

Self/nonself recognition in Cnidaria: contact to allogeneic tissue does not result in elimination of nonself cells in *Hydra vulgaris*

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Summary

Although *Cnidaria* have no specialised immune cells, some colonial forms possess a genetic system to discriminate between self and nonself. Allorecognition is thought to protect them from fusion with genetically different individuals and to prevent germ line parasitism. Surprisingly, when grafting tissue of two species of the solitary freshwater polyp *Hydra*, we found within the contact zone phagocytosing epithelial cells which selectively eliminated cells from the other species (Bosch and David, 1986). This led us to speculate that *Hydra*, which never undergoes “natural transplantation”, can differentiate between self and nonself (Bosch and David, 1986). In a previous paper (Kuznetsov et al., 2002) we described that cells which accumulate in the contact region of these interspecies grafts are apoptotic and that apoptosis is induced by impaired cell matrix contact. Thus, observations in such interspecies grafts did not give hints concerning the presence of a discriminative allorecognition system. To clarify whether this fundamental aspect of immunity is present in these phylogenetically old animals, we examined epithelial interactions between different strains of *Hydra vulgaris*. Here, we show that contact to allogeneic tissue does not evoke any response in terms of phagocytosis and elimination of allogeneic cells. We, therefore, question *Hydra*'s ability to discriminate between self and nonself and propose that, in contrast to colonial cnidarians, the solitary polyp *Hydra* has either lost or substantially reduced this ability.

Key words: *Hydra*, histocompatibility, allorecognition, phagocytosis, RAPD

Introduction

The ability for self-/non-self (allogeneic) recognition is a principal feature of multicellular organisms, allowing them to maintain individuality and species specificity. This phenomenon has been reported in almost all groups of the animal kingdom (Buss, 1987; Humphreys and Reinherz, 1994) and can be traced back to the cnidarians as the phylogenetically oldest Eumetazoa. Examples include the anthozoans *Stylophora pistillata* and *Montipora verrucosa*. There, branches within one colony can easily fuse while branches of genetically

different individuals never undergo fusion (Muller et al., 1984; Hildemann et al., 1980; Chadwick-Furman and Rinkevich, 1994). Fusion of two conspecific individuals is occasionally referred to as “natural transplantation”. Observations in sea anemones (*Anthopeura elegantissima*, *Phymactis clematis*) and gorgonians (*Eunicella stricta*) indicate that individual colonies possess unique sets of histocompatibility elements, which are recognized as nonself by all other conspecific colonies (Lubbock, 1980; Meinardi et al., 1995). In Hydrozoa,

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the same phenomenon was reported for *Millepora dichotoma* (Frank and Rinkevich, 1994) and studied in great detail in the colonial marine hydroid *Hydractinia echinata* (Frank et al., 2001).

Hydractinia was among the first invertebrates shown to display a genetically based system of intolerance against allogeneic tissue. The stolons within one *Hydractinia* individual are able to fuse, forming a complex net on the substratum. The ability to fuse between stolons of genetically different colonies is proportional to their degree of relationship. Contact of genetically different colonies leads to the aggregation of a special type of cnidocytes, termed microbasic mastigophores, in the area of contact (Lange et al., 1989). Firing of these highly specialized cells will eventually result in the destruction of one or both competitors. Allorecognition in *Hydractinia* undergoes a maturation process and is fully developed only after planula larvae have undergone metamorphosis (Fuchs et al., 2002). Genetic experiments involving crossbreeding of the colonies showed that the ability to fuse is genetically controlled by a single genetic locus with multiple, co-dominantly expressed alleles (Mokady and Buss, 1996, 1997). Nothing is known about the nature of the recognition molecules involved and the genes associated with this locus.

