IMMUNOLOGICAL TOLERANCE IN THE AMPHIBIAN

XENOPUS LAEVIS (DAUDIN).

Being a Thesis submitted for the degree of Doctor of Philosophy
to the Council for National Academic Awards by

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in partial fulfilment of the requirements for this degree.

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IMMUNOLOGICAL TOLERANCE IN THE AMPHIBIAN _XENOPUS LAEVIS_ (DAUDIN).

by

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ABSTRACT

Observation of some of the phenomena of tolerance to soluble protein antigens and allogeneic tissue transplants in _Xenopus laevis_ has formed the framework of the present study.

The method of larval induction of high-zone tolerance used in this laboratory has been confirmed and further analysed. Larvae treated with high doses of Human-γ-globulin (HGG) were unable to produce anti-HGG antibody after challenge. The proliferative response demonstrated in the spleens of tolerant toadlets 21 days after challenge was, however, of similar magnitude to that in normally responding animals.

Adoptive transfer of high-zone tolerance specific to HGG was demonstrated by intravenous inoculation of tolerant histocompatible splenocytes simultaneously with an antigenic challenge via the dorsal lymph sac. This is indicative of the active involvement of a suppressor T-cell population.

The induction of high-zone tolerance in _X. laevis_ results in changes in spleen cell populations as demonstrated by buoyant density gradient separation. Spleen cell sub-populations taken from the separated layers were not, however, effective in the adoptive transfer of tolerance.

A normal lymphocyte transfer reaction was observed in _X. laevis_ to show a number of characteristics seen in the mammalian reaction. The use of mitomycin-C treated donor cells and early thymectomized hosts has demonstrated that the phenomenon is composed of donor and host components which are largely distinct from each other.

Implantation of allogeneic larval spleens resulted in the induction of transplantation tolerance or impaired rejection in a significant proportion of skin grafted toadlets in which both the donor and host larvae were up to and including stage 51 at the time of transplantation. The implication of these results is that immunomaturity of the donor and host is important in the induction of transplantation tolerance but that other factors must also be involved.
DECLARATION

This work has not been accepted in substance for any other degree and is not concurrently being submitted in candidature for any other degree.

This is to certify that the work here submitted was carried out by the candidate herself. Due acknowledgement has been made of any assistance received.

Signed

(Candidate)

Signed

(Supervisor)
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CHAPTER I

GENERAL INTRODUCTION
Immunological tolerance may be defined as the acquisition of specific unresponsiveness to antigenic determinants presented to the system. The phenomenon has evolved in answer to the requirement for the organism to tolerate self tissues and proteins whilst retaining the capacity to eliminate foreign material. Hence it is intimately linked with the capability of the organism to discriminate between self and non-self. The major histocompatibility system which governs the recognition of foreign antigenic determinants likewise controls many of the cellular interactions of the immune response. Once antigen is presented to the immune system and recognized by the immunocompetent cells a cascade of reactions is triggered. Whether the endpoint of this stimulation is tolerance of the antigenic determinants or the production of specific neutralising activity depends on the balance between a number of factors (Fujiwara, Qian, Satoh, Kokudo, Ikegami & Hamaoka, 1986). Broadly these are the maturity of the recipient, or more accurately of its lymphoid system (Billingham & Brent, 1956; Szewczuk & Siskind, 1977), and its general physiological condition, the form of the antigen, the dose and the route of administration. In transplantation tolerance the maturity of the donor cells and the level of histocompatibility are also of prime concern. The phenomenon cannot be satisfactorily divided into discrete subsections as they are intimately linked in many phases of the response.

Reconstitution experiments in lethally irradiated mice have shown that tolerance can be induced in both the B- and the T-cell populations. Since both populations are required to cooperate in the fully developed response the inactivation of either could result in apparent tolerance.
in both. This is distinct from the phenomenon of split tolerance in which functionally discrete subsets of T-lymphocytes may differentially succumb to tolerogenesis. Characteristically the T- and B-lymphocytes differ with respect to their relative susceptibility to the induction of tolerance (Nossal, 1983). The early experiments by Mitchison (1964; 1968) showed that in the adult mammal tolerance could be induced by low and high doses of soluble protein antigen. Subsequently Chiller, Habicht & Weigle (1970) found that it was the T-cells which rapidly became tolerant in the presence of low doses of antigen. Considerably higher doses of antigen were required by the B-cells to become specifically tolerant. The onset of tolerance took longer and lasted for much shorter periods than the T-cell tolerance (Rajewsky & Brenig, 1974).

The cooperation of B- and T-cells in the regulation of the mammalian immune response has been well established both in vivo and in vitro (Miller & Mitchell, 1967; Kindred & Schreffler, 1972; Katz & Benacerraf, 1974; Von Boehmer, Hudson & Sprent, 1975; Woodland & Cantor, 1978; Miller, 1981; Howie & McBride, 1982; Tite, Kaye, Saizawa, Ming, Katz, Smith & Janeway, 1986). This helper function has been shown to be MHC restricted with receptors on the helper T-cells for proteins encoded by the MHC class II I-A sub-region in addition to those antigen specific receptors. The cytotoxic T-cells involved in immunoregulation have been shown to be MHC restricted in their interactions with other cell types by the class I antigens of the H-2D and H-2K sub-regions. More recently, evidence has suggested a similar role for the class II H-2I-J sub-region in directing suppressor T-cell activity, particularly in neonatal tolerance (Streilein & Gruchalla, 1981; Czitrom, Sunshine & Mitchison, 1981; Bromberg, Delovitch, Kaufman & Greene, 1983). The presence of a
lymphocyte type with suppressive properties was first indicated by the adoptive transfer experiments of Gershon, Cohen, Hencin & Liehaber (1972). The experiments by Rich & Pierce (1973) failed to isolate suppressor from helper activity, but found a mixed population mainly in the spleen and lymph nodes which suppressed primary and secondary humoral responses in vitro. Herzenberg, Chan, Ravitch, Riblet & Herzenberg (1973) found in transfer experiments an active suppressor population in which the thymus and the spleen were equally suitable sources of these cells. The presence of an active suppressor cell maintaining the suppressive effect in the adoptive transfer of tolerance was also shown by Segal, Weinstein, Melmon & McDevitt (1974). Thus the suppressor cell important in the regulation of the humoral response, was found to be a short lived cell, functionally distinct and separable from helper T-cells. It was implicated in the regulation of cellular immunity and autoimmunity. The existence of non-specific suppressor cells, recent emigrants from the thymus (T1), as opposed to older, specific (T2) cells (Raff & Cantor, 1971) was suggested by Pierce, Peavy & Tadakuma (1975) to be another possible factor. Asherson & Zembala (1976) found a suppressor factor released by these cells. Some further characterization of the suppressor T-cell activity was made by Brent, Brooks, Medawar & Simpson (1976) who found the cell population to be involved in partial tolerance to transplantation antigens, in conjunction with the suppressor factors which they produced. The T1 cells in the periphery are seen to be large, adherent, dividing cells mainly in the spleen. The T2 cells on the other hand, are small, non-dividing and non-adherent, rapidly recirculating cells (Kappler, Hunter, Jacobs & Lord, 1974; Stutman, 1975). Dorsch & Roser (1977) found that transplantation tolerance
was maintained in adult rats by a long lived, rapidly recirculating suppressor cell. Two distinct suppressor cell types have also been shown by Duncan, Stepkowski & Bitter-Suermann (1986), who presented evidence that the presence of suppressor cells in long term tolerant rat recipients is of considerable importance in the maintenance of this tolerance. Dorsch & Roser (1982) used sequential transfer experiments to show that the population of rapidly recirculating suppressor cells supporting tolerance in the adoptive recipients, was able to expand on transfer. This appears to contrast with the findings of Kappler et al. (1974) and Stutman (1975) who found this population to be non-dividing.

The B-lymphocytes have been suggested to be rendered tolerant by four basic mechanisms. The first, clonal deletion, was originally advanced by Burnet (1959). In his proposition the maturation sequence for clones of immature B-cells, which are particularly susceptible to tolerogens, was aborted by the presence of such tolerogens. These clones were not subsequently able to respond normally to antigenic challenge. Studies on the ease of induction of B-cell tolerance have found that neonatal animals require much lower levels of antigen stimulation for B-cell tolerance than the adult (Szewczuk & Siskind, 1977; Kay, Pike & Nossal, 1980). The pre-B-cells themselves appear to be most vulnerable at the transition stage to the virgin B cells when the surface immunoglobulin receptors are first emerging (Pike, Kay & Nossal, 1980). Pre-B-cells in the adult are probably equally susceptible; the overall ease of tolerance induction in neonates then depends on the immaturity of other cell populations involved in the response. Nossal & Pike (1975) used the term clonal deletion to describe the process occurring in adult mice. The
mechanisms by which B-cells become tolerized are not altogether clear. B-cells may be inactivated temporarily or permanently by the binding of antigen to the surface receptors (Aldo-Benson & Borel, 1974). Exposure to high doses of multivalent antigens could result in capping of the surface receptors. The consequent shedding or internalizing of capped receptors is normally followed by resynthesis of the surface receptors in mature cells. Virgin B\(_u\) cells, however, have a limited ability to resynthesize surface Ig receptors (Raff, Owen, Cooper, Lawton, Megson & Gathings, 1975), and so these cells will be rendered inactive by the absence of surface Ig. The vulnerability of T-lymphocytes to tolerogenesis has been discussed. Their susceptibility does not alter markedly over the course of the maturation sequence as do the B-cells, but the immature cells may experience clonal abortion in a manner similar to these B-cells.

The second basic mechanism is functional deletion of immunocompetent activity against T-dependent antigens. This results from the removal or inactivation of helper T-cells thereby reducing or eliminating functioning of the B-cells. The T-independent antigens bypass the need for T-cell help and so functional deletion in this instance relates to the blockade of surface receptors with the large, polymeric molecules of this type of T-independent antigen. It has been observed that individual T-cell subsets may be deleted, which results in the loss of a particular functional activity, whilst leaving other T-cell systems intact.

Clonal exhaustion as the third basic mechanism, can result from the repeated immunization of the animal with antigen. This is particularly so in the presence of immunosuppressants. The T-independent antigens will trigger exhaustive differentiation of mature B-cells resulting eventually in a system devoid of mature responder
The fourth mechanism involves the blockade of antibody-forming cells. It is the final phase at which B-cell tolerance may be induced. It is relatively difficult to obtain this effect at the level of the fully differentiated cell and so requires very high concentrations of antigen.

Allograft rejection is moderated by the influences of donor and host maturity and cell dose (Terasaki, Longmire & Cannon, 1957; Brent & Gowland, 1961; Billingham & Silvers, 1962). These factors contribute to the normal response, in addition to the degree of histocompatibility disparity between the donor and host, which is the major barrier to successful allograft survival (Brooks, 1976). The induction of transplantation tolerance to allogeneic grafts requires either that the graft should be made to appear less immunogenic or that the host response should be impaired.

The immune response to allogeneic tissue transplantation is directed by the presence of both class I and class II MHC alloantigens. T-helper cells are stimulated by the class II antigens and are the principal initiators of rejection. The cytotoxic T-cells are mainly directed at the class I antigens and are unlikely to be major mediators of graft rejection (Loveland & McKenzie, 1982). Class I antigens are present on most cell surfaces. The class II antigens have a far more restricted distribution but are present on all leukocytes. Billingham (1971) examined the passenger cell concept in the context of controlling the graft versus host response. Removal of the passenger leukocytes from allografted tissues such as skin or renal grafts, could mean that these grafts would then be presenting class I MHC alloantigens only to the host system. The perfusion of graft tissue prior to trans-
plantation, or *in vitro* culture, were thus considered in order to remove these passenger leukocytes. Lafferty, Bootes, Killby & Burch (1976) found that after 2–3 weeks *in vitro* culture of murine thyroid tissue these grafts were acceptable to the hosts, retaining their antigenicity on the one hand, and the host retaining the full repertoire of immunocompetence, on the other. An examination of long surviving rat renal grafts by Hart & Fabre (1981) revealed a marked absence of intensely Ia+ donor type dendritic cells, which were normally present, and these were proposed as the hitherto unidentified passenger cells.

The immunogenicity of allografts has been shown to be modulated by other factors. A small proportion of tolerated grafts appeared to lose the capacity for expressing their own H-2 antigens in an immunogenic form. This phenomenon of graft adaptation, first referred to by Woodruff & Woodruff (1950), indicates that the permanent survival of allografts as the classical measure of transplantation tolerance is not altogether reliable. A number of suggestions have been made to account for this phenomenon. The most feasible of these is that it results from the loss of dendritic cells and other passenger leukocytes from the graft, in addition to the gradual replacement of endothelial cells with host tissue (Streilein, Strome, Gruchalla & Wood, 1983).

The production of embryonic stem-cell chimaeras is the most satisfactory means of obtaining permanent allograft survival. This will be discussed in more detail in chapter 6. Although clonal abortion was thought to be the single mechanism active in neonatal transplantation tolerance induction, it is now considered that varying degrees of clonal abortion and regulatory suppression provide a more
accurate picture. Indeed the intermingling of any number of the mechanisms involved is possible in divers instances, and the particular mechanisms activated will depend upon balance of regulating factors previously detailed. The complementary action of clonal deletion and active suppression is also seen in adult tolerance induction (Gruchalla & Streilein, 1981; Streilein, Gruchalla, Wood & Strome, 1982; Good & Nossal, 1983; Good, Pike & Nossal, 1983; Slavin, Morecki, Weigensberg, Bar & Weiss, 1986). Brooks (1975) suggested that complete tolerance, in which the chimaeric state persists, required the absence of cells reactive to the MHC alloantigens of the graft. This clonal elimination (or inactivation) may not be completely effective but might be reinforced by active suppression of the potentially reactive clones (Charpentier, Bach, Lang, Martin & Fries, 1983; Hall, 1985). An active suppressor population was obtained from mouse foetal liver cells (Globerson, Rabinowich & Umiel, 1981). It was suggested that this population played a role in controlling the development of the immune system (perhaps with specific reference to autoimmunity). A second suppressor population has been found by Streilein, Gruchalla & Strome (1981), active in the effector phase of the response to transplantation antigens. The two suppressor T-cell populations separated on a velocity sedimentation gradient by Gorczynski & MacRae (1979), were suggested to be responsible for the induction and maintenance of the tolerant state. Separate suppressor T-cell populations acting at the inducer and effector phases have also been noted in the regulation of the immune response to soluble antigen (Damle, Mahaghegpour & Engleman, 1984).

It has been suggested that tolerance to foreign proteins and allogeneic tissue has developed incidentally to the necessity for self-
tolerance, benefitting from the utilization of normal functions of the immune system. Phillips-Quaglia (1980) found that the presence of the thymus was necessary for the production of neonatal tolerance to the soluble protein Bovine Serum Albumin. This tolerance was not dependent upon the presence of specific or non-specific suppressor cells. It was suggested that the peripheral T-cells recirculating through the thymus were tolerized when coming into contact with the antigen complexed with Ia antigens in the thymic epithelium. The importance of the thymic epithelium was underlined by Jordan, Robinson, Hopkinson, House & Bentley (1985) using the induction of transplantation tolerance in nude mice. It is possible that this forms the basis of the mechanism for the development of self recognition.

The maintenance of tolerance requires the continued presence of the stimulating antigen (Mitchison, 1962) so that new antigen-sensitive clones may be tolerized as they come on-line. The process of localization of foreign proteins in specific areas of lymphoid tissues, in particular the spleen follicles, has been described by a number of workers (Brown, Schwab & Holborow, 1970; Bianco, Dukor & Nussenzweig, 1971; Van Rooijen, 1974; Chen, Adams & Steinman, 1978; Steinman & Nussenzweig, 1980). It is known that the mammalian follicular dendritic cell performs a vital role in antigen presentation to the immune system (Klaus, Humphrey, Kunkl & Dongworth, 1980). For the most part antigen is trapped at the cell surface in the form of immune complexes (Van Rooijen, 1980; 1981). Immune complex trapping in continued antigen presentation has been indicated to be important to the anamnestic response (Mandel, Phipps, Abbot & Tew, 1980; Stavitsky, 1980; Tew, Phipps & Mandel, 1980; Phipps, Mandel, Schizlein & Tew, 1984) and the manner in which memory B-cells accumulate in the follicles in the presence of localized antigen has been examined (Ponzio, Baine &
Thorbecke, 1980). These antigen/antibody complexes have been shown to induce the production of specific suppressor T-cells (Caulfield, Luce, Proffitt & Cerny, 1983) and to be important in the removal of antigen reactive cells (Hutchinson, 1980). The importance of suppressor T-cell memory in tolerance has been discussed by Basten, Loblay & Pritchard (1982).

An overview of the mechanisms of tolerance induction and maintenance in mammals must stretch from central failure of the immune response on the one hand (Billingham, Brent & Medawar, 1953; Burnet, 1959), to such peripheral mechanisms as suppression and blockade on the other (Beverley, Brent, Brooks, Medawar & Simpson, 1973; Steinmuller, 1978). It could be that the network theory of the immune system proposed by Jerne (1974) will provide the framework to draw these contrasting elements together.

In the present work the amphibian *Xenopus laevis* was used to make a study of immunological tolerance. It provides an excellent laboratory model for developmental studies of the immune system. Although relatively primitive, it possesses the basic features of the more advanced vertebrate systems. For example, the T-B-lymphocyte dichotomy and collaboration (Ruben, Van der Hoven & Dutton, 1973; Ruben, Mette, Cochran & Edwards, 1980; Ruben, Welch & Jones, 1980), separate immunoglobulin classes (Hadji-Azimi, 1971; Jurd, Luther-Davies & Stevenson, 1975; Hsu, Flajnik & Du Pasquier, 1985), the anamnestic response (Marchalonis & Germain, 1971), mixed leukocyte reaction (Weiss & Du Pasquier, 1973), allograft rejection (Horton, 1969) which is sub-acute in *Xenopus*, the production of molecules with Il-1-like activity (Watkins, Parsons & Cohen, in press) and the Il-2-like molecules (Cohen & Watkins, 1986), major histocompatibility
complex restriction and polymorphism of the class I and class II MHC antigens (Flajnik, Kaufman, Hsu & Du Pasquier, 1984; Flajnik, Kaufman, Reigert & Du Pasquier, 1984; Flajnik, Kaufman & Du Pasquier, 1985; Kaufman, Flajnik & Du Pasquier, 1985). The free-living early stages of *X. laevis* lend themselves readily to embryonic and larval manipulation as shown by the early thymectomy work of Manning (1971), which did not impair the normal development of larvae and adults.

In the experiments of Horton & Manning (1972) the paired thymi were destroyed by microcautery 8 days post-fertilization, at which time few small lymphocytes had invaded the thymic tissue. Thymectomy at this time, stage 47 - 48 (Nieuwkoop & Faber, 1967), produced an impaired allograft rejection response in the adult, which was more severely impaired by thymectomy at stage 46 (Horton & Horton, 1975). Tochinai & Katagiri (1975) stated that thymectomy at the earlier stage 45, completely abrogated the allograft rejection response in the adult. Tompkins & Kaye (1981) were able to perform thymectomy down to stage 42 with animals exhibiting a progressive increase in the number able to accept allografts permanently. Thymectomy at stage 47 - 48 severely reduced the localization of HGG in the antigen-trapping zone of the spleen and the production of circulating antibody to HGG (Horton & Manning, 1974a; Collie, 1976). Both high and low molecular weight immunoglobulins were inhibited by early thymectomy (Turner & Manning, 1974).

It is not technically feasible to parallel this early thymectomy work in mammals. It was, however, shown by Miller & Osoba (1967) and Miller & Mitchell (1969) that neonatal thymectomy resulted in severe depletion of T-cell activity if performed within 24 hours of birth. Access to the nude mouse has facilitated the study of
the effects of T-cell depletion at early developmental stages. Using this model, some workers have found evidence to suggest that pre-thymic stem-cells can undergo maturation outside the thymus (Gillis, Baker & Smith, 1979), although it is not clear how such populations would learn to discriminate between self and non-self. It would be interesting to speculate on whether the impaired responses viewed in early thymectomized *Xenopus* might be produced in a similar manner. Nagata & Kawahara (1982) presented data suggesting that pre-thymic stem-cells in early thymectomized *Xenopus* could differentiate along a T-cell pathway when an allogeneic thymus was implanted to provide the appropriate microenvironment. This was also shown by Maeno, Nakamura, Tochinai & Katagiri (1985).

The development of *X.laevis* clones, first produced by Kobel & Du Pasquier (1975) is an important manifestation of the value of the early embryonic manipulation techniques available. These clones are widely used in histocompatibility studies. A variety of chimaeras have been produced, by grafting different tissues on to the neurulae, which have been valuable both in adult transplantation experiments and in the elucidation of some early developmental features. Flank grafts from stage 21 - 22 neurulae have been used to create chimaerism in the adult (Clark & Newth, 1972). Flajnik, Du Pasquier & Cohen (1984) used head and tail grafts in these neurulae to create a different form of chimaera, one in which all of the haemopoietic stem-cell tissue would be of different type to the thymus. Tompkins, Volpe & Reinschmidt (1980) used both grafting methods to determine the site of potential haemopoietic stem-cells. Ploidy markers in the neurulae grafting experiments have been used (Maeno, Tochinai & Katagiri, 1985; Maeno, Todate & Katagiri, 1985) to determine the input of dorsal and ventral lateral plate mesoderm in haemopoietic stem-cells.
Turpen & Smith (1986) also used ploidy markers in the neurulae grafting work, which suggested a bipotential precursor for thymocytes and thymic accessory cells.

The free-living larvae of *X. laevis* have made certain evolutionary adaptations in order to survive a potentially hostile environment at an extremely immature stage. These have included the early production of specific positive immunity and the capacity for tolerance. Horton, Rimmer & Horton (1976) observed that the thymus is required throughout much of larval life for the full maturation of the humoral response to T-dependent antigens. However, the allogeneic graft rejection response has been shown to require a more limited period of thymic input in the larvae. This suggested that the different T-cell subsets mature at different times in larval development. Mughal (1984) found that although larvae were readily tolerized by high doses of antigen, the lower doses, which are effective tolerogens in young mammals, produced a positive immune response in *Xenopus*. This was a further indication of the possibility that distinct T-cell subsets mature at different stages in larval development. Such a capacity could be advantageous to free-living larvae which might require tolerance to certain foreign protein antigens encountered regularly or in high doses.

