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Review

Allorecognition and chimerism in an invertebrate model organism

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Abbreviations: *alr*, allorecognition locus; MHC, major histocompatibility locus

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The presence of highly specific histocompatibility reactions in colonial marine invertebrates that lack adaptive immune systems (such as the sponges, cnidarians, bryozoans and ascidians) provides a unique opportunity to investigate the evolutionary roots of allorecognition and to explore whether homologous innate recognition systems exist in vertebrates. Conspecific interactions among adult animals in these groups are regulated by highly specific allorecognition systems that restrict somatic fusion to self or close kin. In Hydractinia (Cnidaria:Hydrozoa), fusion/rejection responses are controlled by two linked genetic loci. Alleles at each locus are co-dominantly inherited. Colonies fuse if they share at least one haplotype, reject if they share no haplotypes, and display transitory fusion if they share only one allele in a haplotype—a pattern that echoes natural killer cell responses in mice and humans. Allorecognition in Hydractinia and other marine invertebrates serves as a safeguard against stem cell or germline parasitism thus, limiting chimerism to closely related individuals. These animals fail to become tolerant even if exposed during early development to cells from a histoincompatible individual. Detailed analysis of the structure and function of molecules responsible for allorecognition in basal marine invertebrates could provide clues to the innate mechanisms by which higher animals respond to organ and cell allografts, including embryonic tissues.

Introduction

Allorecognition—defined as the ability to distinguish between self tissues and those of another member of the same species—is the fundamental obstacle to safe and effective transplantation of life-saving organs in humans.[1] In solid organ transplantation, recognition of donor alloantigens by the host's immune system leads to vigorous host vs. graft rejection responses. In bone marrow transplantation, recognition of host antigens by the transplanted hematopoietic stem cells causes debilitating graft vs. host disease (GVHD).[2] Non-hematopoietic stem cells are also met with rejection if transplanted between non-identical individuals thus, limiting the clinical applicability of allogeneic stem cell therapies.

The quest for safe and effective management of transplant rejection and GVHD has traditionally relied on the study of mammalian allorecognition systems, specifically those of mice and humans.[1] Mammals, like all jawed vertebrates, are endowed with adaptive immune systems characterized by T cells, B cells, and the somatic gene rearrangement machinery necessary for generating diverse B cell and T cell receptors for antigens.[3,4] Recognition of highly polymorphic major histocompatibility complex (MHC) molecules by the diverse repertoire of T cell receptors (TCR) is the principal allorecognition pathway of the adaptive immune system. It is presumed that allorecognition is an unintended byproduct of MHC and TCR diversity, originally evolved to recognize myriad microbial peptides.[5]

Mammalian immunity is not solely dependent on the adaptive immune system but also on the vital functions of the innate immune system. The innate immune system is composed of cells (such as neutrophils and phagocytic leukocytes capable of antigen presentation) and of molecules (for example, the complement system) that respond to microbial non-self.[6] Activation of the innate immune system leads to an acute inflammatory response that rapidly contains microbial expansion, and importantly, alerts the adaptive immune system to the presence of foreign antigen. It is the adaptive system that then eliminates the pathogen and provides longterm protection of the host against re-infection (immunological memory).

How the innate immune system recognizes allogeneic non-self, and the mechanisms by which this early recognition step triggers the adaptive alloimmune response that leads to graft rejection are not well understood. Answering these questions is essential for understanding the fundamental rules of organ, tissue and cell transplantation, including the grafting of embryonic tissues. Here, we will summarize evidence that studying a basal invertebrate model organism could shed light on the fundamentals of allorecognition in higher animals.

Why Study the Evolution of Allorecognition?

