Axolotl Immunology: Lymphocytes, Cytokines, and Alloincompatibility Reactions

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Introductory Remarks

One approach to understanding the evolution of the immune system is to examine this system in taxonomically diverse extant species. A particularly important group of vertebrates in this regard are the Amphibia, for, based on a variety of morphological and physiological characteristics that distinguish them from fish on the one hand and reptiles on the other, this class is generally thought of as a phylogenetically pivotal group. The anuran amphibian (e.g., *Xenopus laevis)* possesses an immune system that is remarkably similar, in terms of its structure and function, to that of mammals (for reviews, see Du Pasquier et al., 1989; Kaufman et al., 1991). This tells us that the basic components of the immune system (e.g., organized and sympathetically innervated lymphoid tissues, T and B lymphocytes, cytokines, antibody isotype diversity, a polymorphic major histocompatibility complex (MHC) whose class I and class II gene products function in restricted antigen presentation and cell interactions, a complement system, cytokines) were all probably in existence 150 million years ago. Among the Amphibia, however, it may be the Urodela rather than the Anura, that offer the more interesting model in the comparative approach to studying evolution of the vertebrate immune system. This is because some features of the salamander immune system appear surprisingly different from those of the frog (Table 1) and of mammals. Our particular contribution to the newsletter will be restricted to those facets of salamander immunology we've been studying in Rochester for several years, namely mitogen-driven proliferation of, and cytokine production by, axolotl lymphocytes *in vitro*, and mixed lymphocyte reactions (MLRs) and transplantation reactions in the Urodela as they relate to the structure and function of a putative salamander MHC.

Lymphocyte Proliferation

Polyclonal activation with mitogens: One characteristic, indeed essential, property of mammalian (and anuran) lymphocytes is that they undergo antigen-specific clonal proliferation when they encounter the antigen for which they express a receptor. Polyclonal proliferation, which involves many of the same events as antigen-driven amplification, can be triggered by phytohemagglutinin (PHA) and concanavalin A (Con A), mitogenic plant lectins that preferentially activate thymus-dependent T-lymphocytes.

Historically, the previously reported lack of routine significant *in vitro* PHA- or Con A-induced proliferative responses of lymphocytes from axolotls (Collins et al., 1975) and newts (Cohen, 1980) provided the basis for arguments that salamanders lack classic T-lymphocytes (Cohen, 1980). This phylogenetically intriguing possibility, however, is no longer tenable owing to the following observations made in several laboratories within the past two years. First, we (Koniski and Cohen, 1992) convincingly demonstrated that PHA and Con A can indeed be used to routinely stimulate *in vitro* proliferation of axolotl splenic lymphocytes, providing that the splenocytes are cultured in the appropriately supplemented tissue culture medium. These observations have been confirmed by Kaufman and by Tournefier (personal communications). Although we may not have yet achieved optimal culture conditions, in our experience, Leibovitz-15 (L-15) supplemented with 0.25% bovine serum albumin (BSA) "works," whereas L-15 supplemented 1% fetal bovine serum (FBS) does not (Table 2; see discussion below). Second, unpublished data from experiments using anti-axolotl immunoglobulin (Ig) M heavy- and light chain-specific monoclonal antibodies (Tournefier et al., 1988a, b) in cell separation procedures such as panning (our lab, antibodies provided by Tournefier) and magnetic beads (Tournefier, personal communication), argue that PHA- and Con A-responsive cells are surface Ig negative (i.e., T cells). Finally, recent studies using antibodies (Kerfourn et al., 1992, 1993) and molecular techniques (Fellah et al., 1993) reveal that axolotl lymphocytes express a putative T-cell receptor (TCR).

