Development of the immune system

Post-metamorphosis maturation of indices of immunity in *Xenopus laevis*

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Culture of lymphocytes from adult *Xenopus laevis* (Daudin) (a pipid anuran amphibian) with mitogens (PHA, ConA, LPS and PWM) reveals a wide variation in the mitogen sensitivity of these cells: relatively high concentrations of mitogen are required to stimulate *Xenopus* lymphocytes compared with the concentrations appropriate for mammalian lymphocytes. Significantly better responses are obtained using spleen cells from 'young' adult (metamorphosed) *Xenopus* aged 6–10 months from 'mature', 4–5 year-old *Xenopus*.

There is evidence for a population of suppressor cells, probably with functional T lymphocyte characteristics, in the spleens of 'mature' *Xenopus*: mitogen responsiveness of 'young' splenocytes is depressed when 'mature' splenocytes are included in 72 h cultures; non-nylon wool adherent cells from 'mature' *Xenopus* spleens exert a similar suppressive effect. A supernatant factor prepared from cultured, 'mature' spleen cells may mediate such suppression.

In unidirectional mixed lymphocyte reactions using 'young' and 'mature' spleen cells, poor stimulation is obtained when 'mature' cells are used as responders, but good stimulation is obtained with 'young' responders.

Our data suggest that there is considerable post-metamorphosis maturation of the cell-mediated arm of the immune response in *Xenopus laevis*.

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Haematopoietic clonal succession in normal and bone marrow transfused mice

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Some problems relating to the organisation of the haematopoietic stem cell pool may be approached by the analysis of changes in the patterns of mosaicism of X-chromosome linked products in the peripheral blood of female mice. We have used the A and B allozymes of the X-chromosome linked enzyme phosphoglycerate kinase (PGK-1) for this purpose. Estimates of the proportions of the two allozymes have been made in whole blood of female mice bled at intervals for up to two years, and also in granulocyte and lymphocyte populations purified on a fluorescence activated cell sorter. The intramouse variance in the proportions of PGK-1A and PGK-1B was shown to be low, indicating that many stem cells were contributing to haematopoiesis at any one time. In contrast the inter-mouse variance was relatively high, indicating that the founder number for the bone marrow compartment of the haematopoietic system was of the order of 20.

Mice of the CBA/Ca strain with a PGK-1B phenotype were lethally irradiated and reconstituted with a low ($10^5$) or high ($10^7$) dose of congenic bone marrow cells from female mice heterozygous for the $a$ and $b$ alleles of $Pgk-1$. In addition the donor and host mice differed at the $Gpi-Is$ locus, the former carrying the $a$ allele the latter the $b$ allele. Thus any host involvement in the repopulation could be measured. In mice repopulated with the higher dose little intra-mouse variance in the proportions of PGK-1A and PGK-1B was seen; with the lower dose, fluctuations in the patterns of mosaicism were very large, indicating that few haematopoietic stem cell clones were involved in the repopulation and that these clones tended to succeed each other during the lifespan of these animals. This was demonstrated not only in whole blood, but also in the purified granulocyte and lymphocyte populations.
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The omental rudiment: a source of embryonic lymphoid stem cells

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The region of the presumptive omentum of the 13-day mouse embryo has previously been shown to contain precursors of lymphoid stem cells that can differentiate to form Thy 1+, Lyt 1+,-2— lymphocytes after prolonged growth in the anterior eye chamber of adult, syngeneic, irradiated mice.

Our current studies include the analysis of long-term stable cell lines of embryonic preomental origin. In addition to these lines we have also generated hybrids between omentum-derived cells and the BW 5147 lymphoma which have been shown by flow cytometric analysis to express both the H-2 and Thy-1 allelic markers introduced into the hybrid by the omentum-derived fusion partner. Hybrids between preomental cells and the B-cell lymphoma SP 2/0 have also been prepared and are now under study.