The freshwater polyp *Hydra* never undergoes transplantation reactions naturally. However, grafts between different *Hydra* species were long considered as a model for the ancestral form of transplantation immunity (Kolenkine, 1958; Kanaev, 1969). In heterografts between *Hydra vulgaris* and *Hydra oligactis* we previously have observed an increased number of phagocytosing epithelial cells located within the contact zone that selectively eliminate cells from the other species (Bosch and David, 1986). This observation suggested that a fundamental aspect of immunity – the ability to distinguish self from nonself – is present in the solitary polyp *Hydra*. This was surprising since the risk for cell lineage parasitisms appears to be low in *Hydra*. In a subsequent study, we have reported (Kuznetsov et al., 2002) that in such interspecies grafts a large number of apoptotic cells accumulate in the contact region. Histological analysis of the graft site suggested that apoptosis is induced by the detachment of epithelial cells from the extracellular matrix and impaired cell-cell contacts and not by a discriminative allorecognition system (Kuznetsov et al., 2002). Because of the failure to obtain clear evidence for the existence of a self/nonself recognition system in those interspecies grafts, and to resolve the issue of the presence of a discriminative allorecognition system in *Hydra*, we prepared intraspecies grafts and investigated tissue interactions between three different strains of *Hydra vulgaris*. Here, we report that contact to allogeneic tissue did not evoke any response in

terms of phagocytosis and elimination of nonself cells. We, therefore, question *Hydra*'s abilities to discriminate between self and nonself and propose that, in contrast to sedentary, colonial marine hydrozoans, the solitary polyp *Hydra* has either lost or substantially reduced this ability.

Materials and methods

Hydra strains

Experiments were carried out with *Hydra vulgaris* (Pallas) strain Basel originally obtained from Dr. Charles David, Munich. *Hydra vulgaris* strain PA2 was kindly provided by Dr. Ulrich Technau, Darmstadt. *Hydra vulgaris* strain Plön was isolated by Dr. Henrik Stotz from a lake in Plön, North Germany. Animals were cultured according to standard procedures at 18 °C. Experimental animals were starved 24 hours before use.

Analytical procedures

Grafts were produced as described (Kuznetsov et al., 2002). The species-specific mouse polyclonal antiserum raised against membrane fraction of *H. vulgaris* strain Basel was characterised in the previous paper (Kuznetsov et al., 2002). To detect strain-specific differences, the antiserum was pre-incubated with fixed cells of *H. vulgaris* Plön in PBS for 14 hrs at 4 °C and then used to stain *H. vulgaris* Basel/*H. vulgaris* Plön grafts. This procedure allowed us to deplete the antiserum of most of the antibodies reacting with both strains.

For identification of epithelial cells containing phagocytotic vacuoles and apoptotic bodies, maceration preparations were stained by the Feulgen method as described previously (Bosch and David, 1984).

Randomly amplified polymorphic DNA (RAPD) analysis was used to estimate gross genomic differences between polyps of three different strains of *H. vulgaris*. Genomic DNA was extracted from one tentacle of a single polyp using chelating resin Chelex-100 (Walsh et al., 1991). For RAPD fingerprinting, we amplified DNA using commercially available decamer primers (Operon Technologies INC, Alameda CA 94501, USA). Primers were biased to contain 60–70% G+C content. For identification of different *H. vulgaris* strains we used primer OPA 4 (5'-AAT CGG GCT G-3'). Reaction mixtures (25 µl) contained 67 mM Tris-HCl pH 8.8, 2 mM MgCl₂, 100 µM each dATP, dGTP, dCTP and dTTP, 200 nM primer, 25 ng of genomic DNA, and 0.3 units of Taq DNA polymerase. Amplifications were carried out using a Primus-96-plus-cycler (MWG Biotech) programmed for 45 cycles of 15 sec at 94 °C, 90 sec at 36 °C, and 2 min at 72 °C. Amplification

products were separated on 1% agarose gels in the presence of ethidium bromide and visualised with UV light. Some primers, such as OPA-2 (5'-TGCGAGCTG-3'), revealed individual differences between polyps within one strain, while others, such as OPA-3 (5'-AGTCAGCCAC-3'), produced a stable pattern for each strain. Appropriate control reactions were performed to ensure that informative fragments were not generated by differences in the concentration or quality of DNA used in PCR reactions. Three to four independent PCR amplifications were carried out using DNAs prepared from different polyps. RAPD patterns were identical in independent DNA preparations.

