The Salamander Immune System: Right and Wrong

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Introduction: not all vertebrates have the same immune system

Many of the features of the immune system that have been worked out in such detail in mammals probably evolved by the time of the bony fish, and the descendant tetrapod lineages apparently all retain structural and functional features of this "mainstream immune system" (Du Pasquier, 1993; Kaufman et al., 1990b; 1991). However, not all vertebrate groups respond to antigenic challenge in the same way. As Nick Cohen pointed out years ago (Cohen, 1980), it has been relatively easy for comparative immunologists to demonstrate strong, rapid and specific immune responses in mammals, birds, frogs and bony fish, in stark contrast to reptiles, salamanders, cartilaginous fish, and jawless fish. What bases are there for the modest immune responses in the latter four vertebrate groups? Cartilaginous fish and jawless fish, diverging from the early vertebrate lineages before or at the same time as bony fish, may use defense strategies that are in some senses alternative, simple, incomplete, or primitive compared to other vertebrates (McCumber et al., 1982). Reptiles almost certainly do have the potential for vigorous immune responses; the contradictory reports about reptiles in the literature can be reconciled by findings of an enormous seasonal variation in immune responses, apparently due to changes in the numbers of T (and possibly B) lymphocytes and thymocytes, which in turn may be due to endogenous corticosteroid levels (Zapata et al., 1992).

That leaves the salamanders as the only animals in the postulated vertebrate "mainstream immune system" that have subdued immune responses compared to mammals. We would like to understand what the immune systems of salamanders fail to do compared to mammals and most other vertebrates, and what their immune systems do instead to protect them from pathogens. To put it simply, what is wrong with them and what is right with them? In this overview, we briefly review certain features of mammalian immune systems (covered in much more detail in virtually any textbook of immunology), summarize the evidence that salamanders have a subdued immune system (based on functional evidence mostly from the work of Delanney, Cohen, Charlemagne, and Tournefier), describe the unpublished data that we and our collaborators have generated to show that the axolotl has both a Major Histocompatibility Complex (MHC) and functional T lymphocytes, and conclude with our present interpretation of why the axolotl and other urodeles have subdued immune responses.

What do we know about the mammalian immune system?

In order to appreciate the interesting features of salamander immune responses, one needs to know something about immune systems in general and that of mammals in particular. Theoretically, there are two ways of recognizing a molecule—recognition of features that are not self (as is done by T and B lymphocytes), and recognition of the absence of features that are self (as may be done by at least some Natural Killer cells). Experimentally, the recognition of molecules by the immune system has three important properties—diversity (many different molecules can be recognized), specificity (each molecule is recognized independently of the others), and memory (the immune response towards a particular molecule is both faster and stronger upon repeated encounters with that antigen). The molecules recognized by the immune system are often called antigens.

For a given individual, non-self molecules are practically limitless. In order to be prepared for any non-self molecule that might come along, T and B lymphocytes rely on so-called antigen receptors: T cell receptor (TcR) and Antibody (also called Immunoglobulin: Ab or Ig). These are cell surface glycoprotein heterodimers that are composed of membrane distal ends that have regions of variable sequence (the V domains) and membrane proximal ends that are relatively constant (the C domains). The V domains bind antigen and the C domains, among other activities, effect the results (for instance, allow signaling to the cell). Different variable sequences in the V domains lead to different molecular shapes of the antigen receptor that can bind different

antigens. For the purpose of this discussion, each lymphocyte has but a single antigen receptor (that is, there is only one version of the variable sequences on the surface of that cell), so that each cell should respond only to a limited number of antigens that bind to the single antigen receptor on the surface of that cell.

Immunologists refer to the binding capability of a particular antigen receptor as the "specificity," presence of a single kind of antigen receptor on each cell as "clonal distribution," the collection of different receptors on a population of cells as the "receptor repertoire," and the overall variability of the collection of different receptors on a population of cells as the "diversity." Immunologists also speak of "memory," which is the faster and better response of the immune system to an antigen that is seen a second time. While it is yet unresolved (and differs depending on the type of cell and antigen), the "secondary response" may be a complicated mix of the expansion of cells that participated in the "primary response," possibly with new properties, and the recruitment of new cells with better affinity or different properties.

A large repertoire of antigen receptors with more-or-less random variable sequence should generate enough disparate shapes to bind to any antigenic shape; the difficulty is that some of the antigens will be self molecules. There seem to be a number of ways to avoid the subsequent autoimmune reactions. For instance, the immature T cells (or "thymocytes") are selected so that those that bind self molecules are deleted in the thymus; the survivors are then allowed to emigrate to the rest of the body (the "periphery" which includes the intestine and a major organ of mature lymphocytes, the spleen). As another example, in order to respond to antigen, B cells in the periphery generally require not only antigen binding to the antigen receptor, but also signals from certain T cells; if there are no autoreactive T cells to "help" the B cells, then under most conditions there is no immune response.

Even though TcR and Ab have very similar protein structures, organization of genes, and mechanisms of generating diverse sequences, they bind very different kinds of antigens and have very different effects. Ab have fairly random repertoires, meaning that they are not under much selection; as a result they can bind all sorts of molecules with different shapes and features, including carbohydrates, proteins, nucleic acids, organic molecules, and metals. B cells that bind antigen with Ab on the cell surface and that receive signals from helper T cells proliferate and differentiate into so-called "plasma cells" that secrete Ab with the same specificity as the Ab that was on the cell surface. This soluble Ab can bind antigen as well as a variety of other molecules that are involved in the immune response. For instance, Ab molecules can cover a virus (and thus "neutralize" it so that it fails to infect cells) and then bind to molecules on the surface of macrophages, to be internalized for degradation.

In contrast, the T cell repertoire (at least for αβ TcR) is highly selected in the thymus of a particular individual, so that the TcR binds primarily peptide antigens associated with certain molecules encoded in the Major Histocompatibility Complex (MHC) of that individual. That means that (at least αβ) TcR only recognize particular antigens on cell surfaces. TcR have never been found as soluble molecules, so the TcR binds the antigen and the attached T cell secretes collections of molecules, either various "cytokines" and "interleukins" from a helper T cell (to regulate the immune response), or "granzymes" and "perforin" from a cytotoxic T cell (to kill another cell). To understand these T cells, it is important to understand the MHC molecules.

The MHC was first described in mice as the major genetic locus responsible for rapid allograft rejection. We now know that this is due to the polymorphic (that is multi-allelic) members of two multigene families, the class I and class II molecules. These are transmembrane glycoprotein heterodimers that are composed of membrane distal ends that have regions of variable sequence (the peptide-binding domains) and membrane proximal ends that are relatively constant (the immunoglobulin-like domains). The variability of antigen receptors is between cells within one individual, but the variability of MHC molecules is, for this discussion, between different individuals of a population (that is, the individuals express different MHC alleles); within a single individual, many or even most cells express the same MHC molecules. Of course, there are differences between the members of the multigene family along a chromosome; immunogeneticists refer to a combination of alleles along one chromosome as an "allelic haplotype" and two different haplotypes as "allogeneic."

