were from inbred X. laevis G-line animals aged 7-10 months.

Four irradiated animals injected with SRBC alone did not produce anti-SRBC PFC.

Table 4.5 Generation of plaque-forming cells in the spleens of lethally irradiated animals injected with MHC-compatible irradiated or non-irradiated lymphocytes

Source of complement used in PFC assay	Treatment of reconstituting cells	Reconstituting cell type							
		Thymocytes		Splenocytes		Primed splenocytes		Splenocytes/ PBL	
		PFC/ spleen	PFC/10^6 splenocytes	PFC/ spleen	PFC/10^6 splenocytes	PFC/ spleen	PFC/10^6 splenocytes	PFC/ spleen	PFC/10^6 splenocytes
Guinea-pig	Non-irradiated	2 5	6 12	137 26	165 22	3163 1260	1917 1235	192	157
	Irradiated 3,000 rads	0 0	0 0	0 0	0 0	205 69	790 229	0 0	0 0
Xenopus laevis	Non-irradiated	0 7	0 18	625 184	753 155	9624 2210	5833 2167	880	721
	Irradiated 3,000 rads	0 0	0 0	0 0	0 0	647 414	2490 1380	0 0	0 0

CHAPTER FIVE

MIGRATION OF INJECTED LYMPHOCYTES TO THE SPLEEN: EXPERIMENTS WITH RADIOISOTOPE-LABELLED AND PLOIDY-MARKED CELLS

Introduction

The experiments in this Chapter were carried out in order to study the migratory properties of lymphocytes injected into the dorsal lymph sac of Xenopus. The purpose of this was to ascertain whether reconstituting lymphocytes injected into thymectomized Xenopus (see Chapter 6) would quickly reach the spleen and remain in this major peripheral lymphoid organ for at least 6 days, and hence have an opportunity to actively interact with host cells to produce an antibody response to sheep erythrocytes.

In order to show that injected lymphocytes quickly imigrate to the spleen (within 24 hours), ^{51}Cr labelling of these lymphocytes was employed. This method has frequently been used to study the homing of lymphocytes in mammals (for example see: Degos, Pla and Colombani, 1979; Heslop and Hardy, 1971; McNeilage and Heslop, 1980; Zatz, Gingrich and Lance, 1972). Chromium-51 becomes bound principally to intracellular protein. It is a useful radioisotope to use for such distribution studies as it is re-utilized very little, if at all. It is also quickly excreted after release from cells, thus minimizing any potentially misleading effects of radioactivity which is retained in the tissue, but is no longer associated with the lymphocytes (Ford, 1978).

A ploidy-marker system was used to demonstrate that injected lymphocytes are still present in the spleen after 6 days. Triploid Xenopus can readily be obtained by cold-shock treatment of fertilized eggs (Kawahara, 1978). Triploid thymocytes injected into diploid,

thymectomized Xenopus have been used to show that the injected cells are a component of the lymphocyte population of the spleen 46-110 days after injection (Kawahara, Nagata and Katagiri, 1980). In this Chapter triploid splenocytes were injected into diploid thymectomized Xenopus and the spleen lymphocyte population assayed for the presence of triploid cells 6 days later. This was done by staining the cells with Feulgen's nuclear stain and determining ploidy by microspectrophotometric methods. The assay time of 6 days was chosen, as this is the interval between lymphocyte injection and antibody assays (on the spleen) used with the thymectomized, reconstituted animals in the next Chapter.

Materials and Methods

Chromium-51 labelling of cells

Lymphocyte or erythrocyte cell suspensions (prepared as described in Chapter 2) at 20 x 10^6/ml were radioactively labelled by incubation with 200 µCi/ml of chromium-51 (^{51}Cr, 350-600 mCi/mg; Radiochemical Centre, Amersham). Cell suspensions were incubated in 12 x 75 mm round-bottom tissue culture tubes (Falcon Plastics, A.J. Beveridge) at 28°C for 2 hours. The ^{51}Cr-labelled cells were then washed twice and resuspended in amphibian strength L-15 for injection.

Production of triploid animals

Triploid (3N) Xenopus were produced by the method of Kawahara (1978). Sperm suspensions were prepared by teasing apart a pair of testes in a watchglass containing amphibian strength L-15 medium. This was then transferred to a test-tube and further sperm released from the testes tissue by gentle pipetting. The cell debris was allowed to settle, and the sperm suspension transferred to a fresh

test-tube, and made up to a volume of approximately 2 ml. The sperm was activated by dilution with an equal volume of double-distilled water, then distributed into several watchglasses. Mature eggs were stripped into the sperm suspensions. After 2 minutes the sperms were removed and replaced with a 1:10 Ringers solution. Twelve minutes post-fertilization, the watch glasses were placed in a large volume (approximately 1 l.) of 1:10 Ringers solution at 3°C for 15 minutes. The eggs were then transferred to aerated standing water at 23 ± 2°C and allowed to develop at this temperature.

The subjection of the eggs to this cold temperature prevents the expulsion of the second polar body, thereby creating triploid eggs, which possess the diploid genetic complement from the female plus the haploid genetic complement from the male. Not all cold-shock embryos are triploid - many must still expel the second polar body and hence remain diploid. Thus the ploidy of these animals must be carefully checked.

Determination of ploidy by silver staining

Toadlets were tested for ploidy by the silver staining of nucleoli (Olert, 1979). Animals were foot bled, and blood smears were prepared on clean glass microscope slides, then allowed to air-dry. Seven parts of an $AgNO_3$ solution (50% w/v in double-distilled water) was mixed with one part of 0.2% formic acid (in double-distilled water, adjusted to a pH of 2.5 with sodium formate), immediately pipetted onto the slides and covered with a cover glass. The slides were left for several minutes for the stain to develop. The ploidy of the animal was then determined by observation of the percentage of diploid or triploid erythrocytes, that is, those with two or three visible nucleoli respectively. The nucleoli stain

black with precipitated silver, while the surrounding chromatin and cytoplasm appear in various shades of yellow to pale brown.

Feulgen's nuclear stain

Cytospin preparations which had undergone glutaraldehyde fixation (see Experimental design) were rinsed in double-distilled water, before being placed in 1 M HCl for 1 minute at room temperature. The slides were then transferred to 1 M HCl maintained at 55°C in a water bath and there incubated for 20 minutes. They were again placed in 1 M HCl for 1 minute, at room temperature, then rinsed in double-distilled water. The slides were then immersed in Schiffs reagent (BDH), in the dark, for 2 hours before being treated with three changes of a bleaching solution, at 1 minute each. [The bleaching solution consisted of 10 ml concentrated HCl: 4 g sodium metabisulphite (BDH): 1 l. double-distilled water.] After washing in double-distilled water, the slides were dehydrated using two changes each of 70%, 95% and 100% alcohol consecutively, cleared in two changes of Xylene and mounted.

Experimental design

(a) ^{51}Cr-labelled cell injections. Outbred Xenopus were injected via the dorsal lymph sac with 10 x 10^6 MHC-incompatible ^{51}Cr-labelled splenocytes, thymocytes or Xenopus erythrocytes (XRBC) in a volume of 0.2-0.3 ml amphibian strength L-15, and left for 6 or 24 hours. After this time the animals were killed, and muscle, spleen, thymus, liver, kidney and gut were removed. Each tissue was rinsed in 10% formaldehyde, which fixes any external blood on the tissues, so that it can be easily washed off in the following two changes of double-distilled water. Each tissue was then weighed, and placed inside

individual vials for gamma counting. The samples were counted, together with 1:10 standards of the injected radiolabelled cells, on an automatic gamma well solid scintillation counter (Philips PW4580; Medical Physics Department, Dryburn Hospital, Durham). Data is expressed as % dose per tissue or organ, and % dose/g tissue or organ. (This is a standard way of expressing such data - see e.g. Taylor, Lallone and Hagan, 1980.) These experiments were performed to show whether lymphocytes injected into the spleen could rapidly home to the spleen and be detected there within 24 hours.

(b) Triploid-labelled cell injections. To determine whether injected lymphocytes could be detected in the spleens of thymectomized animals after 6 days, triploid splenocytes were injected into diploid animals. The ploidy of the donor and host animals was first checked using the silver-staining method. Diploid (2N) inbred G-line animals that had been thymectomized at 7-8 days of age were given an injection of 10×10^6 MHC semi-allogeneic splenocytes from triploid (3N) donors (G-line female Xenopus x outbred male). The cells were injected via the dorsal lymph sac, in 0.2-0.3 ml amphibian strength L-15. Six days later, the spleens were removed, and cell suspensions prepared as previously described (Chapter 2). Cytocentrifuge preparations were made, and fixed overnight in a 2.5% glutaraldehyde solution, in 0.1 M sodium cacodylate buffer, pH 7.4. The slides were then stained using Feulgen's nuclear reaction. Relative absorption of Feulgen-stained lymphocyte nuclei was measured at 560 nm, using a microdensitometer (M85-Vickers, Wellcome Laboratories, Royal Victoria Infirmary, Newcastle) with a x40 objective lens.