Lymphocyte-derived cytokines: One way in which the clonal expansion of antigen stimulated lymphocytes is regulated is by cyto-

Table 1
Some Immunological Features of adult *Xenopus* and *A.mexicanum*[1]

Immunological feature	*Xenopus*	*Axolotl*
Thymus morphology: cortex/medulla	defined	undefined
Spleen morphology: red/white pulp	defined	undefined
Graft rejection (thymectomy)	acute (abrogated)	chronic (abrogated)
In vitro MLR (thymectomy)	strong (abrogated)	poor (?)
T-cell mitogen response	good (FBS[2] or BSA) good (BSA)	poor (FBS)
B-cell mitogen response (LPS)	modest	modest (BSA or FBS)
PMA response	good	good (BSA or FBS)
Antibody response (thymectomy)	good (restricted diversity) poor (thymus dependent)	poor (limited diversity) enhanced (SRBC/HRBC)[3]
Isotype switching (thymectomy)	yes no (IgY)[4]	no
Affinity maturation	modest	none
MHC class II antigens	B cells, T cells, thymocytes thymocyte areas of thymic stroma	B cells, T cells, erythrocytes, thymic stroma, endodermal epithelium, skin glands, brain
MHC class I antigens	ubiquitous cell surface	a chain on erythrocytes

[1] adapted from Kaufman et al., 1991
[2] Refers to protein source in culture medium (BSA = bovine serum albumin; FBS = fetal bovine serum)
[3] SRBC = sheep red blood cells; HRBC = horse red blood cells
[4] Du Pasquier & Wabl, 1977

kines and their receptors. Cytokines are low molecular weight polypeptides or glycoproteins that are produced by avariety of cell types and have pleiotropic effects on cell proliferation, differentiation, and function. Cytokines produced by leukocytes (i.e., interleukins) regulate many facets of an immune response including its intensity, duration, and whether it is predominantly cell-mediated or humoral (Dugan, 1991). For example, in the presence of interleukin (IL)-1, mammalian T cells are triggered, by the binding of PHA to the TCR complex, to synthesize and release IL-2 and express IL-2 receptors (Chilson and Kelley-Chilson, 1989). IL-2 then activates any cell bearing an IL-2 receptor in an autocrine and paracrine fashion, causing those cells to progress from the late G1 phase of the cell cycle into the S phase (Weiss, 1989; Kuziel and Greene, 1991).

Although many cytokines of mammalian leukocyte derivation have been cloned and exhaustively studied (Thomson, 1991), relatively little is known about cytokines in fish,

amphibians, and reptiles (Secombes, 1991; Cohen and Haynes, 1991). With respect to amphibians, small peptides with the functional properties of IL-1 (Watkins et al., 1987), IL-2 (Haynes and Cohen, 1993a), and TGF-β5 (Haynes and Cohen, 1993b) have been identified as products of leukocytes from the anuran, *Xenopus laevis*. Given that lectins can stimulate growth of axolotl T cells, we then asked whether a cytokine with T-cell growth factor (TCGF) activity is involved in mitogenesis of axolotl lymphocytes. Aside from the obvious phylogenetic importance of this question, an absence of an IL-2-like cytokine could explain several features of the axolotl immune response that are discussed later in this article (i.e., the lack of significant mixed lymphocyte reactivity (MLR) *in vitro* (Collins et al., 1975) and chronic skin allograft rejection *in vivo* (Cohen, 1968, 1974, 1980). Such an explanation, however, does not appear to be valid since we have recently described a cytokine with IL-2-like activity in supernatants of PHA-stimulated axolotl lymphocytes (Koniski and Cohen, 1994). These PHA-generated culture supernatants (PHA-SNs) stimulate proliferation of homologous blasts but not resting splenocytes. As would be predicted from studies of IL-2 receptor expression on mammalian cells, axolotl lymphoblasts absorb biologic activity of SNs to a far greater extent than do freshly harvested splenocytes. *SDS*-PAGE gel analysis of metabolically-labeled axolotl PHA-SNs revealed a band at approximately 14-21 kD. This corresponds to the gel fraction with biological activity eluted from axolotl PHA-SNs, and is also consistent with the Mr for mammalian IL-2 (Kuziel and Greene, 1991). Although axolotl lymphoblasts cultured in medium supplemented with 1% FBS do not proliferate in response to PHA, they do secrete a cytokine with lymphoblast growth-promoting activity. Furthermore, PHA-induced lymphoblasts initially cultured in medium supplemented with 0.25% BSA, can proliferate in response to PHA-SNs, even in 1% FBS-supplemented medium. Thus, not only are the properties of a cytokine produced by mitogen-stimulated axolotl splenocytes similar to mammalian IL-2 and *Xenopus* TCGF, but biosynthesis of this cytokine may be separable from processes associated with lymphoproliferation.