The demands of a free-living larval stage result in a number of developmental characteristics in the *Xenopus* immune system which might appear anomalous in comparison with that of the mammal. The metamorphic period encompasses a number of these. During this period the thymic and splenic lymphocyte populations regress (Du Pasquier & Weiss, 1973) and the synthesis of adult major histocompatibility antigens which differ from the larval MHC antigens is begun (Du Pasquier, Blomberg & Bernard, 1979). In particular class I MHC antigens,
absent from normal larvae, are synthesized at this time.

Transplantation tolerance to minor histocompatibility antigens may be induced more readily by the application of allografts in this metamorphic period and the process of rejection of MHC-disparate allografts is apparently suspended at this time (see review by Cohen, DiMarzo, Rollins-Smith, Barlow & Vanderschmidt-Parsons, 1985). Following metamorphosis the switch from larval to adult life also involves changes in the composition of various serum proteins including immunoglobulin and complement classes (Lallone, Chambers & Horton, 1984).

The current understanding of immune tolerance in *X. laevis* has been largely directed towards the study of allogeneic material. It is perhaps surprising that tolerance to xenogeneic antigens has not been more closely examined, when the free-living nature of *Xenopus* larvae is considered. Thus the present work makes an examination of some of the features of tolerance to foreign protein antigens as well as to allogeneic tissues in this amphibian. In chapter 3, the aspects of high zone tolerance to Human-β-globulin studied, centre on the early phase of the response to challenge in tolerant animals and the detection of a suppressor population supporting this tolerance.

Chapter 4 is concerned with a more detailed examination of the cell populations involved in this response. Chapter 5 introduces the normal lymphocyte transfer reaction in the amphibian as a possible alternative to the more traditional methods of testing for transplantation tolerance. Since this amphibian model owes much of its current favour to its embryonic accessibility, full advantage of this feature should be taken. Thus, alternative tests more suited to the low cell numbers present in larvae would be of value. In chapter 6, attempts have been made to induce transplantation tolerance in the
larval stages of *X. laevis* using transplanted spleens. Some of the regulatory factors incident on the inductive phase of transplantation tolerance were thereby examined.
CHAPTER 2

GENERAL METHODS
Three lines of *Xenopus laevis* (Daudin) were used in this study. These were (a) outbred animals, laboratory bred to the third generation; (b) wild, caught *X. laevis* imported through Xenopus Ltd., U.K. and (c) partially inbred *X. laevis* (Katagiri, 1978), donated by C. Katagiri and now in their second and third generations in this laboratory. These animals have been shown to be histocompatible at the major loci and the MHC homozygosity has been designated JJ, (Barlow, DiMarzo & Cohen, 1981; DiMarzo & Cohen, 1982a). Within the strain these animals exhibit varying numbers of differences at the minor loci as shown by the variability in chronic rejection times.

Ovulation was induced in the female by an injection of Human Chorionic Gonadotropin (Chorulon, Intervet Laboratories, Cambridge, U.K.) of approximately 4 iu g\(^{-1}\) body weight, administered via the dorsal lymph sac in sterile water at a concentration of 500 iu cm\(^{-3}\). This dose could be split into a priming dose of 100 iu followed 8 - 12 hours later by the second, larger dose.

Spermiation was induced in the male by the injection of 4 iu g\(^{-1}\) body weight of Human Chorionic Gonadotropin administered via the dorsal lymph sac at the same concentration of 500 iu cm\(^{-3}\). Where the black nuptial pads were not well developed, a priming injection of half the total dose could be given 8 to 12 hours before the main dose, which would then be reduced accordingly. The mating pairs were then placed in standing water at 20° C warming to 23 ± 1° C overnight, with netting placed in the water to retain the eggs.
Fertilized eggs were then removed to aerated, standing tap water and kept at 20 - 23° C until hatching when they were again removed to clean aerated water until the commencement of feeding at day 4 - 5 post-fertilization.

2. (ii) Feeding.

Larvae were fed with a suspension of pea and/or nettle soup supplemented with an Algae/Protozoa culture and milk weekly.

The toadlets were fed daily with Tubificid worms and the adults were fed once weekly on minced ox heart. Occasional supplements of earthworms and Tubificid worms were given to all animals, particularly the brood stock.

2. (iii) Stocking density.

The larvae were thinned out at around 7 days post-hatching to a stocking density of approximately 50 per 15 dm³ tank. From stage 50 - 52 (Nieuwkoop & Faber, 1967) the density was reduced to 20 - 25 per 15 dm³ tank, and finally from stage 58 the density was reduced to 10 per 15 dm³ tank.

Toadlets were maintained at a density of 6 per 15 dm³ tank and the larger toads held in 100 dm³ tanks at an approximate density of 1 per 10 dm³.

2. (iv) Treatment of disease.

_Xenopus laevis_ is a relatively hardy laboratory animal, provided that water quality, temperature and feeding are adequately maintained. They may, however, suffer occasionally from some fungal or bacterial infections which can be successfully treated in the early stages.
Fungal infections were most often seen on the hind and forelimb webbing. They were treated with concentrated Protozin (Waterlife Research Ltd., Heathrow, U.K.) for 2 minutes daily until the affected skin sloughed off. At the same time Penicillin and Streptomycin (Gibco, Paisley, Scotland), was added to the tank water at a concentration of 10,000 iu dm$^{-3}$ to prevent or curtail possible bacterial infection. Isolated cases of systemic infection were treated by the injection of these antibiotics into the dorsal lymph sac at a concentration of 100 iu g$^{-1}$ body weight.
INOCULATION PROCEDURE

2. (v) Administration of antigen.

The standard challenge dose of antigen was 0.025 mg g\(^{-1}\) body weight dissolved in 0.85% Sodium Chloride and emulsified with an equal volume of Freund's Complete Adjuvant (FCA, Difco, Detroit).

The tolerogenic dose used in larvae was 40 times the adult immunogenic dose of 0.025 mg g\(^{-1}\) body weight. Thus 1 mg g\(^{-1}\) body weight of antigen was administered in 0.85% of sterile Sodium Chloride solution.

2. (vi) Administration of [\(^3\)H] -Thymidine.

Animals were injected with 1\(\mu\)Ci g\(^{-1}\) body weight of [\(^3\)H] -Thymidine (Amersham U.K., sp. act. 2Ci\(\times\)Mol\(^{-1}\)) which was usually diluted 1:10 from the stock solution with sterile 0.85% Sodium Chloride solution.

2. (vii) Routes of inoculation.

The larvae were injected as a matter of routine into the peritoneal cavity. The injection was made dorsally through the epaxial muscle, in order to minimise loss of the measured dose. They were injected with a volume of 10 \(\mu\)l g\(^{-1}\) body weight which caused a slight but visible distension of the abdomen. It was administered by a glass needle attached to an Agla syringe and measured with a micrometer gauge.

Toadlets and adult *X. laevis* were injected via the dorsal lymph sac in a volume of 10 \(\mu\)l g\(^{-1}\) body weight. Intravenous injections were made into the *dorsalis pedis* vein, which was visible through the skin of the young toadlets.
GENERAL PROTOCOLS

2. (viii) Preparation of spleen cell suspensions.

Animals were killed in 3-aminobenzoic acid ethyl ester (MS 222, SIGMA, U.K.) at a concentration of 5 g dm\(^{-3}\). The animals were then packed with ice to slow blood clotting and bled from the heart. The organs required were then removed aseptically and stored on ice in Amphibian Culture Medium (see appendix I).

The spleens were homogenized on ice in 3 cm\(^3\) of ACM. Large particles were allowed to settle out and the suspended cells removed and centrifuged at 470g for 5 minutes and 4\(^\circ\)C. The supernatant was then aspirated off and the pellet resuspended in 1 cm\(^3\) of *Xenopus* Culture Medium (XCM, see appendix I) and retained on ice. Cell viability and numbers were assessed using 1% Trypan blue dye. The cell concentrations were then adjusted using XCM.

The *Xenopus* Culture Medium was only used when the cells were to be cultured. When cells were to be inoculated into animals or prepared for cytospin smears, the final suspensions were made up in an Amphibian dilution of Leibovitz medium (L\(_{15}\) with L-glutamine, Gibco, Paisley, Scotland), 100 cm\(^3\) L\(_{15}\) : 45 cm\(^3\) double distilled, sterile water.

For the preparation of thymus suspensions see chapter 3.

2. (ix) Preparation of sera.

Experimental and control animals were killed in MS 222 and blood collected from the heart as described. Blood from untreated animals was also collected as a source of normal *Xenopus* serum. This was taken from the dorsalis pedis vein of animals anaesthetized in 1 gm dm\(^{-3}\) MS 222. The blood was left to clot at room temperature for
30 minutes and then left at 4°C overnight to allow the clot to retract. The serum was then removed and tested for circulating antibodies in the case of test sera. Normal serum was pooled with that of other donors and heated to 56°C for 30 minutes to inactivate the complement. Aliquots of normal sera were stored at -20°C.

2. (x) Passive haemagglutination.

This method for the detection of serum antibody has been modified from the method of Stavitsky, 1954.

Test sera were collected from experimental and control animals by the method described above. These sera were not heat inactivated as this procedure is known to reduce the immunoglobulin content of the sera. The test sera were generally used fresh and not frozen.

Fresh Sheep Red Blood Cells (SRBC) in Alsever's solution (InterMed, Slough, U.K.) were washed 3 times in 0.85% Sodium Chloride spinning each time at 530g on an MSE Centaur centrifuge. Equal volumes of these packed cells were then added to the normal and test sera and incubated at room temperature for 40 - 60 minutes to absorb the natural, non-specific agglutinating factors from the sera.

At this time 4 cm$^3$ of a 2.5% suspension of SRBC in Phosphate Buffered Saline (PBS) pH 7.2 (see appendix II) was made up and 4 cm$^3$ of 0.005% Tannic acid added to it. These cells were incubated at 37°C for 15 minutes, washed in PBS 7.2, centrifuged at 470g for 5 minutes and resuspended at 2.5% in PBS 7.2. This tanning of the cells was done firstly to enhance the uptake of antigen onto the surface of the SRBC's and secondly to increase the sensitivity of the test.

The tanned cells were then coated with the specific antigen to be detected by mixing 4 volumes of PBS pH 6.4 (see appendix II),
1 volume of tanned cells and 1 volume of antigen (1 mg cm$^{-3}$ in 0.85% Sodium Chloride) or 1 volume of 0.85% Sodium Chloride solution for the control, uncoated cells. These cells were incubated at room temperature for 15 minutes, centrifuged at 470g for 5 minutes, and washed in serum diluent (1% v/v heat inactivated, normal, absorbed serum in 0.85% Sodium Chloride). They were then resuspended to a concentration of 0.25% in the serum diluent. The serum diluent was used to prevent the spontaneous agglutination of the tanned cells.

In a microtitre plate with U-shaped wells the agglutination reaction was set up with:

- 50 μl serum diluent per well
- 50 μl control or test sera, serially diluted
- 50 μl antigen-coated or non-coated cells (in the controls).

The plate was then sealed, agitated gently and incubated at room temperature for 3 hours and overnight at 4°C before the agglutination was recorded. Negative reactions were recorded by a button at the bottom of the wells. The presence of antibody in the test sera was shown by a ring of agglutinated SRBC.

2. (xi) Autoradiography.

The incorporation of [3H] -Thymidine into the DNA of proliferating cells has been widely used to assess the proliferative phases in the immune response. The histological method used here allows the spatial distribution of proliferation to be detected as well as the levels. The method employed was modified from that reported by Turner & Manning (1973).

Animals were injected with 1μCi g$^{-1}$ body weight of [3H] -Thymidine as previously stated. The injections were given either 4 or 18 hours
before sacrifice. Tissue was then removed and fixed either in Carnoy's fixative or in 10% Neutral Buffered Formalin. The tissue was then wax-embedded and sections, cut at 5 μm, mounted on 0.5% gelatin coated slides. The slides were gelatin coated to aid the adhesion of sections and the photosensitive gel emulsion.

Sections were dewaxed, hydrated and dipped in a mixture of 0.5 cm$^3$ glycerol, 23.5 cm$^3$ double distilled water and 24 cm$^3$ of K5 emulsion in gel form (Ilford Nuclear Research, U.K.) at 43°C. They were then left to dry on ice-cooled trays before being packed into light-proof boxes with silica gel to maintain low humidity. Slides were stored for 4 weeks before developing.

The slides to be developed were moistened in tap water and placed in D-19 Developer (Kodak) for 4 minutes at 18-20°C. before being rinsed and moved to the fixative for 5 minutes. The fixative was 500 cm$^3$ AMFIX (May & Baker Ltd., Dagenham, U.K.), 3 dm$^3$ water and 87.5 cm$^3$ 'S' type hardener (May & Baker Ltd., Dagenham, U.K.). The sections were then rinsed in running tap water for 15 minutes and stained with Methyl Green-Pyronin. Proliferation was then assessed by counting nuclei marked with aggregations of silver grains.

All dark room procedures were performed under the brown safelight Ilford S 902.

2, (xii) Methyl Green-Pyronin staining.

Hydrated sections were stained in Methyl Green-Pyronin Y for 20 - 30 minutes, differentiated in ice-cold water and dehydrated by two 30 second washes in 2-methylpropan-2-ol. Then they were cleared in Xylene and mounted in DPX.
APPENDIX I

**Amphibian Culture Medium**

L 15, Leibovitz medium with L-glutamine (Gibco, Paisley, Scotland).

Double distilled water
2 cm³

0.2M NaHCO₃
7 cm³

IM HEPES, pH 7.3 (Gibco, Paisley, Scotland).

2-mercaptoethanol, 0.34% v/v in water
0.2 cm³

Foetal Calf Serum (Gibco, Paisley, Scotland).

Penicillin & Streptomycin, 10,000 iu cm⁻³ (Gibco, Paisley, Scotland).

**Xenopus Culture Medium**

Sterile Amphibian Culture Medium 40 cm³

Normal heat inactivated *Xenopus* serum 0.2 cm³

All culture media were sterilized by passing through a 0.22 μm Millipore filter.
APPENDIX II

Phosphate Buffered Saline pH 7.2

NaCl 8.50 g
Na₂HPO₄•12H₂O 3.22 g
KH₂PO₄ 0.20 g

Make up to 1 dm³ in distilled water and adjust pH.

Phosphate Buffered Saline pH 6.4

0.15M KH₂PO₄ 67.8 cm³
0.15M Na₂HPO₄ 32.2 cm³
0.85% NaCl 100.0 cm³

Adjust pH.
CHAPTER 3

An Examination of the Effect of High Zone Tolerance to Human-γ-globulin on the Serum Antibody Titres and Proliferative Responses in the Spleen.
INTRODUCTION

The humoral immune response in *Xenopus laevis* has been extensively studied by a number of workers and a picture has emerged of a system which appears to possess the basic capabilities of the more advanced vertebrates. Thus *Xenopus* is able to recognize foreign antigen determinants and mount specific antibody responses to them, albeit more slowly than mammals, with the primary immune response taking approximately 4 - 5 weeks to develop (Lykakis, 1969; Manning & Turner, 1972). The adult toad is able to synthesize both IgM and the low molecular weight IgY (Marchalonis, Allen & Saarni, 1970; Hadji-Azimi, Brandt, Bossus & Michea-Hamzehpour, 1976). These two immunoglobulin classes are also evident in larvae, with IgM being predominant and IgY, where detectable, of low affinity (reviewed by Du Pasquier, 1982). A third immunoglobulin class has now been detected in *X. laevis*, designated IgX (Hsu, Flajnik & Du Pasquier, 1985). It has been found (Kidder, Ruben & Stevens, 1973) that larvae were unable to mount a rosette-forming cell response against sheep erythrocytes (SRBC) until larval stages 49 - 53 of Nieuwkoop & Faber (1967). The incomplete switch to low molecular weight immunoglobulin production occurs in the secondary response and thereafter both classes are produced. There is some evidence (Hadji-Azimi & Parrinello, 1978) that in *X. laevis* a high proportion of antibody producing cells produce both the 19S (IgM) and the 7S (IgY) immunoglobulin classes simultaneously throughout the primary and secondary response.

The switch from 19S to 7S antibody production following secondary antigen stimulation has been used as the criterion for the memory response in *Bufo marinus* by Marchalonis & Germain (1980). This anamnestic response is well documented in *Xenopus* with larvae capable of being primed at stage 48 (Jurd, Luther-Davies & Stevenson, 1975).
This response may be conserved through metamorphosis as shown by Du Pasquier & Haimovich (1976) and Du Pasquier (1982) and is also manifest in the adult response to TNP-Ficoll as with the mammals (Balls, Ruben & Clothier, 1986).

While positive anamnesis to foreign protein antigens is well documented in amphibians, the mechanisms of induction and maintenance of tolerance to foreign protein antigens have not been so extensively examined. Marchalonis & Germain (1980) found that high doses of soluble protein administered to the adult Bufo marinus resulted in suppression of antibody production. In work done in this laboratory Mughal (1984) and Al Johari (1985) produced tolerance to high doses of soluble protein antigen injected into larval X.laevis according to the procedure shown in Fig. 3.1. It is necessary at this stage to qualify the term 'tolerance' as used in the context of this chapter. It describes the condition in which the stimulated immune system does not proceed through the response to the specific production of antibody. It does not necessarily imply that the phenomenon is the same as that shown by tolerant mammals.

The spleen of X.laevis is organized into follicular (white pulp follicles) and non-follicular (red pulp) regions. The white pulp follicles are discrete areas with a central arteriole, delineated by the boundary layer. Each is surrounded by a perifollicular lymphocyte rich zone (Turner & Manning, 1973). The white and red pulp areas were examined individually in the present experiments.

In X.laevis, antigen is localized in the spleen (Manning & Turner, 1972; Collie, 1974; Horton & Manning, 1974a), in a manner which appeared very similar to that in rats (Nossal, Austin, Pye & Mitchell, 1966). Persistence of antigen in association with high
antibody titres 9 weeks after the administration of HGG in FCA, is a possible indication of antibody/antigen complexes formed in the Xenopus spleen (Collie & Turner, 1975). A primitive dendritic splenocyte has been found by Baldwin & Cohen (1981) in Xenopus, which appears to trap foreign protein antigens. These cells were located in the splenic white pulp follicles near the boundary layer and had developed their mature morphology and location by approximately larval stage 59 (Baldwin & Sminia, 1982). Mughal (1984) showed a peak of antigenic localization in the periphery of the white pulp follicles 3 - 4 weeks after antigenic challenge. The localization of antigen was more rapid when antibody/antigen complexes were administered instead of the antigen alone. This accelerated localization of antigen indicated immune complex trapping.

Proliferation in the spleen in response to antigen was noted 3 - 4 weeks after challenge (Turner & Manning, 1973), at the same time as the peak of antigen localization. A proliferative response has also been observed in the spleens of carp. This response was still apparent in carp rendered tolerant to HGG (Mughal, 1984). In the present work the X.laevis spleen was examined for the presence of a proliferative response in the high-zone tolerant animals.

A number of workers have indirectly shown differences in distribution of T- and B-lymphocyte types in the spleen. Early thymectomy of X.laevis larvae inhibits T-dependent antibody production in toadlets (Turner & Manning, 1974), and has been shown to result in a striking reduction of lymphocytes from the perifollicular zones of the spleen (Horton & Manning, 1974b; Tochinai, 1976), thereby indicating a T-dependent zone. There is also some evidence of a difference of distribution from the spleens of normal challenged animals
which show high concentrations of proliferation in the white pulp follicles (Turner & Manning, 1973), and Obara (1982) has suggested the preferential localization of T-independent lymphocytes in splenic white pulp, with the possibility of T-dependent lymphocyte localization in the red pulp.

The presence of the suppressor T-cell is now well documented in the mammalian literature (Gershon & Kondo, 1971; Feldmann, 1973; Asherson & Zembala, 1976; Waksman, 1977), see chapter 1. Consequent to this the immunoregulatory function of suppressor T-cells is now widely recognized in both cell-mediated and humoral mammalian responses (Pierce et al., 1975). It appears that there are two types of suppressor, each having its own mode of action (Taylor & Basten, 1976). These are the antigen specific suppressor T-cells, which can interact with helper T-cells or with the target B-cells, and the idiotype specific suppressor cell, which binds directly to the receptors of target cells and does not require the presence of antigen to produce suppression. It was noted by Caulfield et al. (1983) that the presence of antibody/antigen complexes appeared to be important in the stimulation of suppressor cells; this observation is consonant with the immunoregulatory function of these cells.

In X.laevis a suppressor T-cell population was implicated in the adoptive transfer experiments of Du Pasquier & Bernard (1980), in which active suppression of adult allograft rejection was achieved by the inoculation of metamorphic lymphocytes. The possibility of adoptively transferring high-zone tolerance to the T-dependent antigen, HGG, has been examined in the present experiments. The successful transfer of this tolerant state using stimulated lymphocytes would point to the presence of a suppressor population supporting it (Gershon & Kondo, 1971;
The time schedule for the inoculation of donor cells and the administration of antigenic challenge in the present experiments was based upon two sets of data. Firstly, the stimulated donor cells, previously rendered tolerant to HGG, were inoculated into the MHC-compatible hosts 21 days after antigenic stimulation. This timing was selected owing to the presence of a proliferating population at this time in tolerant animals (see chapter 4, section 411). Secondly, Cribbin (1984) found that thymocytes injected via the dorsal lymph sac localized rapidly in the spleen and remained for at least 6 days. This has formed the basis for the administration of antigenic challenge to the transferred cells.
MATERIALS AND METHODS

3. (i) Duration of high-zone tolerance to Human-γ-globulin.

High-zone tolerance to Human-γ-globulin (HGG) was induced in Xenopus laevis by injecting the dose of 1 mg g⁻¹ body weight of HGG in saline into the peritoneal cavity (as stated in chapter 2) at larval stage 52-54 (Nieuwkoop & Faber, 1967). The dose was repeated 28 days later at stage 54-56 before the animals began to metamorphose, according to the method used by Mughal (1984) and Al Johari (1985), (see Fig. 3.1).