Embryological analysis of the preomental region of mouse embryos has now been extended from day 12-16 of development. Precursors of lymphocytes are found at all these times, while the adjacent splenic rudiment remains devoid of such stem cells throughout this period of development. The ultimate destination of the omentum-derived cells has not yet been determined.

Functional studies with cells derived from grafts of preomental rudiments as well as from the various long-term cell lines are in progress. To date, however, no clear functional activities have been associated with the lymphocytes or lymphocyte-tumor hybrid lines developed from the preomental region of the mouse embryo.

Transplantation immunity in hydra

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We have previously shown that phagocytosis of overproduced cells is involved in regulating the growth of hydra tissue (Bosch, T. C. G. & David, C. N., 1984, Dev. Biol. in press). We have now obtained evidence in heterografts of hydra that epithelial cells recognize and phagocytose foreign cells suggesting that there is immunoincompatibility in these phylogenetically old animals.

Upper halves of H. attenuata were grafted to lower halves of H. oligactis and vice versa. These heterografts heal normally and remain stable although there is continuous slow displacement of H. attenuata tissue. This displacement is not due to differences in growth rate as determined by 3H-thymidine labelling but to phagocytosis of one partner by the other. When the graft site was macerated and the cells stained with Feulgen, phagocytic vacuoles were identified in epithelial cells. Phagocytic activity was found in ectoderm as well as in endoderm preparations and was high near the graft site. A monoclonal antibody has been prepared which appears to be specific for these phagocytic vacuoles. Ectodermal and endodermal epithelial cells near the graft junction contain vacuoles labelled by this antibody.

This report presents evidence that immunorecognition followed by an incompatibility reaction occurs in hydra and that the epithelial cell is the effector cell in this transplantation reaction. The data are consistent with the idea that immunocompetence first appeared during evolution in coelenterates as suggested by Hildemann, W. H. et al. (1979, Transplant. Proc. 11, 1136–1142).

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Flow microfluorometric analysis of mouse thymus development
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Using monoclonal antibodies to cell surface determinants and flow microfluorometry (FMF), the phenotypic properties of the major subpopulations of cells in the adult mouse thymus have been characterized. In addition, the appearance of these subpopulations within the developing embryonic mouse thymus has been investigated. Using monoclonal antibodies to the Lyt-2 and mouse T4 antigen, it was found that the embryonic thymus was initially colonized by blast cells expressing neither antigen. By the day 16 of embryonic development, blast cells expressing both antigens were detected whilst on day 17, small cortical thymocytes of similar surface phenotype appeared. Using a combination of DNA and cell surface marker analysis it was shown by FMF that these three subpopulations were lineally related. Two major subpopulations of cells expressing either Lyt-2 or T4 antigen appear in the developing thymus at about 19–20 days gestation. Coincident with the appearance of these two thymocyte subpopulations is the presence within the thymus of precursors of immunocompetent T lymphocytes. The possible lineage interrelationships between these two immunocompetent subpopulations of thymocytes and other cells in the thymus are unclear at the present time.

Image analysis of cell proliferation and differentiation in the thymus of the newt by Samba 200 cell image processor
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The relationships between cell kinetics and nuclear transformations related to cell differentiation were investigated in the thymus of the newt by means of image analysis. A Samba 200 cell image processor was used to compute 18 densitometric, textural and morphological parameters on Feulgen-stained thymic nuclei, from a few days after hatching of larvae (stage 40) to 1 month after metamorphosis (150 days old). During the first step, cell nuclei were automatically identified as lymphoid or epithelial with a 93.4%–98.7% confidence level, when compared with the cytological diagnoses. During the second step, four cell classes were recognized in both epithelial and lymphoid cell populations and assumed to correspond to G₀, G₁, S and G₂ cell subpopulations, on the basis of both the nuclear DNA content and the chromation pattern. The variations in the percentages of cells in these four classes, in addition to the evolution of growth fraction and cell number, indicate that the thymus is basically an exponentially growing epithelial bud, which reaches a steady state during metamorphosis. A few lymphoid G₀ stem cells penetrate the epithelial bud up to stage 42, enter the G₁ phase of the mitotic cycle, and give rise to lymphoblasts. Then, lymphoblast cells produce lymphocytes, which perform intensive proliferation until metamorphosis, while an increasing proportion of them leave the thymus. During metamorphosis, a steady state is reached in the lymphoid cell population as in the epithelial one, and statistically half the number of new lymphocytes emigrate.
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The development and control of the TNP-Ficoll response in *Xenopus laevis*, the South African clawed toad