As an alternative to the RAPD method, we quantified nucleotide sequence differences between the three *H. vulgaris* strains by direct sequencing of an intron located between G₅₁₅ and T₅₁₆ of the mRNA sequence of the *Cnox2* gene from *Hydra vulgaris* (GenBank accession number AJ277388). The corresponding DNA template was obtained by PCR using 1× PCR buffer (Gibco), 200 μM dNTPs, 2 mM MgCl₂, 1 μM forward primer (5'-GAACCAGGTGAAGGTACCTCTTC-3'), 1 μM reverse primer (5'-CTGAATGCTAGTAACGCGTTTCG-3'), 0.5 μl genomic DNA (see above), and 1 U Taq polymerase (Gibco) in a total volume of 20 μl. After initial denaturation for 2 minutes at 95 °C, 35 cycles of PCR were performed with 45 seconds denaturation at 95 °C, 45 seconds annealing at 63 °C and 45 seconds elongation at 72 °C each, followed by 2 minutes at 72 °C. PCR products were subcloned and sequenced. Sequences were aligned with one another and the published sequence of *H. vulgaris* *Cnox-2* gene. Alignment was carried out using ClustalW multiple sequence analysis software, version 1.8 (Thompson et al., 1994) available at the ClustalW internet server under URL: <http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html>.

Results

To address the question whether *Hydra* cells are able to recognize and eliminate allogeneic cells we isolated three different *H. vulgaris* strains and tested their ability to induce apoptosis and phagocytosis in response to allogeneic contact. Along with polyps of the *H. vulgaris* strain Basel, we used *H. vulgaris* strain PA2 and a *H. vulgaris* strain recently isolated from a lake in Plön (North Germany).

Identification and characterization of three genetically distinct strains of *H. vulgaris*

By morphological criteria, all three strains were unequivocally identified as *H. vulgaris*. Thus, molecular

data were sought to reveal differences between the three strains. First, we used a polyclonal antiserum against membrane fraction of *H. vulgaris* Basel, pre-treated as described in Materials and methods, to stain *Hydra vulgaris* Basel/*Hydra vulgaris* Plön intra-species grafts. As shown in Fig. 1, the antiserum detects epitopes which are present on the cell surface of *Hydra vulgaris* Basel and absent on the cell surface of *Hydra vulgaris* Plön indicating significant differences