Allorecognition is not restricted to jawed vertebrates but is common to most metazoans, including invertebrates that lack T cells, B cells and MHC, but have sophisticated innate defense mechanisms.[7] Allorecognition has been documented in representatives of at least four different colonial marine invertebrate phyla or subphyla: Porifera (sponges),[8,9] Cnidaria (corals and hydroids),[10,11]

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Bryozoa (sea mats)[12,13] and Urochordata (tunicates or sea squirts).[14] Conspecific interactions among adult animals in these groups are regulated by highly specific allorecognition systems that restrict somatic fusion to self or close kin. Work in both *Hydractinia symbiolongicarpus* (a hydroid), the subject of this review, and *Botryllus schlosseri* (a tunicate), extensively investigated by Irving Weissman and Anthony deTomaso,[14] has established that allorecognition in colonial invertebrates serves as a safe-guard against stem cell or germline parasitism.[15-17] It is intriguing to note that mammals sometimes encounter allogeneic stem cells under natural conditions. One example is the migration of semiallogeneic fetal stem cells across the placental barrier and into the maternal circulation during pregnancy.[18,19] Another is tumor spread between individuals by physical or sexual contact, documented in Tasmanian devils (devil facial tumor disease) and in dogs (canine transmissible venereal tumor).[20-22] The suggestion that these phenomena are an evolutionary echo of ancestors that routinely engaged in conspecific interactions have led to anticipation that molecular analysis of invertebrate allorecognition could bring important insights to allorecognition in the innate and adaptive immune systems of higher animals.[23]

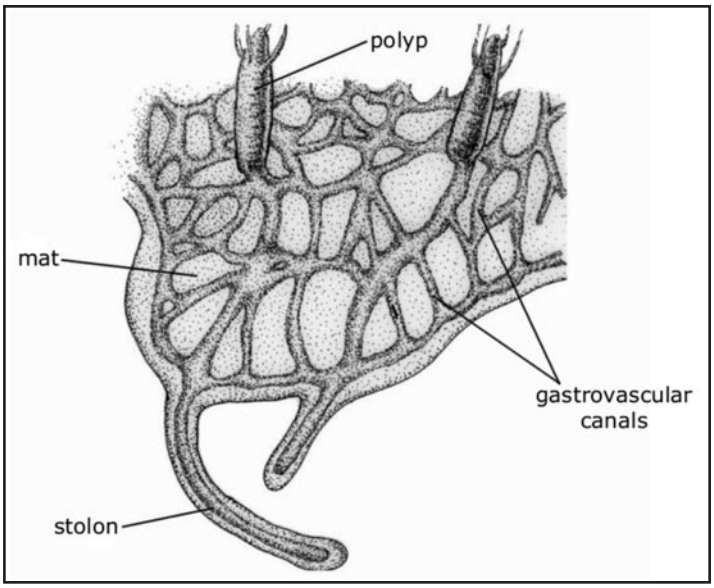

Figure 1. Hydractinia morphology. P, Polyp; M, Matt; S, Stolon.

The Hydractinia Allorecognition System

The allorecognition response of Hydractinia is perhaps the most extensively characterized of any invertebrate animal.[24-29] *Hydractinia symbiolongicarpus* is a colonial athecate hydroid (Cnidaria: Hydrozoa) which grows as a surface encrustation. In nature, it occurs as an epibiont on the shells carried by hermit crabs. Colonies are comprised of three tissue systems: polyps (P in Fig. 1), which feed, bear gametes and defend the animal; stolonal mat (M), an extension of the body column of the polyp which grows as 2-dimensional plate over the substratum; and stolons (S), which are embedded within and extend beyond the stolonal mat and provide vascular continuity between the digestive cavities of the polyps.

Hydractinia displays an unambiguous fusion/rejection response (Fig. 2). Encounters between colonies occur when the stolons of one colony (or, equivalently, the leading edge of a stolonal mat) encounter the stolons of another. Stolonal tips are locomotory organs which elongate by rhythmic pulsation. An approaching stolonal tip induces dissolution of the periderm and the development of new tip in the flank of stolon it approaches,[30] leading to contact between the ectodermal epitheliomuscular cells (EMC) that comprise the tips of the two opposing stolons. Contact between compatible tissues results in adhesion of EMC's, cessation of pulsations in both tips, the realignment of the now adherent EMC's to a common mesogleal surface, fusion of endodermal EMC's, and the establishment of functional gastrovascular continuity.[26,30] Incompatible encounters, in contrast, fail to adhere following initial contact of the ectodermal EMC's[24-26,30,31] and both tips continue periodic pulsation.[30] Incompatible stolons hypertrophy as cnidocytes migrate into them (Fig. 3).[26,30,31] Cnidocytes eventually discharge nematocysts, an effector system unique to cnidarians, into the foreign tissue, causing extensive local tissue destruction. The repetition of these events at each stolonal contact yields a broad zone of hyperplastic stolons and, typically, the eventual demise of one of the two colonies (Fig. 2B).[10,26] Confrontations between stolonless colonies produce passive rejections, which are likewise characterized by nematocyst discharge, but are typically accompanied by cessation of growth along the contact margin (Fig. 2C).[10,26] Fusion (Fig. 2A) results in long-term genetic chimeras.