Feulgen staining, rather than silver staining, was used for these experiments, as Xenopus splenocytes did not stain very well with the latter method (see Table 5.2 and Results).

Results

(a) Distribution of ^{51}Cr-labelled cells

The percentage of ^{51}Cr-labelled cells that reach various tissues and organs 6 or 24 hours after injection, are shown in Table 5.1. The total percentages of the dose are less than 100%, because many labelled cells will be distributed in other areas of the body not examined here, most especially the blood.

Splenocytes. The greatest percentage of the injected dose of ^{51}Cr was found in the liver 6 hours after injection - this was 31.28%. The other organs and tissues looked at only retained a small amount of the injected dose, and only 0.97% of the dose had reached the spleen. However, it was obvious when the % dose/g was calculated that the injected cells were concentrated in the spleen. The % dose/g in the spleen was 242.5%/g, whereas it was only 131.43%/g in the liver. The ^{51}Cr-labelled cells did not accumulate in muscle, thymus, kidney or gut to any great extent. Similar results were obtained 24 hours after injection with the % dose accumulated in the liver of 27.95%, while the spleen only contained 1.32% of the dose. When the % dose/g was calculated however, the spleen had accumulated 330%/g, whereas the liver contained only 180.32%/g. The other tissues/organs had still only comparatively small values for % dose/g.

Thymocytes. The thymocytes were seen to home to the spleen in a similar fashion to that of the splenocytes, described above. Six hours after injection, 41.77% of the dose was contained in the liver, and only 0.97% in the spleen. The % dose/g however, was 323.33%/g in the spleen and 245.71%/g in the liver. After 24 hours there was 30.91% of the dose in the liver and 0.95% in the spleen, but the %

dose/g was 316.67%/g in the spleen and 196.88%/g for the liver. As with the splenocytes, there was little accumulation of the ^{51}Cr-labelled cells in the muscle, thymus, kidney or gut.

Erythrocytes. The total percentage of the erythrocyte dose to reach the tissues examined was only 11.09% after 6 hours, and 13.77% after 24 hours. Most of the labelled cells injected were thus elsewhere in the body, presumably in the blood. After 6 hours 8.64% of the dose was found in the liver, 0.18% in the spleen, and small amounts in the other tissues, ranging from 0.06% in the thymus to 1.68% in the gut. The erythrocytes did not accumulate to any great extent in the spleen - the % dose/g was 60%/g. The liver contained 51.12%/g. Similarly, after 24 hours the liver contained 12.15% of the dose, and the spleen had 0.14%. The liver had accumulated the dose to a higher extent than the spleen this time, with 75.94%/g in the liver and 35.0%/g in the spleen.

These results show that splenocytes and thymocytes preferentially "home" to the spleen, and can be detected there 6 hours after injection. The erythrocytes were used as a control to show that lymphocytes accumulate in the spleen, whereas non-lymphoid cells do not, to any great extent.

(b) Ploidy-marker experiments

(i) Silver staining. The distribution of silver-stained nucleoli in blood erythrocytes and spleen lymphocytes of a representative sample of diploid and triploid animals is shown in Table 5.2. The majority of erythrocytes in diploid animals ($51.3 \pm 22.1\%$, mean $\pm$ S.D.), have two visible nucleoli, while only a few erythrocytes ($1.2 \pm 0.8\%$) were recorded as possessing three nucleoli. A large number of erythrocytes ($46.5 \pm 21.8\%$) appear to have only one

nucleolus, probably because the two nucleoli are lying very close together, or behind one another, thus appearing as one. Although most erythrocytes in triploid animals have three visible nucleoli ($58.2 \pm 7.2\%$), a large number of erythrocytes ($36.3 \pm 8.8\%$) appear diploid, with two nucleoli, again probably due to one nucleolus being obscured by another. It is thus relatively easy to distinguish diploid and triploid animals from one another, by taking a blood sample and observing the ploidy of the erythrocytes. This can be done by eye, due to the low number of apparent triploid cells possessed by diploid animals.

Distinguishing diploid and triploid animals on the basis of silver-stained spleen lymphocytes proved rather more difficult. Thus many splenocytes from both diploid and triploid animals appeared polyploid (in terms of nucleolar number) or had indeterminable ploidy. Moreover the percentage of cells recorded as having three nucleoli was greater than 5% in diploid animals and less than 50% in triploid individuals. Hence it was decided to use Feulgen-staining in conjunction with microdensitometry in the cell transfer studies.

(ii) Feulgen-staining. Triploid and diploid cells can be distinguished from each other by the relative absorption of Feulgen-stained nuclei, as triploid cells have a higher nuclear absorbance at 560 nm. Frequency distributions of the absorption of Feulgen-stained splenic lymphocyte nuclei from three triploid and three diploid animals are shown in Fig. 5.1. The histograms drawn for the diploid and triploid cells are clearly distinguishable from one another. There was little or no overlap in the relative absorption of diploid and triploid lymphocyte nuclei when measured on the same day (see Discussion).

Observations were then made on the frequency distribution of the ploidy of spleen lymphocytes from diploid thymectomized animals that received triploid splenocytes 6 days previously. In each of three animals studied, the spleens displayed two subpopulations of cells (Fig. 5.2a, b, c), which corresponded to diploid and triploid splenocytes. The animals depicted in Fig. 5.2 (a) and 5.2 (b) had large numbers of triploid splenocytes in their spleens 6 days post-triploid splenocyte injection. The spleen shown in Fig. 5.2 (c) contained fewer triploid splenocytes, but the injected cells were there, as judged by the bimodal distribution of the absorbance of the cells, and the wider scatter of absorbances as compared with the diploid and triploid controls in Fig. 5.1. Thus triploid splenocytes injected into a thymectomized toadlet can readily be detected in the spleen 6 days after injection.

Discussion

The results obtained in this Chapter show that MHC-incompatible splenocytes and thymocytes are able to migrate to the spleen of Xenopus within 6 hours of injection. It is also demonstrated that MHC semi-allogeneic splenocytes are generally present in good numbers in the spleens of thymectomized animals 6 days post-injection.

The initial migration of ^{51}Cr-labelled lymphocytes was directed mainly to the spleen. However large numbers of labelled cells also accumulated in the liver. These latter cells probably represented damaged lymphocytes, as mammalian lymphocytes which are deliberately damaged by various methods before injection localize mainly in the liver (Ford, 1978).

The donor lymphocytes used in the ^{51}Cr-labelling experiments were MHC-incompatible to the injected hosts. However it might be

expected that the number of labelled cells reaching the spleen would have been even higher using MHC-compatible combinations. This is because the migration of mammalian lymphocytes to the spleen and lymph nodes is reduced in animals injected with MHC-incompatible lymphocytes as compared to MHC-compatible lymphocytes (Degos, Pla and Colombani, 1979; McNeilage and Heslop, 1980; Zatz and Gingrich, 1972).

The ^{51}Cr-labelling experiments were performed using intact, rather than thymectomized, adoptive transfer recipients. In contrast, thymectomized animals were used as hosts in the ploidy-labelled transfer experiments. This was because the spleens of thymectomized animals generally contain fewer lymphocytes than unoperated animals (data not shown). Thus any injected triploid lymphocytes remaining in the spleen would be easier to detect. Moreover, the major purpose of these experiments was to determine the extent to which injected lymphocytes have colonized the spleens of thymectomized animals by 6 days, in order to obtain background information for the antibody experiments on such animals, described in Chapter 6.

A problem was encountered with these ploidy-marker experiments, as the mean relative absorption values for diploid and triploid splenocytes did not remain constant from day to day (Figs 5.1 and 5.2). Hence it was necessary to take measurements on the micro-densitometer for both diploid and triploid splenocytes at the same session. The readings for Fig. 5.1 (a), (b) and (c) were taken on three different days, hence the variation in the position of the scale in the horizontal x axis. This problem has also occurred in other studies (Kawahara, Nagata and Katagiri, 1980; Turpen, Volpe and Cohen, 1973).