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The response to TNP-Ficoll (a TI-2 antigen in mammals) has been shown to be thymus dependent in *Xenopus* (Horton et al. 1979). While Concanavalin A is capable of restoring responsiveness to TNP-Ficoll in adult-thymectomised animals, other lectins, such as wheat germ agglutinin, cannot (Clothier et al., in press). Early-thymectomised *Xenopus* respond to another mammalian TI-2 antigen, polyvinylpyrrolidone (PVP, Tochinai, 1976), but PVP is not capable of restoring the capacity of thymectomised animals to respond to the TNP-Ficoll. Also, unlike the response to TNP-Ficoll the antibody response to PVP has been found to be susceptible to immunological suppression in *in vitro* studies on spleen/thymus allogene combinations.

The capacity to respond to TNP-Ficoll arises with the development of a thymus medulla (Ruben et al. 1984), but is not dependent on helper T-cells, since carrier priming is not required (Horton et al. 1979).

The identification of this difference between mammals and an amphibian raises interesting questions about the evolution of thymic regulation of immune responses.


Emergence of the intraembryonic hemangioblastic lineage studied in avian chimeras by means of monoclonal antibodies

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The site and timing of blood stem cell segregation from intraembryonic mesoderm was investigated by grafting presumptive hemogenic rudiments from the quail embryo into microenvironments which promote hemopoiesis. Cells belonging to the blood or endothelial lineages were traced by means of a monoclonal antibody which recognizes quail cells from these lineages, to the exception of cells engaged in the erythroid pathway. These cells arise during day 2 of incubation within the area pellucida lateral plate according to a cephalo-caudal gradient. Whether they become blood precursors or endothelial cells seems dictated by the location they engraft into.
Development of the immune system

Xid expression in the B lymphocyte lineage of (CBA/HN × CBA/PGK-1A) F1 mice

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The mechanism of action of the X-chromosome-linked-immunodeficiency (xid) gene on B lymphocyte development is unknown. CBA/HN mice which are homozygous for the xid trait show abnormalities in B lymphocyte function and have been shown to be deficient in at least one recognised B lymphocyte subpopulation. Female mice, heterozygous at the xid locus, show similar, but less pronounced effects.

We have used mice which are heterozygous both for xid and for the A and B alleles of the X-linked enzyme phosphoglycerate-kinase (PGK-1A), to answer questions relating to the effects of the xid gene on the differentiation of B lymphocytes. Due to the phenomenon of X chromosome inactivation, any B cell clone will express either the xid and Pgk-1B bearing X chromosome or the normal X chromosome carrying the Pgk-1A allele, but not both. The level of PGK-1B or PGK-1A activity in any cell suspension is thus an indirect measure of xid or normal gene expression respectively.

B lymphocytes were isolated from bone marrow, peripheral blood and various other lymphoid tissues using monoclonal antibodies against B lymphocyte lineage antigens and fluorescence-activated-cell-sorting. The PGK phenotypes of these purified cell populations were estimated after electrophoresis. The data suggest that the proportions of the A and B alloenzymes of PGK in the B lymphocyte compartment of bone marrow of xid heterozygotes are similar to those in normal mice. However, in the subcutaneous lymph nodes, spleen and blood the proportion of the A alloenzyme (non-xid-associated) increases in the surface immunoglobulin-bearing lymphocyte populations and approaches 100% in some individuals. This suggests that the xid lesion acts during maturation of B lymphocytes after egress from the bone marrow. Further data will be presented of the analysis of specific B lymphocyte subpopulations.