The peptide-binding domains bind antigenic peptides, whereas the immunoglobulin-

like domains are involved in other aspects of MHC molecule function, including transduction of signals. Different variable sequences in the peptide-binding domains form pockets that allow binding of peptides with different sequences, but only a few residues of the peptide are important for the binding, with much of the peptide antigen exposed on the surface of the MHC molecule. It is these complexes of peptide and MHC molecules on the surface of cells that are recognized by (at least αβ) TcR of the T cells.

There are two kinds of MHC molecules; they bind different kinds of peptides and are recognized by different kinds of T cells. Class I molecules on a particular cell bind peptides produced by degradation of proteins synthesized within that cell, so-called "endogenous peptides." Complexes of class I molecules and endogenous peptides on a target cell are recognized by "cytotoxic T lymphocytes" (CTL) and the target is then killed. (CTL in general bear the so-called co-receptor CD8; this molecule binds to class I molecules interacting with TcR.) The tissue distribution of class I molecules is wide (said to be "ubiquitous") in keeping with functions including immune surveillance—even a clever virus that fails to express viral proteins on the surface of such a cell may be detected because of the endogenous peptides derived from viral cytoplasmic proteins and the infected cell (a viral factory) can then be destroyed.

On the other hand, class II molecules bind peptides that are acquired from outside the cell, so-called "exogenous peptides." In general, complexes of class II molecules and exogenous peptides on cell are recognized by helper (or regulatory) T lymphocytes; often the result is stimulation of the target cell as well as the helper T cell (chemotactic factors may also be secreted to summon destructive cells, as in delayed-type hypersensitivity or DTH). (Helper T cells in general bear the so-called co-receptor CD4; this molecule binds to class II molecules interacting with TcR.) The tissue distribution of class II molecules is relatively narrow, mostly restricted to so-called "antigen presenting cells" or APCs (including macrophages, certain kinds of dendritic cells, B lymphocytes, and some others) in keeping with a function of regulation of responses to exogenous pathogens. For example, bacteria that invade the blood stream might be phagocytosed by macrophages and degraded, some of the bacterial peptides end up complexed with class II molecules on the surface of the macrophage, the macrophage presents these antigens to helper T cells with the correct TcR, and both the macrophage and T cells are activated for a variety of effector functions as a result. In the meanwhile, some B cells will use their surface Ab to bind and ingest these bacteria, the bacteria will be degraded and some of the bacterial peptides end up complexed with class II molecules on the surface of the B cells. These B cells will be recognized by the activated T cells and stimulated to proliferate and differentiate into plasma cells that secrete soluble Ab directed to the bacteria. As the bacteria are destroyed, there is less to pick up by APCs, and therefore fewer helper T cells (and macrophages) will be stimulated, and finally fewer B cells turned into plasma cells.

Both CD8 and CD4 bearing T lymphocytes undergo two kinds of selection in the thymus. The initial receptor repertoire is quite broad (that is, there are many kinds of variable sequences in the V domains, one to each cell) and more-or-less shared by most individuals in a population. Only those T cells that bind to an MHC molecule in the thymus of a particular individual are positively selected for survival, and those T cells that recognize self peptides bound to self MHC molecules in the thymus are negatively selected, so that all T cells in the periphery of an individual can recognize an MHC molecule in that individual, but not bearing self peptides. Thus, this "education" in the thymus may prevent autoimmune recognition of some self molecules. However, the T cells in one individual are not selected with regard to the MHC molecules in another individual, so many T cells may recognize self peptides complexed with nonself MHC molecules. This is the common explanation for the high level of alloreactivity by T cells, leading to rapid allograft rejection as well as strong proliferation in the "mixed lymphocyte reaction" (MLR) *in vitro*.

MHC molecules are by far the most polymorphic molecules known in vertebrates; the polymorphism is thought to be selected due to their central role in the recognition of nonself molecules and the fact that a particular MHC molecule can bind many peptides but certainly not all peptides. The general argument is to imagine a population of individuals bearing only one MHC gene with one allele (a "monomorphic" single gene MHC); a clever pathogen that encoded only proteins with no peptides that bound that MHC molecule would devastate that population. By having multiple MHC molecules, the individuals would be protected

from multiple variants of the pathogen. One way to protect all individuals is to have a large number of MHC genes along a chromosome (that is, a large multigene family). Unfortunately, the more MHC molecules in an individual, the more self peptides are bound by these MHC molecules and the fewer T cells survive negative selection in the thymus. As the number of MHC molecules in an individual goes up, the number of TcR that are autoreactive goes up as well, finally reaching the point at which the individual has no T cells left. So the alternative is to have a few MHC molecules with multiple alleles, so that any particular individual may not survive a certain pathogen variant, but some individual in the population will.

A last important point is that this description of the immune system as composed of Ab, TcR and MHC molecules, B and T lymphocytes, thymus and spleen is incomplete and misleading for any number of reasons. For example, there are many features of disease resistance that are due to molecular and cellular systems lumped under the term "innate immunity"; these contribute to the polygenic nature of disease resistance. Also, there are the γδ T lymphocytes that may represent T cells which recognize antigen like Ab; there is more and more evidence for their importance, but most details are simply unclear. Finally, there are the Natural Killer (NK) cells; these have the effector machinery of a cytotoxic T cell, but different receptors. There is evidence that at least some NK cells recognize absence of self, in this case the absence of self class I molecules, as the stimulus to kill the target. Since a virus may be ferreted out by CTLs that recognize class I molecules bearing viral peptide, one strategy of the virus would be to simply down-regulate the class I molecule (as in fact happens with adenovirus and cytomegalovirus); NK cells that recognize cells with lowered class I levels would be a host adaptation to such viruses. There are certainly other features of the mammalian immune system, as well as alternative strategies used by other vertebrate groups, that await discovery.

Compared to frogs, salamanders have a subdued immune system

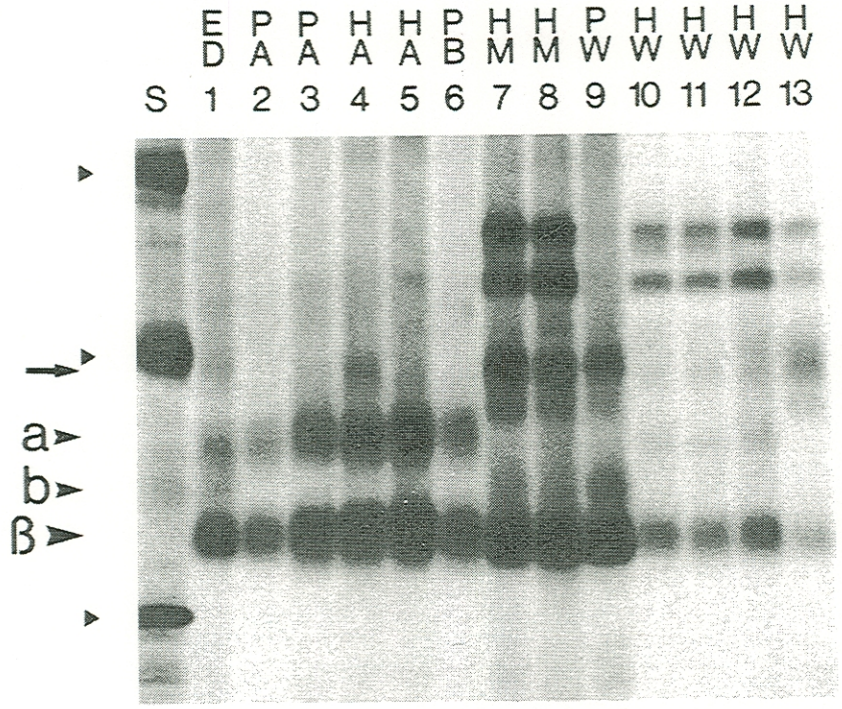
In general, anuran amphibians (frogs and toads) have immune responses that are quite reminiscent of mammals, whereas the immune responses of urodele amphibians (salamanders and newts) are somehow more subdued. (The immune responses of the third great group of amphibians, the legless salamanders or Caecilians, are not well studied.)