In this experiment the animals were then challenged at 1, 3, 4 and 7 months post-metamorphosis with the standard immunogenic dose of 0.025 mg g⁻¹ body weight of HGG in Freund's Complete Adjuvant (FCA), administered via the dorsal lymph sac. The animals were sacrificed 28 days later and the serum tested for antibody specific to HGG by the passive haemagglutination method described in chapter 2.

3. (ii) Tolerogenic effect of a single high dose of HGG.

A single antigenic administration of HGG in saline was given to larvae at stages 54-56 at the standard tolerogenic dose of 1 mg g⁻¹ body weight of antigen, (see Fig. 3.2). These animals were allowed to metamorphose and were challenged one month later with HGG in FCA to test for the presence of circulating antibodies to HGG. Controls were previously untreated animals challenged with HGG in FCA or saline in FCA.

3. (iii) Examination of the proliferative response following challenge in the spleens of animals rendered tolerant to HGG.
FIG. 3.1  Protocol for the induction of high-zone tolerance to Human-γ-globulin in *X. laevis*.
FIG. 3.2  Protocol for the investigation of the tolerogenic effect of a single injection of antigen.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>54-56</td>
<td>0</td>
</tr>
<tr>
<td>62</td>
<td>28</td>
</tr>
<tr>
<td>56</td>
<td></td>
</tr>
</tbody>
</table>

**Key**
- Injection into *X. laevis*
- HGG/Saline
- HGG/FCA
- Metamorphosis
- Ip: Intraperitoneal injection
- Dis: Injection via dorsal lymph sac
- X: Sacrifice
Outbred *X. laevis* were rendered tolerant to Human-γ-globulin by the method described in section 3 (i) and shown in Fig. 3.1.

To examine the proliferative activity in the spleens of these animals, toadlets of approximately 10 g were then challenged with the standard immunogenic dose of HGG in adjuvant. These animals were then injected 20 days later with 1 μCi g⁻¹ body weight of [³H] -Thymidine and sacrificed 18 hours later. The blood was removed and serum from each sample collected to be tested by passive haemagglutination for the presence of circulating antibodies specific to HGG. The spleens were fixed in 10% Neutral Buffered Formalin, wax embedded and sectioned for autoradiography as described in the general methods, chapter 2. Sections of 5 μm were cut serially through the spleens and examined every 40-50 μm. Proliferating cells were counted at ×400 magnification over the whole section. Results have been recorded as means per mm² with the field of view of the Zeiss microscope used being 0.15 mm² at ×400 magnification.

Control animals in the experiment included a group injected with saline as larvae and given the standard challenge dose as toadlets. A second group consisted of untreated animals.

3. (iv) **Differential distribution of proliferating nuclei subsequent to antigenic challenge in the spleens of animals rendered tolerant to Human-γ-globulin as larvae and in their saline injected controls which were able to respond to this challenge with antibody production.**

An examination of the autoradiographic material prepared in section 3 (iii) was made using the Kontron Image Processing System (used by courtesy of the Institute for Marine Environmental Research, Plymouth, U.K.).
Sections were studied every 40-50 μm through the spleens and 5 fields of view per section examined at x400 magnification. For each field of view (an area of $4.2 \times 10^{-2} \text{ mm}^2$) the percentage of the follicular areas was measured, see Plate 3.1. Proliferating nuclei in the follicular and non-follicular areas were counted under phase-contrast lighting. The animals assessed by this method were:

a) Those rendered tolerant to HGG as larvae and challenged with HGG in FCA as toadlets.

b) Those injected with saline as larvae and challenged with HGG in FCA as toadlets.

c) Untreated animals given no challenge.

3. (v) Adoptive transfer of high-zone tolerance to HGG using pre-stimulated cell populations.

Inbred JJ-strain *X. laevis* were rendered tolerant to the high dose of HGG as larvae, by the method shown in Fig. 3.1. These animals were used as toadlets of size 4-15 g, four months post-metamorphosis. They were stimulated with the standard challenge dose of HGG in adjuvant injected into the dorsal lymph sac.

The spleens and thymi from these animals were removed 21 days after challenge and pooled cell suspensions of these histocompatible animals made up within the groups as described in chapter 2 in a sterile amphibian dilution of L15. The thymus cell suspensions were made by macerating the paired organs in 1 cm³ of the sterile amphibian dilution of L15 in a solid watch glass. The debris was allowed to settle for approximately 20 seconds and the cell suspension then withdrawn to be pooled with those from other members of the group. The concentrations were adjusted to approximately $8.5 \times 10^7$ cells cm$^{-3}$.
PLATE 3.1 Autoradiograph of T.S. through spleen of normal *X. laevis* toadlet, 21 days after antigenic challenge, showing the follicular area studied, fo. (Assessed by Kontron Image Processing System).
for the spleens and $1.2 \times 10^8$ cells cm$^{-3}$ for the thymi. They were injected at a volume of 10 $\mu l$ g$^{-1}$ body weight into histocompatible hosts of like size and age. The hosts were challenged with HGG in FCA at the same time as the cellular inoculation, (Group A), 7 days after (Group D) and 7 days before (Group E), see Figs. 3.3 and 3.4.

The hosts were injected with [$^3$H] -Thymidine 27 days after the challenge injection and sacrificed 18 hours later. The blood was collected to be tested for circulating antibody by the passive haemagglutination method.

Most of the cellular inoculations were made through the dorsal lymph sac. However, in Group A the cells were inoculated at the same time as the antigen/adjuvant emulsion and so had to be administered by an alternative route. The dorsalis pedis vein was chosen.

Control groups were: Group B. The HGG challenge to the hosts was followed 7 days later by the inoculation of cells via the dorsalis pedis vein. This was done to check that any effect produced in Group A was due to the simultaneous timing of HGG challenge and cellular inoculation and not only to the use of the intravenous inoculation route compared to the dorsal lymph sac route.

Group C. The specificity of the adoptive transfer of tolerance was checked by challenging animals inoculated intravenously with HGG stimulated histocompatible cells taken from animals rendered tolerant to HGG (at timing A) with Keyhole Limpet Haemocyanin (Behring Diagnostics, La Jolla, CA) in Freund's Complete Adjuvant. These animals were tested 28 days later for the presence of serum antibody to Keyhole Limpet Haemocyanin and to HGG, see Fig. 3.3.
KEY FOR FIGS. 3.3 AND 3.4

Injection into high-zone tolerant donor JJ-Xenopus laevis.

Injection into host JJ-Xenopus laevis.

JJ-strain donor cells rendered tolerant to HGG and stimulated with HGG/FCA.

HGG/FCA administration.

KLH/FCA administration.

iv Intravenous inoculation route.

dls Dorsal lymph sac injection route.

[³H] -Thymidine administration.

Sacrifice.
FIG. 3.3 Protocols for the transfer of high-zone tolerance between histocompatible *X. laevis* toadlets using the intravenous route for cellular inoculations.
FIG. 3.4 Protocols for the transfer of high-zone tolerance between histocompatible *X. laevis* toadlets using the dorsal lymph sac route for cellular inoculations.
To ensure that there was no residual antigen effect on the hosts resulting from the stimulation of the donor cells in vivo with HGG in FCA, the stimulated donor cells were inoculated into the hosts and no subsequent HGG challenge was administered to these hosts. They were tested 28 days after the cellular inoculation for the presence of serum antibody to HGG, see Fig 3.4.

Groups G and H. Outbred *X. laevis* of the same size and age as the experimental JJ-strain *X. laevis* given no larval pre-treatment with antigen were challenged with HGG in FCA or group H, saline in FCA, to check the normal responses 3 and 4 weeks after challenge.

Group I. This group was the same as group G except that Keyhole Limpet Haemocyanin (KLH) was used as the antigen and the animals were killed 4 weeks after challenge only.

* A control to ensure that there was no residual antigen effect was considered to be necessary owing to the work of Mughal (1984) who was able to obtain the production of anti-HGG antibody in host *X. laevis* after the administration of donor antigen-trapping cells with HGG trapped onto these cell surfaces.
RESULTS

3.(i) Duration of high-zone tolerance to Human-γ-globulin.

Animals rendered larvally tolerant to high doses of HGG were tested at 1, 3, 4 and 7 months post-metamorphosis for the presence of circulating antibody, 28 days after a challenge of HGG in FCA. The results in Table 3.1 show that there was no production of antibody in these tolerant animals, a condition which persisted until at least 8 months after the last exposure to the antigen.

The controls, previously untreated animals challenged with HGG in FCA, responded with good levels of antibody production detectable in dilutions up to 1/1024 of the test sera. Those given saline in FCA produced no antibody specific to HGG.

3.(ii) Tolerogenic effect of a single high dose of Human-γ-globulin.

The efficacy of a single high dose of HGG injected at the later larval stage (54-56) for the induction of tolerance to this antigen is shown in Table 3.2. Results show no detectable antibody titres in 8/10 of the experimental toadlets 28 days after challenge with HGG in FCA. The controls all showed reasonable circulating antibody titres detectable in dilutions of up to 1/256 of the test sera.

3.(iii) Examination of the proliferative response following challenge in the spleens of animals rendered tolerant to HGG.

Three weeks after the introduction of the antigenic challenge the spleens exhibited a marked proliferative response in the animals previously rendered tolerant to that antigen as well as the controls which were able to respond to challenge with antibody production. Table 3.3 shows the antibody titres produced in the 3 groups of
<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>Serum antibody -log₂ antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGG tolerised <em>X. laevis</em> challenged with HGG/FCA 1 month post-metamorphosis.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>HGG tolerised <em>X. laevis</em> challenged with HGG/FCA 3 months post-metamorphosis.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>HGG tolerised <em>X. laevis</em> challenged with HGG/FCA 4 months post-metamorphosis.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>HGG tolerised <em>X. laevis</em> challenged with HGG/FCA 7 months post-metamorphosis.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Saline injected <em>X. laevis</em> challenged with HGG/FCA 1-7 months post-metamorphosis.</td>
<td>10, 8, 7, 10, 9, 7</td>
</tr>
<tr>
<td></td>
<td>9, 8, 8, 9, 7, 4</td>
</tr>
<tr>
<td></td>
<td>7, 7, 2, 7, 5, 8</td>
</tr>
<tr>
<td>Untreated <em>X. laevis</em> injected with Saline/FCA 1-7 months post-metamorphosis.</td>
<td>0, 0, 0, 0, 0</td>
</tr>
<tr>
<td></td>
<td>0, 0, 0, 0, 0</td>
</tr>
<tr>
<td></td>
<td>0, 0, 0, 0, 0</td>
</tr>
</tbody>
</table>

**TABLE 3.1** Duration of high-zone tolerance to Human-γ-globulin. Serum antibody titres measured 28 days after challenge.

₀ = no detectable agglutination
Experimental treatment

<table>
<thead>
<tr>
<th>Serum antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-\log_2) antibody titre</td>
</tr>
<tr>
<td>----------------</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Larvae stage 54-56 injected with single dose of HGG in saline of 1 mg g(^{-1}) body weight.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Challenged 1 month post-metamorphosis with HGG in FCA</td>
</tr>
<tr>
<td>0, 0, 0, 0, 2, 3</td>
</tr>
<tr>
<td>0, 0, 0, 0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animals challenged with HGG in FCA approximately 1 month after metamorphosis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>7, 6, 5, 6, 6, 8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animals injected with saline in FCA approximately 1 month after metamorphosis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, 0, 0, 0, 0</td>
</tr>
</tbody>
</table>

**TABLE 3.2** Serum antibody titres measured 28 days after challenge in animals given a single high dose of HGG at larval stage 54 - 56.

0 = no detectable agglutination
### TABLE 3.3  Serum antibody titres in animals tested 21 days after antigenic stimulation.

<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>Serum antibody -log$_2$ antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals rendered tolerant to HGG challenged with HGG/FCA</td>
<td>0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>Saline injected animals challenged with HGG/FCA</td>
<td>3 0 5 0 4 5</td>
</tr>
<tr>
<td>Previously untreated animals given no antigenic challenge.</td>
<td>0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

θ = no detectable agglutination.
animals used. It may be noted that the antibody levels were low in the responding animals at this time (21 days after challenge).

In Table 3.4 the mean number of proliferating nuclei per mm² is given for animals within the 3 groups discussed. From Table 3.4 it can be seen that the highest levels were recorded for those animals rendered tolerant to HGG prior to challenge and the lowest levels for those not exposed to this antigen at any time. An analysis of variance shows a highly significant difference between the 3 groups at a probability level of 0.001.

3, (iv) Differential distribution of proliferating nuclei subsequent to antigenic challenge in the spleens of animals rendered tolerant to Human-γ-globulin as larvae and their saline injected controls which were able to respond to this challenge with antibody production.

The differences in distribution of labelled nuclei in the spleens of animals able to respond to challenge with antibody production and those that cannot produce antibody subsequent to challenge were shown more clearly by the separate examination of the follicular and non-follicular areas. The data obtained from this examination are given in Table 3.5 which shows the proportion of labelled nuclei in the follicular and non-follicular areas of 1 mm² of spleen tissue. These data have been presented graphically in Fig. 3.5 to show the number of proliferating nuclei per mm² of follicular or non-follicular tissue thereby taking into account the change in percentage area of follicular tissue with treatment.

From Table 3.5 it can be seen that the unstimulated animals have considerably lower levels of proliferating nuclei in both the follicular and the non-follicular areas than either of the stimulated
<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>Proliferating nuclei (counts mm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals rendered tolerant to HGG and challenged with HGG/FCA</td>
<td>$\bar{x} = 703.44 \pm 31.39$</td>
</tr>
<tr>
<td>Saline injected animals challenged with HGG/FCA</td>
<td>$\bar{x} = 380.41 \pm 9.32$</td>
</tr>
<tr>
<td>Previously untreated animals given no antigenic challenge</td>
<td>$\bar{x} = 162.00 \pm 17.76$</td>
</tr>
</tbody>
</table>

**TABLE 3.4** Mean autoradiography counts mm$^{-2}$ in *Xenopus laevis* spleen 21 days after challenge with HGG in FCA.  
$\pm$ standard error
<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>% follicular area</th>
<th>Labelled nuclei per mm² spleen tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals rendered tolerant to HGG larvae and challenged with HGG/FCA as toadlets.</td>
<td>$\bar{x} = 34.90 \pm 1.29$</td>
<td>$\bar{x} = 307.13 \pm 44.25$</td>
</tr>
<tr>
<td>Larvae injected with saline and toadlets subsequently challenged with HGG/FCA.</td>
<td>$\bar{x} = 35.80 \pm 2.36$</td>
<td>$\bar{x} = 329.33 \pm 20.60$</td>
</tr>
<tr>
<td>Unstimulated animals.</td>
<td>$\bar{x} = 23.10 \pm 0.63$</td>
<td>$\bar{x} = 142.22 \pm 18.69$</td>
</tr>
</tbody>
</table>

**TABLE 3.5** Distribution of labelled nuclei in 1 mm² of spleen tissue 21 days after antigenic stimulation.

* ± standard error
FIG. 3.5 Concentration of labelled nuclei in 1 mm² of follicular or non-follicular spleen tissue 21 days after antigenic stimulation (± standard error).
groups. This low level of labelled nuclei can be seen clearly in Plate 3.2. Table 3.5 also shows that those animals rendered tolerant to HGG prior to challenge have higher levels of proliferating nuclei in the non-follicular areas than those which were able to respond with antibody production. There was a slight but insignificant increase in the number of labelled nuclei in the follicular areas of the antibody producing animals. The significance of these results was tested using the $\chi^2$ statistic at a probability level of 0.001. The Fig. 3.5 however, lends additional information to this picture. By considering the changes in the percentage follicular area it can be seen that although the total numbers of labelled nuclei per unit area were similar for both types of stimulated animal, those that were able to produce antibody had a significantly greater concentration of proliferating nuclei in the follicles. Plate 3.3 shows this characteristically increased level of proliferating nuclei in the spleen follicles of antibody producing animals. Plate 3.4 is an autoradiograph of the spleen of a challenged animal previously rendered tolerant to HGG. This shows considerably higher levels of labelled nuclei in the non-follicular spleen areas. The peri-follicular lymphoid tissue in particular had a large number of labelled nuclei but some were also scattered throughout the non-follicular "red pulp" tissue.

The maximum diameters of fixed spleen sections for these 3 groups are given in Table 3.6. An analysis of variance of these data does not show a significant difference at the probability level of 0.05. This suggests that the increase in follicular area noted in both the tolerant and the antibody producing animals compared to the unstimulated animals (shown in Fig. 3.5) is for the main part accounted
Autoradiograph of T.S. through spleen of normal unchallenged *X. laevis* toadlet; showing low levels of labelled nuclei (ln) in the white pulp follicle (fo) and little in the perifollicular zone (pz) and the remaining non-follicular tissue.

Autoradiograph of T.S. through spleen of normal *X. laevis* toadlet 21 days after antigenic challenge; showing high levels of labelled nuclei (ln) in the white pulp follicle (fo) and low levels of labelled nuclei in the non-follicular tissue.
PLATE 3.4  Autoradiograph of T.S. through spleen of HGG tolerant *X. laevis* toadlet, 21 days after antigenic stimulation with HGG in FCA; showing high levels of labelled nuclei (ln) in the non-follicular tissue, in particular in the perifollicular zone (pz).
<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>Maximum diameter mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals rendered tolerant to HGG and challenged with HGG/FCA.</td>
<td>$\bar{x} = 2.16 \pm 0.56$</td>
</tr>
<tr>
<td>Saline injected animals challenged with HGG/FCA.</td>
<td>$\bar{x} = 2.59 \pm 0.3$</td>
</tr>
<tr>
<td>Previously untreated animals given no antigenic challenge</td>
<td>$\bar{x} = 1.99 \pm 0.33$</td>
</tr>
</tbody>
</table>

**TABLE 3.6**  Maximum diameters of spleen sections (fixed in 10% Neutral Buffered Formalin) of stimulated and unstimulated animals tested 21 days after antigenic challenge.
for by a decrease in the non-follicular volume. This further indicates that the increase of labelled nuclei per unit area in non-follicular areas of the spleen of tolerant and antibody producing animals was partly due to a reduction in total non-follicular area. That this is not the entire answer is shown by Plate 3.4 which has already been noted to show differential concentration of labelled nuclei in the peri-follicular lymphoid tissue.

3, (v) Adoptive transfer of high-zone tolerance to Human-γ-globulin using pre-stimulated cell populations.

The antibody titres produced by the JJ-strain stimulated donor animals, previously rendered tolerant to HGG, are shown in Table 3.7. It can be seen that all animals previously rendered tolerant to HGG (36), except for one in group A which exhibited a low antibody titre, were producing no antibody at this time 21 days after antigenic challenge. Group G shows that there were significant levels of circulating antibody at this time in animals that had not been rendered tolerant to the antigen prior to challenge. From previous experiments it is known that in _X.laevis_ these serum antibody levels will go on to peak 6 - 8 weeks after challenge. These results serve only to check that the inoculated donor cells were taken from animals which were tolerant at this time - at least at the level of antibody production. The animal in group A producing a low antibody titre may have been a poor responder (group G shows that there is a reasonable degree of variability present in the normal response) or it may have been partially tolerant to HGG.

The serum antibody titres produced by histocompatible host animals after transfer of antigen stimulated cells taken from tolerant animals
<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>Serum Antibody -log₂ antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A JJ-strain <em>X.</em> <em>laevis</em> donors, rendered tolerant to HGG and challenged with HGG/FCA.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Group B JJ-strain <em>X.</em> <em>laevis</em> donors, rendered tolerant to HGG and challenged with HGG/FCA.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Group C JJ-strain <em>X.</em> <em>laevis</em> donors, rendered tolerant to HGG and challenged with HGG/FCA.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Group D JJ-strain <em>X.</em> <em>laevis</em> donors, rendered tolerant to HGG and challenged with HGG/FCA.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Group E JJ-strain <em>X.</em> <em>laevis</em> donors rendered tolerant to HGG and challenged with HGG/FCA.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Group F JJ-strain <em>X.</em> <em>laevis</em> donors rendered tolerant to HGG and challenged with HGG/FCA.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Group G Animals challenged with HGG/FCA.</td>
<td>3, 5, 2, 4, 5, 0</td>
</tr>
<tr>
<td>Group H Animals challenged with saline/FCA.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
</tbody>
</table>

**TABLE 3.7** Serum antibody levels measured 21 days after challenge in donor animals prior to the inoculation of the cells into histocompatible hosts.

0 = no detectable agglutination.
are shown in Tables 3.8, 3.9 and 3.10. These results show that the adoptive transfer of this high-zone tolerance was possible using spleen cells injected via the dorsalis pedis vein at the same time as the challenge of HGG in FCA was administered via the dorsal lymph sac. (see Table 3.8). No antibody titres were recorded using thymus cells in this schedule for 2 out of 5 animals. Other timings for the presentation of antigenic challenge appeared to be ineffective in the transfer of this tolerance as shown in Table 3.9. The transfer of tolerance via the intravenous route was not effective when administered 7 days after challenge although a significant reduction in antibody titres was observed (using an analysis of variance at the probability level of 0.05).

Group F included one animal from which a low antibody titre was registered 4 weeks after the injection of stimulated cells previously rendered tolerant to the antigen, (see Table 3.9.) This suggests the possibility of some residual antigen effect in the responding animals but does not alter the nil response to HGG challenge offered by those animals rendered tolerant to HGG.

The specificity of the adoptively transferred tolerance was tested by challenging with KLH, a group rendered tolerant to HGG. After 28 days the anti-KLH antibody titres in the sera of these animals were found to be greater than 1/262144 see Table 3.10. So those animals incapable of producing antibody to HGG were able to give a good response to KLH. It is apparent from Table 3.10 that antibody levels against KLH in X.laevis were considerably higher than those against HGG in normally responding animals.
### Table 3.8

Serum antibody levels measured 28 days after challenge in animals inoculated with MHC compatible cells rendered tolerant to HGG. All cells injected intravenously.