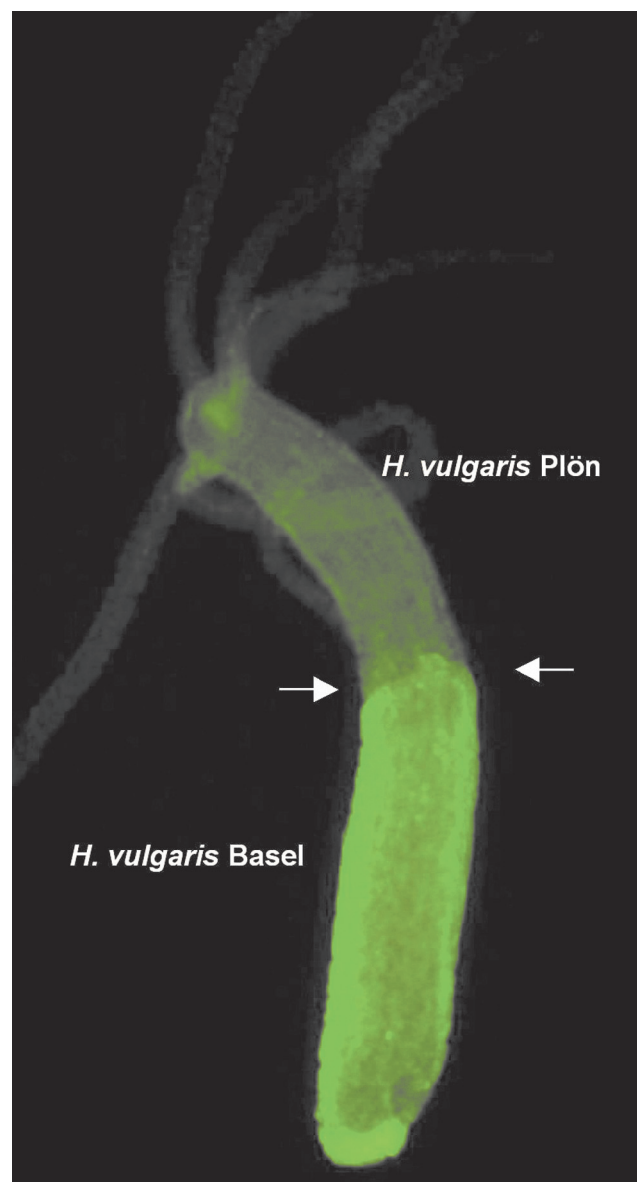


Fig. 1. Allograft between *H. vulgaris* Basel and *H. vulgaris* Plön showing differential binding of a polyclonal antiserum generated against membrane protein of the *H. vulgaris* Basel strain to *H. vulgaris* Basel cells. Note that the antiserum does not detect surface epitopes on cells of the *H. vulgaris* Plön strain.

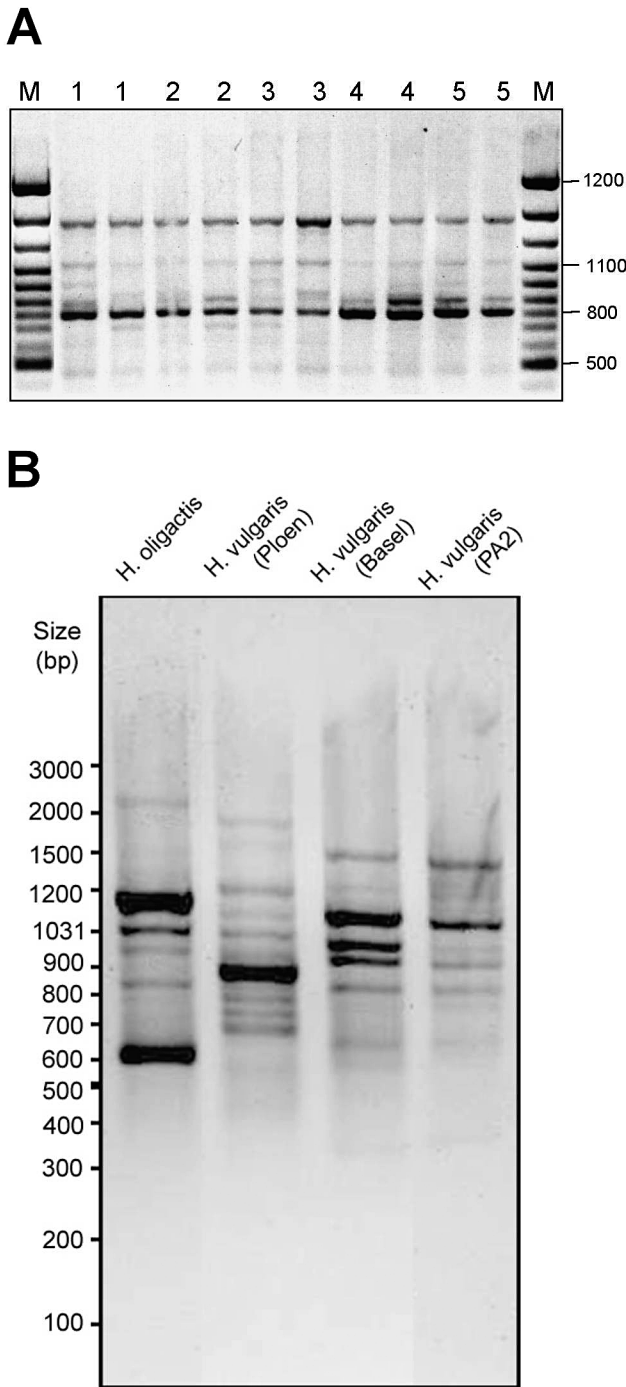


Fig. 2. RAPD fingerprinting detects genetic differences among individuals of three different strains of *H. vulgaris*. (A) Control RAPD profiles generated by using random primer OPA04 are nearly identical in five individuals (numbered 1 to 5) of *Hydra vulgaris* strain Plön indicating high reproducibility. Each PCR was performed in duplicate. Molecular size in base pairs (M) is indicated at the left side of the figure. (B) RAPD profiles in individuals of three different strains of *Hydra vulgaris* and in *Hydra oligactis*. Molecular size in base pairs is indicated at the right side of the figure.