One very simple method for assaying the immune response is the transplantation of skin. For *Xenopus* (the clawed toad or South African frog), skin grafts between outbred wild animals are invariably rejected rapidly, indicating a strong immune response against polymorphic tissue antigens. In most defined *Xenopus* families, the most rapid rejection depends on a single genetic locus that encodes polymorphic MHC class I and class II molecules; these MHC-disparate grafts are rejected in 12-21 days (so-called acute rejection). In families that are the same at the MHC but differ elsewhere in the genome (that is, in so-called minor histocompatibility antigens), the skin grafts are rejected in 21-31 days (so-called "chronic rejection"). This allograft rejection depends on T cells, and is therefore dependent on a thymus, and displays specificity, diversity, and memory (Du Pasquier et al., 1989; Du Pasquier et al., 1975).

In contrast, salamanders are generally reported to reject grafts more slowly (Cohen, 1971; 1977; 1980; Manning and Horton, 1982), both for laboratory strains like the Mexican axolotl (*Ambystoma mexicanum*, Delanney, 1961; 1978; Delanney et al., 1975; Jurd, 1985; Tahan and Jurd, 1978; Tournefier, 1982) and the Spanish newt (*Pleurodeles*

Figure 1. Identification of axolotl class II molecules and class I α-like chains in axolotls from different laboratories (ED, English dark; PA, Paris albino; HA, Hübrecht albino; PB, Paris black; HM, Hübrecht melanoid; PW, Paris white; HW, Hübrecht white). Erythrocytes were cell surface iodinated and class II molecules were immunoprecipitated with a rabbit antiserum. A. Immunoprecipitates were analyzed by SDS gel electrophoresis under reducing conditions with β chains (large arrowhead) and a chains (small arrowheads) of class II molecules indicated for type a/a (lanes 2-6), b/b (lanes 7-9, 13) and a/b (lanes 1, 10-12). Class I α chain-like bands are indicated with an arrow (lanes 1, 4, 7-9, 13); other molecules were not identified. Triangular arrowheads indicate standards (s, 70, 45 and 30 kD). B. Immunoprecipitates analyzed by SDS gel electrophoresis followed by isoelectric focusing show class II molecules of type a/a (PA, HA, PB), b/b (HM, PW) and a/b (ED, HW). Long arrows under the isoelectric focusing gel indicate the direction of SDS gel electrophoresis and the length of the strip from the first dimension; +, acidic end; -, basic end of the second dimension.

A.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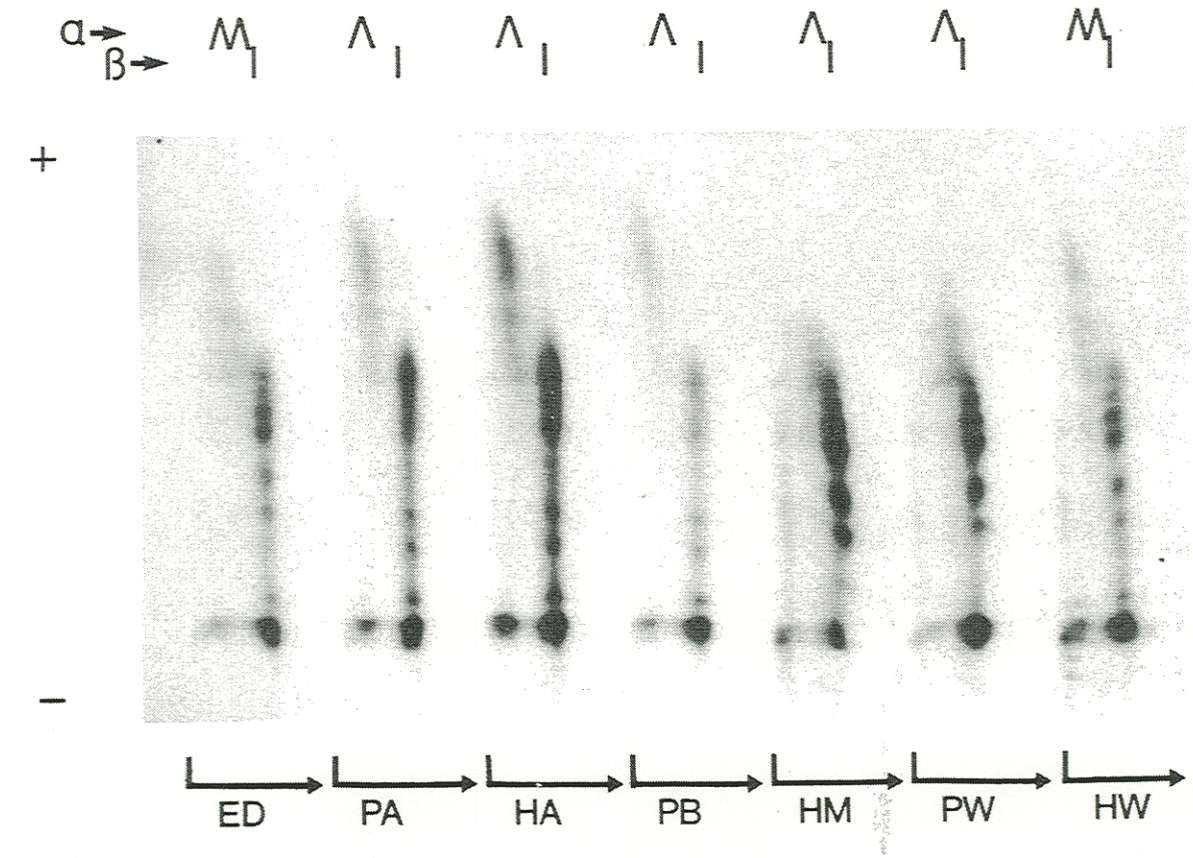
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donor	b/b	b/b	b/b	b/b	b/b	a/a	a/a
recipient	0	2	5	9	12	3	4
b/b 0	a	a	a	a	a*	r	r
b/b 2	a		a (a)	a	a	R!	r
b/b 5	a	a		a	a	r	R
b/b 9	a (a)	a (a)	a	a	a	R	R
b/b 12	a*	a	a	(a)	a	R (a)*	R
a/a 3	R (a)*	R	R	R	R	a	a
a/a 4	(R)	(R)	(r)	(r)	(R)	a* (R)	a

waltlii, Charlemagne and Tournefier, 1974; Fache and Charlemagne, 1975) and for field-collected specimens like the Eastern spotted newt (*Notophthalmus viridescens:* Cohen, 1969; 1971; 1980; Cohen and Horan, 1977) and the European alpine newt (*Triturus alpestris*: Plytycz, 1977; Tournefier, 1973). This slow ("chronic") graft rejection is mediated by T lymphocytes of the immune system (Cohen, 1980), because it has the immunological properties of memory and specificity (that is, after a first graft is rejected, there is a faster response to a graft from the same but not from a different individual, Cohen, 1971; Tahan and Jurd, 1978), and is dependent on the thymus (that is, appearance of the cells that mediate the response is abrogated by neonatal but not adult thymectomy, Cohen, 1969; Fache and Charlemagne, 1975; Tournefier, 1973; 1982).