<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>Serum antibody levels (-\log_2) antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen cells injected</td>
</tr>
<tr>
<td>A. JJ-strain host + JJ-strain donor cells (rendered tolerant to HGG and stimulated with HGG/FCA) injected (i.v.) + HGG/FCA (dls) challenge at the same time.</td>
<td>(0, 6, 0, 0, 0, 0)</td>
</tr>
<tr>
<td>B. JJ-strain host + JJ-strain donor cells (rendered tolerant to HGG and stimulated with HGG/FCA) injected (i.v.) 7 days after challenge of HGG/FCA (dls).</td>
<td>(7, 8, 10, 9, 7, 11)</td>
</tr>
<tr>
<td>C. JJ-strain host + JJ-strain donor cells (rendered tolerant to HGG and stimulated with HGG/FCA) injected (i.v.) at the same time as challenge of KLH/FCA (dls).</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Tested for HGG antibody</td>
</tr>
<tr>
<td></td>
<td>(&gt;18, &gt;18, &gt;18)</td>
</tr>
</tbody>
</table>

\(\emptyset\) = no detectable agglutination  
n. d. = not done
**Experimental treatment** | **Serum antibody levels -log2 antibody titre**
---|---
D. JJ-strain host + JJ-strain donor cells (rendered tolerant to HGG and stimulated with HGG/FCA) injected (dls) + HGG/FCA 7 days later. Tested for HGG antibody | Spleen cells injected: 5, 10, 5, 7, 9, 4 | Thymus cells injected: 6, 7, 7, 6, 5, 7
E. JJ-strain host + JJ-strain donor cells (rendered tolerant to HGG and stimulated with HGG/FCA) injected (dls) 7 days after challenge of HGG/FCA. Tested for HGG antibody | | Spleen cells injected: 0, 8, 7, 6, 6, 8 | Thymus cells injected: 7, 6, 2, 6, 6, 7
F. JJ-strain host + JJ-strain donor cells (rendered tolerant to HGG and stimulated with HGG/FCA) injected (dls). No challenge. Tested for HGG antibody | | Spleen cells injected: 0, 0, 0, 0, 4 | Thymus cells injected: 0, 0, 0, 0, 0

**TABLE 3.9** Serum antibody titres measured 28 days after challenge in animals inoculated with MHC compatible cells rendered tolerant to HGG. Cells injected via the dorsal lymph sac.

Θ = no detectable agglutination
Serum antibody titre

<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>Serum antibody (-\log_2) antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>G: Outbred <em>X. laevis</em> injected with HGG/FCA (dls). Tested for HGG antibody</td>
<td>7, 11, 12, 14, 9, 10</td>
</tr>
<tr>
<td>H: Outbred <em>X. laevis</em> injected with saline/FCA (dls). Tested for HGG antibody</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>I: Outbred <em>X. laevis</em> injected with KLH/FCA (dls). Tested for KLH antibody.</td>
<td>&gt;18, &gt;18, &gt;18, &gt;18, &gt;18, &gt;18</td>
</tr>
</tbody>
</table>

**TABLE 3.10** Serum antibody titres measured 28 days after antigenic challenge in control animals where no cells were injected.

\(\Theta\) = no detectable agglutination.
DISCUSSION

In this laboratory high-zone tolerance to the T-dependent antigen HGG, has been induced by the administration of two doses of antigen into larvae, at a level capable of inducing tolerance in the adult (Mughal, 1984; Al Johari, 1985). Mughal (1984) also showed that these larvae were not susceptible to tolerization by the lower dose of antigen, which is immunogenic in the adult but would be tolerogenic in the young mammal. The current work has suggested that a single large dose of antigen administered in late stage larvae also induces tolerance at the level of antibody production.

High doses of antigen are required to induce tolerance in B-cells owing to the difficulty of rendering mature B-cells specifically unresponsive to antigen. Considerably lower doses will be effective in rendering tolerant the helper T-cell population. Similarly the immature B-cell is highly susceptible to tolerogenesis in mammals. It has been considered that the immature, developing animal will have a higher proportion of immature lymphoid cells within its system and hence the rationale for the larval induction of tolerance in X.laevis.

The X.laevis larvae have not achieved the mature capacity for antibody response at the time of their first antigen dose (stage 52-54). The second dose is likely to persist at a level sufficient to maintain high zone tolerance after the level of relative immaturity has been passed. It would seem then, that using two doses of antigen in these larvae, could result in the activation of two mechanisms of tolerance induction; one based on immunomaturity, and one based on high doses of antigen. The larvae at stage 56 have rising percentages of lymphocytes in their bodies which reach a larval peak at stages 56 - 58
(Du Pasquier, 1982). Thus, the high dose of antigen injected at stage 52 is rapidly diluted in the body tissues, perhaps to levels below those required to be effective on the immature B- or the helper T-cells produced. The large antigen doses administered at stage 56, however, will be introduced into animals with near optimum larval lymphocyte-to-body weight ratios and increasing numbers of mature cells. Under these circumstances the advantage of using an immature system is not apparent, as cells rendered tolerant in this manner will be relatively few in number. Complete tolerance to the high dose of antigen in these older larvae is achieved probably in the same manner as in the adult.

The antigenic challenge causes a proliferative response 21 days later, even in tolerant animals as shown in Table 3.4. This table shows that the levels of proliferation were significantly higher in the tolerant animals than in the antibody producing responders. Table 3.5 shows that in the same animals as used to obtain the data offered in Table 3.4, higher levels of labelled nuclei were recorded. This was due to the use of phase-contrast lighting, which revealed the more lightly labelled nuclei resulting from daughter cell generations. This indicates that absolute numbers should not be compared where different methods of recording the numbers of labelled nuclei have been employed. In chapter 4 an assessment of proliferation, in the spleens of tolerant and antibody producing animals subsequent to antigenic challenge, was made after the spleen cells were separated on a BSA discontinuous density gradient. These results revealed similar proliferative levels in tolerant and responding animals, whereas the data shown in Table 3.4 indicates heightened proliferative levels in tolerant animals. It is not possible to resolve this discrepancy with the experimental data here available. There is a
considerable loss of cells on the BSA gradient, e.g. those entering the pellet, as has also been noted by other workers (Donnelly, Manning & Cohen, 1976; Shortman, 1968). Any selectivity in this cell loss with respect to the layers under investigation is not immediately apparent. At the same time, the analysis of the follicular and non-follicular areas of the spleen could be affected by disproportionate shrinkage of follicular and non-follicular tissue. This could be further complicated by the different experimental procedures to which the animals were subjected. It can be concluded that the proliferation levels present in tolerant animals are at least as high, if not higher, than those present in antibody producing responding animals. Although this is an unexpected result it is a similar phenomenon to that observed by Mughal (1984) in *Cyprinus carpio*.

The proliferative activity observed in the spleens of tolerant animals 21 days after antigenic stimulation, could reflect the presence of any one or a number of T-cell populations, in particular, suppressor T-cells.

When the spleens were looked at more closely it was noted that there was a real difference in the distribution of proliferating cells. Also, the follicular areas were significantly larger in stimulated animals (both tolerant and responding). The antibody producing responder animals had higher concentrations of proliferating cells in the follicular tissue. The tolerant animals had higher concentrations of proliferating cells in the perifollicular zones than the non-tolerant animals 21 days after antigenic challenge. These zones were shown by Manning (1975) to be T-dependent zones. These results would not conflict with the proposition that the proliferating populations in these tolerant animals were either suppressor T-cells or some other
T-cell types; remembering that they are not seen in such numbers at this time in unstimulated animals nor those that are able to respond to challenge with antibody production. This could be because suppressor T-cells proliferate at the same time as other T-cell subsets, thereby producing an overall increase in total numbers. Alternatively, the proliferation observed in the perifollicular zone of tolerant animals could be visible at this time, because the induction of the tolerant state has changed the kinetics of the immune response. Thus, sampling at day 21 after antigenic challenge could mean that two totally different parts of the response were being examined.

Specific tolerance to HGG was transferred, using stimulated spleen cells from animals rendered tolerant to HGG and histocompatible at the major loci, injected intravenously at the same time as the antigenic challenge. This transfer of the tolerant state, by using tolerant cells, indicates the presence of a suppressor T-cell population. The use of thymus cells instead of spleen cells, resulted in an impaired antibody response when the same experimental procedures were used. The effectiveness of thymocytes in this infectious tolerance may have been lower because of the lower proportion of immunocompetent cells present. Previous work by Ruben, Mette, Cochran & Edwards (1980) has shown that thymocytes were able to produce a suppressive effect, reducing splenic antigen-binding cell numbers and antibody production in experiments where adult spleen fragments were cultured in vitro with the thymus.

In conclusion, the presence of a suppressor T-cell population supporting the tolerance to HGG has been indicated by the infectious transfer of this tolerant state. The distribution of proliferative activity in the perifollicular zones of tolerant animals give additional
indication of one or more T-cell subsets, which are closely involved with the induction and maintenance of the tolerant state. Of the parameters examined, the timing was seen to be the most important. It is known that infectious tolerance in mammals, which is dependent on suppressor T-cells, will rapidly disappear when the cells are moved to a new system, if the antigen to which they are tolerant is not present.
CHAPTER 4

Discontinuous Density Gradient Separation of Whole Spleen Cell Populations Taken From Normal and HGG Tolerant Animals.
INTRODUCTION

The mature immune response is most intricate. The necessity for understanding its component mechanisms has generated wide research into its basic contents. To this end, various methods have been employed to isolate or enrich specific cell populations, either functionally or by physical characterization.

In vitro techniques of cell separation have utilized a wide range of cellular characteristics. There are the adherent properties of B-lymphocytes, which have been used to produce enriched populations (Julius, Simpson & Herzenberg, 1973). There is the fractionation of populations on immunoabsorbent columns (Schlossman & Hudson, 1973) and the flotation characteristics which make feasible the separation or enrichment of a number of lymphocyte sub-populations on density gradients. Lastly, an example of cellular characterization which must be included is the identification of a number of cell surface markers which permit the isolation of discrete lymphocyte subsets. The advent of flow cytometric analysis (Herzenberg, Sweet & Herzenberg, 1976), as used by Imai, Oguchi, Nakano & Osawa, (1979) in the production of a mouse suppressor T-cell subset, has facilitated the separation of highly purified cell populations. The development of an extensive repertoire of monoclonal antibody techniques, such as that used by Reinherz, Penta, Hussey & Schlossman (1981) for the isolation of both the human inducer T-cell subset (T4⁺) and a subset showing suppressor activity (T8⁺), and Haskins, Kubo, White, Pigeon, Kappler & Marrack (1983) in the isolation of the MHC-restricted antigen receptor on T-cells, has been of immense value in this area. These technical possibilities are now becoming available to X.laevis work.
A mouse monoclonal antibody XT-1 has been produced for JJ-strain *Xenopus* thymocyte surface determinants. Small lymphocytes are seen to be XT-1+ after stage 56 (Nagata, 1985).

Separation of cell populations on continuous and discontinuous density gradients has been performed using a number of buoyant density media. This can result in the production of populations whose flotation characteristics can be linked to their activity. For example, mouse Concanavalin A-induced helper and suppressor cells, separated on a Ficoll discontinuous gradient by Tse & Dutton (1976). Mughal (1984) was able to demonstrate an enriched population of splenic antigen-trapping cells obtained from *X. laevis* using a discontinuous Percoll density gradient. The Bovine Serum Albumin linear density gradient method used by Shortman (1968) showed that it was possible to separate a large number of rat lymphoid sub-populations on the basis of their density. Subsequent work by Kraft, Shortman & Marchalonis (1971) attempted to relate the separated sub-populations to specific activity. It was found possible, by Kraft & Shortman (1972) to suggest a sequence of antibody-forming cell maturation in the spleen of *Bufo marinus* by this method. The antibody-forming cells produced early in the response were relatively homogeneous and confined to the lighter density bands. This profile became more complex as the response developed, with antibody forming cells in the denser bands appearing later in the response. This method was adapted for use in *Xenopus laevis* by Donnelly, Manning & Cohen (1976). They were able to separate four lymphoid sub-populations on the basis of density and showed a density profile similar to that in mice. These workers also showed that thymectomy produced a marked reduction in mitogen responsiveness in the cell populations of the lighter density bands.
The present work is concerned with a closer study of the activity of *Xenopus* spleen cell populations enriched by this discontinuous density gradient centrifugation, both in cell-mediated responses and in populations taken from normal and HGG-tolerant unchallenged and challenged animals.
4.1 Discontinuous density gradient separation of spleen cells.

A stock solution of 35% w/v BSA (Bovine Serum Albumin, SIGMA fraction V, extensively dialysed), was made up in sterile amphibian culture medium (ACM). This was dissolved overnight at 4°C using a magnetic stirrer and then sterilized by passing through a 0.45 μm Millipore filter.

From the stock solution, dilutions of 31%, 27%, 23% and 15% w/v were made up in sterile ACM and 0.56 cm³ of each layered into a 5 cm³ polycarbonate MSE centrifuge tube and retained on ice, see Fig 4.1.

The spleen cell suspension was made up, as stated in chapter 2, in 1 cm³ of sterile ACM and carefully pipetted onto the BSA gradient. The gradient was then centrifuged for 30 minutes at 20,000g and 4°C.

This method produced 5 distinct cell populations at the density interfaces and a small pellet. The majority of the pellet was composed of erythrocytes. It was discarded. No erythrocyte contamination was found in the interface bands.

The cell populations were removed from each density interface and sterile ACM added to make the cell suspension volumes up to 2 cm³. These cell suspensions were then centrifuged for 5 minutes at 500 g and 4°C. The supernatants were then aspirated off and the remaining cells were washed once more with sterile ACM, and again centrifuged for 5 minutes at 4°C, this time at 350 g. It was found that the BSA was relatively easy to remove from the cells as compared to the colloidal PVP coated silica particles of the separation medium.
FIG. 4.1 Discontinuous Bovine Serum Albumin density gradient for the separation of spleen cell populations.
PERCOLL (SIGMA, St. Louis, MO). The cells were then counted and the viability assessed using 1% Trypan Blue dye exclusion. The viability was usually greater than 95% and the cell suspensions were not used when it was less than 90%.

4. (ii) **Autoradiographic study of proliferation in spleen cell populations separated on a BSA discontinuous density gradient.**

Outbred *X. laevis* were rendered tolerant to a high dose of HGG as described in chapter 2. These toadlets weighed 5-10 g at 4 months post-metamorphosis. At this stage they were challenged with HGG in FCA at the standard immunogenic dose of 0.025 mg g\(^{-1}\) body weight. This challenge was followed 20 days later by an injection of 1μCi g\(^{-1}\) body weight of \[^{3}H\] -Thymidine administered via the dorsal lymph sac. The animals were sacrificed 18 hours later and their spleens removed. Spleen cell suspensions were then made up in the Amphibian dilution of L\(_{15}\) (100 : 45) and separated on the BSA gradient. The blood was also collected at this time and the serum removed to test for HGG specific antibody by passive haemagglutination.

Cells collected from each interface of the density gradient were then resuspended in the diluted L\(_{15}\) and the concentration adjusted to 1x10\(^{6}\) cells cm\(^{-3}\). Cytospin smears were made by loading 0.1 cm\(^{3}\) of the cell suspension per cuvette funnel onto the cytocentrifuge head (Shandon Southern Cytospin centrifuge) and spinning at 600rpm for 5 minutes. Adherence of the cells was improved by first spinning a 1% BSA solution onto the slides cleaned with concentrated Hydrochloric acid. This was found necessary to ensure the adherence of cells through the processes of autoradiography. Gelatin coated slides, which are used as a matter of routine for
autoradiography, were not used here as the cells would not adhere satisfactorily to such dried, coated slides. Wet gelatin coated slides were also found to be unsuitable as the cells became partially obscured by a thin coating of gelatin.

The smears obtained were then air dried and fixed with methanol, hydrated and coated with photosensitive gel emulsion as described in the section in chapter 2 regarding autoradiography. They were stored for 4 weeks at 4°C before developing and staining with Methyl Green-Pyronin Y.

4.(iii) Changes in BSA separated spleen cell populations taken from the HGG stimulated animals.

Cytospin smears of the cell suspensions obtained from the five interfaces of the BSA gradient were produced using the 1% BSA adherence method as previously described. The smears were again air dried and fixed in methanol. They were then stained in May-Grunwald stain (BDH Chemicals, U.K.). After 1 minute an equal volume of distilled water was added to the stain with a pipette and mixed by rocking the slides gently. This diluted stain was left to act for a further 1 minute before draining. The smears were then counter-stained for 10-15 minutes in 10% Giemsa (R66, BDH Chemicals, made up in Giemsa buffer pH 6.5, BDH Chemicals, U.K.). The smear cells were then differentiated for approximately 5 seconds in distilled water, blotted and air dried before mounting in DPX.

The changes in cell populations were monitored for animals with an induced high zone tolerance to HGG, both unchallenged and 21 days after challenge with 0.025 mg g⁻¹ body weight of HGG in FCA.

These were compared with control non-tolerant animals also including challenged and unchallenged groups.
Efficacy of separated populations in the adoptive transfer of high-zone tolerance to HGG.

JJ-strain *X.laevis* toadlets rendered tolerant to a high dose of HGG as larvae (see chapter 3), were given a challenge injection of HGG in FCA via the dorsal lymph sac to stimulate proliferation of the population that has been detected in tolerant animals (see chapter 3). Following the same procedure as used in chapter 3, the spleens were removed from these donor animals 21 days after challenge and a cell suspension prepared as detailed in section 4(i). The blood was collected at this time from the donor animals and tested for the presence of serum antibody specific to HGG to check that they were tolerant at the level of antibody production.

The spleen cell suspensions were then separated on the discontinuous density gradient. The cells were pooled from interfaces 1 and 2 and mixed together in the amphibian dilution of L15 for each animal. These cells were then adjusted to a concentration of approximately $8 \times 10^7$ cells cm$^{-3}$. The cells were also pooled from interfaces 3 and interfaces 4 of each animal, and were treated in the same manner as those from 1 and 2 except that these populations were not mixed together. These cells were then injected into histocompatible hosts via the *dorsalis pedis* vein at a volume of 10 $\mu$l g$^{-1}$ body weight. At the same time the host animals were challenged with HGG in FCA via the dorsal lymph sac.

These animals were tested for the presence of circulating antibody specific to HGG, 28 days after antigenic challenge.
Activity of BSA density gradient separated spleen cell populations in mixed leukocyte culture.

After the separation of a whole spleen cell population on the BSA density gradient, 5 populations were removed from the gradient, washed clean of BSA and counted. At this stage cell viability was assessed using 1% Trypan Blue dye exclusion. This dye exclusion tested only for those cells with disrupted membranes, however, and so a more accurate assessment of viability in culture was necessary.

For this purpose the separated populations were adjusted to a concentration of $1 \times 10^6$ cm$^{-3}$ and 200μl of each population were cultured separately for 5 days in the same manner as the unstimulated controls, as in the normal MLR system used by Manning & Botham (1980).

In order to provide a base line of normal stimulation indices between JJ-strain and outbred *X. laevis*, a number of normal two-way mixed leukocyte reactions were set up as described by Manning & Botham (1980). Having obtained this data it was possible to proceed with the use of the BSA separated populations in conjunction with the normal MLR system. This was done in order to observe whether there was any detectable level of enrichment of cell populations following separation that could be responsible for the enhancement of the suppression of the normal mixed leukocyte reaction.

The spleens were removed from histocompatible animals (1 outbred animal and 2 JJ-strain animals) for this *in vitro* assessment of possible enriched cell populations and whole spleen cell suspensions or BSA density gradient separated cell suspensions prepared in *Xenopus* culture medium. The final cell concentrations were adjusted to $1 \times 10^6$ cells cm$^{-3}$. In some cases there were insufficient cells...
recovered from BSA density interface bands and these cell suspensions (usually from the first density interface) were discarded. Two types of cell culture were then set up.

The first type of culture consisted of a mixed leukocyte reaction between a whole spleen cell population of outbred *X. laevis* (population N) and a pooled spleen cell population of JJ-strain *X. laevis* (population Jp) to which was added one of the BSA density gradient separated interface populations of JJ-strain *X. laevis* (populations J1-5). The pooled spleen cell populations were made up from the 5 density interfaces according to the proportion of cells retrieved from each density interface band.

(a) 67 μl (N) + 67 μl (Jp) + 67 μl (Jp)
Stimulated culture controls.

(b) 67 μl (N) + 67 μl (Jp) + 67 μl (J1-5)
Experimental cultures.

(c) 200 μl (N)

(d) 200 μl (Jp)
Unstimulated controls.

(e) 200 μl (J1-5)

The second form of culture used was an intra-layer cross-culture. Both the outbred *X. laevis* and the JJ-strain *X. laevis* spleen cell populations were separated on the BSA density gradient for this. The following cultures were set up between corresponding interface populations.

(f) 100 μl (Np) + 100 μl (Jp)
Stimulated culture controls.

(g) 100 μl (N1-5)+100 μl (J1-5 )
Experimental cultures.

(h) 200 μl (N1-5)

(i) 200 μl (J1-5)
Unstimulated controls.
4. (ii) **Proliferation in spleen cell populations separated on a BSA discontinuous density gradient.**

Animals rendered tolerant to a high dose of HGG and then challenged with this antigen in the presence of Freund's Complete Adjuvant have been shown, in chapter 3, to exhibit a proliferative response which was strongly marked 3 weeks after the challenge dose. The flotation characteristics of these proliferating cells have been looked at more closely by using the BSA density gradient separation and the results of these are shown in Table 4.1. Assessment of the numbers of proliferating nuclei in cells from each density interface were obtained from a mean of 10 fields of view from each of 3 cytospin smears per animal. There were generally 6 animals per group. These results are shown in Figs. 4.2 and 4.3. Analysis of these data showed 5 separate populations at the BSA density interfaces for each of the 3 treatments used, at a significance level of 0.05.

As in all the experiments with animals putatively rendered tolerant to HGG, the sera were tested for the presence of specific serum antibody. These antibody titres are given in Table 4.1. Only those animals untreated before the administration of the challenge dose of HGG in FCA exhibited an agglutinating antibody titre. This was a low titre in 4 out of 6 animals, which would be expected at this early time.