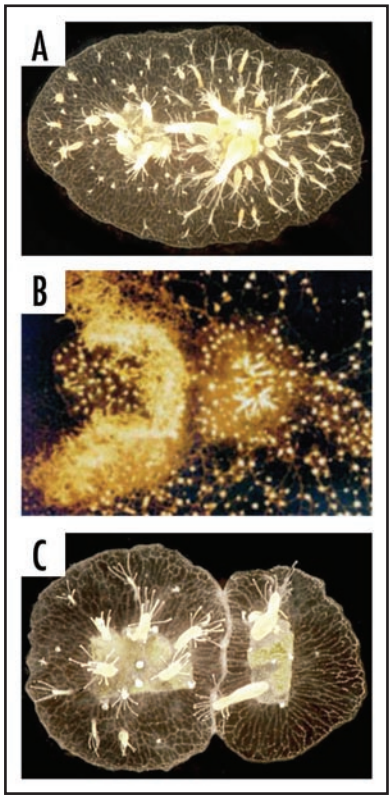

Figure 2. Fusion and rejection responses in Hydractinia. (A) fusion, (B) aggressive rejection and (C) passive rejection.

A third outcome of intraspecific encounters, distinct from fusion and rejection, is that of transitory fusion (TF).[24,25,28,32] Colonies fuse upon initial contact to establish a common endodermal gastrovascular system in a fashion indistinguishable from a permanent fusion. After 12–24 hours post-contact, a necrotic band appears at the point of initial contact, which subsequently spreads to form a line

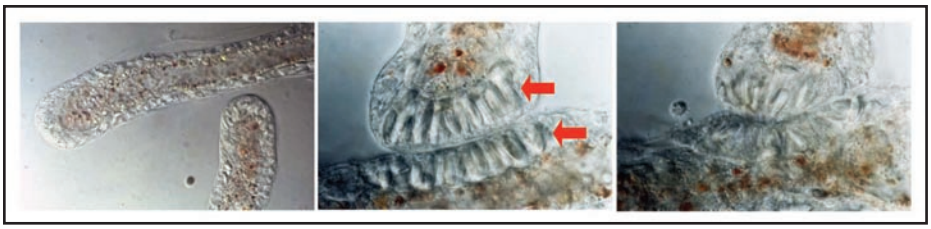

Figure 3. Encounter between incompatible (allogeneic) Hydractinia colonies. (A) Two stolons of allogeneic colonies prior to contact, (B) at point of contact and (C) at the moment of nematocyst discharge (arrows point to nematocytes). Photo credit: Rolfe Lange.

spanning the original contact zone. The emergence of the necrotic line is accompanied by occlusion of the once fused endodermal canals. One to two days after the first appearance of the necrotic line, colonies separate from one another. From this point on, the response is either indistinguishable from a rejection reaction (Type 1 TF) or repeatedly cycles between fusion and separation (Type 2 TF).[33]

Alloresponsivity is not restricted to EMCs located at the tip of stolons or periphery of the stolonal mat. Grafting experiments have established that all post-metamorphic tissue types of the colony (i.e., polyps, stolonal mat and stolons) exhibit allorecognition behavior;[34,35] embryos do not.[34,36] No second set response is detectable.