The main conclusion to be drawn from this Chapter is that

injected lymphocytes readily migrate to the spleen of thymectomized toadlets and therefore should be potentially capable of interacting with the recipient's own immunologic cells (e.g. B lymphocytes and antigen-presenting cells) in this important site of immune reactivity.

Table 5.1 Tissue distribution of ^{51}Cr-labelled injected cells

Cells injected	Tissue/ organ	6 hours post-injection		24 hours post-injection	
		% Dose/ organ	% Dose/g	% Dose/ organ	% Dose/g
Splenocytes	Muscle	0.08	0.68	0.13	1.09
	Spleen	0.97	242.50	1.32	330.0
	Thymus	0.22	11.0	0.13	21.67
	Liver	31.28	131.43	27.95	180.32
	Kidney	1.43	2.70	0.55	17.19
	Guts	2.52	6.09	1.0	3.47
		Total 36.50		Total 31.08	
Thymocytes	Muscle	0.20	1.57	0.14	1.89
	Spleen	0.97	323.33	0.95	316.67
	Thymus	0.63	39.38	0.39	48.75
	Liver	41.77	245.71	30.91	196.88
	Kidney	1.21	44.81	0.94	32.41
	Guts	0.59	4.31	0.97	6.38
		Total 45.37		Total 34.30	
Xenopus erythrocytes	Muscle	0.12	1.21	0.25	2.45
	Spleen	0.18	60.0	0.14	35.0
	Thymus	0.06	5.0	0.01	2.0
	Liver	8.64	51.12	12.15	75.94
	Kidney	0.41	7.32	0.55	19.64
	Guts	1.68	10.06	0.67	3.81
		Total 11.09		Total 13.77	

The animals used were outbred X. laevis aged 6 months.

The injected thymocytes were from outbred X. laevis aged 6 months, and the splenocytes and erythrocytes from an outbred X. laevis adult aged 1-2 years.

Table 5.2 Distribution of silver-stained nucleoli in blood erythrocytes and spleen lymphocytes of diploid and triploid animals

Ploidy of animal	Tissue/ organ (cell types)	Number of nucleoli visible			
		1	2	3	other*
Diploid	Blood+ (erythrocytes)	46.5 ± 21.8	51.3 ± 22.1	1.2 ± 0.8	1.0 ± 1.1
	Spleen++ (lymphocytes)	41.5 ± 0.7	40.0 ± 5.7	5.5 ± 2.1	14.0 ± 4.2
Triploid	Blood+ (erythrocytes)	2.3 ± 1.4	36.3 ± 8.8	58.2 ± 7.2	3.0 ± 4.0
	Spleen++ (lymphocytes)	1.5 ± 2.1	23.0 ± 2.8	43.5 ± 2.1	32.0 ± 2.8

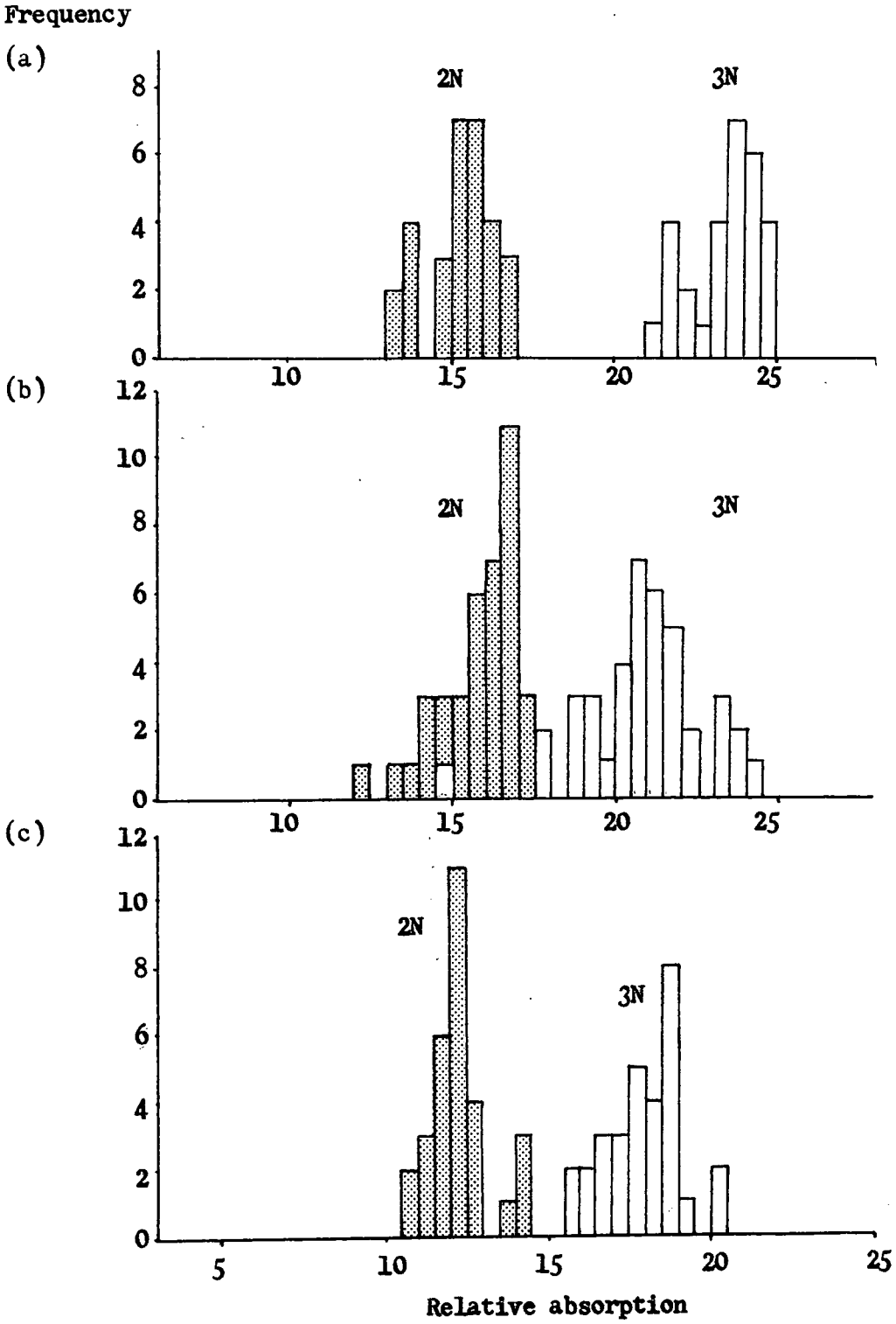
Data is expressed as per cent (mean ± S.D.).

* Nuclei containing more than 3 nucleoli, and those of which the ploidy is indistinguishable.

\+ $n = 6$.