From Fig. 4.3 it can be seen that the pattern of distribution of proliferating nuclei was the same for both antibody producing and antibody non-producing animals.
**TABLE 4.1** Autoradiography counts showing the number of labelled nuclei cm\(^{-3}\) in spleen cell populations (separated on a BSA density gradient) of HGG antibody producing animals, and animals tolerant to HGG at this level. In this table the data represented in Fig. 4.3 was adjusted in order to take into account the total cell numbers retrieved from each interface (see Fig. 4.2). The total counts mm\(^{-2}\) were adjusted by the factor used to dilute original interface populations to the uniform \(10^6\) cells cm\(^{-3}\).

<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>Mean number of labelled nuclei cm(^{-3}) in BSA density gradient bands</th>
<th>-log(_2) antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Animals rendered tolerant to HGG and challenged with HGG/FCA.</td>
<td>257.64 ± 30.59</td>
<td>2280.00 ± 91.58</td>
</tr>
<tr>
<td>Normal animals challenged with HGG/FCA.</td>
<td>38.00 ± 4.18</td>
<td>6515.67 ± 423.89</td>
</tr>
<tr>
<td>Normal animals injected with saline/FCA</td>
<td>79.80 ± 10.64</td>
<td>65.28 ± 7.60</td>
</tr>
</tbody>
</table>

\(0\) = no detectable agglutination
\(\pm\) = standard error
<table>
<thead>
<tr>
<th>Cell suspension</th>
<th>Normal toadlets</th>
<th>Normal toadlets + HGG/FCA</th>
<th>HGG Tolerant toadlets</th>
<th>HGG tolerant toadlets + HGG/FCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15% w/v BSA</td>
<td>0.61% (0.21x10^6)</td>
<td>0.46% (0.25x10^6)</td>
<td>-</td>
<td>1.26% (0.30x10^6)</td>
</tr>
<tr>
<td></td>
<td>2.89%</td>
<td>4.25% (2.30x10^6)</td>
<td>9.62% (2.34x10^6)</td>
<td>4.19% (1.00x10^6)</td>
</tr>
<tr>
<td></td>
<td>17.06%</td>
<td>22.90% (12.40x10^6)</td>
<td>24.00% (5.84x10^6)</td>
<td>20.55% (4.90x10^6)</td>
</tr>
<tr>
<td></td>
<td>32.18%</td>
<td>38.69% (20.95x10^6)</td>
<td>51.19% (12.45x10^6)</td>
<td>54.09% (12.90x10^6)</td>
</tr>
<tr>
<td></td>
<td>47.27%</td>
<td>33.70% (18.25x10^6)</td>
<td>15.17% (3.69x10^6)</td>
<td>19.91% (4.75x10^6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 4.2** Discontinuous Bovine Serum Albumin density gradient separation of stimulated spleen cell populations; showing approximate % of total leukocytes retrieved per layer and mean cell numbers. Challenged animals examined 21 days after administration of antigen.
FIG. 4.3  The proportional distribution of proliferating nuclei in BSA density gradient separation of spleen cell populations, ± standard error. ( ■ normal toadlet + saline/FCA; □ normal toadlet + HGG/FCA; ▲ HGG tolerant toadlet + HGG/FCA).
Animals administered adjuvant only, showed a relatively low level of response throughout. The animals rendered tolerant to HGG showed proliferative levels in excess of the antibody producing animals in cells recovered from the first and fifth interfaces using an analysis of variance and the probability level of 0.05.

The cytospin smears were prepared from cell suspensions adjusted to a uniform concentration of $1 \times 10^6$ cells cm$^{-3}$ so it is probable that the distribution of cells shown in Fig. 4.3 was modified by this dilution process. Fig. 4.2 shows that in normal animals the largest cell population is obtained from interface band 5 (the most dense) and the smallest from interface band 1 (the least dense) of the gradient. Considering the relatively large number of leukocytes retrieved from the fifth density interface, it can be seen that the excess of labelled nuclei in challenged tolerant animals compared to normal challenged animals, is accentuated when this dilution process is taken into account. The importance of the third density interface as the density at which the largest proportion of proliferating cells aggregate 21 days after challenge is also emphasized by taking into account the cell dilution factor.

4. (iii) Changes in stimulated BSA separated spleen cell populations taken from HGG stimulated animals.

Three groups of cells were monitored in each of the 5 density interface populations collected. These were the lymphocytes, Figs. 4.4 and 4.5, the granulocytes, Fig. 4.6 and the macrophages; they represented the largest leukocyte populations present in the total number of cells retrieved from the interface band after the total spleen population was loaded on to the gradient.
FIG. 4.4a Distribution of large lymphocytes in the density interface bands of spleens separated on a BSA density gradient from normal toadlets unchallenged (O......O), or 21 days after challenge with HGG/FCA (●—●), ± standard error.

FIG. 4.4b Distribution of large lymphocytes in the density interface bands of spleens separated on a BSA density gradient from HGG tolerant toadlets, unchallenged (□......□) or 21 days after challenge with HGG/FCA (■——■), ± standard error.
FIG. 4.4a

FIG. 4.4b
FIG. 4,5a Distribution of small lymphocytes in the density interface bands of spleens separated on a BSA density gradient from normal toadlets, unchallenged (O····O) or 21 days after challenge with HGG/FCA (●—●), ± standard error.

FIG. 4,5b Distribution of small lymphocytes in the density interface bands of spleens separated on a BSA density gradient from HGG tolerant toadlets, unchallenged (□····□) or 21 days after challenge with HGG/FCA (■—■), ± standard error.
FIG. 4.5 a

FIG. 4.5 b
FIG. 4.6a Distribution of total granulocytes in the density interface bands of spleens separated on a BSA density gradient from normal toadlets, unchallenged (○····○) or 21 days after challenge with HGG/FCA (●——●), ± standard error.

FIG. 4.6b Distribution of total granulocytes in the density interface bands of spleens separated on a BSA density gradient from HGG tolerant toadlets, unchallenged (□····□) or 21 days after challenge with HGG/FCA (■——■), ± standard error.
Considering firstly the large lymphocytes, it can be seen from Fig. 4.4 a) and 4.4 b) that there were two general trends of distribution between the density interfaces, one for the normal animals and the other for those animals rendered tolerant to HGG. The normal animals showed an increase in the number of large lymphocytes in the middle density band after challenge with HGG in FCA (significant at a probability level of 0.01 by analysis of variance). Those animals rendered tolerant to HGG had considerably smaller populations of large lymphocytes over the whole gradient than their normally responding counterparts. They showed little change 21 days after stimulation with the antigen to which they were rendered tolerant.

The maximum numbers of small lymphocytes in the normal animals, shown in Fig. 4.5 a) were recovered from the fourth density interface. Challenge with HGG in FCA caused a two-fold increase in the size of this population. Animals rendered tolerant to HGG showed a gradient distribution profile the same as responding animals. The total number of cells were not altered by challenging the tolerant animals with HGG in FCA.

Figs. 4.6 a) and 4.6 b) again showed two separate trends for the normal and the tolerant animals. The normal animals had a large population of granulocytes in the most dense interface band; surprisingly they reacted to challenge with HGG in FCA by producing a much greater cell population in density interface band 3. The tolerant animals, on the other hand, showed total cell numbers across the gradient significantly lower than normal animals and were little affected by antigenic challenge.

Tables 4.2 - 4.5 showed changes in the proportions of cell types
<table>
<thead>
<tr>
<th>Density gradient interface</th>
<th>Macrophages</th>
<th>Polymorphonuclear granulocytes</th>
<th>Other granulocytes</th>
<th>Small lymphocytes</th>
<th>Large lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.27 ± 1.33</td>
<td>0.27 ± 1.33</td>
<td>50.69 ± 10.14</td>
<td>4.00 ± 3.47</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2.67 ± 2.74</td>
<td>15.14 ± 6.87</td>
<td>188.96 ± 19.34</td>
<td>53.83 ± 11.47</td>
</tr>
<tr>
<td>4</td>
<td>4.87 ± 3.87</td>
<td>39.35 ± 7.00</td>
<td>27.35 ± 4.34</td>
<td>429.75 ± 32.88</td>
<td>52.49 ± 7.94</td>
</tr>
<tr>
<td>5</td>
<td>3.34 ± 1.47</td>
<td>10.01 ± 1.47</td>
<td>212.11 ± 21.61</td>
<td>254.13 ± 14.81</td>
<td>135.4 ± 12.41</td>
</tr>
</tbody>
</table>

**TABLE 4.2** Bovine Serum Albumin density gradient separation of major leukocyte types in the spleen (number of cells mm⁻²). Toadlets were normal unchallenged animals.

± standard error
<table>
<thead>
<tr>
<th>Density gradient interface</th>
<th>Macrophages</th>
<th>Polymorphonuclear granulocytes</th>
<th>Other granulocytes</th>
<th>Small lymphocytes</th>
<th>Large lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>10.67 ± 3.61</td>
<td>54.69 ± 5.81</td>
<td>22.68 ± 5.00</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.34 ± 1.40</td>
<td>14.67 ± 4.60</td>
<td>442.22 ± 28.48</td>
<td>15.01 ± 4.60</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2.00 ± 1.47</td>
<td>18.01 ± 4.94</td>
<td>26.01 ± 2.60</td>
<td>20.68 ± 6.47</td>
</tr>
<tr>
<td>4</td>
<td>1.00 ± 1.93</td>
<td>2.34 ± 2.40</td>
<td>6.00 ± 4.14</td>
<td>216.44 ± 23.61</td>
<td>14.34 ± 3.80</td>
</tr>
<tr>
<td>5</td>
<td>3.00 ± 3.20</td>
<td>6.00 ± 4.74</td>
<td>156.08 ± 17.21</td>
<td>299.82 ± 22.41</td>
<td>112.06 ± 13.87</td>
</tr>
</tbody>
</table>

**TABLE 4.3** Bovine Serum Albumin density gradient separation of major leukocyte types in the spleen, (number of cells mm⁻²). Normal toadlets were challenged with HGG in FCA and examined 21 days after challenge.

± standard error
<table>
<thead>
<tr>
<th>Density gradient interface</th>
<th>Macrophages</th>
<th>Polymorphonuclear granulocytes</th>
<th>Other granulocytes</th>
<th>Small lymphocytes</th>
<th>Large lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.20 ± 1.27</td>
<td>0.67 ± 1.67</td>
<td>2.67 ± 2.67</td>
<td>318.16 ± 28.41</td>
<td>6.87 ± 3.86</td>
</tr>
<tr>
<td>3</td>
<td>0.53 ± 1.93</td>
<td>6.40 ± 4.07</td>
<td>8.00 ± 4.74</td>
<td>196.9 ± 22.68</td>
<td>42.96 ± 8.20</td>
</tr>
<tr>
<td>4</td>
<td>0.20 ± 1.27</td>
<td>0.60 ± 1.47</td>
<td>1.73 ± 2.20</td>
<td>356.58 ± 31.35</td>
<td>13.94 ± 5.34</td>
</tr>
<tr>
<td>5</td>
<td>1.33 ± 1.84</td>
<td>1.33 ± 1.22</td>
<td>36.02 ± 9.01</td>
<td>61.36 ± 8.25</td>
<td>32.02 ± 7.00</td>
</tr>
</tbody>
</table>

**TABLE 4.4** Bovine Serum Albumin density gradient separation of major leukocyte types in the spleen (number of cells mm⁻²). Toadlets rendered tolerant to a high dose of HGG as larvae; no challenge given.

± standard error
<table>
<thead>
<tr>
<th>Density gradient interface</th>
<th>Macrophages</th>
<th>Polymorphonuclear granulocytes</th>
<th>Other granulocytes</th>
<th>Small lymphocytes</th>
<th>Large lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.00 ± 3.43</td>
<td>2.67 ± 2.75</td>
<td>20.14 ± 12.03</td>
<td>39.49 ± 10.97</td>
<td>12.27 ± 8.40</td>
</tr>
<tr>
<td>2</td>
<td>0.67 ± 1.47</td>
<td>0.34 ± 1.40</td>
<td>12.67 ± 8.40</td>
<td>76.37 ± 11.01</td>
<td>91.71 ± 22.55</td>
</tr>
<tr>
<td>3</td>
<td>4.74 ± 3.34</td>
<td>15.27 ± 6.80</td>
<td>70.17 ± 11.61</td>
<td>286.28 ± 19.88</td>
<td>81.37 ± 12.27</td>
</tr>
<tr>
<td>4</td>
<td>0.67 ± 1.93</td>
<td>1.33 ± 1.73</td>
<td>10.34 ± 6.67</td>
<td>364.18 ± 11.21</td>
<td>18.68 ± 4.00</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>4.00 ± 5.60</td>
<td>40.02 ± 8.69</td>
<td>104.05 ± 18.42</td>
<td>32.02 ± 9.50</td>
</tr>
</tbody>
</table>

**TABLE 4.5**  Bovine Serum Albumin density gradient separation of major leukocyte types in the spleen (number of cells mm⁻²). Toadlets rendered tolerant to a high dose of HGG as larvae were challenged with HGG in FCA and examined 21 days after challenge.

±  standard error
in the 5 density interfaces 21 days after antigenic challenge. They also showed the macrophage populations obtained in each density band for the experimental treatments used. From these data the macrophages appear to be distributed at random throughout the density gradient.

4, (iv) Efficacy of separated populations in the adoptive transfer of high-zone tolerance to HGG.

The MHC compatible donor animals rendered tolerant to HGG and stimulated with this antigen were tested 21 days after this administration of HGG for the presence of serum antibody to HGG. The results from this routine check for tolerance at the level of antibody production showed that there were no antibody titres registered from the donor animals.

The host serum antibody titres obtained 28 days after the inoculation of cells from tolerant donors, and the simultaneous HGG in FCA challenge, are shown in Table 4.6. All 3 groups of cell recipients shown in Table 4.6 yielded serum antibody titres of up to approximately 1/512. The normal animals responding to HGG in FCA also produced serum antibody titres of this level 28 days after challenge. These titres have been found to be normal for 4 week antibody responses to HGG in adjuvant. Thus, unlike the whole cell populations reported in chapter 3, the cell populations separated on the density gradient were unable to transfer tolerance.

4, (v) Activity of BSA density gradient separated spleen cell populations in mixed leukocyte culture.

The normal unstimulated levels of \textit{in vitro} [^{3}\text{H}] -Thymidine
<table>
<thead>
<tr>
<th>Experimental treatment</th>
<th>Serum antibody -log$_2$ antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>JJ-strain hosts + JJ-strain donor cells rendered tolerant to HGG and stimulated with HGG/FCA. Cells from BSA gradient interface bands 1 and 2 inoculated i.v. at the same time as HGG/FCA challenge.</td>
<td>9, 10, 7, 8, 11, 6</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>JJ-strain hosts + JJ-strain donor cells rendered tolerant to HGG and stimulated with HGG/FCA. Cells from BSA gradient interface band 3 inoculated i.v. at the same time as HGG/FCA challenge.</td>
<td>10, 10, 9, 10, 9, 9</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>JJ-strain hosts + JJ-strain donor cells rendered tolerant to HGG and stimulated with HGG/FCA. Cells from BSA gradient interface band 4 inoculated i.v. at the same time as HGG/FCA challenge.</td>
<td>10, 7, 8, 14, 10, 3</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Outbred X.laevis challenged with HGG in FCA.</td>
<td>7, 14, 11, 12, 9, 10</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Outbred X.laevis challenged with saline in FCA.</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
</tbody>
</table>

**TABLE 4.6** Serum antibody titres measured 28 days after antigenic challenge in animals where BSA separated cell populations were inoculated i.v. at the same time as the challenge dose was administered.

$0$ = no detectable agglutination
incorporation into cell populations separated on the BSA density gradient are shown in Table 4.7. These results show that the cells were viable in 5 day cell culture and did not appear to be adversely affected by the preparative treatment afforded them.

Table 4.8 gives a range of normal stimulation indices between JJ-strain and outbred *X. laevis* adults in two-way mixed leukocyte culture. The stimulation indices produced were normally in the order of 5 times that of the relevant unstimulated cultures.

Table 4.9 shows a variation in MLR stimulation indices using pooled populations, prepared as indicated on page 79 of the Materials and Methods, from the values normally obtained. (see Table 4.8). The mean stimulation index for these pooled populations was 3.32 ± 0.63. This variation was partly due to the change in ratio of the two different cell populations, here 1:2 instead of the optimal 1:1. The ratio could have been maintained at 1:1 by changing the cell proportions shown on page 79 part b. to:

100 μl (N) + 50 μl (Jp) + 50 μl (J1.5).

However, this would reduce the test cell populations (i.e. those separated on the BSA density gradient) to only a 25% representation within the culture system. Due to this imbalance in the proportions of cell populations in the culture system, only those results where the change in stimulation index was >33% have been considered as significant. A second reason for the variation seen in the standard MLR stimulation indices could have been the possibility of inconsistencies arising from the method of production of the pooled cell populations. The possibility of inconsistencies arising here could have been avoided by removing a fraction of the whole cell population before centrifuging it on the BSA gradient, but it was
### TABLE 4.7

Levels of $[^3H]$-Thymidine incorporation by unstimulated, density gradient separated spleen cell populations after a 5 day *in vitro* culture. Results are recorded as mean cpm per $10^5$ leukocytes ± standard error (n=5). Pooled cell populations were collected from the BSA density gradient and whole cell populations were not subjected to the BSA density gradient separation.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$\bar{x} = 1738$</td>
<td>$\bar{x} = 3069$</td>
<td>$\bar{x} = 3021$</td>
<td>$\bar{x} = 3427$</td>
<td>$\bar{x} = 1465$</td>
<td>$\bar{x} = 6108$</td>
<td>$\bar{x} = 5264$</td>
</tr>
<tr>
<td>± 220</td>
<td>± 669</td>
<td>± 648</td>
<td>± 1140</td>
<td>± 67</td>
<td>± 460</td>
<td>± 600</td>
</tr>
<tr>
<td>Stimulated cultures (cpm)</td>
<td>Mean unstimulated cultures (cpm)</td>
<td>Stimulation index</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------------------------</td>
<td>-------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38077</td>
<td>6852</td>
<td>5.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49229</td>
<td>6505</td>
<td>7.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20974</td>
<td>3916</td>
<td>5.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36674</td>
<td>6789</td>
<td>5.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25587</td>
<td>7604</td>
<td>3.37</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44818</td>
<td>6250</td>
<td>7.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x = 35893</td>
<td>x = 6319</td>
<td>x = 5.74</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± 4053</td>
<td>± 470</td>
<td>± 0.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 4.8  Levels of \([^3]H\) -Thymidine incorporation by whole spleen cell populations stimulated in two-way mixed leukocyte culture, or unstimulated in 5 day culture. Results recorded as cpm per \(10^5\) leukocytes ± standard error

Stimulation Index = \(\frac{\text{cpm of stimulated culture (a + b)}}{\text{mean of cpm of unstimulated cultures a and b}}\)
<table>
<thead>
<tr>
<th>Standard MLR stimulation index</th>
<th>MLR + J₂ stimulation index</th>
<th>MLR + J₃ stimulation index</th>
<th>MLR + J₄ stimulation index</th>
<th>MLR + J₅ stimulation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.00</td>
<td>2.77 *</td>
<td>6.94</td>
<td>7.83</td>
<td>3.30</td>
</tr>
<tr>
<td>5.41</td>
<td>2.56 *</td>
<td>7.35 *</td>
<td>3.09 *</td>
<td>-</td>
</tr>
<tr>
<td>2.14</td>
<td>-</td>
<td>4.48 *</td>
<td>2.10</td>
<td>1.10 *</td>
</tr>
<tr>
<td>2.47</td>
<td>-</td>
<td>3.18</td>
<td>3.10</td>
<td>0.04 *</td>
</tr>
<tr>
<td>1.66</td>
<td>-</td>
<td>-</td>
<td>2.62 *</td>
<td>1.80</td>
</tr>
<tr>
<td>2.88</td>
<td>-</td>
<td>4.55 *</td>
<td>3.23</td>
<td>0.78 *</td>
</tr>
<tr>
<td>3.14</td>
<td>2.13</td>
<td>4.49 *</td>
<td>2.72</td>
<td>1.21 *</td>
</tr>
<tr>
<td>1.86</td>
<td>-</td>
<td>1.71</td>
<td>2.77 *</td>
<td>1.32</td>
</tr>
</tbody>
</table>

**TABLE 4.9** Two-way mixed leukocyte reactions with the addition of BSA separated spleen cell populations. Mean stimulation indices ± standard error. (* indicates cultures exhibiting >33% change in stimulation index compared to the standard).
thought better to treat both parts of the histocompatible population in the same manner.

An analysis of variance for the results given in Table 4.9 showed a significant difference between the density interface bands at a probability level of 0.001, indicating that particular cell types were accumulating at specific density interfaces. This has been shown by previous work on the BSA density gradient separation, (see section 4.iii). Using the criterion of a >33% change in stimulation index, a different picture emerges. The addition of cell populations from the density interfaces 2 and 5 to the culture system resulted in a significant reduction of the stimulation index in 2 out of 3, and 5 out of 8 cultures respectively. The use of cell populations from density interface 3 resulted in an increase in half of the cultures and parity with whole cell suspensions in the remainder. The addition of cell populations from density interface 4 produced no significant change in stimulation overall.

Two trends have then become apparent; the depressed stimulation using cell populations from density interfaces 2 and 5, and the absence of a statistically significant change using cell populations from density interfaces 3 and 4. The intra-layer cultures were therefore performed to examine whether these depressed levels were due to the reduced numbers of active cells in these population bands rather than to the presence of active suppressor cells in the populations.