Interestingly, there have been a few reports of significant and reproducible proliferation of splenocytes from the newt (Suzuki and Cohen, 1987) and axolotl (Koniski and Cohen, 1992), following their incubation in FBS-supplemented medium, with nanogram quantities of phorbol 12-myristate 13-acetate (PMA). PMA directly activates protein kinase C (PKC; Niede et al., 1983), whereas PHA binds to a carbohydrate residue on the mammalian T-cell receptor (Weiss, 1989) and triggers proliferation by cross-linking that receptor. Our observations, together with additional studies (Suzuki and Cohen, 1987 and unpublished observations) with H7, an inhibitor of PKC (Kawamoto and Hidaka, 1984), and with retinol acetate, which blocks activity of PMA in mammalian systems (Wertz et al., 1979), suggest that: a) PMA induces salamander lymphocyte mitogenesis by direct stimulation of the PKC second messenger system (Niede et al., 1983), and b) PHA (or Con A) does not provide an appropriate signal for proliferation under culture conditions that can support proliferation of lymphocytes cultured with PMA. This apparent lack of *in vitro* mitogenesis in FBS-supplemented culture medium, to classic T-cell mitogens, but not to PMA, suggests that axolotl and frog T lymphocytes may differ by some immunologically fundamental feature(s), perhaps related to lectin binding, signal transduction, or T-cell receptor structure. With this in mind, it may be informative to speculate if, and how, FBS might block PHA activation. For example, FBS (which is not cytotoxic) might be blocking PHA binding to and signaling through the TCR complex (Weiss, 1989). Alternatively, FBS may: a) nonspecifically bind to IL-2 secreted by axolotl T cells; b) prevent the binding of IL-2 to its receptor; or c) prevent IL-2 receptor expression. It is important to note that once splenocytes are activated in BSA-supplemented medium, they can proliferate equally well in response to IL-2-containing supernatants in medium supplemented with either FBS or BSA. In addition, as mentioned previously, PHA-treated axolotl cells can secrete an IL-2-like cytokine in the absence of proliferation (Koniski and Cohen, 1994). These observations rule out the possibilities that FBS blocks axolotl IL-2 synthesis and secretion, or that FBS is nonspecifically absorbing the IL-2-like cytokine, or preventing its binding to the IL-2 receptor. These data, however, do not rule out that the negative effect of FBS on PHA-induced proliferative responses could result from FBS preventing IL-2 receptor expression.

The following preliminary data from several experiments recently conducted in our lab suggest still another alternative explanation of the absence of mitogenesis of spleen

Table 2

Stimulation indices (SIs) and background CPM±SE of PHA-stimulated splenocytes from individual axolotls cultured for 5-days in L-15 medium supplemented with either FBS or BSA[1]

	L-15 culture medium supplemented with either			
	1% FBS		0.25% BSA	
Axolotl #	SI[2]	Background CPM ±SE	SI	Background CPM ±SE
1	1.3	2733 ± 645	5.2	1726 ± 189
2	1.6	1047 ± 196	8.3	749 ± 62
3	0.8	2388 ± 211	4.8	4863 ± 305
4	3.0	1118 ± 148	5.5	2150 ± 305
5	1.3	307 ± 43	4.2	695 ± 26
6	1.0	1202 ± 24	14.1	515 ± 61

[1] Data from Koniski & Cohen (1992); Mean counts per minute (CPM) ± standard error (SE) are values of tritiated thymidine incorporation in a 5-day PHA mitogen assay.

[2] SIs are calculated by dividing the CPM of PHA-stimulated cultures by the CPM of cultures without mitogen (i.e., background CPM); SIs are associated with optimal PHA concentration (0.5-4 µg/ml) for an individual animal in either BSA- or FBS- supplemented medium.