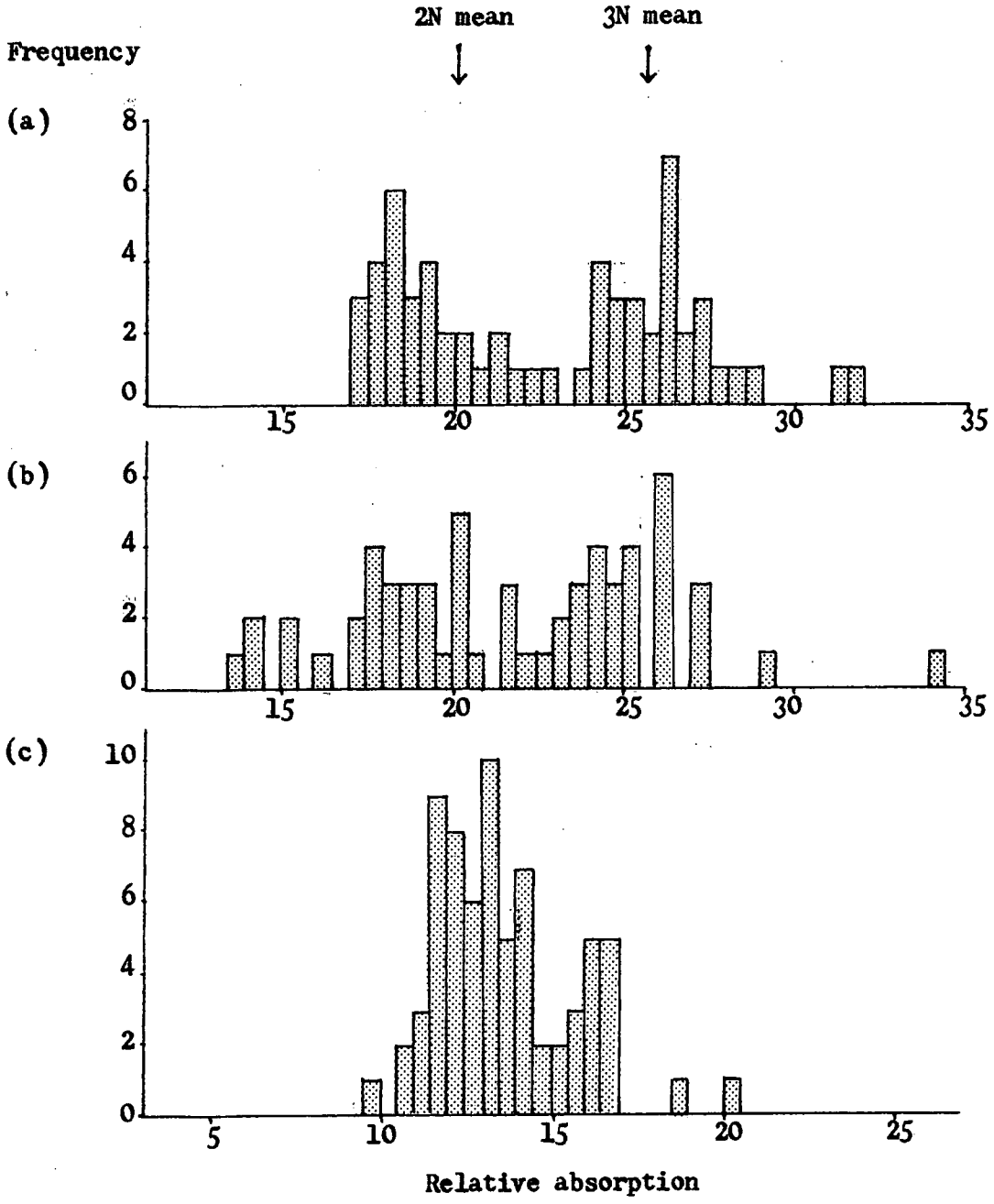
++ $n = 2$.

Fig. 5.1 Frequency distribution of absorption at 560 nm by Feulgen-stained nuclei of spleen lymphocytes from diploid and triploid animals.



(a)-(c) represent three separate experiments, carried out on separate days, comparing individual diploid (2N) and triploid (3N) animals.

Fig. 5.2 Frequency distribution of absorption at 560 nm by Feulgen-stained nuclei of spleen lymphocytes from thymectomized diploid animals 6 days post-injection of triploid splenocytes.



(a)-(c) represent three separate experiments. (a) and (b) were carried out on the same day. (c) was run alongside Fig. 5.1 (c). The positions of the mean values for diploid (2N) and triploid (3N) spleen lymphocytes shown (arrows) are derived from the mean values of Fig. 5.1 (c). The abscissae of (a) and (b) were aligned with that of (c) so that the mean of a 3N spleen lymphocyte population measured at the same time as those in (a) and (b) co-incided with the mean 3N value from that of Fig. 5.1 (c). No control 2N population was measured alongside those in (a) and (b).

CHAPTER SIX

RESTORATION OF THE ANTIBODY RESPONSE TO SHEEP ERYTHROCYTES IN THYMECTOMIZED XENOPUS INJECTED WITH MHC-COMPATIBLE OR MHC-INCOMPATIBLE LYMPHOCYTES

Introduction

It has already been demonstrated (see Chapter 2) that the antibody response to SRBC in thymectomized Xenopus can be restored by the implant of an MHC-incompatible thymus. However, as discussed in Chapter 2, the Xenopus thymus may not be centrally involved in self-restriction of helper T cells, i.e. host T cells that develop within the donor thymus do not become restricted to the MHC type of the donor. On the other hand, it is possible that T cells, originating from the thymus donor, can effect such restoration by migrating to the periphery and there collaborating with host B cells. It therefore seemed important to determine whether or not antibody responsiveness to SRBC can take place in vivo when T and B cells of the animal are known to be MHC-disparate.

A lasting restoration of the antibody response to SRBC in nude mice can only be achieved with MHC-compatible thymocytes (Kindred and Schreffler, 1972). A transitory response only, lasting less than 2 weeks, is found when MHC-incompatible thymocytes are injected along with SRBC (Kindred, 1971b; Kindred, 1975). Kindred (1971b) suggested that this initial response might be due to a hormonal effect on existing cells capable of producing antibody to SRBC, whereas the development of new antibody-producing cells involves cell surface interactions that require compatibility. Subsequent experiments employing splenocytes also showed that MHC-incompatible cells can give only a transitory restoration of the antibody response to SRBC

in nude mice, whereas MHC-compatible splenocytes will produce a lasting restoration (Kindred and Weiler, 1972).

In contrast to these mammalian experiments, recent studies have revealed that both MHC-compatible and MHC-incompatible thymocytes may partially restore the antibody-producing capacity of thymectomized Xenopus to the T cell-dependent antigens rabbit erythrocytes and human gamma-globulin, when a course of injections of these antigens is given 4 weeks after the reconstituting cells. With respect to human gamma-globulin, only IgM antibody could be found in restored animals, whereas in control toads both IgM and Ig"G" antibodies were produced. In contrast to the response to human gamma-globulin, MHC-incompatible thymocytes generally resulted in a poorer restoration of the anti-rabbit erythrocyte response than MHC-compatible thymocytes (as measured by serum antibody titres) (Nagata, 1980). Since ploidy-marker experiments with histocompatible thymocytes revealed that the B cells that produce the anti-rabbit erythrocyte antibody are of host origin (Kawahara, Nagata and Katagiri, 1980), it seems possible that in vivo anti-red cell antibody production in Xenopus can be effected, to some extent, by co-operation between histoincompatible T and B lymphocytes. One should note, however, that the number of anti-rabbit erythrocyte PFCs generated in the spleen of thymectomized Xenopus, following reconstitution with histoincompatible thymocytes and erythrocyte immunization, were extremely low and variable (only $14.2 \pm 13.6/10^6$ spleen cells) (Nagata, 1980).

The major purpose of the experiments performed in this Chapter was therefore to examine in more depth the ability of MHC-incompatible lymphocytes to restore the antibody response of thymectomized animals to foreign erythrocytes. Histoincompatible or MHC-compatible

lymphocytes of various sources were injected - thymus, spleen, SRBC-primed spleen or a splenocyte/PBL mixture. Some of the reconstituting lymphocyte injections were first γ-irradiated to remove functional B cells (see Chapter 4). An attempt was also made in this Chapter to determine the origin of any anti-SRBC PFCs produced as a result of restoration of thymectomized animals, using ploidy-marked cells and the poly-L-lysine plaque assay (Kennedy and Axelrad, 1971), followed by Feulgen-staining and ploidy determination by microspectrophotometric methods (Kawahara, Nagata and Katagiri, 1980). An ontogenetic survey of the responses of control animals to SRBC was first carried out, to determine the ages at which control toadlets give good splenic PFC responses - donors and hosts for the reconstitution experiments were then chosen accordingly.

Materials and Methods

Poly-L-lysine plaque assay

The haemolytic plaque assay described by Kennedy and Axelrad (1971), modified by Kawahara, Nagata and Katagiri (1980) was used. Two millilitres of 0.025 mg/ml poly-L-lysine hydrobromide (Sigma, mol. wt. 260,000) in amphibian strength PBS, was pipetted onto clean glass slides which had been thoroughly washed in an acetone/alcohol mixture (1:1). After 15 minutes at room temperature, the slides were thoroughly rinsed in PBS, and 2 ml of 1% SRBC in PBS added immediately. The SRBC were allowed to settle for 15 minutes at room temperature, then gently agitated and left for a further 15 minutes to settle again. The slides were then washed three to four times with PBS to remove any unbound SRBC, taking care not to let the monolayer dry out between washes. The monolayers of bound SRBC were kept under PBS until used. Spleen cell suspensions were prepared as described

in Chapter 2, but adjusted to final concentrations of 0.5-2.0 x 10^6/ml, in a medium consisting of 5 parts L-15: 3 parts double-distilled water: 1 part FCS. Guinea-pig complement, previously absorbed with SRBC, was added to 1 ml aliquots of the spleen cell suspensions to a final concentration of 2% vol/vol. The spleen cell/complement mixtures were then gently pipetted onto the slide monolayers. The slides were incubated for 1 hour at 37°C in a moist chamber, then carefully placed in an air-tight container together with a small dish of 25% vol/vol glutaraldehyde solution. The preparations were then left overnight for fixation in the glutaraldehyde vapour, before being stained using Feulgen's nuclear reaction (see Chapter 5).