Results obtained from the intra-layer cultures are shown in Table 4.10. An analysis of variance for these results displays a significant difference, again at the probability level of 0.001 between cell populations from density interfaces (1, 2 and 5) and (3 and 4). The latter have stimulation indices similar to those
<table>
<thead>
<tr>
<th>Cross</th>
<th>Jpool + Npool stimulation index</th>
<th>J₁ + N₁ stimulation index</th>
<th>J₂ + N₂ stimulation index</th>
<th>J₃ + N₃ stimulation index</th>
<th>J₄ + N₄ stimulation index</th>
<th>J₅ + N₅ stimulation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.43</td>
<td>-</td>
<td>4.21</td>
<td>2.99</td>
<td>2.72</td>
<td>0.43</td>
</tr>
<tr>
<td>2</td>
<td>4.02</td>
<td>0.07</td>
<td>1.89</td>
<td>3.02</td>
<td>2.84</td>
<td>1.10</td>
</tr>
<tr>
<td>3</td>
<td>2.66</td>
<td>0.12</td>
<td>1.55</td>
<td>2.40</td>
<td>2.95</td>
<td>0.93</td>
</tr>
<tr>
<td>4</td>
<td>3.14</td>
<td>-</td>
<td>1.06</td>
<td>4.27</td>
<td>3.03</td>
<td>0.57</td>
</tr>
<tr>
<td>5</td>
<td>1.89</td>
<td>-</td>
<td>-</td>
<td>2.33</td>
<td>1.46</td>
<td>0.10</td>
</tr>
</tbody>
</table>

\[ \bar{x} = 3.43 \pm 0.38 \]  \[ \bar{x} = 0.10 \pm 0.01 \]  \[ \bar{x} = 2.18 \pm 0.61 \]  \[ \bar{x} = 3.00 \pm 0.22 \]  \[ \bar{x} = 2.60 \pm 0.18 \]  \[ \bar{x} = 0.63 \pm 0.11 \]

**TABLE 4.10**  Two-way mixed leukocyte reaction between BSA density gradient separated spleen cell populations.  Mean stimulation index ± standard error.
of the pooled cell suspensions, and the former show greatly reduced stimulation.

It has been observed that there are not always sufficient cell numbers recovered from the first density interface to culture. This, together with the very low levels of stimulation produced by these cells, indicates that the depression of [3H]-Thymidine incorporation is due to a virtual absence of active cells. The results for the cell populations derived from the second and fifth density interfaces are more difficult to interpret. It is possible that there may be some suppressor activity in the second density interface band. It seems improbable that the degree of depression of the stimulation index achieved by the cell populations from the fifth density band would be forthcoming from a population that had not been pre-sensitized. A mixture of active cells appear to have been collected from the third and fourth density bands. They were capable of producing stimulation indices equal to the standards for each experiment, which would suggest that the appropriate balance of active cells is present in these bands.
DISCUSSION

The BSA density gradient method for the separation of whole spleen populations has been shown by workers in the mammalian field (Shortman, 1968 and Kraft et al., 1971) and in amphibians (Kraft et al., 1971 and Donnelly et al., 1976) to produce discrete bands of cells which are comprised of populations of separate morphological identity.

In previous work on *Xenopus laevis* four bands of cells were produced (Donnelly et al., 1976) at the density interfaces 1.045 - 1.070 g cm$^{-3}$, 1.070 - 1.082 g cm$^{-3}$, 1.082 - 1.094 g cm$^{-3}$ and 1.094 - 1.106 g cm$^{-3}$. The largest cell population was recovered from the interface band between density layers 1.082 g cm$^{-3}$ and 1.094 g cm$^{-3}$. This is in contrast to the separation of murine spleen cells which separated on the BSA gradient with the largest population at the interface between densities 1.074 g cm$^{-3}$ and 1.082 g cm$^{-3}$ (Takaguchi, Adler & Smith, 1971; Mugraby, Gery & Sulitzeanu, 1974). In the present study on *X. laevis* the largest cell population was again recovered from the interface between densities 1.082 g cm$^{-3}$ and 1.094 g cm$^{-3}$ showing the *X. laevis* splenocytes to be of higher density than their murine counterparts. Five density interface populations were retrieved from these gradients. The cell band not previously recorded was found on the least dense BSA layer of 1.045 g cm$^{-3}$. Cells were normally recovered from this band but the numbers involved were low.

Results obtained by Donnelly et al. (1976) in *Xenopus* showed that cell populations recovered from the lighter density bands had a considerably higher phytohaemagglutinin reactivity in culture than the populations from the denser bands, with a maximum reactivity.
in the cells from the second density interface band. The phyto-
haemagglutinin activity was almost completely ablated by thymectomy at
stage 47 (Nieuwkoop & Faber, 1967), suggesting that the highest
proportion of T-cells would be recovered from the lighter density bands.

In the present study, Fig. 4.5a shows that the maximum number of
small lymphocytes was retrieved from the fourth density interface
band, although the largest total population was found in the fifth
density band, (see Fig. 4.2) in normal unchallenged animals. Normal
animals sacrificed 21 days after challenge with HGG in FCA, yielded
twice as many small lymphocytes from this fourth density band as the
unchallenged animals. The tolerant unchallenged animals also had a
large population of lymphocytes in the fourth density band. The total
number of these was observed to remain unchanged when the animals were
killed 21 days after the administration of HGG in FCA. The total
number of small lymphocytes recovered from the fourth density interface
band of tolerant unchallenged animals was significantly greater than
those recovered from normal unchallenged animals. This increase might
have been the result of low level antigenic stimulation in the immune
system caused by the persistence of antigen, which is known to be
necessary for the maintenance of the tolerant state, (Mitchison, 1962).

The administration of antigenic challenge (HGG in FCA) is
accompanied by the production of a considerably larger population of
large lymphocytes at the third density interface of normal animals,
but again has no similar effect on those rendered tolerant to HGG.
The same trends were noted in the total granulocyte populations.

The data in Figs. 4.4 - 4.6 were obtained from cell populations
adjusted to $10^6$ cells cm$^{-3}$ thereby producing proportional cell
representations within each band. Whilst this treatment does not
allow the comparison of cell populations from one density band with another, it does point to the movement of cells into and out of the spleen. This state of change should be remembered when closer examinations of heterogeneous cell populations are being made. This cellular migration has been suggested by a number of observations. They include the presence of large pyroninophilic cells in the dorsal mesogastrium of *X. laevis* (Manning & Horton, 1982), and the migration of splenic antibody forming cells in *Bufo marinus* (Kraft & Shortman, 1972). Analysis of the nature of these cell populations is difficult because of the diverse migratory patterns of different lymphoid populations in and out of the spleen. Another restraint is the relatively unsophisticated techniques available for the identification of *Xenopus* lymphocyte subsets compared with the cell markers used in mammalian studies.

In the experiments using $[^3H]$ - Thymidine autoradiography, the profile of distribution of proliferating nuclei through the populations separated on the BSA density gradient shown in Fig. 4.3 is for animals sacrificed 21 days after the administration of antigenic challenge. The graph shows two main points of relevance. The first is, that normal animals injected with saline in FCA produced a profile of proliferation in the five density interface bands similar to the stimulated animals, but at considerably lower levels. The second point is, that the levels of proliferation in both the normal and the HGG tolerant animals after challenge with HGG in FCA, were similar in all of the density interface bands. The maximum proliferative levels were found in the interface band between BSA densities $1.070$ g cm$^{-3}$ and $1.082$ g cm$^{-3}$. This proliferation in challenged tolerant animals has also been found in *Cyprinus carpio* (Mughal, 1984). The exception to the similar proliferative activities in tolerant and normal animals
was found in the fifth density interface band. Proliferation in the HGG challenged, tolerant animals was significantly higher than in the normal animals, (using an analysis of variance at the probability level of 0.05). The data presented in Tables 3.5 and 3.6 also show an excess of proliferation in tolerant animals after antigenic challenge in comparison with normal animals. However, the data shown in Tables 3.5 and 3.6 refer to proliferation throughout the spleen, whereas the data recording an increased proliferation in tolerant animals shown in Fig. 4.3, refers to one population separated on BSA density interface 1.094 - 1.106 g cm$^{-3}$. Total proliferation from all the density interface bands is not increased in Fig. 4.3. A number of possible reasons for this discrepancy have been offered in chapter 3. The conclusion to be drawn from the data in Figs. 3.5 and 4.3 is, that there was no reduction of proliferative activity in tolerant challenged animals. There may or may not have been an actual increase. The resolution of this inconsistency would be possible with more rigorously quantitative methods.

The excess proliferation in the fifth density band of tolerant challenged animals compared to normal challenged animals would be emphasized if the total cell populations were considered, remembering that these cell suspensions were diluted to a uniform concentration of $10^6$ cells cm$^{-3}$ for the production of cytospin smears. When the total cell numbers are taken into account, the excess of proliferation in the tolerant challenged cells in the fifth band is significant.

This proliferating population could be composed of cells whose reactivity has been modified by a suppressor T-cell population or it could conceivably be a suppressor T-cell population itself.

Although the proliferation levels in the spleens of normal and
tolerant animals challenged with HGG in FCA were generally similar, there was a marked increase in the number of lymphocytes present in the spleens of normal challenged animals compared to their tolerant counterparts. It is not known what happens to the progeny from the proliferation of lymphocytes in HGG tolerant animals. It may be that this is an additional indication of the movement of cell populations out of the spleen.

Table 4.6 shows the agglutinating antibody titres obtained from animals inoculated simultaneously with tolerant spleen cells and HGG in FCA, 21 days after the donors were stimulated with antigen. The effect obtained by transferring whole populations of tolerant donor cells (i.v.) at the same time as the HGG challenge, i.e. the adoptive transfer of tolerance, (see chapter 3, Table 3.8) was lost when the spleen cell populations were separated on the discontinuous density gradient. This may have been caused by an alteration in the cell balance between the effector cells and the cells on which they act. Since stimulation with antigen causes the populations involved to enter a maturation sequence, the density characteristics of the cells may have been altered (Shortman, Diener, Russell & Armstrong, 1970) and this could have resulted in a dispersal of cells across the density gradient. On the other hand, Gershon (1975) found an effect in an adoptively transferred heterogeneous T-cell population which acted to suppress the immune response to tumour growth in the mouse. When these cells were separated on a BSA gradient, the suppressive effect was lost. This may indicate the requirement for cellular cooperation in suppressor cell activity.

The mixed leukocyte reaction was used to outline some of the activity of unstimulated BSA separated populations in vitro. The
experiment recorded in Table 4.9 was set up to observe whether any of the populations obtained from the discrete density interface bands contained an enriched population that would enhance or suppress the normal mixed leukocyte reaction. The data in Table 4.9 showed that cells from the third density interface band produced slightly higher stimulation indices than the controls, and these cells were shown in Table 4.10 to be producing levels of stimulation almost equal to the pooled controls. These data indicate that although the whole spleen population has been separated, the cells contained within the third and fourth density interface bands provided the range of cells necessary for the production of a moderate degree of stimulation in mixed leukocyte culture.

In Table 4.9 it was noted that the population obtained from the third density interface band produced marginally better stimulation than that from the fourth density interface band, despite the latter containing more small lymphocytes, (see Fig. 4.5a). This might indicate that the optimum balance of small lymphocytes to large lymphocytes and other non-lymphoid accessory cells, was achieved in this third density band; it is also possible that there was some functional separation of this large heterogeneous lymphocyte population occurring across the gradient. By the same argument, the depressed stimulation indices obtained from the addition of the fifth density interface population to the mixed leukocyte culture, could have been the result of the large proportion of granulocytes present in this population, interfering with the reaction and reducing the number of reactive cells present.

In conclusion, the separation of whole spleen cell populations on the discontinuous BSA gradient produced large heterogeneous
lymphocyte populations which showed real differences in separation according to whether they were able to respond normally to antigenic challenge or had been rendered tolerant. Some further functional clarification of the lymphocyte subsets is possible within this system, but in doing so it must be remembered firstly, that the spleen as a lymphoid organ is in a state of flux with the organism as a whole. Secondly, it is possible that the dynamics of lymphoid maturation differ with the type of stimulation afforded the immune system and its ability to respond. Therefore, when employing the physical characteristic of cell density to sample the immune system, it must be allowed that the flotation characteristic is not necessarily a static property of the cell.
CHAPTER 5

THE NORMAL LYMPHOCYTE TRANSFER
REACTION IN XENOPUS LAEVIS.
INTRODUCTION

The normal lymphocyte transfer reaction as described by Brent & Medawar (1963), was proposed as a means of quantifying the immunological reactivity of allogeneic lymphocytes against antigens of the host animal into which they were injected. This was undertaken with a view to the selection of homograft donors. Its use as a selection procedure in mammalian studies was, however, rapidly superceded by other lymphocyte typing techniques. In the human (Gray & Russell, 1963) and the guinea-pig, the reaction exhibits some features of the delayed hypersensitivity reaction (Brent & Medawar, 1964). However, the hosts have had no previous contact with donor antigens, nor the donor with the host, which would be necessary for the production of specific T-delayed hypersensitivity cells. Indeed, Brent & Medawar (1966) noted specifically that the reaction must be distinguished from the immune lymphocyte transfer reaction, in which the donor lymphocytes have been pre-sensitized to host tissue.

The majority of the work of Brent & Medawar on NLT in guinea-pigs, used irradiated hosts in order to eliminate the onset of a second inflammatory episode, which was usually the host response to the donor antigens. Under these conditions the response was divided into the first inflammatory episode (the recognition event), and the flare-up, in which the cell proliferation is at its greatest; these two events comprised the graft versus host response. The fade-out period, which was thought to be due to the revival of the host lymphocytes, follows swiftly (Brent & Medawar, 1967). The amplitude of the first inflammatory response was dependent on the cell dose and the genetic disparity of the inoculated cells; the onset and duration of the
response were not altered. This difference of amplitude was thought to be due to the number of cells involved in the response.

The test has not previously been performed on poikilotherms. If animals such as *X. laevis* could be shown to exhibit a small but definite graft versus host reaction on the inoculation of lymphocytes (as in the guinea-pig, Brent & Medawar, 1967), then the relative strength of these reactions could be used as a measure of MHC disparity and immunocompetence, as has already been stated. This would be of great advantage, as small host larvae could be tested, thereby disposing of the longer growing period required to raise these animals to the size where they could normally be used.

In the experiments reported here, untreated donor lymphocytes, early thymectomized hosts (in which the allograft response was impaired, Horton & Manning, 1972), and mitomycin-C treated donor cells (with impaired proliferative capacity), were used to outline the general form of the response and to define its donor and host components. A pilot test on putatively tolerant animals was subsequently performed.

In the work of Brent & Medawar (1966), the level of the response was assessed by an arbitrary rejection score based primarily on the diameter of the inflammatory lesions. Autoradiography was used by Terebey (1973) to look at events in the normal response to skin allografts. This latter method was used here to quantify the response.

The capacity of different mammalian test species to exhibit the normal lymphocyte transfer reaction has been shown by a number of workers to be variable. It is possible that the less complex nature of the immune system in *X. laevis* would produce results from which useful interpretations may be made, especially if these results were obtained using clonal lines of *Xenopus* to eliminate the minor histo-
compatibility disparities. Clonal *Xenopus* were not available for the present study, which was made using inbred, JJ-strain animals. These animals introduced minor histocompatibility disparities into the experimental design. Nevertheless, it was hoped that an initial understanding of the NLT in *Xenopus* would permit this reaction to be assessed as a possible test for immunocompetence in our studies on transplantation tolerance.
MATERIALS AND METHODS

A group of five toads were killed and their spleens removed aseptically. Spleen cell suspensions were prepared from these animals as described in the general methods, pooled and resuspended at a concentration of $3 \times 10^7$ cells cm$^{-3}$ in the amphibian dilution of sterile L15.

Larvae were selected at stage 52-54 (Nieuwkoop & Faber, 1967) because they have been shown by Horton (1969), to be immunocompetent at the level of allograft rejection by this stage. They were injected with 2 µl of the cell suspension ($6 \times 10^6$ cells per animal) into the connective tissue lying directly underneath the ventral musculature of the buccopharyngeal region, which was attached to the right and left Meckel's cartilages. A drawn glass needle was used as in the microinjection method described in chapter 2.

A total of 10 experimental and 10 control larvae per day were used in the experiments described under sections 5(i) - 5(iv) below. On days 1 - 10 after the cellular inoculation, 10 larvae per group were injected intraperitoneally with 1μCi g$^{-1}$ body weight of $[{\text{H}}]$ -Thymidine and sacrificed 4 hours later by immersion in Carnoy's fixative at 4°C. After 18 hours they were re-hydrated for storage in 70% ethanol.

Serial sectioning of wax embedded specimens was done through the area of the intermandibularis muscle and the areas directly adjacent to it, see Fig. 5.1. Sections 5 µm thick were processed for autoradiographic observation of labelled nuclei as described in chapter 2. The labelled nuclei of proliferating cells in the connective tissue under the intermandibularis muscle, were counted every 40 - 50 µm through the area shown in Fig. 5.2.
FIG. 5.1 Diagram of ventral view of stage 53 *X. laevis* larval head, showing intermandibularis muscle (im) site of cell inoculation (si), and the region serially sectioned for autoradiographic analysis (rs).

FIG. 5.2 Diagram of transverse section through stage 53 *X. laevis* larval head from region shown in Fig. 5.1. Showing the intermandibularis muscle (im), the left and right Meckel's cartilages (mc), the hyoid cartilage (hc), the buccopharyngeal cavity (bp) and the connective tissue region in which labelled nuclei were counted (shown as a stippled area).
EXPERIMENTAL PROTOCOLS

5. (i) Inoculation of untreated allogeneic lymphocytes.

Adult JJ-strain *X. laevis* donor spleen cells were prepared as described and 2 µl injected into the connective tissue lying beneath the intermandibularis muscle of outbred host *X. laevis* larvae. On days 1 - 10 groups of 10 larvae per day were injected with 1 µCi g⁻¹ body weight of [³H]-Thymidine into the peritoneal cavity and sacrificed 4 hours later. The control animals for this group consisted of an equal number of outbred larvae injected with 2 µl of the amphibian dilution of L₁₅ in the same position.

5. (ii) Inoculation of mitomycin-C treated allogeneic lymphocytes.

Adult JJ-strain *X. laevis* donor spleen cell suspensions were prepared and pooled as previously. These cells were then incubated for 60 minutes with an equal volume of 0.05 mg cm⁻³ mitomycin-C, made up in the amphibian dilution of L₁₅ at 26°C. The cells were then washed 3 times in the diluted L₁₅ and resuspended in this medium at the concentration of 3 x 10⁷ cells cm⁻³. Treatment of *Xenopus* splenocytes with mitomycin-C by this method, has been shown to impair the ability of the cells to incorporate [³H]-Thymidine in mixed leukocyte cultures, (Johari, Botham & Manning, 1981). As previously, 2 µl of this cell suspension were injected under the intermandibularis muscle of outbred *X. laevis* host larvae. Groups of 10 larvae per day were injected with [³H]-Thymidine 4 hours before sacrifice on days 1 - 10 after the inoculation of cells. The control animals for this experiment consisted of an equal number of outbred larvae injected with 2 µl of diluted L₁₅ under the intermandibularis muscle.
5. (iii)  Inoculation of allogeneic donor cells into early thymectomised hosts.

JJ-strain X. laevis larvae were thymectomized at stage 47 - 48 (Nieuwkoop & Faber, 1967) by microcautery of the paired thymus glands as described by Horton & Manning (1972). The positive electrode, a sharpened tungsten needle, was placed onto the surface of the thymus gland and a 0.5A high frequency pulse discharged across the gland, coagulating the tissue. The thymectomized larvae were then placed into aerated amphibian Ringer's solution diluted 1:3 with standing tap water for 1 day. In sham-thymectomized larvae, the skin was penetrated but no pulse was discharged across the thymus. On the following day the thymectomized and sham-thymectomized larvae were removed to standing tap water and grown up to stage 52 - 54 under husbandry conditions described in chapter 2.

At stage 52 - 54, all early thymectomized larvae were examined under a stereoscopic microscope, for the presence of any residual thymic tissue. Larvae found possessing any such material were added to the sham-thymectomized control group. There was no difference in growth between the thymectomized and the sham-operated larvae.

Adult outbred X. laevis donor spleen cell suspensions were prepared as described and injected into the connective tissue under the intermandibularis muscle of the larval hosts at the dose of $6 \times 10^4$ cells per animal. The thymectomized and the sham-thymectomized groups each consisted of 40 animals. On days 4 - 7 they were injected intraperitoneally with $1\mu Ci \, g^{-1}$ body weight of $[^3H] -$Thymidine 4 hours before sacrifice.
5. (iv) **Inoculation of a putatively chimaeric donor spleen cell population.**

Putative chimaeras were made between JJ-strain and outbred *X. laevis* according to the method of Clark & Newth (1972) and Manning & Botham (1980).

*X. laevis* embryos developing at approximately 23°C were taken at the neurula stage approximately 48 hours after fertilization. The jelly coat and the vitelline membrane were removed from the JJ-strain donor and the outbred host and these naked neurulae were floated into grooves cut into the agar of a Stendor dish well covered with Holtfreter's solution. The graft bed was prepared on the host flank. A graft of dorsal and ventral lateral plate mesoderm was removed from the JJ-strain donor from the same position and of the same size as the host graft bed, with fine tungsten needles, and floated on to the host graft bed. The graft was lightly pressed into position and held in place with a fragment of a glass coverslip. This was removed after 2 - 3 hours at which time the graft was seen to have grown securely into position. The position of the graft is shown in Fig. 5.3. After the removal of the coverslip fragment, the Holtfreter's solution was gradually diluted so that the embryos could be moved to aerated, standing tap water 24 hours later. The embryos were then grown on according to the usual husbandry procedures.

These putatively chimaeric toadlets attained a suitable size in approximately 6 months after metamorphosis. A group of 6 were sacrificed, the spleens removed and cell suspensions prepared as before, adjusting to the concentration of $3 \times 10^7$ cells cm$^{-3}$. As previously, 2 μl of the cell suspension were injected into the connective tissue underlying the intermandibularis muscle of 100
FIG. 5.3  Diagram of *X. laevis* neurula, stage 22 showing position of lateral plate mesoderm graft (mg).
JJ-strain host larvae at stage 52 - 54. The control animals consisted of a group of 100 JJ-strain host larvae inoculated with $6 \times 10^4$ cells per animal from a JJ-strain donor population. Both groups of animals were injected intraperitoneally with 1μCi g$^{-1}$ body weight of [3H] -Thymidine on days 1 - 10 after the cellular inoculation and sacrificed 4 hours later as described.
RESULTS

In the original experiments on the effects of normal lymphocyte transfer by Brent & Medawar (1964), the allogeneic lymphocyte inoculations were delivered into the dermis of the guinea-pig. The connective tissue area was chosen in the *Xenopus* larvae because of the need for overlying muscle tissue to close over the needle entry point and minimise loss of the inoculated cells.