Genetics of Allorecognition in Hydractinia

Work by Hauenschild in the 1950s on the transmission genetics of allorecognition suggested that fusibility was controlled by a single locus.[24,25] His work, like some later efforts using wild-type stocks,[29] was not definitive.[37] The departures from single locus segregation noted by Hauenschild (and others) might be attributed to the fact that his studies involved four (and others more) different wild type genetic backgrounds. Therefore, defined genetic lines within which the effects of background were homogenized were generated—these include two near inbred genetic lines and one congeneic line.[28,33,38] Using these lines, a standard incross-intercross-backcross analysis was performed.[28,38] With rare exceptions, results were consistent with single locus Mendelian expectations, wherein fusibility is controlled by a single co-dominant locus such that colonies fuse if they share one or both alleles and reject otherwise. The rare exceptions displayed the transitory fusion response. The low frequency of transitory fusions in a cross otherwise segregating in a Mendelian fashion suggested two tightly linked loci. Crosses between offspring displaying transitory fusion confirmed that the chromosomal interval originally identified is comprised of at least two loci, designated *allorecognition 1* (*alr1*) and *alr2.* Colonies fuse if they share at least one haplotype, reject if they share no haplotypes, and display transitory fusion if they share only one allele in a haplotype (Fig. 4). Note that genetic rules of fusion and rejection in these animals echo the response of natural killer cells to missing self in mice and human bone marrow transplantation. Work is currently underway to positionally map and identify the genes responsible for allorecognition at the *alr1* and *alr2* loci.

Chimerism in Hydractinia

Natural chimerism has been documented in the wild among representatives of at least four different colonial marine invertebrate phyla: Porifera, Cnidaria, Bryozoa and Chordata.[39] As outlined above, conspecific interactions among adult animals in these groups are regulated by highly specific allorecognition systems that restrict somatic fusion and thus, chimerism, to self or close kin.[7] This natural occurrence of invertebrate chimerism is thought to result in benefits such as increased genetic variation, size and survivorship. Conversely, the self/non-self discriminatory ability acts to limit germ cell and somatic cell parasitism.[15]

Among vertebrates, chimerism occurs less frequently but has important immunological consequences. In 1945 Ray Owen described a naturally occurring state of stable hematopoietic chimerism in fraternal bovine twins that shared a common placental circulation (so-called freemartin cattle).[40] The clinical significance of this observation became clear when in 1951 Peter Medawar and colleagues showed that these chimeric twins were specifically tolerant to skin grafts of each other.[41,42] They were also able to experimentally induce donor-specific tolerance in prenatal and neonatal, but not adult, mice by injecting a large number of donor splenocytes in utero.[43] Therefore a strong relationship between mixed hematopoietic chimerism established prior to birth and allotolerance exists in vertebrates.

While allorecognition is a characteristic of many colonial invertebrate groups, until recently little was understood about how invertebrate allorecognition systems respond to chimerism formed across histocompatibility barriers. Two approaches have been used in the past to evaluate this question. First, histo-incompatible hematopoietic chimerism in adult invertebrate chordates has been attempted in *Botryllus schlosseri* by transplanting hemocyte suspensions between incompatible colonies. Chimerism in this case was highly unstable, resulting in complete loss of the donor cells within weeks post-transplantation, as measured by allelic markers.[17] Since Hydractinia lacks a circulating blood system, a second approach, that of establishing embryonic chimeras, has been used to evade natural incompatibility barriers. This approach involved grafting two histo-incompatible blastomeres or larvae and observing subsequent development. For the most part, chimerism generated in this fashion was also unstable, demonstrated by visible separation of histo-incompatible grafts shortly after metamorphosis.[34,44] In a few cases, embryonic grafts did not separate, suggesting variable tolerization to both donors immediately post-metamorphosis. The potential for tolerance was an exciting possibility, but the lack of molecular markers in these early studies prevented documentation of genetic chimerism in later ontogeny.

More recently, we studied the fate of embryonic chimerism and its effects on allorecognition in Hydractinia by generating embryonic chimera from well-mixed blastomeres and developing genetic markers to help characterize the fate of each cell line and determine the contribution of the *alr* gene complex to the stability of chimerism.[36] We found that histocompatible chimeras, generated from embryos matched at both *alr1 and alr2*, exhibited markedly higher growth rates and survivorship than histo-incompatible pairings (those mismatched at both *alr* loci). Histo-incompatible chimeras were unstable—they progressively lost chimerism after metamorphosis, with complete absence of chimerism by four weeks of age. In contrast, colonies generated from histocompatible pairings remained chimeric at markedly higher frequencies and longer durations. Histo-incompatible chimeras that lost detectable chimerism retained the fusibility/rejection characteristics of the remaining component

(genotype) of the chimera, but not that of the lost component. Therefore, embryonic chimerism across histocompatibility barriers in Hydractinia is unstable and does not induce tolerance to allogeneic non-self. This 'intolerance' to chimerism in a colonial marine invertebrate may be a reflection of the evolutionary pressure to prevent stem cell or germline parasitism in these animals.