Ontogeny of Fc γ receptors in the gut of suckling rats

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Transmission of antibodies (IgG) across the gut of suckling rats is known to involve specific receptors (Fc γ receptors) present on enterocytes. One way in which these receptors have been demonstrated is by means of an erythrocyte antibody (EA) rosette assay in which enterocytes isolated by means of an EGTA-containing buffer are reacted with indicator sheep red blood cells sensitized with rabbit IgG (Wild, 1982). In the present investigation observations have been extended to various stages of development in order to study the ontogeny of Fc γ receptors as reflected by E-A rosette formation. E-A rosetting of gut enterocytes occurred up to 21 days after birth with the percentage of cells exhibiting rosette formation falling off rapidly at this stage and individual variation increasing. E-A rosetting of enterocytes was first seen in low incidence in the gut of 20 day old foetuses and the percentage of rosette forming cells rose rapidly on subsequent days. At all ages studied indicator red blood cells bound only to the abluminal plasmalemma, this presumably being a reflection of the deep seated location of Fc γ receptors on the apical microvilli. Binding of indicator red blood cells was found to be acid pH dependent at all stages studied and confined to enterocytes of the proximal part (duodenum and jejunum) of the small intestine. These findings are similar to previous ones made on 12 day old rats (Wild, 1982) and where the pH dependency of IgG-receptor binding to enterocytes seems to be an adaptation to the acid pH of the proximal small intestine gut lumen.

Development of the immune system
The frog – A comparative model for investigating immunologic development and thymus function
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The free-living amphibian embryo offers a unique opportunity for probing ontogenetic aspects of immunity in the absence of direct maternal influence. The usefulness of this comparative approach for immunological research has been greatly increased in recent years, following the development of inbred lines and isogenic strains of anurans, and through experiments revealing that immune cell interactions in anurans are, as in mammals, genetically restricted by major histocompatibility complex (MHC) antigens. This paper reviews some of the anuran studies emanating from laboratories around the world (see articles in Cohen & Sigel, 1982), which further our understanding of: (i) the embryological origin of lymphocytes, (ii) the functional development of B cells and T cell subsets, (iii) the acquisition of self- and allo-tolerance, and (iv) the role of the thymus in immune development. Special attention is focussed on ongoing allotransplants 'chimera' experiments with Xenopus being performed in this laboratory and elsewhere. To date, involvement of the thymus in conferring MHC restriction on the T cell populations involved in antibody production and the rejection of minor histocompatibility antigen-disparate skin grafts is suggested from work on embryonically-established thymus chimeras (Flajnik, 1983), but not in those experiments using the Xenopus thymectomy/thymus implant model system (Du Pasquier & Horton, 1982; Gearing et al. 1984).

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Precursors of B lymphocytes and factors which affect their differentiation
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Little is known about how the enormous daily production of B lymphocytes within mammalian bone marrow is regulated. The immediate precursors of B cells can be identified by their content of an incomplete immunoglobulin molecule and presumptive earlier cells of this lineage can be distinguished by means of monoclonal antibodies directed to surface macromolecules. Maturation of such precursors occurs in culture and this has made it possible to investigate cellular interactions and factors which might affect this process. Our studies indicate that the final stages of B cell formation can be enhanced in vitro by the lineage nonspecific cytokines interleukin-1 and immune (gamma) interferon. Another factor was discovered by study of a strain of mice (NZB) which has hyperactive lymphopoiesis early in life. The biochemical and biological properties of this material indicate that it may be a novel regulatory substance. Study of a cyclic neutropenia patient with dysregulated pre-B cell formation recently led to the identification of another factor(s) which seems to act on very early precursors of B cells to stimulate their proliferation and/or differentiation. The physiologic importance of these substances to development of the immune system and in thereafter maintaining a steady output of newly formed immunocytes has not yet been formally evaluated. Of equal interest will be the characterization of cells which elaborate these factors and identification of other substances that might counter their action.
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Studies on the developmental potential of thymus stem cells