cells in FBS-supplemented medium, namely that in this medium, the PHA-treated spleen cells produce the cytokine, transforming growth factor beta (TGF-ß), which, in turn, actively suppresses mitogenesis. TGF-ß is secreted in a 100kD biologically inactive latent form that, when cleaved by a change in pH or proteolysis, yields a 25kD active homodimer (Roberts and Sporn, 1990). Specifically, we have observed that when goat anti-TGF-ß2 antibody was added to splenocytes cultured with PHA in L-15 medium supplemented with 1% FBS, the cells were then responsive to PHA. Such responses did not occur with the addition of either the control goat IgG or goat anti-TCGF-ß5. In another preliminary experiment, we have demonstrated that acid-activated PHA-SNs from cultures of axolotl splenocytes had profound inhibitory effects on PHA-induced proliferation of splenocytes cultured in 1% BSA, whereas untreated PHA-SNs had no such effect. That this inhibition was not reversed by the addition of either anti-TGF-ß5, anti-TGF-ß2, or goat IgG, suggests that either another isoform of TGF-ß may exist in the axolotl, or that another inhibitory cytokine may be involved. Finally, our unpublished data reveal that PHA-induced proliferation of axolotl splenocytes and thymocytes is sensitive to the addition of *Xenopus* recombinant TGF-ß5, an effect that can be reversed by the addition of anti-TGF-ß5, but not anti-TGF-ß2 or goat IgG.

Alloincompatibility Reactions and MHC Structure and Function

Although we now know that axolotls have T cells that can polyclonally respond to mitogenic signals and synthesize an IL-2-like cytokine, these and other species of salamanders still exhibit strikingly different immunological features from either anurans or mammals (Table 1). Among these are a lack of significant mixed lymphocyte culture (MLC) reactivity *in vitro* (Collins et al., 1975; Cohen, 1980) and the routine chronic graft rejection of skin and organ grafts *in vivo* (Cohen, 1968, 1969). At least some of these features appear related to the phylogenetically important issue of whether axolotls and other urodeles have an MHC; this topic will be addressed in the remainder of this article.

The MHC is a collection of closely linked gene loci whose molecular products are thought to be critical for the proper functioning of the immune system of mammals, birds and anuran amphibians (Stites and Terr, 1994). MHC class I molecules consist of a polymorphic α chain (Mr 45kD) that is noncovalently paired with a nonpolymorphic $ß_2$-microglobulin light chain (M_r 12kD) coded for by a gene located outside the MHC. In mammals, MHC class I molecules are expressed on all nucleated cells examined, although their level of expression can vary significantly. MHC class I molecules serve as restricting elements in T-cell-mediated cytotoxic reactions directed against virally-infected and allogeneic cell targets. Class MHC class II molecules are composed of a polymorphic α and ß chain, both of which are encoded within the MHC. In mammals, class II expression is limited to antigen-presenting cells such as macrophages, monocytes, B-cells, (activated T cells in humans and adult *Xenopus*), dendritic cells, Langerhans cells, and some epithelial cells (Sigal and Ron, 1994). Class II molecules "restrict" antigen presentation and T-B cell collaboration.

One theory concerning the phylogeny of the MHC is that it evolved to ensure that self- and allorecognition can coexist without autoimmune consequences. Indeed, a polymorphic gene locus that controls fusion and rejection reactions in colonial protochordates has been proposed as the primordial MHC (Du Pasquier, 1989). A clearly recognizable structural and functional homologue of MHC, however, can be traced back at least as far as the emergence of anuran amphibians, 150 million years ago (Du Pasquier et al., 1989). As in mammals, the MHC of *Xenopus*, known as XLA, exhibits extensive class I (Flajnik et al., 1984, 1986) and class II polymorphism (Kaufman et al., 1985a, 1985b, 1991). Acute graft rejection *in vitro* and cell-mediated cytotoxicity, MLR and antigen-specific T-cell proliferation *in vitro* are but a few immunologically significant phenomena that involve products of XLA class I and II loci (Bernard et al., 1979, 1981; Harding, 1990; Harding et al., 1993)