Another way to analyze immune response is with *in vitro* assays. The "mitogen reaction" is an assay to measure the response of most if not all T lymphocytes to a general stimulus; cells including lymphocytes are cultured with a plant lectin (the "mitogen") that crosslinks molecules (presumably including TcR) on the surfaces of the cells. The "Mixed Lymphocyte Reaction" (MLR) is an assay to measure the response of those T lymphocytes (usually 1-10% of T cells) that recognize a particular set of nonself class II MHC molecules; two populations of cells (including lymphocytes) are cultured. In both of these assays, the amount of proliferation after some days is measured by uptake of radioactive thymidine; a "Stimulation Index" (S. I.) indicates the fold increase (or decrease) upon stimulation (the amount of radioactive thymidine taken up after a specific stimulus divided by the amount of radioactive thymidine taken up with no specific stimulus).

The *in vitro* immune responses of urodeles are also modest in comparison with those of anurans. MLR cultures of MHC-disparate spleen or blood cells from outbred or inbred *Xenopus* result in high specific incorporation of ^{3}H-thymidine (S. I. from 6 to 20), which is abrogated by early larval thymectomy (Du Pasquier et al., 1975; 1989; Du Pasquier and Horton, 1976). In contrast, the S. I. of axolotl and Eastern spotted newt MLRs were generally below 3; these poor MLR responses do not correlate with the speed of graft rejection (Cohen, 1980; Cohen and Horan, 1977; Collins et al., 1976; Collins and Cohen, 1976; Delanney et al., 1975; Manning and Horton, 1982). *Xenopus* lymphocytes respond to both the B cell mitogen lipopolysaccharide (LPS) and T cell mitogens like Concanavalin A (Con A) and Phytohemagglutinin (PHA) (Du Pasquier et al., 1989; Du Pasquier and Horton, 1976), whereas lymphocytes of the two urodele species were reported to respond to high doses of LPS but not to Con A and PHA (Collins et al., 1976; Collins and Cohen, 1976).

Finally, the *in vivo* antibody responses of urodeles are subdued in comparison with anurans. Immunization of *Xenopus* with either soluble antigens (proteins like ovalbumin or fowl gamma globulin) or particulate antigens (like viruses or red blood cells) leads to the rapid appearance of high levels ("titers") of specific antibody; the titers and affinities of the IgM antibody increase after a second immunization. The appearance of the second antibody isotype (IgY) is dependent on the presence of T cell help (which is absent in animals thymectomized as tadpoles) (Du Pasquier et al., 1989; Du Pasquier and Horton, 1982). In contrast, axolotls produce little specific antibody response to soluble antigens and the antibody response to particulate antigens are reported to display restricted diversity. There is no reported secondary response (that is, a faster appearance or increase in titer of antibody, or a switch from IgM to IgY isotype) (Charlemagne, 1979; 1987; Charlemagne and Tournefier, 1977; Jurd, 1985;

Figure 2. An F2 cross of laboratory axolotls shows that the class I α-like chain and both class II chains are determined by the functionally-defined MHC. Axolotls from an F2 cross (a/b x a/b derived from PA and PB axolotls) were typed by SDS gel electrophoresis (arrow indicates class I α-like chain, small and large arrowheads indicate class II α and β chains) with typing results next to the identification number of the animal. Each axolotl shown was grafted with belly skin from all of the others with the results for this first graft indicated as follows: R!, rejected by 20 d (rapidly); R, rejected by 30 d (normally); r, rejected by 80 d (slowly); a, still tolerated at 160 d (accepted); blank, no data due to technical problems. Some grafts were repeated later and the results for these second grafts indicated as follows: (R), rejected by 10 d; (r), rejected (white) at 50 d; (a), tolerated at 50 d. The asterisks indicate the following technical notes. First grafts of animal 12 on animal 0 and vice versa were healthy, but showed thickened epithelium. All first grafts fell off of animal 4, except the autograft and a small portion of the animal 3 graft, which was tolerated. All second grafts on animal 4 were rejected, including the animal 3 graft. Second grafts of animal 0 on animal 3 and animal 3 on animal 12 showed edema and pigment cell contraction by 50 d, but were not yet rejected.

Manning and Horton, 1982; Tournefier, 1982). In axolotls, both larval and adult thymectomy as well as hydrocortisone treatment are reported to lead to slightly enhanced antibody responses (Charlemagne, 1979; Charlemagne and Tournefier, 1977; Tahan and Jurd, 1978; Tournefier, 1982), as though T cell help is not involved.

If the modest responses in urodeles all have the same basis, it is likely to be in some aspect of the helper T cell response to class II molecules, since that is largely responsible for MLR and allograft rejection as well as for the antibody response to soluble antigens and the secondary antibody response to particulate antigens in mammals, chickens and *Xenopus* (Du Pasquier, 1993; Kaufman et al., 1990b; 1991). Among the possibilities that have been suggested to explain the modest urodele responses are the lack or incompetence of T helper cells, presence of a strong suppressor cell response, inappropriate culture conditions (for the *in vitro* assays), minimal polymorphism (for the graft rejection and MLR) or even a complete lack of MHC class II molecules (Charlemagne, 1979; Charlemagne and Tournefier, 1977; Cohen, 1977; 1980; Collins et al., 1976; Collins and Cohen, 1976). So it becomes interesting to know whether salamanders have an MHC and T cells.