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The mature T-cell population consists of a number of sub-populations which differ in function and in the array of cell surface markers they possess. These sub-populations are derived from pre-thymic cells which must take up residence in the thymus in order to mature into functional T-lymphocytes. At present the identity of the stem cells, whether the different sub-populations diverge pre-thymically or intra thymically, and when they acquire their antigen receptor repertoire are unknown.

As an approach to these questions, we have developed an in vitro system using organ culture in which embryonic lobes are depleted of their own lymphoid stem cells, by exploiting the fact that these cells are selectively susceptible to the toxic effects of deoxyguanosine added to the culture medium. These empty lobes in which the epithelial stroma remains unharmed are then recolonized by association in hanging drops with known numbers of T cells precursors from another early embryonic thymic rudiment from a strain carrying different alleles at Thyl or Lyt 1 and 2 loci.

Using this system we have investigated the proliferative potential of thymic stem cells and shown that very small numbers of precursors and even a single cell can recolonize empty lobes. We have also found that a single thymic stem cell, isolated by micromanipulation and allowed to colonize an empty lobe can give rise to three different phenotypes defined by combinations of the lymphocyte surface markers Lyt 1 and Lyt 2. These results imply that divergence into the marker defined sub-populations does not occur pre-thymically and should provide a basis to investigate the point in T-cell development when the antigen receptor repertoire is generated.

The maturation of immunocompetence in young carp, *Cyprinus carpio* L.

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Commercial interests in immunizing young fish against disease emphasize the need for a better understanding of the maturation of the immune system, so that young fish can be vaccinated in an appropriate manner and at the best age. Our work on the carp, *Cyprinus carpio*, shows that the cell-mediated component of the immune system develops rapidly, enabling the young fry to respond to allografts by day 16 post-hatching (at 20 ± 2 °C). By the age of 4 weeks, these young carp show a proliferative response of their lymphocytes to bacterial antigens and display second-set alloimmunity. At this age, (but not by 9–10 weeks old) they could, however, still be rendered tolerant in certain humoral antibody responses.

Experiments will be described in which 4-week old carp were immunized with a soluble protein antigen, human gamma globulin (HGG), or a bacterial antigen, formalin killed cells of *Aeromonas salmonicida*, the causative agent of furunculosis. The HGG was either injected or administered by bath immersion. A dose of 25 µg/g body weight was used for injection and this was either administered in solution or coated onto latex beads. For direct immersion, the bath contained either 5 mg/litre soluble HGG or 5 mg/litre HGG coated onto 0.8% w/v latex beads. It was found that administration of HGG by injection induced tolerance (non-reactivity after secondary challenge at an age when carp can normally produce antibody against HGG) and that the use of a latex bead carrier did not prevent this tolerogenic effect. Immunization by direct immersion produced different results: in this case little or no tolerogenic effect was observed. All fish, even the injected fish which had been rendered tolerant in their antibody responses, were able to react to HGG on challenge by a proliferative lymphocytic response. Thus not all components of the immune system were rendered tolerant by the antigen in these young fish. Furthermore, administration of the bacterial antigen, either by injection of 10⁸ cells/g body weight or by directly immersing the 4-week old fish in a bath containing 10⁸ cells per litre, yielded positive responses in all experiments, and the priming led to improved responses on secondary exposure. These bacterial cells, in contrast to HGG, are thymus-independent antigens. The results therefore lend some support to the suggestion that young fish respond better to thymus-independent antigens than to those which require T-cell help in mammals.
Development of the immune system