Plate 5.1 shows a section through the intermandibularis muscle and underlying tissue 24 hours after the inoculation of allogeneic cells. This plate shows that these cells had spread out under the muscle and that the numbers of labelled lymphocyte nuclei were low at this time.

Plate 5.2 shows the change in distribution of allogeneic cells by day 3 after inoculation. The remaining cells were now more dispersed through the connective tissue and there were higher numbers of labelled cells in evidence.

Later in the response there was some indication of a movement of lymphocytes deeper into the host tissue. Plate 5.3 of an animal killed on day 6, illustrates this movement with pockets of lymphocytes in deeper tissue as well as the cells in the same area as seen in Plate 5.1 and 5.2. These pockets of lymphocytes had developed into large aggregates by day 10 as shown in Plate 5.4.

5.1 Inoculation of untreated allogeneic lymphocytes.

The inoculation of untreated allogeneic lymphocytes into normal
PLATE 5.1 Autoradiograph of T.S. through intermandibularis muscle (im) of host larva 24 hours after the inoculation of allogeneic lymphocytes; showing low levels of labelled nuclei (ln) in the cells visible in the connective tissue area (ct). Stained with Methyl-Green Pyronin-Y

PLATE 5.2 Autoradiograph of T.S. through intermandibularis muscle (im) of host larva 3 days after the inoculation of allogeneic lymphocytes; showing that the higher numbers of labelled nuclei (ln) visible in the connective tissue area are also more dispersed at this time.
PLATE 5.3  Autoradiograph of T.S. through intermandibularis muscle (im) of JJ-strain sham thymectomized host larva 6 days after the inoculation of allogeneic lymphocytes; showing increased levels of labelled nuclei (ln) deeper in the connective tissue area.

PLATE 5.4  Autoradiograph of T.S. through intermandibularis muscle (im) of JJ-strain host larva 10 days after the inoculation of putatively chimaeric donor lymphocytes; showing an aggregate of cells with labelled nuclei (ln) in the connective tissue area.
PLATE 5.3

PLATE 5.4
host larvae, resulted in the production of 2 rises in the number of proliferating lymphocytes in the connective tissue beneath the intermandibularis muscle. The first reached a maximum 3 days after the cellular inoculation, and the second 7 days afterwards. These results are shown in Fig. 5.4.

5. (i) Inoculation of mitomycin-C treated allogeneic lymphocytes.

Mitomycin-C treated allogeneic cells exhibited a very different response to that of the untreated allogeneic cells. In Fig. 5.5 it can be seen that the first proliferative episode was reduced to almost half of that obtained when untreated allogeneic lymphocytes were injected. The second proliferative episode, at day 8 after the administration of cells, was higher than that obtained from untreated donor cells. Fig. 5.6 shows the excess of proliferating nuclei in experimental groups compared to the controls. It shows that both untreated and mitomycin-C treated donor cells yielded a burst of proliferative activity between days 2 and 4, followed by a further rise in activity in the second week of the response. Significance was tested using an analysis of variance and the probability level of 0.05.

5. (iii) Inoculation of allogeneic donor cells into early thymectomized hosts.

Owing to the restricted number of early-thymectomized host animals available, samples were taken between day 4 and day 7, i.e. between the first and second peaks of proliferative activity as determined by the inoculation of untreated lymphocytes. Fig. 5.7 shows that the control animals (sham-thymectomized), produced two proliferative episodes at the same time, and of the same order of magnitude as the untreated animals described in section 5(i), on
FIG. 5.4
Mean number of labelled nuclei in the connective tissue area shown in Fig. 5.2 after the inoculation of allogeneic spleen cells, studied by $[^3\text{H}]$-Thymidine autoradiography; showing injection of allogeneic cells, showing injection of L15 (± standard error).

FIG. 5.5
Mean number of labelled nuclei in the connective tissue area shown in Fig. 5.2 after the inoculation of mitomycin-C treated allogeneic spleen cells, studied by $[^3\text{H}]$-Thymidine autoradiography; showing injection of mitomycin-C treated allogeneic cells, showing injection of L15 (± standard error).

FIG. 5.6
Analysis of the excess of labelled nuclei in animals inoculated with allogeneic cells compared to their L15 injected controls when studied by $[^3\text{H}]$-Thymidine autoradiography in the connective tissue area shown in Fig. 5.2, (●——● showing mitomycin-C treated allogeneic cells injected, ○····○ showing untreated allogeneic cells injected).
injection with allogeneic cells. The thymectomized animals, on the other hand, produced lower levels of proliferating nuclei and gave no indication of a rise in proliferative levels around day 3 or day 7. An analysis of variance using a probability level of 0.05 showed that there was no significant difference between the levels of proliferation on days 4 - 7 in the thymectomized animals.

5. (iv) Inoculation of a putatively chimaeric donor spleen cell population.

The inoculation of MHC-histocompatible cells was shown to produce low levels of proliferation in the tissue area being considered, over the whole 10 day period, see Fig. 5.8. These levels of proliferation were not demonstrably different from those obtained by the injection of culture medium alone, as shown in Fig. 5.4. By contrast, the putatively chimaeric donor cells produced a response (shown in Fig. 5.8), which was essentially similar to that shown by the inoculation of JJ-strain cells into outbred hosts (see Fig. 5.4), and an analysis of variance confirms this similarity at the probability level of 0.05.
Mean number of labelled nuclei in the connective tissue area shown in Fig. 5.2 after the inoculation of allogeneic spleen cells into early thymectomized and sham-thymectomized hosts, studied by $[^{3}H]$-Thymidine autoradiography; showing sham-thymectomized hosts, showing early thymectomized hosts (± standard error).

Mean number of labelled nuclei in the connective tissue area shown in Fig. 5.2, after the inoculation of putatively chimaeric spleen cells, studied by $[^{3}H]$-Thymidine autoradiography; showing injection of putatively chimaeric donor cells, showing injection of MHC-compatible donor cells (± standard error).
FIG. 5.7

FIG. 5.8
DISCUSSION

The data presented in this chapter allowed for some comparisons to be made between the normal lymphocyte transfer reaction (NLT) in mammals and a lower vertebrate, the amphibian *Xenopus laevis*. The three phases of the mammalian NLT response described by Brent & Medawar (1963) are not, however, as distinct in *X. laevis* as they are in the mammal. In *X. laevis* the time schedule for the response appeared to be surprisingly similar to that in the mammal, considering the lower temperatures involved. The first proliferative phase was seen at days 3 - 4 and the second at days 7 - 8 after cellular inoculation.

In *X. laevis*, the initial experiment (Fig. 5.4) of injecting untreated allogeneic cells into the connective tissue area, showed that allogeneic cells produced a proliferative response in excess of that produced by the injection of sterile culture medium alone from days 1 - 8. A subsequent experiment (Fig. 5.5) using mitomycin-C treated donor cells, indicated that a large proportion of the first proliferative phase was due to the donor lymphocytes, thereby paralleling the mammalian response. In *X. laevis* the peak of the second proliferative phase did not appear to be enhanced over the first phase peak. A difference was shown, but this was not significant when the variability within the groups was taken into account, (analysis of variance at probability level of 0.05).

The data obtained from early thymectomized and sham-thymectomized animals, (Fig. 5.7) was consistent with the idea that the first and second proliferative phases indicated in previous experiments, were predominantly of donor and host origin respectively. In the early
thymectomized animals the levels of proliferative activity on days 4-7 after the cellular inoculation, showed no significant change, verified by an analysis of variance using a probability level of 0.05. In the early thymectomized host, in which the response to alloimmune cells would be expected to be impaired, (Horton & Manning, 1972; Rimmer & Horton, 1977; Tochinai & Katagiri, 1975) this observed proliferative response was of donor origin. This contrasts with results from sham-thymectomized controls where the expected decline in donor response was observed. In this interpretation, the maintenance of the donor response in the early thymectomized host is accounted for by the lack of response against the donor cells by the early thymectomized host. The immunological deficiencies of the early thymectomized host could also account for the lack of the second proliferative phase in the reaction. The experiment displayed in Fig. 5.5 shows that there was some residual proliferative response at day 3, although the donor cells were treated with mitomycin-C. This suggests that the first proliferative phase comprised a host response in addition to the major donor component. The lower levels of proliferation in the early thymectomized animals, when compared with their sham-thymectomized controls, could be accounted for by the lack of this host component as only the donor cells were participating.

In the mammal, on the other hand, irradiation of the host tissue eliminated host interference with the first phase of the response, (Brent & Medawar, 1966). Bearing in mind the lower temperature in the amphibian, the onset of the host response before the completion of phase 1 (after Brent & Medawar, 1964) does not seem improbable.

Putatively chimaeric animals which had received JJ-strain grafts as embryos, were used as donors in a pilot experiment to assess the
suitability of the NLT reaction as a test for tolerance. Similar chimaeras produced by mesodermal grafting of allogeneic neurulae have been tested for tolerance to the donor tissue by skin grafting, (Clark & Newth, 1972; Horton, Horton & Rimmer, 1977) and in mixed leukocyte reaction (Botham & Manning, 1980).

In the following test, recorded in Fig. 5.7, the putatively chimaeric donors did not appear to be tolerant of host tissue since the proliferation levels were well in excess of those of the MHC compatible controls throughout the experiment. The peak of the first phase of proliferation was of the same magnitude as that obtained from the inoculation of MHC incompatible animals. Therefore it cannot have been caused by proliferative variability in the part of this phase attributable to activity of the host. It is conceivable that there could have been a situation of split tolerance here, such that the populations involved in normal lymphocyte transfer, differed from those concerned with skin allograft rejection or mixed leukocyte reaction. This point could be clarified by the use of cytotoxicity assays. It is more probable, however, that the group of putative chimaeras contained one or more animals which were not chimaeric, and which were responsible for the activity detected in the pooled cell population as a whole.

In the experiments of Brent and Medawar, the dermis was selected as the injection site because the inoculated cells would here tend to be confined within a limited area. Cells inoculated intradermally were localized to an extent which was not possible in _X. laevis_ as there is no equivalent tissue area, particularly in the larvae, in which inoculated cells would be as readily retained. Even in the adult, the skin structure of the amphibian is different from that of
the mammal. This highlights the problem in phylogenetic studies of anatomical variations which introduce mechanical as well as immunological differences. The problem of finding a suitable site in young larvae was further complicated by the fact that it was difficult to introduce the cells into an area not affected by local muscular movement. There is a potential net loss of cells from this connective tissue area for these reasons; indeed, attempts to estimate the numbers of cells seen in the sections compared to the numbers injected, suggested that this had occurred.

Other difficulties with the NLT test as applied to larvae, limit its practical use. Although it resembles the mammalian normal lymphocyte transfer in many ways, there is a degree of variability present in the X.laevis system which could obscure more marginal responses. Figs. 5.4 and 5.5 show that the injection of diluted LIS into the connective tissue of the control animals was accompanied by the appearance of varying numbers of cells, many of which were dividing, in the tissue concerned. This area was not one which normally contained cells. This host invasion was widespread in response to the trauma effected by the injection of a relatively large volume of sterile culture medium. The nature of the dividing cells was unclear as most of the cells involved in the inflammatory response do not normally proliferate at the site of damage. The variable nature of this response to the injection of culture medium could also have been affected by a number of environmental factors incident on the larvae, for example, temperature and stress.

In conclusion, the transfer of lymphocytes into the connective tissue underlying the intermandibularis muscle of larval X.laevis appeared to have some of the characteristics of the normal lymphocyte
transfer in mammals. The much greater variability observed in *X. laevis*, however, makes results less clear cut. This variability is thought to be introduced from three main sources which are not as prominent in mammals. They are (a) the greater movement of cells in and out of the area resulting in a net deficit; (b) a host inflammatory response to tissue damage, and (c) environmental factors. These three sources of variability would be extremely difficult to quantify.

This precludes a more versatile approach to the NLT as a general test for immunocompetence. Nevertheless, it remains of some value in situations involving small larvae and low cell numbers.
CHAPTER 6

The Induction of Transplantation Tolerance in Larvae by the Transfer of Allogeneic Spleens.
INTRODUCTION

The induction of specific transplantation tolerance requires that the host immune system must either identify foreign material as self, eliminate clones reactive to the foreign material or suppress the response to them in the peripheral system as has been discussed in chapter 1.

Transplantation tolerance has been produced by methods including avian yolk sac grafting (see review, Le Douarin, Jotereau, Houssaint, Martin, Dieterlen-Lievre, 1982; Dieterlen-Lievre, 1984) and embryonic parabiosis in the leopard frog, Rana pipiens (Volpe & Gebhardt, 1966; 1968). The inoculation of histoincompatible cells at early developmental stages has also been used to induce this type of tolerance (Billingham & Silvers, 1962; Roser & Dorsch, 1981, in mammals), as has the exchange of skin grafts (Terasaki, Longmire & Cannon, 1957; in mammals, and Cohen et al., 1985; Nakamura, Maeno, Tochinai & Katagiri, 1985, in X. laevis). In anuran amphibians the perimorphic stage provides an additional period of increased susceptibility to tolerance induction; the relative ease of induction of tolerance to skin grafts is well documented in X. laevis at this time (Chardonnens & Du Pasquier, 1973; Du Pasquier & Chardonnens, 1975; Cohen, DiMarzo & Hailparn-Barlow, 1980; Barlow, DiMarzo & Cohen, 1981). The reconstitution of lymphocyte depleted organs with foreign tissue has been used for the induction of transplantation tolerance, for example the mammalian allogeneic bone marrow chimaeras produced by Singer, Hathcock & Hodes (1981) and the allogeneic thymic chimaeras produced by the same group (Singer, Hathcock & Hodes, 1982). Similarly, allogeneic organ grafts such as the allogeneic thymi grafted into nude
mice (Kindred, 1978) showed that transplantation tolerance could be induced in this manner. Kawahara & Nagata (1980), implanted histoincompatible triploid thymi into early thymectomized diploid X.laevis hosts and found tolerance to thymus donor skin grafts and a low level of chimaerism. Embryonic mesoderm grafting has been shown to effectively produce transplantation tolerance in X.laevis (Clark & Newth, 1972; Botham & Manning, 1980). Experiments of Tompkins, Volpe & Reinschmidt (1980) and Flajnik, Du Pasquier & Cohen (1985) produced transplantation tolerance by exchanging the head and tail in amphibian neurulae. Tetraparental mice, as used by Von Boehmer, Hudson & Sprent (1975), formed by whole embryo fusion at the 8 cell stage are tolerant to tissue from either parent. The injection of peripheral blood leukocytes into larval X.laevis, however, resulted neither in tolerance nor in a heightened response (Johari, Botham & Manning, 1981).

The state of chimaerism within the host animal is common to the majority of these examples as shown by Tompkins et al. (1980) and Flajnik et al. (1985). Beverley et al. (1973) described a method for obtaining rough guidelines to the level of chimaerism within the animal. Botham & Manning (1980) have stated that it is as yet unclear whether this chimaeric state supports transplantation tolerance or results from it.

Attempts to induce tolerance can result in anything from an enhanced immune response to partial or complete non-responsiveness. The ultimate outcome has been shown to be affected by a number of factors such as the level of histocompatibility differences between the donor and the host. Calne (1976) has reviewed this within the mammalian system. The experiments of DiMarzo & Cohen (1982b) showed that in X.laevis the incidence of tolerance was dependent upon the
degree of MHC disparity between the donor and the host at the minor loci as well as the major loci. Arnall & Horton (1986) have indicated that the degree of polymorphism at the minor loci is limited in *X. laevis* and that impaired rejection of allografts will occur in the presence of minor antigen disparities.

The size of cell dose is of considerable importance to the rate of rejection of allografts. In 1969 it was proposed by Lappe, Graff & Snell, that in mammals large allografts would have prolonged survival compared to small grafts particularly where only non-H-2 disparities were present between the donor and host. In *X. laevis* Bernardini, Chardonnens & Simon (1970) showed that the size of the allograft played a fundamental role in the capacity of the host to reject the foreign tissue with the largest skin grafts (9 mm²), producing prolonged rejection times in 68% of the recipients compared with 25% in animals given 1 - 2 mm² grafts. Volpe (1970) also found that large doses of neural fold tissue grafts would induce the tolerant state to that tissue.

The gene-dosage effect at the major H-2 loci might be expected to cause more rapid allograft rejection where there is a 2 haplotype disparity between the donor and the host. In *X. laevis* it has been proposed by Tompkins, Reinschmidt, Wilson & Volpe (1980) that this does not occur. Other workers, however, have considered this gene-dosage effect to be responsible for the enhanced rate of allograft rejection (Du Pasquier, Chardonnens & Miggiano, 1975), but the extent to which the minor histocompatibility antigens were affecting the system was not quantified.

Lastly, the stage of maturation appears to be important to the survival of allografts. Ruben, Stevens & Kidder (1972) showed
retardation of allograft rejection when adult organ fragments were implanted into early larval stages of *X. laevis* and spleens were found to be more effective than thymi. DiMarzo & Cohen (1982b) reported that larval *X. laevis* stages 48/49 frequently became partially or fully tolerant to adult skin allografts applied at this time. The level of maturity of the donor tissue was not examined. This is investigated in the experimental work reported here, where the maturation level of both the donor and the host has been considered whilst attempting to induce transplantation tolerance.
MATERIALS AND METHODS

Spleen transplants were carried out using JJ-strain donor larvae from stages 50 - 56 (Nieuwkoop & Faber, 1967) and outbred host larvae from stages 47 - 57. The donor larvae were killed quickly in 2 g dm\(^{-3}\) of 3-aminobenzoic acid ethyl ester (MS 222), the spleens being removed and placed into sterile amphibian culture medium at 4°C. The host larvae were anaesthetized by immersing for approximately 5 seconds in 2 g dm\(^{-3}\) of MS 222 and washed immediately afterwards in standing tap water. The larvae would then lie quietly on a damp cotton-wool bed. The spleen was grafted ectopically in the position used by Horton & Horton, (1975) and Cribbin, (1984) for thymus transplants. A small incision was made in the epidermis just anterior to the right hand thymus gland using a sharpened tungsten needle. The donor spleen was pushed through this incision to sit in the underlying connective tissue as shown in Plate 6.1. Plate 6.2 is a higher magnification of this transplanted spleen at 13 days after grafting, showing it to be well vascularized, healed in and covered with a dense accumulation of melanophores. The host larvae were then placed in aerated standing tap water and grown on according to normal husbandry procedures.

After the animals had metamorphosed they were grown on for 1 month before their tolerance to JJ-strain tissue was tested by the application of a skin graft.

Adult JJ-strain toads 12 - 18 months of age were anaesthetized in 2 g dm\(^{-3}\) of 3-aminobenzoic acid ethyl ester. They were washed in standing tap water and a strip of the white ventral skin removed. This was stored in L15 at 4°C. The donor toad was then returned to standing tap water and antibiotics added to the water at the concentration
PLATE 6.1  Larval *X. laevis* showing the transplanted larval spleen (as) *in situ*, anterior to the thymus (t), 13 days after implantation.

PLATE 6.2  Higher magnification of the grafted area seen in Plate 6.1, showing the transplanted spleen (as) to be well vascularized.
stated in chapter 2. The host toadlet was anaesthetized and an incision made in the skin of the dorsal surface and the graft bed prepared inside this 'pocket'. A piece of the donor tissue was slipped under the host skin flap. The host animal was then placed in a small darkened container to restrict its movement, and left over-night for the graft to grow into place. The next day the animals were anaesthetized again, and the skin flap covering the skin graft was removed so that the graft area was readily visible. The skin grafts were monitored to observe three phases in the graft rejection process according to Horton, (1969). These were:-

Phase 1. The enlargement of capillaries supplying the graft.

Phase 2. The breakdown of pigment cells (guanophores), usually beginning at the periphery.

Phase 3. The end point of graft rejection judged by the time at which all guanophores had been broken down.
RESULTS

During the course of operations to transplant larval spleens into larval hosts the mortality rate was low with only 4.4% of all animals grafted, dying at this stage. The highest proportion of mortalities were found in groups where either the host or the donor were developed beyond stage 51 (Nieuwkoop & Faber, 1967). This may have been a physical problem caused by a disparity in sizes. Table 6.1 records the mortalities in the various groups of larval spleen transplant recipients. It shows that the majority of deaths occurred around the metamorphic period. This period appeared to be particularly important in those groups of animals where the donor spleen was up to and including stage 51 and the host post-stage 51 at the time of spleen transplant. The mortalities were also high at this time in the animals where the donor and the host larvae had both developed beyond stage 51 at the time of spleen transplant.

Stage 51 (Nieuwkoop & Faber, 1967) was chosen as the dividing line between mature and immature spleens for the purpose of this experiment. This was based on work by Horton (1969), which showed that stage 50 larvae had no capacity to respond to skin allografts but had developed this response by stage 52.