MLC reactions: The immunobiological, immunogenetic, and molecular evidence for a true polymorphic MHC in any urodele is far less convincing than that for *Xenopus*. For example, when lymphocytes from two outbred anurans (or mammals) are co-cultured, a robust bi-directional response involving blast-cell transformation and cell proliferation response is typically triggered by MHC class II-encoded antigen disparities (Harding et al., 1993; Kaufman et al., 1985a). This so-called MLR is thought of as an *in vitro* correlate of *in vivo* allograft recognition. It has been known for many years that MLRs of splenocytes from either axolotls (Collins et al., 1975; DeLanney et al., 1975) or *Notophthalmus* (Cohen and Horan, 1977; Cohen, 1980) are either nonexistent (stimulation indices or SIs <1.6) or of borderline significance (e.g., SIs of 1.6-3.0). In newts, attempts either to correlate the incidence or magnitude of MLR with rapidity of skin graft rejection or to increase the incidence or magnitude of MLRs by *in vivo* immunization, have been unsuccessful (Cohen and Horan, 1977). Recently, we have demonstrated a similar lack of reproducible MLRs of cocultured axolotl lymphocytes, even in the BSA-supplemented L-15 medium that supports mitogen-driven proliferation to which we also added homologous IL-2 (unpublished observations). These data have suggested that either the axolotl has no class II homologue, or if it does, either: a) the molecules that it encodes do not stimulate lymphocyte proliferation; b) the locus is minimally polymorphic; or c) axolotl lymphocytes lack a receptor for allo-class II MHC. Are there data that support or negate any of these alternatives? Recently, molecules that closely resemble mammalian MHC class II gene products have been identified in the axolotl by using cross-reactive antibodies against human MHC class II antigens (Kaufman et al., 1990, 1991). In concert with speculations derived from studies of *in vivo* and *in vitro* alloreactions (Cohen, 1980), only two molecular forms ("alleles") of this glycoprotein have been detected (Kaufman et al., 1990). It is noteworthy, however, that even when lymphocytes from pairs of axolotls, each of which was "typed" for different "alleles" at this putative class II locus, were co-cultured in medium that supports mitogenesis, Kaufman (personal communication) observed MLRs that although statistically significant, were of very low magnitude. Although one might explain this minimal polymorphism by referring to a possible founder effect in the axolotl (Kaufman et al., 1991), similarly poor or nonexistent MLC responses have also characterized *in vitro* alloimmune responses of newts (*Notophthalmus*) from geographically diverse populations. Data from MLR experiments with such populations are also consistent with the possibility of what Cohen (1980)

called a "predominant MLR locus" with two alleles. However, even when lymphocytes from animals thought to be homozygous for different alleles at this so-called predominant locus were cocultured, the magnitude of statistically significant proliferative responses was minimal (Cohen, 1980).

The implications that salamander MHC class II genes have restricted polymorphism is profound, both for survival of the species and for considerations of immune system phylogenesis. MHC polymorphism in mammals strongly impacts on the immune response to nominal antigens in that each allelic form of an MHC antigen is able to bind a finite number of peptides. Lack of class II polymorphism leads to restricted capacity to recognize diverse antigens. In an outbreeding population, polymorphism is thought to prevent "holes" in the TCR repertoire, and, thereby, facilitate antigen presentation/recognition on a population as well as on an individual level. In certain inbred strains of mice where the repertoire of MHC antigens is limited, animals are unable to respond to antigens that are normally strong antigens in other strains. According to current dogma, since it seems unlikely that an entire population would be unable to respond to a given antigen, antigen diversity has been thought to provide the selection pressure that maintains the polymorphism. If like mammals, the salamander T-cell receptor for allo-MHC is really a receptor for self-MHC plus nominal antigen, and if class II polymorphism on a population level is really limited to two alleles, then the fact that axolotls respond poorly, if at all, to the few protein antigens that have been examined, suggests that the antigen receptors on their T cells may indeed have a remarkably restricted T-cell repertoire. How this restricted repertoire has affected survival and speciation of the urodeles is but one of the fascinating questions engendered by this possibility.

Transplantation reactions: In mammals, rejection of skin grafted among outbreeding animals is acute (i.e., it is completed within the first 1-2 weeks postgrafting) and is mediated primarily by T cells responding to both MHC and minor histocompatibility (H) antigens. During acute graft rejection, T cells in the initial infiltrate contact alloantigens and synthesize and secrete various cytokines. These, in turn, cause a cascade of cellular events. IL-2 secretion, for example, effects an autocrine and paracrine clonal expansion that culminates in the generation of effector cytotoxic T cells. Interferon gamma (IFN γ) secretion leads to a delayed-type hypersensitivity response that promotes the influx of macrophages and their subsequent activation. TNF-β mediates cytotoxic activity against the cells of the graft, and the induced secretion of IFN α, β, and γ upregulates expression of donor cell-surface MHC class I and II antigens. Acute rejection of vascularized organ grafts is manifested by endothelial damage, necrotizing arteries, and mononuclear cell infiltrate-induced fibrosis (Stites and Terr, 1994).