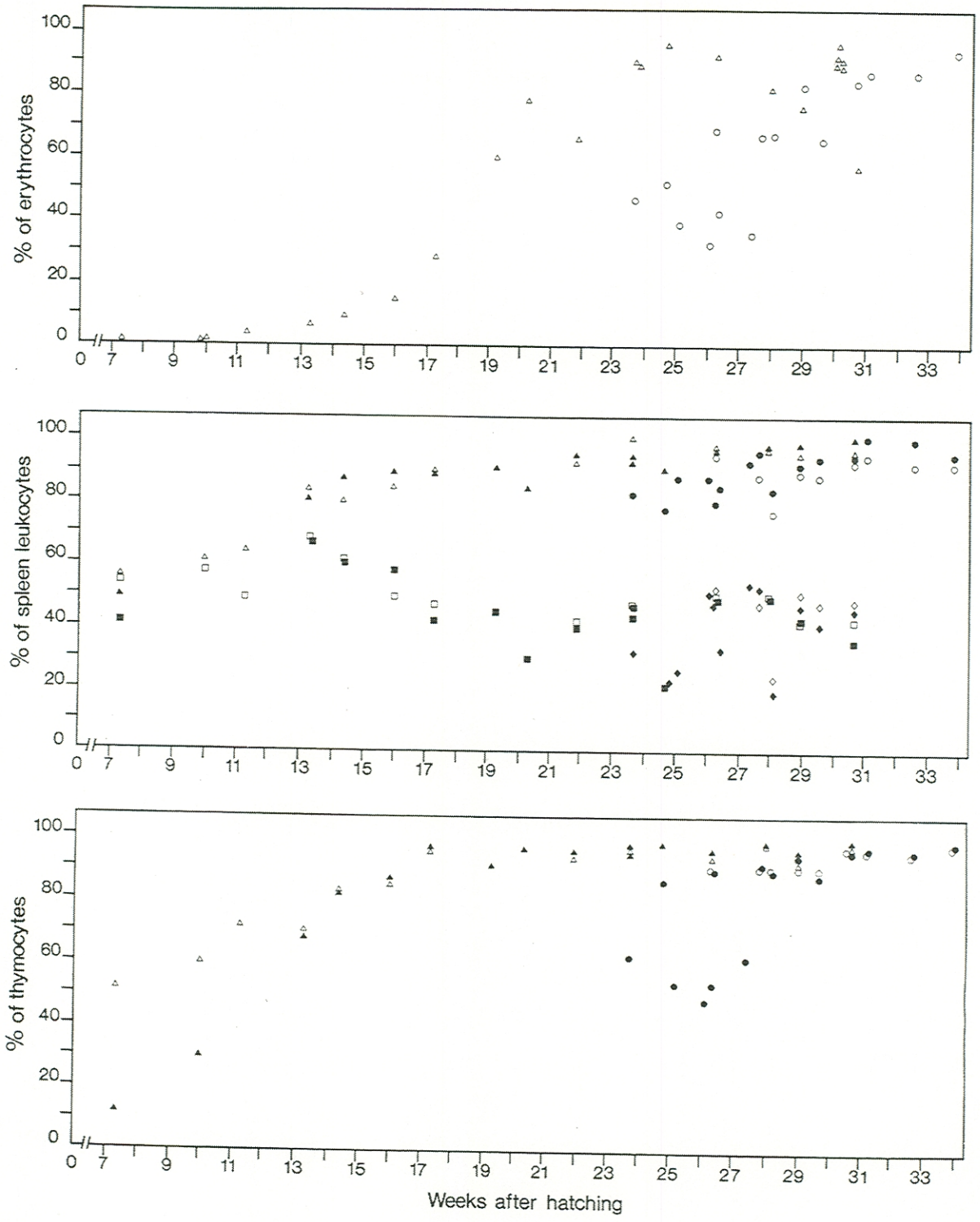
Axolotls have an MHC that encodes oligomorphic MHC molecules

Many years ago, we began to use xenoantibodies to search for MHC-like molecules, first in the frog *Xenopus* (with Louis Du Pasquier and Martin Flajnik), and then in representatives of many vertebrate groups (Kaufman et al., 1985a; 1985b; 1990a; 1990c). In 1985, we found some rabbit antisera (and later mouse monoclonal antibodies) to human class II molecules that immunoprecipitated axolotl molecules that had most of the structural features expected for mammalian class II molecules (see fig. 1). Like mammalian class II molecules, the axolotl molecules were transmembrane glycoprotein heterodimers of around 30 kDa with characteristic sequences and there were size and charge variants in different animals. Two-dimensional gels (an SDS polyacrylamide gel separating by size followed by an isoelectric focusing gel separating by charge) showed a number of spots for class II molecules, indicating a multigene family. We found only two allelic haplotypes—one with an overall more acidic isoelectric point and a 31 kDa α chain (the "a" or acidic haplotype) and the other with an overall more basic isoelectric point and a 33 kDa α chain (the "b" or basic haplotype).

Subsequently, we found rabbit antisera directed to human class I molecules that would immunoprecipitate a molecule from some axolotls (see fig 1); this molecule had some of the features of a mammalian class I α chain in the absence of β_2-microglobulin: a transmembrane glycoprotein of 45 kDa that was protease sensitive. [As a technical aside, we have found these cross-reactive reagents generally recognize buried determinants; separation of the PBDs in class I molecules leads to separation of the two chains whereas in the class II molecules the two chains stay together presumably due to the transmembrane regions (Kaufman et al., 1990a; 1990c). Therefore, the only class I molecules that we could identify are separated from β_2-microglobulin]. Two-dimensional gels showed a single array of charge species for the class I, suggesting only a single gene product. We found only two alleles—the single array of spots and a null allele (with either no class I α-like chain or one that failed to react with our antisera).

Jacques Charlemagne and Annick Tournefier had an old F2 family of axolotls (white/dark x white/dark), so we analyzed the class II and class I α-like chains by SDS and isoelectric focusing gels at the same time that they examined skin graft rejection between eight animals (see fig. 2). We found that there were no heterozygotes left in this old family—

Figure 3. The appearance of class II molecules on hemopoietic cells is developmentally regulated in the axolotl. Blood cells (virtually all erythrocytes) in the top panel, Ficoll density gradient-purified spleen leukocytes in the middle panel, and thymus cells in the bottom panel were stained with a cross-reactive monoclonal antibody to class II molecules (and for spleen cells, double-stained with a biotin-coupled monoclonal antibody to axolotl light chain) followed by a fluorescein-coupled second antibody of sheep anti-mouse Ig absorbed with axolotl spleen and blood cells (and for spleen cells, rhodamine-coupled strepavidin), and then was analyzed by flow cytometry. Each point represents a pool of individuals of a particular family in the 1989 experimental series; the size of the pool depended on the size of the animals at that stage (for instance, pools of 100 animals were used at 7 weeks after hatching). PI, propidium iodide to gate out dead cells.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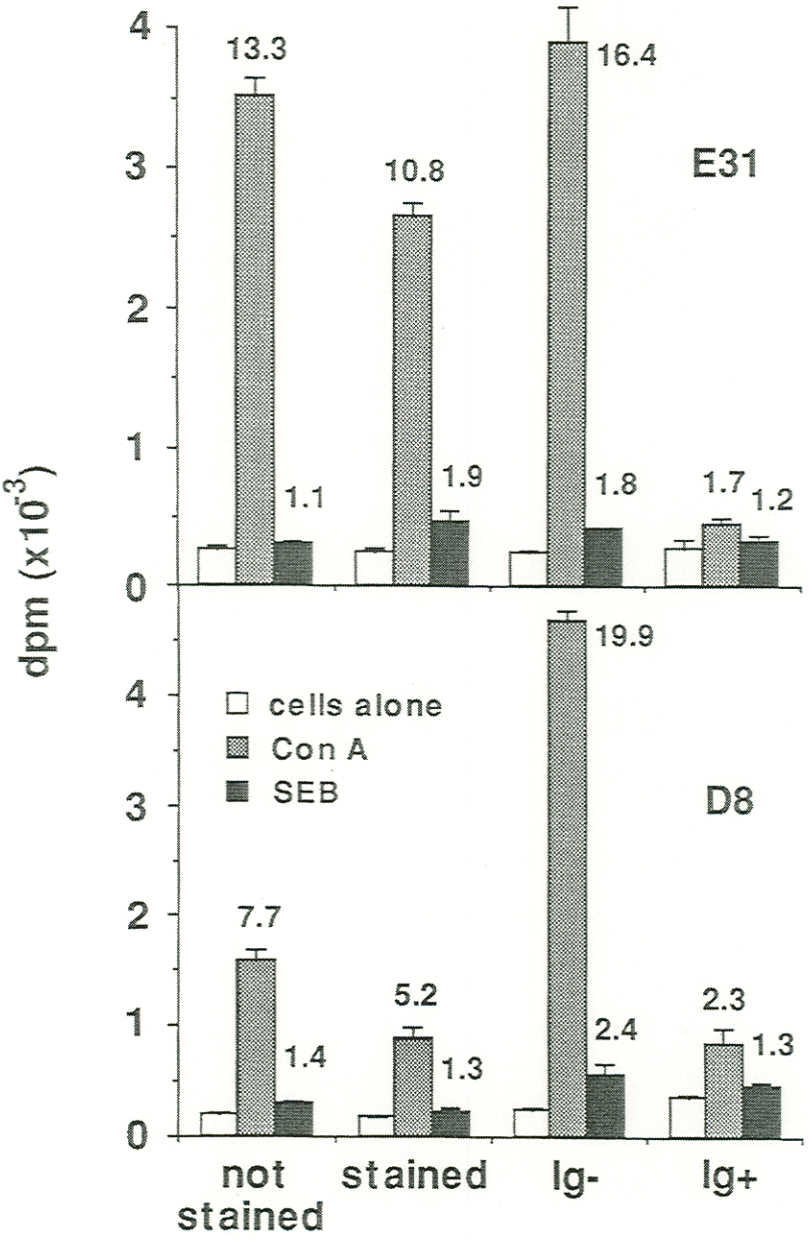