between the two strains at the protein level. Second, the view that *H. vulgaris* strain Plön is genetically distant from the other *H. vulgaris* strains was supported by results from RAPD (random amplification of polymorphic DNA) fingerprinting. RAPD analysis represents a PCR based method to randomly amplify a number of genomic fragments in a single reaction. Subsequent analysis of the pattern of amplified products allows to quantitatively estimate the differences between samples (Williams et al., 1990). We individually amplified DNA from five polyps of each of the three *H. vulgaris* strains. As shown in Fig. 2A, individual primers produced highly reproducible patterns consisting of a multitude of bands of different sizes and intensity. The patterns were different from primer to primer. While many primers revealed differences between *H. oligactis* and *H. vulgaris* (data not shown), some primers yielded identical patterns only within all polyps of a given *H. vulgaris* strain and thus could serve as strain-diagnostic traits. For example, Fig. 2B shows the fingerprints produced with primer OPA-4 with clear differences between the different *Hydra vulgaris* strains. Interestingly, the patterns obtained by using DNA from *H. vulgaris* Basel and *H. vulgaris* PA2 is rather similar although not identical. In contrast, the pattern produced by using genomic DNA from *H. vulgaris* Plön has little in common with the pattern in the two other *H. vulgaris* strains indicating that the Plön strain is more distantly related to the other *H. vulgaris* strains. Finally, the conclusion that these strains are genetically distinct was underlined by differences in the nucleotide sequence of an intron from a randomly chosen gene, the *Hydra* homeobox gene *Cnox-2*. As shown in Fig. 3, the intron is positioned after guanine 515 according to the published (Gauchat et al., 2000) cDNA sequence of the *Cnox-2* gene of *H. vulgaris*. The intron sequence of the *H. vulgaris* strain used in the above mentioned study (Gauchat et al., 2000) comprised 344 bases. The corresponding intron in *H. vulgaris* PA2 was of the same length. Only 1% nucleotide differences were observed between *H. vulgaris* strain Basel and strain PA2. In contrast, *H. vulgaris* strain Plön had a 339 base pair intron and contained 9% nucleotide substitutions when compared to *H. vulgaris* Basel strain (Fig. 3). Using the same set of primers no amplification product could be obtained when using DNA from *H. oligactis* indicating distinct species differences at the *Cnox-2* nucleotide level. Taken together, three independent molecular approaches to estimate the degree of divergence between different strains of *H. vulgaris* indicated unambiguously that all three strains are genetically different and that *H. vulgaris* strain Plön is more distantly related to the Basel strain than the PA2 strain.