Experimental design

Protocol for cellular reconstitution of thymectomized Xenopus. Thymectomized Xenopus aged 6-12 months were reconstituted with either MHC-compatible or MHC-incompatible lymphocytes (irradiated or non-irradiated) from a variety of sources - thymus, spleen, primed spleen or a 4:1 splenocyte: PBL mixture. The donors were also aged 6-12 months. Two methods were used for reconstitution as follows:

(a) Short-term protocol. Thymectomized animals (thymectomized at 7 days, as elsewhere in the Thesis) were given a single injection of 1 x 10^7 lymphocytes (prepared as previously described), suspended in 0.2-0.3 ml amphibian strength L-15, via the dorsal lymph sac. The animals were simultaneously given a single injection of 10% SRBC via the intraperitoneal route, as described in Chapter 2. Cellular antibody production was assayed 6 days post-injection, using the PFC assay described in Chapter 2. Both guinea-pig and Xenopus complement were used in assays on the same spleen cell suspensions whenever possible, as described in Chapter 4.

(b) Long-term protocol. Here thymectomized animals were given the injection of 1 x 10^7 lymphocytes 28 days prior to an injection of 10% SRBC and the PFC assay performed 6 days later. The PFC assays on individual spleens were again done with both guinea-pig and Xenopus complement where possible. Some animals (see Table 6.4) received multiple SRBC injections 28 days post-thymocyte injection, and were assayed for splenic PFCs and serum antibody 2 weeks after the final injection (see Chapter 2).

Ploidy-marker experiments. Diploid thymectomized animals aged 6-12 months were reconstituted with triploid splenocytes, from donors that had been primed with an injection of SRBC 6 days previously, using the short-term protocol described above. The cellular antibody production was measured using the poly-L-lysine plaque assay, described above. The purpose of these experiments was to determine the ploidy of the plaque-forming cells, thus showing whether these latter cells were of donor or host origin. Ploidy estimations were attempted by measuring the relative absorption of the Feulgen-stained nuclei of the PFCs using a microdensitometer, as described in Chapter 5. These latter absorption values were then compared with the average relative absorption of diploid and triploid splenocytes.

Results

(a) Plaque-forming cell responses in the spleens of control and thymectomized toadlets of varying age

Controls. The cellular antibody response of control toadlets was very variable. The PFC responses of animals aged 5-18 months are shown in Table 6.1. Although the mean number of PFC/10^6 spleen leucocytes appeared to be similar in animals aged 6, 7, 8 and 9 months, the individual responses differed widely. In general though, animals aged 5 and 6 months did not respond very well to SRBC, when

assayed using guinea-pig complement. (Although at 6 months the mean PFC number was 214 ± 529, this mean was only produced because of the high response of one animal of 1523 PFC/10^6 leucocytes.) The animals aged 7 and 8 months that responded poorly to SRBC were, generally speaking, small for their age, and possibly were at an earlier stage, developmentally. The cellular antibody response of the one 4 month old animal tested was zero PFC with guinea-pig complement (Table 6.2). However, this animal gave a good response of 894 PFC/10^6 leucocytes when assayed using Xenopus serum. The responses of other animals was also dramatically improved by the use of Xenopus serum as a source of complement. Thus Xenopus complement not only increased the sensitivity of the plaque assay, but also enabled responsiveness to SRBC to be detected at an earlier age (see Lallone, 1984). Hence the animals chosen for the reconstitution experiments (donors and hosts) were generally aged at least 7 months. Xenopus complement was also used when possible.

Thymectomized. Thymectomy removed the ability of toadlets aged 7-9 months to respond to SRBC with cellular production of antibody 6 days post-injection. No plaques could be detected using either guinea-pig serum (Table 6.1) or Xenopus serum (data not shown) as a source of complement.

(b) Reconstitution of thymectomized animals with thymocytes

Thymocytes were unable to reconstitute thymectomized animals to any great degree. Histocompatible and MHC-incompatible thymocytes used in both long and short-term experiments restored the plaque response to a low level only (Table 6.3). A number of experiments were also performed following the protocol of Nagata (1980), i.e. the long-term reconstitution period was followed by three injections

of antigen 3 days apart, and the assay was performed 2 weeks after the final injection. (The antigen used here was SRBC, rather than the rabbit erythrocytes employed by Nagata.) This protocol gave a poor restoration of some thymectomized animals, but not others (see Table 6.4). Thus two out of five animals showed a low level of restoration as judged by the PFC response, but the other three animals did not respond to SRBC. The total serum antibody responses also showed that reconstitution was variable. One animal had a total serum antibody titre of 10, which is comparable to the best levels produced by control animals (see Table 2.3). However the total serum antibody titre of the other four animals was no greater than that of any thymectomized non-reconstituted animal (Table 2.3), although the titre of 6 given by one reconstituted animal compares with the minimum titre seen in some SRBC-injected controls (Table 2.3). There was no 2-mercaptoethanol-resistant antibody produced by any of the animals tested. Two other animals restored by the method of Nagata died 42 and 46 days after the thymocyte injection, presumably through a graft versus host disease (see Discussion).

(c) Reconstitution of thymectomized animals with splenocytes

Non-irradiated splenocytes. Histocompatible and MHC-incompatible splenocytes were unable to restore the ability of thymectomized animals to mount a splenic PFC response to any great extent in the short-term experiments, with mean values of 13 and 15 PFC/10^6 leucocytes respectively (see Table 6.5). However MHC-compatible splenocytes used in the long-term experiments enabled the two thymectomized animals tested to produce good PFC levels (mean = 217), presumably due to an expansion of the donor splenocytes over the longer term reconstitution period (Table 6.5). The animals reconstituted with

MHC-incompatible splenocytes over a long-term reconstitution period died on days 13, 18 and 27, presumably through a graft versus host disease (see Discussion).

Irradiated splenocytes. When the MHC-compatible and MHC-incompatible splenocytes used in short-term reconstitution experiments were first irradiated (3,000 rads) to destroy B cell activity (see Chapter 4), they were unable to restore the ability of the thymectomized animals to respond to SRBC (Table 6.5). Hence the PFCs seen in animals reconstituted with non-irradiated splenocytes in the short-term experiments (both MHC-compatible and MHC-incompatible) were possibly produced by B cells coming from the injected splenocytes and not by co-operation between helper T cells of the donor and the B cells of the thymectomized host. The likelihood that injected T cells can co-operate with host B cells is, however, suggested by the long-term experiments with irradiated donor splenocytes. Thus there are low numbers of plaques produced in one out of two animals reconstituted with irradiated MHC-compatible splenocytes and in two out of two animals reconstituted with irradiated MHC-incompatible splenocytes. Spleen PFC numbers in the latter case were amplified when assayed using Xenopus complement.

(d) Reconstitution of thymectomized animals with primed splenocytes

Non-irradiated primed splenocytes. These experiments were only performed using the short-term protocol for reconstitution. Both MHC-compatible and MHC-incompatible 6 day SRBC-primed splenocytes appeared to restore the PFC responses of thymectomized animals to a good level - 175 PFC/10^6 and 173 PFC/10^6 leucocytes respectively (Table 6.6). (These responses were magnified using Xenopus serum in the plaque assay.) The PFC numbers in a thymectomized animal

injected with SRBC-primed MHC-incompatible splenocytes, but not given an SRBC injection along with the reconstituting cells, were only 15 PFC/10^6 spleen leucocytes. (Before injection into the thymectomized host, the primed splenocytes were tested for their ability to generate anti-SRBC antibody and 210 PFC/10^6 leucocytes were recorded.) Thus the much higher PFC numbers generated in SRBC-injected thymectomized hosts reconstituted with primed splenocytes appear to be induced by antigen administration to these hosts.

Irradiated primed splenocytes. When the primed splenocytes were first irradiated (3,000 rads) before being used for reconstitution, the PFC responses of thymectomized animals injected with MHC-compatible and MHC-incompatible irradiated primed splenocytes were reduced to mean values of 22 and 15 PFC/10^6 leucocytes respectively (using guinea-pig serum). This response was again magnified when the PFC assay was performed using Xenopus complement (Table 6.6). Hence the majority of the PFCs produced by animals reconstituted with non-irradiated primed splenocytes, could be due to the primed B cells in the splenocyte injections. Furthermore, it was shown in Chapter 4 that the dose of 3,000 rads irradiation does not destroy the activity of all primed B cells. So the PFCs seen in animals reconstituted with irradiated primed splenocytes could also have been produced by donor B cells. It is therefore impossible to tell from this experiment whether any of the PFCs produced by animals reconstituted with irradiated primed splenocytes were formed through co-operation between donor helper T cells and host B cells.