Figure 4. Proliferation by purified populations of Ig$^-$ and Ig$^+$ spleen leukocytes stimulated with Con A or SEB. Spleen leukocytes from E31 and D8 axolotls were isolated by A-Ficoll density gradient centrifugation, and stained with a monoclonal antibody to axolotl antibody light chain and fluoresceine-coupled sheep anti mouse Ig. The stained cells were sorted under sterile conditions using a FACstar Plus gated on small lymphocytes, giving cells of greater than 99% purity upon reanalysis. Equal numbers of cells before staining, after staining but before sorting, and after sorting were cultured with either 5 µg/ml Con A or with 2.5 µg/ml SEB (Sigma) in IMDM adjusted to amphibian osmolarity , and then analyzed for ^{3}H-thymidine incorporation on day 6. Means of S. I. from triplicate cultures (bars), SE (error bars) are shown.

only animals with a/a class II molecules and no class I α-like chains and animals with b/b class II molecules and a class I α-like chain. Jacques and Annick found that the a/a animals accepted grafts from a/a animals, the b/b animals accepted grafts from b/b animals, but the a/a animals rejected grafts from the b/b animals and vice versa. This shows that the class II β, class II α and class I α-like chain all co-segregate with the locus that determines the most rapid graft rejection in this family. Thus the axolotl has an MHC (a *Major* Histocompatibility complex) that encodes MHC molecules just as in mammals.

However, the level of polymorphism came as a complete surprise—for both class I and class II molecules, there were only two alleles in all the animals that we tested (see fig. 1). These included animals from laboratories in Europe and the United States (including F1 of laboratory animals crossed at the IU Axolotl Colony with wild animals brought from Lake Xochimilcho). Sometimes the class I array would be found with the class II acidic haplotype and sometimes with the basic haplotype, as though there is occasional recombination in the MHC (as is found with mammals). One explanation for this low number of MHC haplotypes might be that the axolotl population underwent a genetic bottleneck (due to few founders in the lakes around Mexico City or to few founders of the neotenic population), but isoenzyme electrophoretic polymorphisms are in the normal range for amphibians (Shaffer, 1984). This suggests that only the MHC has unexpectedly low levels of variability in the axolotl.

The tissue distribution of axolotl MHC molecules is developmentally regulated and unusual

Another surprise was the tissue distribution of the axolotl MHC molecules—adult axolotls have the widest class II tissue distribution and the narrowest class I tissue distribution yet reported (Völk, 1990). We should point out that in those vertebrates carefully examined, the expression of MHC molecules is developmentally regulated, and the precise tissue distribution of the adults is not a very stable feature between species.

Most salamanders, including many of the genus *Ambystoma*, metamorphose from an aquatic juvenile with a broad finny tail and external gills to a terrestrial adult with a thin tail and no external gills. The axolotl apparently makes too little of the thyroid hormone thyroxin to undergo this metamorphosis, but still switches from fetal to adult globin expression at the appropriate time (around 3-4 months) in a so-called "cryptic metamorphosis" (Armstrong and Malacinski, 1989; Ducibella, 1974a; 1974b). We found that the expression of MHC class II molecules on blood cells is also developmentally regulated (see fig. 3), but lymphocytes bearing class II molecules appear before cryptic metamorphosis: B cells before 7 weeks after hatching, thymocytes around 7 weeks, T cells around 10 weeks, and finally, erythrocytes around 12 weeks, along with the change in globin.

We have not determined whether class II-negative mature cells in the periphery directly become class II positive or are replaced by new cells originating from class II-bearing precursors. Waves of T cells are known to populate and exit the thymus in mammals and birds; it may be that a wave of class II bearing thymocytes first replaces the earlier class II-negative thymocytes, and then emigrates to the periphery where they replace the class II negative T cells. The replacement of juvenile erythrocytes with adult erythrocytes is known in many vertebrates, so it may be that the class II negative erythrocytes bearing fetal globin are replaced with class II positive-erythrocytes bearing adult globin. We have found that both the transition from juvenile to adult globin and the appearance of hematopoietic cells with class II molecules are not affected by drugs such as sodium perchlorate, thiourea, methimazole or 1-methyl imidazole; these data suggest that the production of low levels of thyroxin are unlikely to play a role in these changes (contrary to the conclusions in Ducibella, 1974b).

The class I α-like chain does not appear until later; thus far we have only detected it on erythrocytes. This may mean that the expression of this molecule is restricted to erythrocytes; alternatively, only on erythrocytes does enough class I α separate from β_2-microglobulin to be detected by our reagents.