When murine inbred donors and hosts are identical at the MHC but disparate at so-called minor or "weak" histocompatibility loci, graft rejection is typically more chronic. Studies of such murine alloimmune reactions has led to the formulation of certain rules associated with minor histoincompatibilites (Hildemann and Cohen, 1967). For example: a) the later the median survival time of such weakly disparate grafts, the broader the range of individual graft survivals; b) grafts that differ from hosts by one or two minor H-loci can survive for many months or even indefinitely; and c) grafts that differ from recipients by multiple minors may be rejected as vigorously as are MHC disparate grafts.

In a compilation of several different studies, typical median survival times of first-set skin allografts in the newt *Notophthalmus viridescens viridescens* ranged from 33-45 days. In those same studies, survival times of several thousand *N. v. viridescens* allografts ranged from the acute to chronic (e.g., 7-155 days) (Cohen, 1971), and in some donor-host combinations, permanent graft survival (>400 days) was noted. Antigenically-specific accelerated rejection of second-set grafts in newts (as well as in other urodele species studied) reveals that first-set allografts affect classic immunological memory (Cohen, 1970).

In addition to several species of newts of the family, Salamandridae, outbreeding axolotls (Tahan and Jurd, 1983; Charlemagne and Tournefier, 1977) and other Ambystomidae (Cohen, 1968) as well as salamanders from other urodele families (e.g., Proteidae, Plethodontidae) also typically reject skin allografts in this chronic fashion (Cohen, 1968). Surprisingly, xenografts exchanged in intra- and inter-familial combinations within the Urodela are also rejected chronically. Specifically, in a study involving reciprocal skin grafting between four families of urodeles, six combinations exhibited chronic responses,

whereas two were characterized by subacute reactions. As was seen for allografts, some xenografts also survived indefinitely (Cohen, 1971). In some instances, third-party xenografts, as well as true second-set original donor xenografts were rejected in an accelerated fashion, suggesting shared H-antigens among members of the order as well as among members of an individual species.

Owing to the lack of highly inbred salamander strains, insights into the immunogenetic basis of subacute and chronic rejection have been derived by studying the rejection patterns of *Notophthalmus* allografts transplanted in interpopulation and intrapopulation combinations (Cohen, 1969). Data from these studies, together with those from the previously cited investigations, are consistent with the proposition that allograft rejection in salamanders involves only minor H-locus systems. Acute and subacute rejections have been attributed to the cumulative interactions of multiple minor H-locus antigenic disparities. Alloimmunity involving only minor H-loci could be explained by at least three different alternatives, each of which would be important from a phylogenetic perspective. First, it could reflect the complete absence of a homologue of the class I locus. If so, then by definition, salamanders can not be thought of as having a classical MHC which is a cluster of class I, class II, and other tightly linked loci. Second, it could reflect highly restricted polymorphism of a class I locus, as appears to be the case for the axolotl MHC class II gene locus. Finally, it could reflect a very limited tissue distribution of class I antigens that would not include cells of a skin graft, a possibility that, in terms of graft histoincompatibility, would be equivalent to the absence of class I in the organism. Are there data to substantiate any of these alternatives? Kaufman et al. (1991) have reported that class I-like molecules (of unknown polymorphism) do exist in the axolotl, but they are expressed solely on erythrocytes from older animals. Whether these animals really lack class I molecules on most cells, whether Kaufman has been unsuccessful in detecting these antigens on non-erythroid cells, or whether the molecule identified as a putative class I homologue really is a classical class I molecule with limited polymorphism and limited tissue distribution (Shawar et al., 1994), are but a few of the salient questions that clearly need to be resolved before one speculates further on the importance of the Urodela to an understanding of the evolution of the MHC. Perhaps the use of PCR with a low-stringency heterologous probe, as was done with the axolotl TCR (Fellah et al., 1993), might provide additional information on this subject. At this juncture of ignorance, however, it is still of heuristic value to question the biological importance of class I MHC molecules in terms of the life of the urodele. That class I might not be essential to developmental events and/or to physiological processes in the adult salamander is, in fact, supported by recent studies with class I "knockout" mice (Koller et al., 1990) as well as by the fact that in *Xenopus*, class I does not appear to be expressed either in embryos or in immunocompetent larvae (Flajnik and Du Pasquier, 1990).