In Table 6.2, the skin allograft rejection times are given for JJ-strain ventral skin grafted onto outbred *X.laevis* hosts and for JJ-strain ventral skin grafted onto JJ-strain hosts. The former has provided the timing of acute graft rejection based on MHC-histocompatibility, and the latter has provided the timing for MHC-compatible graft rejection based on minor loci disparities. These graft rejection times were measured at 20 ± 2°C. as were the experiments recorded in Fig. 6.1.
<table>
<thead>
<tr>
<th>Spleen transplant types</th>
<th>Total successful spleen transplant operations</th>
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<th></th>
</tr>
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<td></td>
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</tr>
<tr>
<td>Outbred host $\text{st. 51}$</td>
<td>57</td>
<td>0</td>
<td>12.0%</td>
<td>36.0%</td>
<td>36.0%</td>
<td>16.0%</td>
</tr>
<tr>
<td>JJ-strain donor $\text{st. 51}$</td>
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<td></td>
</tr>
<tr>
<td>Outbred host $\text{st. 51}$</td>
<td>37</td>
<td>10.2%</td>
<td>30.6%</td>
<td>44.9%</td>
<td>12.3%</td>
<td>2.0%</td>
</tr>
<tr>
<td>JJ-strain donor $\text{st. 51}$</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Outbred host $\text{&gt;st. 51}$</td>
<td>13</td>
<td>13.9%</td>
<td>8.3%</td>
<td>69.4%</td>
<td>5.6%</td>
<td>2.8%</td>
</tr>
<tr>
<td>JJ-strain donor $\text{st. 51}$</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Outbred host $\text{&gt;st. 51}$</td>
<td>32</td>
<td>5.6%</td>
<td>29.2%</td>
<td>58.3%</td>
<td>4.2%</td>
<td>2.8%</td>
</tr>
<tr>
<td>JJ-strain donor $\text{&gt;st. 51}$</td>
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</tbody>
</table>

**TABLE 6.1** Mortalities in *X. laevis* hosts subsequent to larval spleen transplant.
Skin graft combination | Skin graft rejection |
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>Phase 1</td>
</tr>
<tr>
<td>Outbred host toadlet</td>
<td>10</td>
</tr>
<tr>
<td>+ ventral skin graft</td>
<td>-</td>
</tr>
<tr>
<td>from JJ-strain donor</td>
<td>19</td>
</tr>
<tr>
<td>toadlet.</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>15</td>
</tr>
<tr>
<td>JJ-strain host toadlet</td>
<td>12</td>
</tr>
<tr>
<td>+ ventral skin graft</td>
<td>12</td>
</tr>
<tr>
<td>from JJ-strain donor</td>
<td>8</td>
</tr>
<tr>
<td>toadlet.</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>48</td>
</tr>
<tr>
<td>JJ-strain host toadlet</td>
<td>50</td>
</tr>
<tr>
<td>+ ventral skin graft</td>
<td>-</td>
</tr>
<tr>
<td>from JJ-strain donor</td>
<td>-</td>
</tr>
<tr>
<td>toadlet.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

**TABLE 6.2**  Rejection times for adult JJ-strain skin allografts at 20 ± 2°C in JJ-strain and outbred *X. laevis* toadlets. (Time in days; - = not recorded).
FIG. 6.1 Rejection of skin allografts by toadlets following larval spleen transplants: rejection time shown refers to the end point of rejection, phase 3; 'n' numbers on bars represent the number of animals in each group. (Donor and host ≤ stage 51; Host ≤ stage 51 and donor > stage 51; Host > stage 51 and donor ≤ stage 51; Donor and host > stage 51).
In order to consider the effect of larval spleen transplants on adult allograft rejection, the rejection times have been grouped into four main categories according to the time at which rejection phase 3 was attained, (see Fig. 6.1). The first category of acute rejection referred to animals that attained phase 3 rejection in ≤25 days. This timing was selected after examining the data in Table 6.2 relating to the rejection of JJ-strain allografts by outbred adult _X. laevis_ hosts. These data showed the acute rejection of MHC-disparate allografts, and were in accord with acute graft rejection times obtained by Du Pasquier & Chardonnens (1975) and Horton & Horton (1975). The fourth category used in Fig. 6.1 was that of chronic rejection, and was also obtained from Table 6.2. Rejection was ruled to be chronic if the destruction of allograft pigment cells took longer than 56 days, because in these experiments, this was the period required by JJ-strain toadlets to completely reject JJ-strain allografts. Other workers have used 50 days as the measure for chronic rejection of MHC-compatible allografts, (DiMarzo & Cohen, 1982b; Cohen, DiMarzo, Rollins-Smith, Barlow & Vanderschmidt-Parsons, 1985). These grafts were tolerated due to histocompatibility, but were ultimately rejected through minor loci disparities. Between these two points were the two categories of sub-acute rejection, (in which the end point of rejection occurred 26 - 40 days after application of the allograft), and impaired rejection (in which the third phase of rejection was achieved 41 - 55 days after application of the allograft). Other workers have not generally made this differentiation between sub-acute and impaired rejection.

Fig. 6.1 shows that only those animals in which the larval spleen was transplanted from donors of up to stage 51 into hosts of up to stage 51, showed the induction of tolerance according to the criterion
of a rejection time of >56 days, and this was found in only some of the group. Other animals showed impaired allograft rejection (days 41 -55). Half of the animals in which spleens were transplanted at this stage were accounted for by these two groups. The other half rejected allografts at times consonant with major histocompatibility differences.

In spleen transplanted animals where both the donor and the host had developed beyond stage 51 at the time of the transplant, there was a 31% impairment of graft rejection time but no induction of tolerance, according to the criterion described earlier.

Table 6.3 shows the mean time to the onset of rejection (phase 1) and the mean time to the end of the rejection process (phase 3). The length of the rejection process has been calculated from this data. An analysis of variance shows that there was a small but significant increase in the length of the rejection process (at a probability level of 0.05) with an increasing length of survival for the allograft. The ratio of time to the onset of allograft rejection to the total rejection time was considered and a steady increase of time was noted as the length of survival of the allograft increased. An analysis of variance confirms this as significant at a probability level of 0.01. It indicates that prolonged allograft survival was achieved primarily by a delay in the onset of rejection and secondarily by a slower rejection process.

Table 6.4 shows the levels of vascularization of the donor spleens at metamorphosis in the hosts which survived to the end of the experiment. This table shows that although there was considerable variation in the level of vascularization throughout the groups, there was an overall trend towards the retention of 'healthier' spleen transplants in the animals with the longest surviving allografts. Plates 6.3 and 6.4
<table>
<thead>
<tr>
<th>Phase 3: &lt;25 days</th>
<th>Phase 3: 26-40 days</th>
<th>Phase 3: 41-55 days</th>
<th>Phase 3: &gt;55 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both</td>
<td>Host</td>
<td>Donor</td>
<td>Both</td>
</tr>
<tr>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Length of rejection period (days)</td>
<td>11.0</td>
<td>8.7</td>
<td>9.5</td>
</tr>
<tr>
<td>(Phase 3 - Phase 1)</td>
<td>1.1</td>
<td>1.0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**TABLE 6.3** Mean phase 3 allograft rejection times in toadlets given larval spleen transplants as larvae. (* group containing 2 animals only)

± standard error
<table>
<thead>
<tr>
<th></th>
<th>Level of vascularization of donor spleens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phase 3 in ≤25 days</td>
</tr>
<tr>
<td>Host and donor ≤ stage 51</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3+</td>
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<tr>
<td></td>
<td>0</td>
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<td>0</td>
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<td>0</td>
</tr>
</tbody>
</table>

| Host ≤ stage 51 donor > stage 51 | 0                 | 2                   | 3 3 5             | 5 5              |
|                                   | 1                 | 4- 0                | 4 4 3             | 2 2              |
|                                   | 0                 | 4 5                 | 4 3              | 3                 |
|                                   | 0                 | 5 3                 | 4 2              | 3                 |
|                                   | 0                 | 4 4                 | 4 2              | 3                 |
|                                   | 0                 | 5 0                 | 4 2              | 3                 |

| Host > stage 51 donor ≤ stage 51 | 3                 | 3                   | 2 2 5             | 3+ 1             |
|                                   | 3                 | 0                   | 1 2 1             | -                |
|                                   | 3                 | 1                   | 1 4              | -                |
|                                   | 2+                | 3                   | 0 1              | -                |

| Host and donor > stage 51 | 1 2                | 1 0                  | 1 1 4 5-          | 1 1 4- 4          |
|                          | 0 3                | 0 2+                 | 3 4              | 4 4              |
|                          | 2 1                | 1 1 4-               | 5 3+             | 5 3+             |
|                          | 4 0                | 1 2                  | 4- 5             | 4- 5             |
|                          | 5 0                | 2 0                  | 4- 4             | 4- 4             |
|                          | 0                  | 0 3                  | 3                 | -                |

**TABLE 6.4** Level of vascularization of donor spleens at metamorphosis in hosts subsequently given skin grafts. Animals grouped according to stage of maturity of donor and host at time of spleen transplant and according to rate of graft rejection, recorded in subsequent skin grafting experiments. Scale of 0 (no evidence of donor spleen remaining) to 5 (well vascularized and growing spleen).
show transplanted spleens in their toadlet hosts to be vascularized and well grown.
Young adult *X. laevis* head, showing the well grown and vascularized allogeneic donor spleen (as) adjacent to the eye.

Young adult *X. laevis* showing healthy allogeneic spleen transplant (as) adjacent to the eye with allogeneic ventral skin graft (sg) in position on dorsal surface of toadlet.
DISCUSSION

The use of the spleen transplant at various stages of larval development in order to produce transplantation tolerance in the adult host was proposed to offer an alternative to the dorsal and ventral lateral plate mesoderm grafting method in neurulae. Skin grafting has been used previously as a means of inducing tolerance, (Bernardini, Chardonnens and Simon, 1970; Du Pasquier, Chardonnens & Miggiano, 1975; Cohen, DiMarzo & Hailparn-Barlow, 1980; Barlow, DiMarzo & Cohen, 1981; DiMarzo & Cohen, 1982b), in larval and perimetamorphic *X.laevis*. In this study the spleen was selected as a possible source of suitable numbers of the lymphoid precursors needed to set up and maintain a chimaeric population. 43% of the larval spleen recipients survived to the end of the post-metamorphic skin grafting experiment. This mortality rate was very low compared with that observed in embryonic mesoderm grafting. This reduced mortality rate together with the simplicity of the transplantation technique, demonstrated that the method was physically effective, whether or not the spleen proved to be a satisfactory cell source for the production of chimaeras. The production of the chimaeric state in both the donor and the host larval spleens, has been indicated by work done in collaboration with L.J. Blok in this laboratory. Triploid larvae were produced by the cold shock method of Kawahara (1978), and in a pilot experiment the following spleen transplants were performed:–

Diploid JJ-strain *X.laevis* host (stage 47 - 48) + Triploid outbred donor (stage 50 - 51).

Triploid outbred *X.laevis* host (stage 47 - 48) + Diploid JJ-strain donor (stage 50 - 51).

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The donor spleen in both groups of animals was seen to be penetrated by considerable numbers of host cells 4 weeks after the transplant, whilst lower levels of donor cells were recorded in host spleens. The possible persistence of the chimaeric state to the post-metamorphic stages has not yet been investigated in these spleen transplanted animals.

Table 6.2 records a range of the normal rejection times of allo- grafts of JJ-strain donor skin placed onto outbred recipients at 20 ± 2°C. It also includes JJ-strain recipients of JJ-strain donor skin grafts. This second group rejected their allografts chronically on the basis of disparities at the minor histocompatibility loci. The nature of these disparities was not known. It is possible that they did not comprise a truly representative cross-section of the population as a whole. Thus, these control rejection times should be viewed as guidelines and not as immutable values.

Fig. 6.1 shows that 35% of the spleen transplanted animals subsequently given skin allografts, showed impaired rejection of that graft. 21% of these animals were given larval spleen transplants when both the donor and the host were immature, i.e. before stage 52. This immature group included the only 9 animals to exhibit a mean rejection time similar to that of the JJ-strain control hosts grafted with JJ-strain skin. These animals could have been rendered tolerant to the original donor tissue but differ from subsequent JJ-donors at minor histocompatibility loci.* These animal numbers, (6.5% of the total) are consonant with the incidence of chance MHC-compatibility between the donor and the host, and this possibility must therefore

* These JJ-strain *X. laevis* are compatible at the major histocompatibility loci but will express variation at the minor histocompatibility loci at the normal incidence rate found within our colony.

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be considered. It is improbable, however, that they would all fall within the same group by chance.

Whilst considering those animals in which the donor and the host were immature at the time of spleen transplant, it must be noted that an equal number of animals from this group did not experience impaired rejection. This indicates that although the stage of immunological maturation is important, there are other factors which exert a strong influence on the results.

The impairment of allograft rejection is shown in greater detail in Table 6.3 which provides the mean time taken for the onset of the visible graft rejection process. This table shows that the delayed onset of rejection was of major consequence to the increased graft retention times. The increase in the total rejection period was also significant. The size of the cell dose and the number of disparate MHC-haplotypes of an allograft have profound effects on the rate of graft rejection, (Bernardini et al., 1970; Chardonnens & Du Pasquier, 1973; DiMarzo & Cohen, 1979; Cohen et al., 1980).

The range of sub-acute rejection times obtained from the 4 groups of animals given larval spleen transplants (shown in Table 6.3), could have been large because of an irregular persistence of the tolerogenic antigen in the host body. The maintenance of tolerance is known to require constant stimulation of the host system which may be achieved by the persistence of the specific tolerogen (Mitchison, 1962). The persistence of antigen has also been shown to be important in the maintenance of specific transplantation tolerance as in the chimaeric and ex-parabiotic frogs of Davison (1966), and in the eye and tailbud grafts used by Kaye, Schermer & Tompkins (1983) in *X. laevis*. Although the role of the chimaeric state in the induction of transplantation
tolerance has not been determined, it has been found concurrent with
specific transplantation tolerance in animals tested (Volpe, 1970;
Tompkins, 1977; Volpe, Tompkins & Reinschmidt, 1979; Tompkins, Volpe
& Reinschmidt, 1980; Flajnik, Horan & Cohen, 1984). If the donor
tissue initially transferred to the host had contained insufficient
cell numbers, then it may not have been possible to present new cell
populations being formed, which have the ability to react against
this antigen, with the donor cells. The net result would have been a
gradual loss of tolerance as new clones replaced the old.

In Table 6.4 the levels of vascularization of donor spleens at
metamorphosis have been recorded. Despite considerable variation
there was an overall trend towards the correlation of healthier spleen
transplants with prolonged post-metamorphic allograft retention. The
mortalities amongst the entire spleen transplanted animals have been
recorded in Table 6.1. Mortalities in the perimeetamorphic period
formed a large percentage of the total. They cannot easily be
accounted for by the depression of the immune system at this time
because of the presence of the donor spleen cell populations. These
may or may not have been under the influence of the host thymus and
were therefore compounding an already complex situation. The
mortalities which occurred after the application of the post-metamorphic
skin graft, were considerably higher in the group in which both the
donor and the host Larvae were pre-stage 52 and hence, immunologically
immature at the level of graft rejection at the time of the larval
spleen transplant. It is possible that these animals experienced a
graft versus host reaction which was balanced by the host immune system
until an extra dose of donor material was added to the system, thereby
precipitating the reaction as shown by Clark & Newth (1972).
To summarise, the system of spleen transplantation in young *X. laevis* larvae has revealed an effect of impaired allograft rejection which is linked to the level of maturation of both the host and the donor spleens. There appears to be a chimaeric state produced in the larvae by this transplantation method, which persists at least into the fourth week after transplant. The persistence of donor cells has not yet been followed through into adult hosts. Were this to be observed then the relative importance of other elements, such as the size of the donor transplant, might be ascertained.

* The mortalities were considerably higher than those normally found within our colony at metamorphosis, therefore they cannot simply be accounted for by the depression of the immune system at this time. It is possible that some form of graft versus host response was occurring, but if this were the case then a stronger correlation between the percentage mortality and the maturity of the donor spleen would have been expected.
CHAPTER 7

General Conclusions and Summary
GENERAL CONCLUSION

The present work has been concerned with an observation of some of the phenomena of immunological tolerance to soluble protein antigens and to allogeneic tissue transplants.

The work concerned with larvally induced high zone tolerance to Human-γ-globulin has substantiated the results of Mughal (1984) and Al Johari (1985), showing that *X. laevis* larvae are susceptible to tolerogenesis in the presence of massive antigen doses used here. In addition to this, the long-term persistence of this tolerant effect has been shown. The mechanisms underlying the persistence of the tolerant state will require some form of antigen retention system, according to Mitchison (1962), and remain to be elucidated.

Mughal (1984) showed that carp rendered tolerant to HGG at young stages, responded to subsequent antigen challenge with a proliferative response in the spleen. This effect was observed here in high zone tolerant *X. laevis*. Incomplete tolerance may develop when some lymphocyte populations are able to respond normally to antigen whereas others are rendered unresponsive. This is a well known phenomenon in mammals; for example, split tolerance which occurs when different T-cell subsets either respond actively to antigen or are rendered tolerant. It is not clear whether the observation of tolerant carp and *Xenopus*, proliferating in response to antigen, is analogous to the mammalian situation. The nature of the proliferating cells is not here known. They could be populations multiplying at the beginning of the response, which fail to proceed through the maturation sequence towards the effector cells involved in antibody production. The proliferating population is likely to include T-cell subsets involved in cell-mediated
immunity, as well as those concerned with the humoral response. It is also possible that this proliferation may have included the suppressor T-cell subset itself. In addition, the possibility of proliferation in a non-lymphoid population should not be overlooked.

There has been relatively little evidence of active suppressor populations in amphibians and even less in fish. Du Pasquier & Bernard (1980) detected a suppressor population in cells transferred into metamorphic *X.laevis* hosts, enhancing transplantation tolerance. Histocompatible lines have been available in *X.laevis* since 1978 (Katagiri, 1978), and these have meant that this work is now feasible. Although some gynogenetic lines are currently in the process of development in fish, they have not as yet been made widely accessible for such studies.

Transfer experiments used here have indicated that under certain timing conditions the adoptive transfer of tolerance is possible. This is the first instance of adoptive transfer of high zone tolerance to soluble protein antigens in amphibians or fish and would provide strong evidence for the presence of suppressor T-cells in *X.laevis* were more work to be done to confirm these original findings. These transfer experiments might suggest that suppressor T-cells form part of the populations observed proliferating in tolerant animals, 21 days after antigenic challenge.

The separation of spleen cell populations on BSA density gradients has shown that high zone tolerance is accompanied by changes in the density profiles of spleen cell populations. To carry these studies further it is necessary to make use of the array of cell surface markers that are now becoming available to *Xenopus* work.
The present work has addressed the problem of assaying tolerance in *Xenopus* larvae in order to take full advantage of the free-living nature of this model. To this end the normal lymphocyte transfer reaction was examined, in relation to transplantation tolerance, as a possible test for immunocompetence using low cell numbers. This reaction was found to exhibit some of the characteristics of the mammalian response, which has not previously been shown in amphibians and has yet to be investigated in fish.

The development of a more efficient method of inducing transplantation tolerance in larvae was explored using whole spleen transfer. These experiments showed that the maturity of donor and host animals was important at certain stages, but that a number of other factors came into play when either donor or host began to develop populations of immunocompetent lymphoid cells. Cohen *et al.* (1985) similarly found a number of factors incident on the system that they were using to induce transplantation tolerance at metamorphosis, namely graft size, gene dose and the degree of genetic disparity.

Larvaly induced tolerance in *Xenopus* is a phenomenon which lends itself readily to laboratory study. The advent of isogeneic clones and ploidy markers has heralded the construction of more specifically defined experimental systems. From these it may be hoped that some of the basic, yet elusive components of the toad immune system may be illuminated. Of particular interest to a continuation of the present study would be investigation into the role of thymic education in these early transplantation models and the relevance of chimaerism to the induction and maintenance of tolerance.
SUMMARY

1. *Xenopus laevis* larvae may be rendered tolerant to high doses of HGG and this tolerance is retained through metamorphosis to adult life where it has been shown to persist for at least 7 months. In addition to the induction method used by Mughal (1984) and Al Johari (1985), it has been shown that a single tolerizing dose will produce the tolerant state.

2. High zone tolerant animals exhibit a proliferative response at the same time as normal antibody producing, responding animals, 21 days after antigenic stimulation. There is, however, a difference in the distribution of the proliferating cells. The HGG tolerant animals have higher numbers of proliferating cells in the perifollicular zones of the spleen than the normally responding animals. These normally responding animals show higher levels of proliferation in the follicular areas of the spleen than their tolerant counterparts.

3. The adoptive transfer of tolerance to high doses of HGG has been achieved using pre-stimulated histocompatible whole spleen cell populations inoculated intravenously, simultaneously with the administration of antigenic challenge via the dorsal lymph sac. This indicates the presence of a suppressor T-cell population. The adoptively transferred high zone tolerance to HGG was shown to be a specific effect; the HGG tolerant animals could produce normal levels of anti-Keyhole Limpet Haemocyanin antibody.

4. Buoyant density gradient separation of spleen cell populations has shown that the induction of high zone tolerance to Human-γ-globulin is accompanied by changes in cellular populations within the spleen.
compared with the normally responding animals which are capable of producing antibody. These changes include increased levels of proliferating cells in the second and fifth density interface bands and decreased numbers of large lymphocytes and granulocytes overall. The small lymphocytes appeared to be distributed in the same manner in tolerant and normally responding animals. Antigenic challenge was accompanied by an increase in large and small lymphocytes and granulocytes in normally responding animals only. The HGG tolerant animals did not exhibit this change.

5. The ability to adoptively transfer high zone tolerance to HGG into histocompatible hosts was lost when these cells were separated into sub-populations on the BSA density gradient. This method does not significantly impair cell activity in mixed leukocyte culture. Indeed, cells retrieved from the middle density interface bands produced stimulation indices similar to controls, indicating that the mixture of active cells present in these layers was adequate for this reaction.

6. Host larvae at stage 52 - 54 were inoculated with allogeneic cells into the connective tissue under the intermandibularis muscle. Two proliferative episodes were observed in the region of the injected cells, the first around day 3 and the second around day 7 after cellular inoculation. These were demonstrated to be largely of donor and host origin respectively, by the use of mitomycin-C treated donor cells and early thymectomized hosts. This reaction was similar in a number of respects to the mammalian normal lymphocyte transfer reaction.

7. The induction of transplantation tolerance was attempted using larval spleens implanted ectopically into larval hosts. Donor spleens
from larval stages 50 - 56 were grafted onto histoincompatible hosts of stages 47 - 57. Transplantation tolerance or chronically impaired rejection was observed in a proportion of individuals in which both the donor and the host were at or below stage 51 at the time of spleen implant. Experiments indicated that allograft retention was prolonged primarily by a delay in onset of rejection and secondarily by a slower rejection process.
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