Differentiation of T lymphocytes in the embryonic thymus

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Despite some claims that T lymphocyte maturation can proceed within extra thymic sites for example in lymphomyeloid organs of athymic nude mice, most evidence suggests that the thymus is an essential site of generation of T lymphocytes which are then exported to peripheral lymphoid tissues. However, many questions concerning the role of the thymus in T lymphocyte production remain unanswered; is the thymus the site of generation of diversity of T lymphocyte antigen recognition receptors?; are differentiating T lymphocytes rendered tolerant to autoantigens within the thymus?; is MHC (major histocompatibility complex) restriction of T lymphocyte responsiveness acquired within the thymus?; what role/s do thymic stromal cells play in T lymphocyte differentiation?

Fortunately, some progress has been made recently in providing pointers to the answers to these questions. Large numbers of diverse T lymphocytes are probably derived from a limited number of stem cells within the thymus. This observation provides support for the notion that diversity is generated within the thymus. The thymic stroma contains epithelial cells and dendritic (antigen presenting) cells both of which strongly express class II MHC antigens. Progress is being made in analysing the relative importance of these two cell types in tolerance induction and MHC restriction. Monoclonal antibodies are available which can be used to define subtypes of thymic stromal cells; they may allow an analysis of the respective roles of these cells in T lymphocyte differentiation.

The thymus continues to provide a focus of interest for immunologists. The application of recent knowledge of the nature of the T cell receptor to intrathymic events should prove exciting.

Long-term murine bone marrow cultures: characterization of three stromal cell populations by flow cytofluorimetry and sorting, and studies of their origin in radiation chimaeras

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The bone marrow stroma is essential for the support of haematopoiesis in vivo and in vitro. It can be investigated in vitro using two culture systems, long-term bone marrow cultures (LTBMC) and cultures of fibroblast colony forming cells (CFU-F), which are considered to be part of the stroma. Histochemical and immunohistochemical staining of the adherent layers within LTBMC and CFU-F has shown that fibroblastic cells and macrophages constitute the major stromal elements (Reincke, U., Hsieh, P., Mauch, P., Hellman, S. & Chen, L. B. (1982). J. Histochem. Cytochem. 30, 235–244; Xu, C. X., Hendry, J. H., Testa, N. G. & Allen, T. D. (1983). J. Cell Sci. 61, 453–466).

Fluorescence-activated cell sorter (FACS) analysis using green and red fluorescence of adherent-layer cells from LTBMC, previously incubated with fluorescent green beads, reveals four distinct populations. Histochemical and immunoperoxidase staining of FACS-sorted populations with macrophage-specific F4/80 monoclonal antibody, shows that pop. 1 is F4/80\(^{-}\), nonspecific esterase (NSE)\(^{-}\) (this is the main source of spleen colony-forming cells and granulocyte/macrophage progenitors); pop. 2 is mainly F4/80\(^{-}\), NSE\(^{-}\), is nonhaematopoietic and non-phagocytic, but contains a small proportion of granulocytes; pop. 3 is F4/80\(^{+}\), NSE\(^{+}\) and non-phagocytic; pop. 4 is phagocytic and otherwise resembles pop. 3. FACS analysis of CFU-F cultures reveals a similar picture except that pop. 1 is absent and pop. 2 is free of granulocyte contamination. Pop. 2 thus represents a nonhaematopoietic component of the adherent layer.

It is known that haematopoietic stem cells are transplantable by intravenous injection, but controversial whether the bone marrow stroma is transplantable in the same way. By means of enzyme markers (allelic, electrophoretically distinct forms of phosphoglycerate kinase), radiation chimaeras can be established in which donor and host components can be identified. Currently we are using alloenzyme analysis of FACS-sorted cells to investigate the origin of stromal elements in LTBMC and CFU-F cultures derived from repopulated marrow.