There are a few other considerations, other than minor H-locus incompatibility interactions, that might bear on the mechanisms underlying chronic graft rejection in urodeles. One is the possibility that chronicity reflects some inherent defect in the immune system of the salamander, a defect that might be related to the "poor" humoral immune responses of these animals. However, this idea is not supported by the fact that several instances of acute (<14 days) and subacute allograft rejection (14-22 days postgrafting) have been observed in both newts and axolotls. Therefore, not only does the salamander have the immunological machinery to mount a mammalian type of vigorous response, but the skin transplant itself is not inherently resistant to destruction. That the "immune response" capacity of the host may actually play some role in graft rejection, however, was the conclusion from experiments involving the exchange of skin grafts within and between two subspecies of newts, *N. v. viridescens* and *N. v. dorsalis*. In several studies, newts of the *dorsalis* subspecies were noted to routinely reject grafts significantly more rapidly than newts of the *viridescens* subspecies. Since *dorsalis* also rejected *viridescens* skin grafts rapidly, whereas *viridescens* hosts chronically rejected *dorsalis* as well *viridescens* grafts, it would appear that the host alloresponse itself may be under some poorly understood genetic and/or physiological control (i.e., *dorsalis* may be a "better responder" than *viridescens*).

If one accepts that, at least functionally, urodeles lack MHC class I molecules, one might argue that class II molecules serve as restriction elements/targets for cytotoxic effector T cells in salamanders as they can in mice (Zijlstra et al., 1993). This idea is consis-

tent with a relationship between limited class II polymorphism and chronicity of rejection; however, it is somewhat weakened by our observations, in newts, that there is no apparent relationship between MLR positivity (i.e., a disparity at a postulated predominant MRL locus) and the rapidity of allograft rejection (Cohen and Horan, 1977; Cohen, 1980).

If axolotls were the only species of urodeles to exhibit chronic graft rejection (which, in fact, it is not), then one might argue that in some way, neoteny may be related to this facet of their relatively "primitive" immune system. However, thyroxine-induced metamorphosis of the adult axolotl affects neither graft rejection, changes in class I and II expression, nor the expression of Ig isotypes in the serum (Kaufman et al., 1991; Fellah et al., 1989).

Concluding Remarks

In conclusion, the axolotl has a population of T cells that, in response to PHA stimulation, proliferate and secrete a cytokine with IL-2 activity. This argues that the failure to observe: MLRs; a primary antibody response to some antigens (Ching and Wedgwood, 1967); anamnestic humoral responses (Charlemagne, 1979); a lack of thymus-dependent antibody responses (Charlemagne and Tournefier, 1977); and the absence of class switching from an IgM to an IgY isotype (Fellah and Charlemagne, 1988), are probably not related to an inherent inability of axolotl T-lymphocytes to proliferate and synthesize "IL-2". Although there are published data suggesting that limited MHC class II polymorphism and the absence of MHC class I gene products on target cells are, in some way, responsible for the lack of significant MLR *in vitro* and routinely chronic graft rejection *in vivo*, these two rather remarkable and fundamental differences between frogs and salamanders still demand much more rigorous investigation before they are fully understood. In this regard, it will be important to confirm a lack of MHC class II polymorphism and an apparent lack of MHC class I expression on non-erythroid cells in a urodele species that, unlike the axolotl, exhibits wide geographical dispersion (i.e., maximal genetic diversity). It will also be most interesting to determine the diversity of the T-cell receptor repertoire in any urodele species. A restricted repertoire could explain many of the apparent differences in immune responsivity between frogs and salamanders.

Finally, despite what at least comparatively appears to be a rather "poor" immune system, this phylogenetically important order of animals is physiologically diverse, geographically dispersed, and apparently quite successful. Clearly, if salamanders lack several features of a mammalian or anuran immune system that are thought to be important for survival, it would be of considerable interest to study their nonspecific defense mechanisms.

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