The axolotl has functional T cells but they do not recognize allogeneic cells very well

Once we knew that axolotls have MHC molecules, it seemed likely that they would have functional T cells to recognize them. It

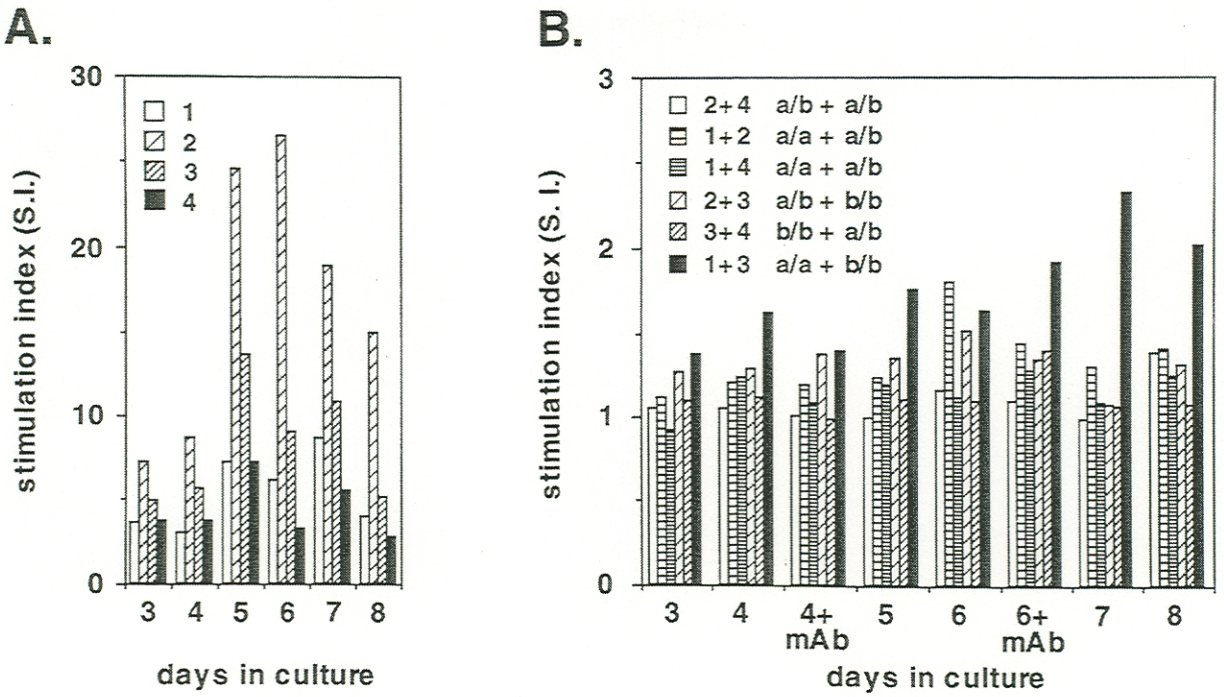

Figure 5. Kinetics of proliferation by spleen leukocytes cultured with 2 µg/ml Con A (panel A) or with allogeneic cells in the presence or absence of 6 µg/ml protein A-purified monoclonal antibody to class II molecules (checkerboard two-way MLR, panel B). The E17 family siblings were typed for class II alleles by iodination of erythrocytes and immunoprecipitation followed by SDS and isoelectric focusing gels. Triplicate cultures of A-Ficoll gradient-purified spleen leukocytes in IMDM adjusted to amphibian osmolarity were analyzed for ^{3}H-thymidine incorporation on days 3-8.

was already known that the slow ("chronic") graft rejection found in salamanders depended on an intact thymus early in life, indicating that thymocytes were educated in the thymus giving rise to functional T cells in the periphery. Leukocytes from mammals, birds, frogs and bony fish respond strongly in *in vitro* assays that mimic graft rejection. In contrast, salamander leukocytes failed to respond strongly to allogeneic cells; one possible reason was that the salamanders tested were the same MHC type. However, salamander leukocytes also failed to respond strongly to general stimuli, so it was possible that they were somehow nonfunctional.

We wanted to use *in vitro* assays to measure the ability of salamander T cells of defined MHC type to respond to antigenic stimuli, both to a general stimulus and to MHC molecules. We got nowhere with this approach until we made the fortuitous observation in 1989 (Völk, 1990) that axolotl spleen cells put in culture directly *ex vivo* without stimulation gave a higher level of thymidine incorporation in media completely lacking fetal bovine serum and axolotl serum. We then found that the plant lectins Con A and PHA stimulated spleen leukocytes to incorporate a lot of thymidine. Of a number of commercial and personal serum-free media, the so-called "Iscove's modified Dulbecco's modified Eagle's minimal medium" (IMDM) adjusted to amphibian molarity gave by far the best results, with the lowest background and the highest specific incorporation. We used monoclonal antibodies to axolotl antibodies to separate B lymphocytes from presumptive T lymphocytes by fluorescence-activated cell sorting (see fig. 4),

and found that T cells rather than B cells responded strongly to Con A and PHA (as well as weakly to preparations of Staphylococcus enterotoxin B, a T cell specific superantigen). Juvenile animals do not seem to respond to the mitogens under these conditions, and some adult animals respond much more strongly than others, with S. I. ranging from 3 to 50 (see fig. 5); the reasons are not clear.

We examined both mitogen responses and MLR in F2 families that were typed for class II molecules (see fig. 5). The mitogen responses were much stronger than the MLR responses and peaked a day or so earlier. The MLR responses were only a little stronger in IMDM than in other media, but they followed the class II typing much better in IMDM than other media. We tested many other conditions, including adaptation to warmer than normal temperatures, without further improvement. Pooling many experiments (see fig. 6), we found that the S. I. of two MHC haplotype differences (an allogeneic combination; a/a cells + b/b cells) was significantly above all others, that the S. I. of one MHC haplotype difference (a semi-allogeneic combination; a/b + a/a or a/b + b/b) was equivalent to differences in background only (a syngeneic combination, a/a + a/a, b/b + b/b or a/b + a/b using different animals), and that the S. I. of no genetic difference (that is, a mixture of self cells) was significantly lower than the rest.