Questions and Answers

Dr. Marc Hammerman, ***(Chromalloy Professor of Medicine, Washington University School of Medicine)*****:** Thank you Fadi for an interesting talk. When you initiated these experiments was your expectation that the chimerism would persist?

Dr. Lakkis: When we started these experiments to be honest, we were simply asking a question without predicting an answer. Is tolerance possible in invertebrates? It related to Charlie Janeway's prediction, shown in my second slide, that the innate immune system doesn't make errors and therefore does not require tolerance. It turns out that in Hydractinia, the invertebrate model organism that presumably has what is equivalent to an innate system but lacks adaptive immunity, neither stable chimerism nor tolerance were possible when we mixed completely mismatched embryos. This may not be the case in higher animals because (A) it appears that there are tolerance mechanisms in the mouse innate immune system, for example in natural killer cells, and (B) invertebrate immune or recognition systems are not necessarily equivalent to mammalian innate immune systems. In addition, it is now known that some invertebrates have immune diversification mechanisms, therefore, blurring the distinction between innate and adaptive and between invertebrate and vertebrate immune systems.

Dr. Wayne Yokoyama ***(Sam and Audrey Loew Levin Professor of Medicine, Washington University School of Medicine)*****:** What do you think is the function of alr?

Dr. Lakkis: We do not know. It doesn't have a mammalian homolog. One hypothesis is that it functions like inhibitory receptors present on NK cells. We are currently pursuing biochemical studies to understand how alr1 and 2 function.

Dr. Hammerman: In the wild, do Hydractinia chimeras always have the same genotypes?

Dr. Lakkis: This is a very good question. The Buss and Dellaporta labs have screened more than 500 animals and found 12 that match at alr2 with the tester laboratory strain used in the fusion/rejection assays. It turns out that out of these 12, some had full fusion, some transitory fusion, and four rejected the tester animal. This means that the alr2 locus can account for the majority of fusion events but not all of them and implies that there may be contribution from unlinked loci or so-called background effect on allorecognition. This is similar to what happens in mice where minor histocompatibility loci can still effect rejection despite matching at the major loci between donor and recipient.

Dr. Hammerman: What is your best guess, at this point in terms of the relationship between the ability of Hydractinia to resist fusion and the innate immune system?

Dr. Lakkis: My best guess would be some kind of a structurally conserved inhibitory receptor system analogous to that present on natural killer cells. Upon recognition of missing self MHC, natural killer cells are activated. This system, I predict, would be important for the rejection of stem cells as is the case in Hydractinia, where allorecognition is a safe-guard against stem cell chimerism or parasitism.