(e) Reconstitution of thymectomized animals with splenocytes/peripheral blood lymphocytes

Non-irradiated cells. These experiments were performed using only the short-term protocol for reconstitution, and using non-primed

cells. Although the splenocytes/PBL were not primed, they produced good PFC levels in both MHC-compatible and MHC-incompatible combinations of 77 and 157 PFC/10^6 spleen leucocytes respectively (Table 6.7) - compare with PFC levels in short-term experiments with non-irradiated splenocytes alone (Table 6.5).

Irradiated cells. Even when irradiated, the splenocyte/PBL mixture of cells was able to restore the ability of thymectomized animals to respond to SRBC (Table 6.7). The cellular antibody response was restored to a mean level of 33 PFC/10^6 spleen leucocytes with MHC-compatible cells and MHC-incompatible cells restored the response to a mean level of 283 PFC/10^6 spleen leucocytes. (The use of Xenopus complement was especially important here, as it resulted in mean PFC responses of 419 and 392 PFC/10^6 spleen leucocytes with MHC-compatible and MHC-incompatible irradiated cells respectively.) Since it was shown in Chapter 4 that the dose of 3,000 rads irradiation is able to destroy the activity of unprimed B cells in a splenocyte/PBL mixture, the PFCs produced upon reconstitution of a thymectomized animal with a mixture of irradiated splenocytes and PBL are likely to be due to co-operation between helper T cells of the donor and B cells of the thymectomized host. Helper T cells therefore appear to be able to co-operate with MHC-incompatible B cells just as efficiently as when the two lymphocyte populations are MHC compatible.

(f) Ploidy-marker experiments on the origins of plaque-forming cells

These experiments did not produce any concrete results, due to technical difficulties. Hence although Feulgen-stained diploid and triploid splenocytes could easily be distinguished on the basis of their relative absorptions when examined as cytocentrifuge preparations

(see Chapter 5), this was not the case in the present experiments using the poly-L-lysine plaque technique. For example, the splenocytes from the diploid and triploid animals shown in Fig. 5.1 (c) had relative absorption means of 12.3 ± 0.9 and 17.9 ± 1.1 (± S.D.) respectively. No overlap was shown in the relative absorptions of these two animals. However, when splenocytes (non-PFC) from the same animals were measured in the poly-L-lysine technique, the relative absorption means obtained were 4.3 ± 0.8 (diploid) and 5.5 ± 0.8 (triploid). Thus a large overlap in the relative absorption values of diploid and triploid cells was now found. When the relative absorption of PFCs in diploid thymectomized animals reconstituted with triploid splenocytes was measured, it was impossible to say whether these cells were diploid or triploid, as the majority of the PFCs gave absorptions in the range of the overlap. Consequently, this technique was abandonned.

The cells on the cytocentrifuge preparations appeared larger than the cells allowed to settle on a monolayer of SRBC, as the former cells became flattened as a consequence of the technique. It is possible that the size of the nucleus being measured by microdensitometry can affect the accuracy of the technique. It was necessary to use a smaller mask size through which to measure the absorbance of lymphocytes in the poly-L-lysine plaque method; this caused a reduction in the relative absorptions of the nuclei, which may have rendered the measurements less sensitive. (Similar ploidy-marker experiments were performed by Kawahara, Nagata and Katagiri in 1980. However their experiments appear to be free from the technical difficulties described above. It is possible that this is due to differences in fixation and staining procedures - they may also have used a more sensitive or accurate microdensitometer for their measurements.)

Discussion

The results presented in this Chapter indicate that thymocytes (both MHC-compatible and MHC-incompatible) are generally poor at restoring the splenic PFC response (and serum antibody response) of the thymectomized animals to SRBC. Although the levels of PFCs given by thymocyte-reconstituted toadlets to SRBC are comparable to those recorded by Nagata (1980) in his experiments (using rabbit erythrocytes as the immunizing antigen), these levels are much lower than the anti-SRBC PFC numbers recorded here for control Xenopus. However in Nagata's experiments, a mean level of only 42.5 ± 10.8 PFC/10^6 splenocytes could be detected in controls. Hence the relative restoration of the cellular antibody response of thymectomized Xenopus appeared greater in Nagata's experiments. Perhaps PFC culture conditions were better in the present studies. (Control Xenopus gives similar PFC numbers in response to SRBC and rabbit erythrocytes in my hands - data not shown.)

The poor restorative potential of thymocytes recorded here may well reflect the fact that the thymus is a poor source of helper T cells compared with the spleen. This has recently been shown to be the case for *in vitro* antibody production (Hsu, Julius and Du Pasquier, 1983). Moreover, the thymus is known to be able to suppress splenic antibody production to thymus-dependent antigens, as shown by organ co-culture experiments (Ruben, Buenafe and Seivert, 1983). Hsu *et al.* (1983) have also shown that non-nylon wool-adherent thymocytes ("T" cells) can suppress the *in vitro* antibody response of nylon wool-adherent splenocytes ("B" cells).

In contrast to the experiments with thymocytes, the results presented here strongly suggest that the PFC response to SRBC in thymectomized animals can be restored with an injection of an irradiated splenocyte/

PBL mixture, regardless of whether these lymphoid cells are MHC-compatible or MHC-incompatible with the thymectomized host. It is perhaps surprising that irradiated splenocytes/PBL can restore the antibody response to SRBC in thymectomized animals, whereas irradiated splenocytes alone cannot, at least in the short-term experiments. It is possible that PBL are enriched for a particular cell type compared with the spleen, such as helper T cells and/or macrophages. It may be that thymectomized animals are deficient in macrophages as well as T cells. There is a suggestion of a low response to SRBC appearing in longer term reconstitution experiments with irradiated splenocytes. This cannot be due to an expansion of the deficient cell type, as when irradiated cells start to divide they are killed by the irradiation-induced damage (Anderson and Warner, 1976; Kettman and Dutton, 1971). Perhaps lymphokines involved in the interactions between T and B cells and/or in the clonal expansion of B lymphocytes are being produced by the irradiated cells and these require a time longer than 6 days for their effects on PFC numbers to be seen. Irradiation has a damaging effect on the ability of mammalian cells to migrate properly to lymphoid organs (Anderson, Sprent and Miller, 1974; Anderson and Warner, 1976). However, this is unlikely to be the cause of the failure of irradiated splenocytes to restore the PFC response to SRBC in the short term, as irradiated splenocytes/PBL are able to bring about the restoration of the response.

It will be important to perform long-term experiments with irradiated splenocytes/PBL to determine whether the restoration obtained with these cells is a lasting response. It has been demonstrated in nude mice that the restoration of the antibody response to SRBC produced with MHC-incompatible splenocytes and thymocytes is a transitory response, which is over within 2 weeks after injection

of the lymphocytes (Kindred and Weiler, 1972; and Kindred, 1971b; Kindred, 1975). There is a suggestion that this is not the case with MHC-incompatible cells in Xenopus because of the small responses to SRBC just beginning to be seen 5 weeks after the injection of irradiated MHC-incompatible splenocytes into the thymectomized host. However further experiments must be carried out to confirm this, using for example irradiated splenocytes/PBL.

Such long-term experiments can be performed using MHC-incompatible irradiated splenocytes/PBL without the risk of a graft versus host response. In reconstitution experiments with MHC-incompatible non-irradiated splenocytes, the thymectomized hosts died 2-4 weeks after injection of the lymphocytes (see Results), presumably due to a graft versus host response. However, proliferation of T cells is required for this response (see Hood, Weissman and Wood, 1978) and, as already mentioned, when irradiated cells divide, they are killed by the irradiation-induced damage (Anderson and Warner, 1976; Kettman and Dutton, 1971). Hence irradiated cells are unable to elicit a graft versus host response. It should be noted that MHC-incompatible splenocytes are more effective than MHC-incompatible thymocytes in a graft versus host response. This is presumably because the spleen contains more functional cytotoxic T cells than the thymus, the latter having many immature and non-functional cells within it (see also Hsu, Julius and Du Pasquier, 1983). Alternatively, the lack of effectiveness of thymocytes in a graft versus host response might be due to the high content of suppressor T cells within the thymus (see above).

The main conclusion to be drawn from the experiments in this Chapter, is that MHC-incompatible lymphocytes can, in the short term, partially restore the anti-SRBC response of thymectomized animals,

suggesting that MHC-incompatible T and B cells can co-operate in an *in vivo* PFC response to SRBC. One possible explanation for this is that the above response is not restricted by the MHC, or at least the production of IgM antibody is unrestricted. It may be that the production of Ig"G" antibody does require compatibility at the MHC, and further experiments should concentrate on determining whether this is the case (see Concluding Remarks).