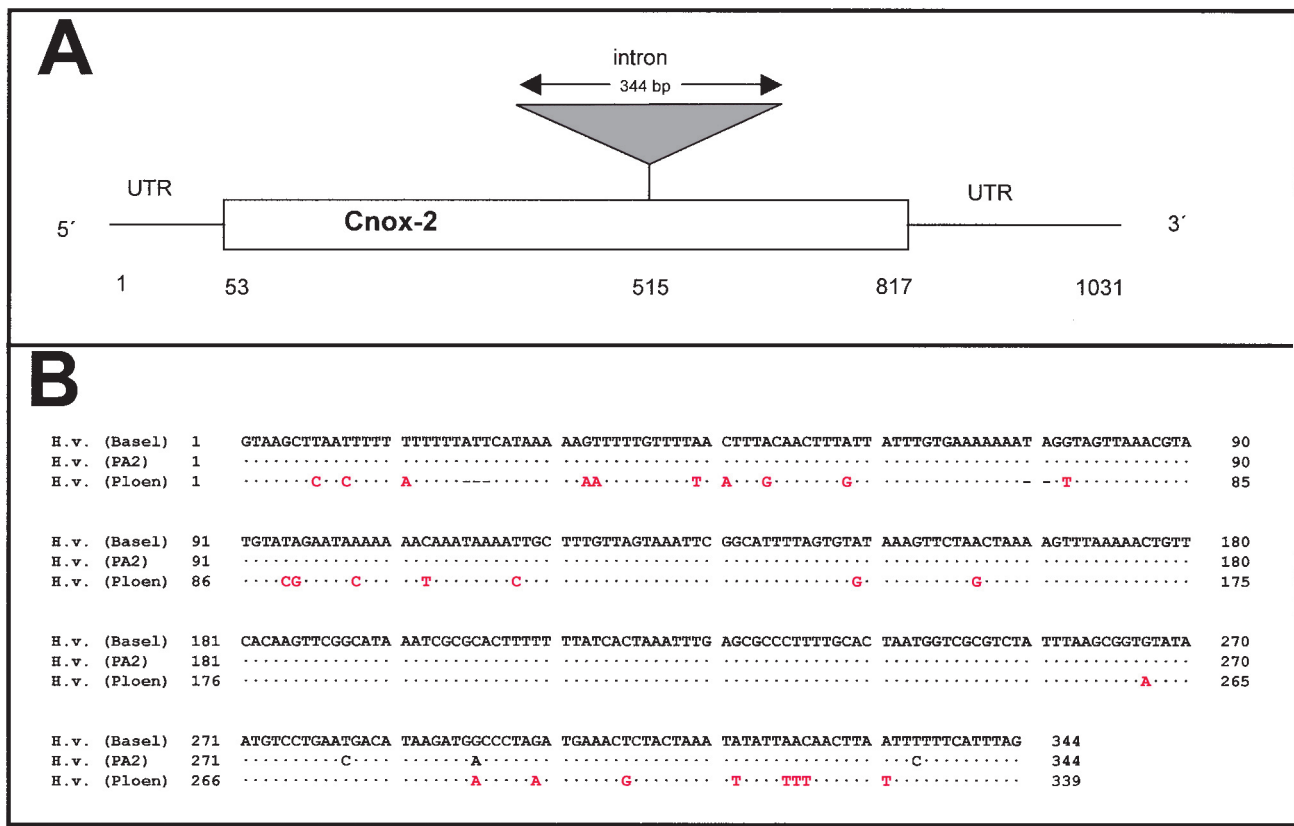


Fig. 3. Intron sequences of *Hydra* Cnox2 reveal differences among three different strains of *H. vulgaris*. **(A)** Structure of the Cnox2 transcript indicating the localization of the intron at position 515. **(B)** Nucleotide sequence of the Cnox2 intron in three strains of *H. vulgaris*. The intron sequences have been deposited in GenBank under accession numbers AY079148, AF486282, AF486283 for the Basel, PA2, and Plön strain respectively. Dots indicate bases identical to that in the Basel strain; dashes indicate gaps inserted by the alignment program.

No evidence for removal of allogeneic cells at the contact site of *Hydra* allograft

To test the ability of *Hydra* polyps to recognize and eliminate allogeneic cells, we induced allogeneic contact between epithelial cells by producing grafts between the three strains of *H. vulgaris* as described above. Standard animals of the strains were selected from a mass culture. Proximal halves of *H. vulgaris* strain Basel were grafted to distal halves of either *H. vulgaris* strain Plön or *H. vulgaris* strain PA2. After 24 hours of parabiosis, 10 intraspecies grafts of each type were cut into head, graft and foot regions and macerated. To identify and quantitatively determine the extent of cellular phagocytosis we used the previously described (Bosch and David, 1986) phagocytosis assay and Feulgen-stained macerates. Figure 4A shows an *H. vulgaris* strain Basel ectodermal epithelial cell containing a phagocytized cell in a vacuole. The percentage

of epithelial cells containing phagocytic vacuoles was scored by counting 600 to 1000 epithelial cells. As positive controls we prepared interspecies grafts between *H. vulgaris* and *H. oligactis* which have two to three times more epithelial cells containing such phagocytic vacuoles at the graft site than in other body regions (see also Bosch and David, 1986). Figure 4 B indicates that allografts between *H. vulgaris* Basel and two other *H. vulgaris* strains did not display an elevated level of phagocytized cells at the graft site. This was particularly striking for grafts between *H. vulgaris* Basel and *H. vulgaris* Plön since these strains were shown above (Fig. 1–3) to differ greatly at the molecular level. Since epithelial contact between genetically distinct individuals of three different *H. vulgaris* strains did not lead to any increase in the phagocytic activity at the site of contact, we conclude that a discriminative allorecognition system, if present, is rather poorly developed in *Hydra*.