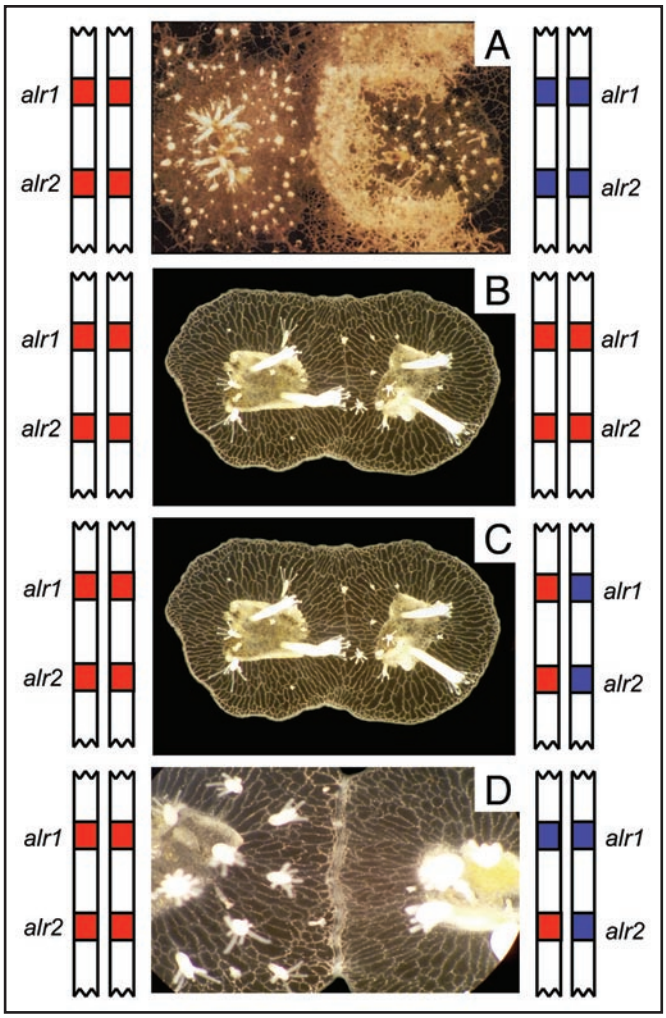

Figure 4. Genetics of fusion and rejection in Hydractinia. (A) Two colonies reject if they do not share any haplotypes, (B and C) fuse if they share at least one haplotype, and (D) undergo transitory fusion if they share only one allele in a haplotype.

In other words, this system may turn out to be more relevant to the rejection of a bone marrow than a solid organ transplant.

Dr. Hammerman: Is there anything in vertebrates analagous to the nematocyst effector system in cnidarians?

Dr. Lakkis: We don't have any cells that shoot spears but we have cells that shoot perforin and granzymes. These cells are the natural killer cells.

Dr. Hammerman: That was my next question. Are there genetic or molecular homologies between nematocysts and anything in vertebrates?

Dr. Lakkis: So called cytotoxic granules that natural killer cells have, would be closest we have. The granules contain perforin and granzymes the kill target cells.

Dr. Feng Chen ***(Assistant Professor of Medicine, Washington University School of Medicine)*****:** We probably assume in Hydractinia that when rejections occur they recognize each other as foreign. However, is it possible that it is the lack of recognition as self that causes rejection?

Dr. Lakkis: This is an important question. We do not know the answer. Whether this is a recognition system based on the missing

self hypothesis or a recognition system more like the T cell receptor, we still have to find out. We have not developed the technology yet to knock down genes in Hydractinia. One disadvantage of working with this model organism is that many techonologies have not been developed yet. Finally, I keep bringing back the missing self hypothesis as a likely possibility because one haplotype mismatch allows fusion to happen—this is very similar to the so called hybrid resistance in the natural killer cell system in mice.

Dr. Jeffrey Miner ***(Professor of Medicine, Washington University School of Medicine)*****:**

What do you think might happen if you synthesize recombinant *alr2* of one genotype and incubate that with an animal of a different genotype?

Dr. Lakkis: The Dellaporta group are currently making recombinant alr2 in insect cells. We hope that we will be able to answer your question soon, specifically to determine whether solube alr2 will inhibit the fusion or the rejection response.

Conclusion

The allorecognition phenomena of colonial marine invertebrates have long captured the attention of immunologists because they resemble the allogeneic interactions that characterize pregnancy and transplantation. Recent studies in the ascidian, *Botryllus schlosseri* and the cnidarian, *Hydractinia symbiolongicarpus*, provide evidence that highly specific allorecognition systems evolved early in metazoan history in response to pressure to prevent stem cell or germline parasitism. Detailed analysis of the structure and function of the molecules responsible for allorecognitoin in these basal animals may provide clues to the mechanisms of allorecognition in the innate immune systems of higher animals.

Note

Edited transcripts of research conferences sponsored by Organogenesis and the Washington University George M. O'Brien Center for Kidney Disease Research (P30 DK079333) are published in Organogenesis. These conferences cover organogenesis in all multicellular organisms including research into tissue engineering, artificial organs and organ substitutes and are participated in by faculty at Washington University School of Medicine, St. Louis Missouri USA.

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