Another possibility is that only the interaction between macrophages and helper T cells is MHC restricted, whereas that between helper T and B cells is unrestricted, as has been suggested by several authors for mammalian cells (see General Introduction). If this is the case, the MHC-compatible helper T cells and any macrophages in the reconstituting injection could interact with each other, and then stimulate the MHC-incompatible B cells of the thymectomized host to produce antibody to SRBC. The immunogenic function of macrophages in the immune response to SRBC appears to be quite radioresistant in mammals (Anderson and Warner, 1976), so irradiation of the reconstituting cells would not prevent such an interaction from taking place. As mentioned in the General Introduction, in mammals polyclonally activated B cell blasts can be stimulated by MHC-disparate antigen-activated helper T cells to secrete antigen-specific antibody (Andersson, Schreier and Melchers, 1980; Schreier, Andersson, Lernhardt and Melchers, 1980). It is possible that B cells in the host thymectomized *Xenopus* that have recently been activated by exposure to environmental antigen could be stimulated by MHC-disparate helper T cells to react in this way to SRBC.

Alternatively, there may exist two subsets of B cells in *Xenopus*, one requiring MHC-restricted interaction with T cells for the production of antibody and another population which can be activated

by MHC-incompatible helper T cells. This appears to be the case in mice, where Lyb-5^+ B cells can be activated by MHC-incompatible helper T cells, but the activation of Lyb-5^- B cells by helper T cells is MHC-restricted (Asano, Singer and Hodes, 1981; Singer, Morrissey, Hathcock, Ahmed, Scher and Hodes, 1981).

One criticism that could be brought against the experiments reported in this Chapter, is that no controls were carried out to ensure that when restoration takes place with MHC-incompatible cells, this is not due to an "allogeneic effect" - i.e. no thymectomized animals were given an injection of reconstituting (non-SRBC primed) lymphocytes without the accompanying injection of SRBC. It is possible that an allogeneic effect did occur with non-irradiated lymphocytes. In mammals, the allogeneic effect is caused by the secretion of various mediators by T cells in response to their interaction with MHC-incompatible B cells. These mediators can then bring about the activation of B cells and the stimulation of antibody production (Katz, 1972). However, no allogeneic effect can take place when the population of T cells is first irradiated (Katz, 1972). Hence the restoration obtained in this Chapter with irradiated MHC-incompatible splenocytes/PBL is not due to an allogeneic effect.

Table 6.1 Splenic PFC responses to SRBC in control and thymectomized Xenopus laevis

Age of animal (months)	Controls (PFC/10^6 leucocytes)		Thymectomized (PFC/10^6 leucocytes)
	Individual values	$\bar{x} \pm$ S.D.	
5	9 6 20 3	10 ± 7	
6	19 10 12 1523 23 37 63 25	214 ± 529	
7	26 210 165 457 145 133 80 352 270 255 476 322 91 51 12	203 ± 149	0 0 0 0 0 0
8	157 270 255 90 476 322 63 187 116 53 46 142 49 1931 503 386 170 193 824 491 272 723 847 191 14 11 15 24 74 71	299 ± 388	0 0
9	576 122 418 80 47 219	243 ± 211	0 0
10	216 281 49	182 ± 120	
11	53 93 416 42 192 29	138 ± 149	
12	183 31 51 78	86 ± 68	
13	148 77 59	95 ± 47	
18	731 1001 740 1120 52 1318	827 ± 442	

The PFC assays were performed 6 days post-injection with SRBC, using guinea-pig complement.

The animals used included both outbred and inbred G-line X. laevis.

Table 6.2 Comparison of the splenic PFC response to SRBC in control Xenopus laevis when assayed using guinea-pig and Xenopus laevis complement

Age (months)	Source of complement	
	Guinea-pig	Xenopus laevis
4	0	894
6	25	731
7	91 51 12 $\bar{x} = 51$	603 431 776 $\bar{x} = 603$
8	15 24 74 71 $\bar{x} = 46$	126 103 423 137 $\bar{x} = 197$
9	47 219 $\bar{x} = 133$	581 450 $\bar{x} = 516$
12	51	431

All values are PFC/10^6 spleen leucocytes.

The PFC assays were performed 6 days post-injection with SRBC, using both guinea-pig and Xenopus complement on the same spleen cell suspension.

The animals used included both outbred and inbred G-line X. laevis.

Table 6.3 Splenic PFC responses to SRBC in thymectomized animals injected with thymocytes, following either short-term or long-term protocols

Reconstituting cell type	MHC-compatible		MHC-incompatible	
	Short term	Long term	Short term	Long term
Thymocytes	1 5 1 84 $\bar{x} = 23$	10 13 $\bar{x} = 12$	15 15 28 1 $\bar{x} = 15$	2 12* 0* $\bar{x} = 5$

All values are PFC/10^6 spleen leucocytes.

Assays were performed 6 days post-injection with SRBC, using guinea-pig complement.

Both donors and hosts used in the MHC-compatible experiments were inbred G-line X. laevis.

For MHC-incompatible experiments the hosts were outbred X. laevis and the donors were either outbred or inbred G-line X. laevis.

All animals, donors and hosts, were aged 6-18 months.

* Donor aged 6 months.

Table 6.4 Cellular and serum antibody production to SRBC in thymectomized animals injected with thymocytes, following a long-term protocol, with multiple antigen injections.

Reconstituting cell type	PFC/10^6 spleen leucocytes	Total serum antibody titre ($-\log_2$)	2-Mercaptoethanol-resistant antibody titre ($-\log_2$)
Thymocytes (MHC-incompatible)	15* 70* 0 0 0+ $\bar{x} = 17$	10* 6* 4 0 5+ $\bar{x} = 5$	0* 0* 0 0 0+ $\bar{x} = 0$

Animals were given three injections of antigen, 3 days apart and the assay was performed 2 weeks after the final injection, using guinea-pig complement.

Donors and hosts were outbred X. laevis.

Donors and hosts were aged 6 months, except for: * host aged 18 months;
+ donor aged 8 months.

Table 6.5 Legend

All values are PFC/10^6 spleen leucocytes.

Assays were performed 6 days post-injection with SRBC, using both guinea-pig and *Xenopus* complement on the same spleen cell suspension, where possible.

Both donors and hosts were inbred G-line *X. laevis* in MHC-compatible experiments.

For MHC-incompatible experiments, the donors were outbred *X. laevis* and the hosts either outbred or inbred G-line *X. laevis*.

All donors and hosts were aged 6-16 months.

* Donor aged 6 months.

Table 6.5 Splenic PFC responses to SRBC in thymectomized animals injected with irradiated or non-irradiated splenocytes, following short-term or long-term protocols

Source of complement	Reconstituting cell type	MHC-compatible		MHC-incompatible	
		Short term	Long term	Short term	Long term
Guinea-pig	Splenocytes	3 22 $\bar{x}$ = 13	137 296 $\bar{x}$ = 217	26 12 8 $\bar{x}$ = 15	Dead (3 animals)
	Irradiated splenocytes	0 0 $\bar{x}$ = 0	7 0 $\bar{x}$ = 4	0 0 0 0* $\bar{x}$ = 0	16 7 $\bar{x}$ = 12
Xenopus laevis	Splenocytes	-	-	-	-
	Irradiated splenocytes	-	1 10 $\bar{x}$ = 6	-	38 78 $\bar{x}$ = 58

Table 6.6 Splenic PFC responses to SRBC in thymectomized animals injected with irradiated or non-irradiated SRBC-primed splenocytes, following the short-term protocol

Source of complement	Reconstituting cell type	MHC-compatible	MHC-incompatible
Guinea-pig	Primed splenocytes	191 246 89 $\bar{x}$ = 175	15 113 390 $\bar{x}$ = 173
	Irradiated primed splenocytes	53 2 10 $\bar{x}$ = 22	38 1 20 0 $\bar{x}$ = 15
Xenopus laevis	Primed splenocytes	332 381 154 $\bar{x}$ = 289	173
	Irradiated primed splenocytes	157 176 63 $\bar{x}$ = 132	91 4 $\bar{x}$ = 48

All values are PFC/10^6 spleen leucocytes.

Assays were performed 6 days post-injection with SRBC, using both guinea-pig and Xenopus complement on the same spleen cell suspension where possible.