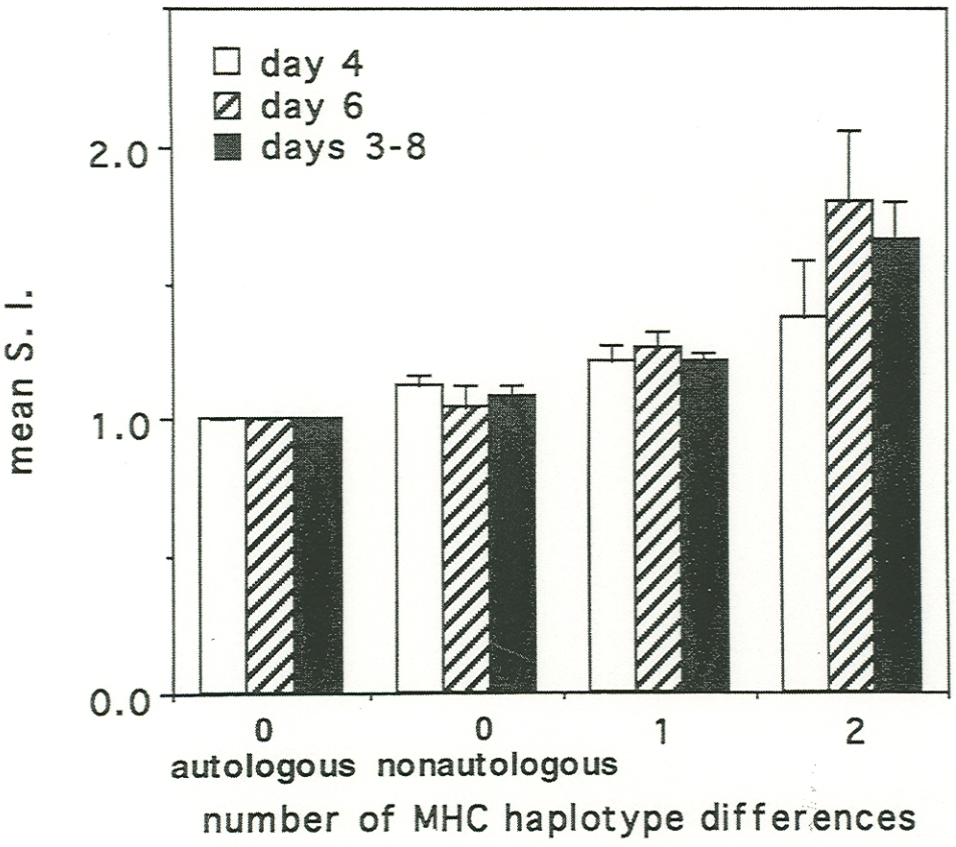

Figure 6. Pooled data from four MLR experiments with animals from F2 families typed for class II alleles. S. I. values from multiple MLR experiments were pooled according to the number of MHC haplotype differences between the two cell populations in culture (autologous with 0 MHC differences, nonautologous with 0 MHC haplotype differences, semi-allogeneic with1 MHC haplotype difference and fully allogeneic with 2 MHC haplotype differences); the S. I. value for each MLR before pooling was the mean of triplicate cultures. Means of S. I. values (bars) with SE values (error bars) are shown for day 4 (18 autologous, 8 nonautologous with no MHC difference, 20 semi-allogeneic and 4 fully allogeneic combinations), day 6 (18, 8, 20, 4 combinations) and days 3-8 (52, 20, 56, 12 combinations).

This indicates that there are T cells, but most of them do not respond to molecules determined by the genetic region that we assess by typing the class II molecules.

What is wrong with the immune systems of salamanders compared to mammals?

There is still much to learn about the way in which salamanders recognize foreign antigen, and so there are many possible explanations for their modest immune responses, which fall into three classes. First, most salamanders actually do not have subdued responses and there is something unique about the ones that have been examined, particularly the axolotl. Second, most salamanders have subdued responses, and there is a common basis. Third, most salamanders have subdued responses, but for many different reasons.

Are the modest responses general for all urodeles? Of all salamanders, the axolotl has been the subject of much of the work on graft rejection, and virtually all of the work on lymphocyte responses *in vitro*, antibody responses *in vivo*, and the biochemistry and molecular biology of MHC molecules, TcR and antibodies. Since the axolotl is a neotenic (also called "nontransforming") salamander, remaining juvenile in appearance due to insufficient production of the thyroid hormone thyroxin, it is not impossible that some of the subdued immune responses are normal characteristics for a juvenile salamander. We think that this is unlikely for two reasons. First, most other salamanders tested (including transforming salamanders) show the same slow ("chronic") graft rejection as the axolotl. Second, administration of thyroxin to axolotls leads to full anatomical metamorphosis, but no measured change in the immune response (Armstrong and Malacinski, 1989; Ducibella, 1974a; 1974b; Jurd, 1985; Völk, 1990). The reason for this is probably that changes have already occurred—despite the lack of anatomical metamorphosis in normal axolotl development, many biochemical and hemopoietic changes occur [sometimes referred to as "cryptic metamorphosis"(Ducibella, 1974a; 1974b)] that are likely unconnected with the thyroxin-dependent developmental program. These changes include hemoglobin, serum proteins, erythrocyte structure, expression of class II molecules, and most importantly, levels of certain immune responses (Ducibella, 1974a; 1974b; Jurd, 1985; Völk, 1990).

What features of the axolotl immune system could be responsible for the modest responses? As mentioned above, there are a host of suggestions already in the literature, some blaming the experimenter (for instance, poor culture conditions for the MLR, use of animals with the same MHC for graft rejection and MLR, dogged insistence on investigating an unusual animal) and others blaming the axolotl (lack or incompetence of T helper cells, presence of a strong suppressor T cell response, lack of MHC molecules); Jacques Charlemagne and Annick Tournefier have also mentioned in conversation that they think that there is a defect in antigen processing and presentation. To us, the likeliest explanation was that there is something awry with the response of helper T cells to class II molecules (since this is important for MLR, allograft rejection, antibody response to soluble antigens and secondary antibody response to particulate antigens).

We have found MHC class II molecules and a class I α-like chain that are determined by the same locus that determines graft rejection and the modest MLR, so an axolotl MHC exists with some functional attributes. Along with Nick Cohen (Koniski and Cohen, 1992), we have shown that Ig-negative lymphocytes exist that respond to T cell mitogens, and Julien Fellah and Jacques Charlemagne have shown that there are expressed TcR β genes in the axolotl (Fellah, et al., 1993), so apparently functional T cells exist as well. We have shown that typed animals respond weakly but with some specificity in both graft rejection and MLR, so that modest response is not due to using the MHC identical animals. Of course, we can not rule out some of these objections conclusively (for instance, we may appropriate culture conditions for mitogen response but not for MLR), but they seem increasingly unlikely.

So, at the moment, our favorite explanation is that there is nothing wrong with the axolotl T lymphocytes or MHC molecules, but that most of the axolotl T cells simply are not directed to recognize the MHC molecules that we have described. This might explain the low polymorphism and funny tissue distribution of axolotl MHC molecules—since they are not used very much, they are not under much selective pressure to diversify or to be expressed with particular cell distributions. It seems very unlikely that we have simply

missed the boat, that is, that there is some other highly polymorphic MHC that determines graft rejection and MLR; we have much data from F2 families showing that our oligomorphic MHC molecules are determined by the same locus that determines the modest graft rejection and MLR. Of course, we still have to determine that exact number of T cells that respond to a particular allogeneic MHC molecule, but to our minds, it is really more important to determine what the majority of axolotl T do recognize. There are a lot of possibilities—T cells that recognize the "medial histocompatibility antigens," which are minimally polymorphic nonclassical class I molecules (like TL, Qa, Hmt and CD1 molecules), γδ T cells that may recognize antigen without MHC-like molecules, or perhaps NK cells of one kind or another. And there is the strong possibility that something new and unexpected may be discovered.

Is there a common basis for the modest responses in all urodeles? As mentioned above, most investigations report slow (or "chronic") skin graft rejection in salamanders, both among individuals in laboratory strains and individuals in wild outbred populations. The reports of faster rejections are of uncertain significance, since they are all from wild populations and are anecdotal in the sense that they have not been repeated nor have attempts been made to understand them in detail. Still, it is possible that there are differences in the level of immune response between salamander species.

Nick Cohen has made the point that all relevant data is compatible with only a few strong histocompatibility alleles within each population of salamanders (Cohen, 1980). This is quite reminiscent of the finding of two class II alleles in axolotls, and could have the same meaning—most salamander populations have few MHC alleles because most of their T cells don't recognize classical MHC molecules and thus they have subdued immune responses. This is another interesting direction for future investigation.

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