Both donors and hosts were inbred G-line X. laevis in MHC-compatible experiments.

For MHC-incompatible experiments, the donors were outbred X. laevis and the hosts either outbred or inbred G-line X. laevis.

All donors and hosts were aged 7-12 months.

Table 6.7 Splenic PFC responses to SRBC in thymectomized animals injected with a mixture of splenocytes and peripheral blood lymphocytes (either irradiated or non-irradiated), following the short-term protocol

Source of complement	Reconstituting cell type	MHC-compatible	MHC-incompatible
Guinea-pig	Splenocytes/PBL	129 24 $\bar{x} = 77$	22 51 298 57 1 515 $\bar{x} = 157$
	Irradiated splenocytes/PBL	25 40 $\bar{x} = 33$	517 49 $\bar{x} = 283$
Xenopus laevis	Splenocytes/PBL	741 112 $\bar{x} = 427$	153 412 $\bar{x} = 283$
	Irradiated splenocytes/PBL	538 300 $\bar{x} = 419$	720 63 $\bar{x} = 392$

All values are PFC/10^6 spleen leucocytes.

Assays were performed 6 days post-injection with SRBC, using both guinea-pig and Xenopus complement on the same spleen cell suspension where possible.

Both donors and hosts were inbred G-line X. laevis in MHC-compatible experiments.

Both donors and hosts were outbred X. laevis in MHC-incompatible experiments.

All donors and hosts were aged 8-12 months.

CHAPTER SEVEN

CONCLUDING REMARKS

In this Thesis, attempts were made to restore the T cell-dependent antibody response to SRBC in early-thymectomized Xenopus laevis, by giving these animals a thymus implant or an injection of lymphocytes from either an MHC-compatible or MHC-incompatible source. The cellular and serum antibody response to SRBC in thymectomized Xenopus was shown in Chapter 2 to be restored by implantation of an MHC-incompatible thymus in later larval life. It was considered unlikely that this restoration was due to the presence of donor B cells derived from the implanted thymus, as 5,000 rad irradiated MHC-incompatible thymus implants were also able to bring about the restoration of the anti-SRBC response. One interpretation to place on these experiments is that host T cells, that develop within the donor thymus, do not become restricted to the thymus donor MHC type. However, it is conceivable that T cells could have originated from the MHC-disparate thymus implant and then co-operated successfully with allogeneic B cells in the periphery. Hence subsequent experiments were designed to determine whether such a non-MHC-restricted co-operation could take place between Xenopus helper T and B cells by injecting an MHC-disparate lymphocyte suspension (depleted of B cells) into thymectomized Xenopus.

However, before such experiments could be performed, it was necessary to examine a number of related aspects. In Chapter 3 the suitability of thymocytes and splenocytes for cellular reconstitution, in terms of the numbers of unwanted B cells present, was examined. It was found that the thymus, far from being a relatively "pure" source of T lymphocytes, contained a substantial population of contaminating

B-lineage lymphocytes. This was shown by treating lymphocytes with the B cell mitogen LPS to induce the differentiation of cytoplasmic IgM-positive cells in culture and employing an immunofluorescence technique to visualise these cells.

The suitability of two techniques for the depletion of B cells from thymocyte and splenocyte populations was then investigated in Chapter 4. Adherence of Xenopus B cells to nylon wool was found to be an unsatisfactory method for preparing a B cell-depleted population of lymphocytes, as nylon wool-passaged thymocytes and splenocytes were still able to display enhanced tritiated thymidine uptake in response to the B cell mitogen LPS and to produce cytoplasmic IgM as measured by immunofluorescence. The majority of immunoglobulin-secreting B cells (obtained by in vivo priming with SRBC) were also not retained on a nylon wool column and were still able to produce plaques in an anti-SRBC assay. On the other hand, a dose of 3,000 rads of γ-irradiation was found to be efficient in removing B cells from unprimed splenocytes, thymocytes and PBL, as measured by the ability of irradiated cells to mount an anti-SRBC PFC response in a lethally irradiated, MHC-compatible adoptive transfer host. However this dose of irradiation did not remove the potential of SRBC-primed splenocytes to produce PFC.

Before the cellular reconstitution experiments could be carried out, it was necessary to first check that lymphocytes injected into a thymectomized animal would be able to migrate successfully to the centre of reactivity with antigen - the spleen (Chapter 5). It was shown, using ^{51}Cr labelling, that MHC-incompatible thymocytes and splenocytes could reach the spleen within 6 hours post-injection and, using ploidy-marked cells, that MHC semi-allogeneic splenocytes were still present at least 6 days later. However further experiments

along these lines will be necessary to check whether or not γ-irradiation affects the ability of Xenopus cells to migrate properly.

Injection of thymectomized Xenopus with MHC-incompatible, 3,000 rad irradiated splenocytes/PBL was found to restore the primary in vivo antibody response of thymectomized Xenopus to SRBC (Chapter 6). This suggests that MHC-incompatible helper T and B cells can co-operate in an in vivo PFC response to SRBC. This is perhaps surprising, in view of the finding that the 6 day splenic PFC response to foreign red cells in vitro seems to require MHC identity of the T and B cell populations (Lallone, 1984).

It will be important in future work to concentrate on Ig"G" responses, where requirements for T-B collaboration may prove to be more stringent and include MHC-restricted reactivity. It has been shown in Xenopus that a secondary Ig"G" response to dinitrophenylated-fowl gamma-globulin will only take place in vitro between T and B cells sharing at least one MHC haplotype, although occasional IgM responses are observed with MHC-incompatible lymphocyte combinations (Bernard, Bordmann, Blomberg and Du Pasquier, 1981). Hence cell-reconstituted animals could be tested for the presence of 2-mercapto-ethanol-resistant serum antibody, as with animals reconstituted with thymus implants (Chapter 2). Alternatively, more sophisticated methods for the detection of Ig"G" antibodies could be used, such as isoelectric focussing assays on immune sera in polyacrylamide gels. Only Ig"G" is detected by this method, as IgM does not penetrate into the gel (Du Pasquier and Horton, 1982). Monoclonal antibodies directed against Xenopus Ig"G" could also be used in an enzyme-linked immunosorbent assay or radioimmunoassay for the detection of anti-SRBC Ig"G" antibodies. It may also be informative to examine T-B collaboration in reconstituted animals by employing

other T cell-dependent antigens such as dinitrophenylated-fowl gamma-globulin. The response to these antigens may prove to be more dependent upon MHC-restricted T cell help than the response to SRBC.

It would be interesting to attempt an investigation of the role of the macrophage in these cellular reconstitution experiments. Mammalian macrophages adhere to nylon wool (Erb and Feldmann, 1975; Henry, Chen, Stout and Swain, 1980); perhaps macrophages could be depleted from a population of Xenopus lymphocytes in this way. (The presence of macrophages could be identified using e.g. non-specific esterase staining - see Stuart, Habeshaw and Davidson, 1978.) If in vivo restoration of anti-SRBC reactivity takes place in thymectomized animals given a macrophage-depleted and B cell-depleted (by irradiation) injection of MHC-incompatible splenocytes/PBL, then this would suggest that this antibody response is not genetically restricted in terms of both helper T cell/macrophage and helper T cell/B cell interactions. If, however, restoration only takes place upon addition of macrophages (perhaps these could be obtained by recovery of a glass-adherent population of Xenopus peritoneal exudate cells) of the same MHC type as the donor T cells, then MHC restriction would appear to exist in Xenopus between helper T cells and macrophages. A lack of restoration here might imply that there is also restriction between Xenopus helper T and B cells. However, these experiments depend upon the purity of the T cell and macrophage populations used. Cell-separation techniques for amphibians must first be improved before such experiments can be performed and the MHC restriction of macrophage-T cell-B cell collaboration be properly examined in Xenopus.

Finally, to determine the nature of helper T cell restriction

in thymectomized animals given an MHC-incompatible thymus implant, it will be important to examine the ability of such T cells to collaborate *in vitro* with macrophages and B cells of various genotypes.

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APPENDIX

Abbreviations Used in This Thesis

$cIgM^+$	Cytoplasmic IgM positive
$cIgM^-$	Cytoplasmic IgM negative
Con A	Concanavalin A
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Ig	Immunoglobulin
L-15	Leibovitz L-15 culture medium
LG	*Xenopus laevis/gilli* hybrid
LPS	*E. coli* lipopolysaccharide
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte response
PBL	Peripheral blood lymphocytes
PBS	Phosphate-buffered saline
PFC	Plaque-forming cell
PHA	Phytohaemagglutinin
SRBC	Sheep red blood cells

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