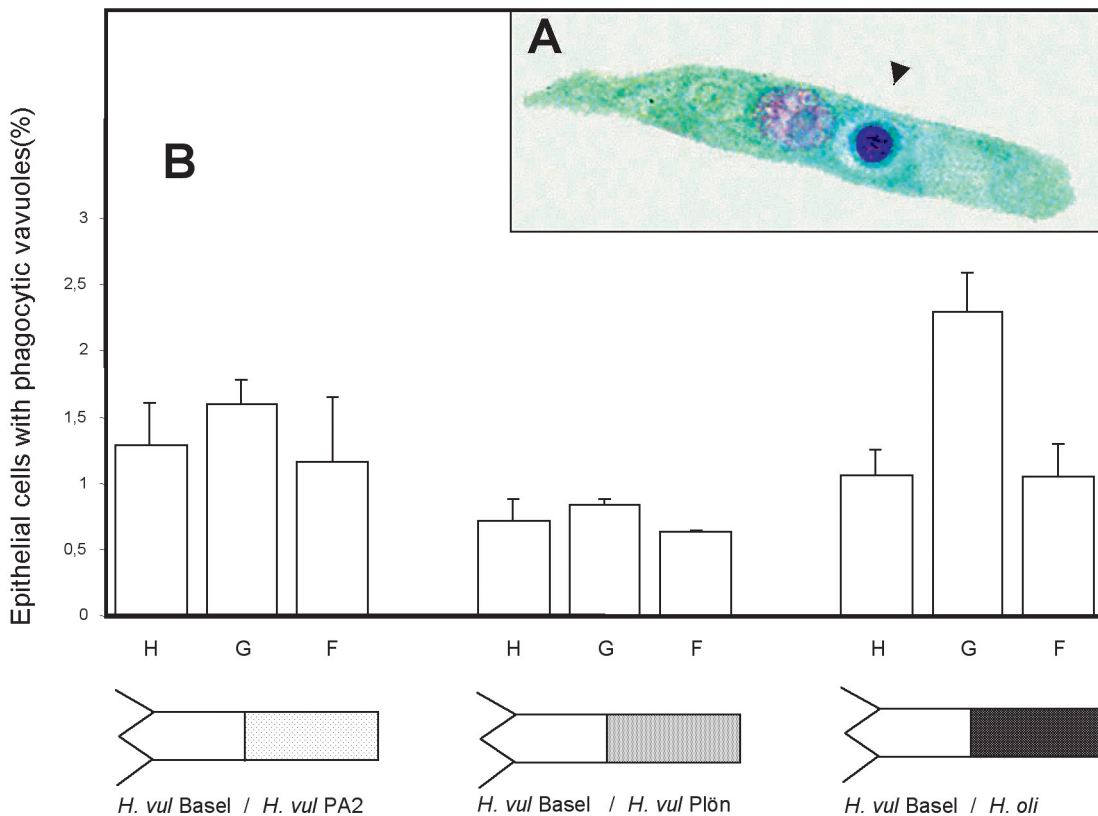


Fig. 4. Phagocytic activity in the contact zone of intra- and interspecific grafts. (A) *Hydra* epithelial cell containing a phagocytic vacuole (arrowhead). (B) Number of phagocytizing epithelial cells in the head (H), graft (G) and foot (F) regions of various graft combinations. Values represent means + S.D. of percent of epithelial cells (n = 800) containing phagocytosed cells.

Discussion

Allorecognition in invertebrates prevents somatic and germ line parasitism

Why do “simple” animals need the ability for allorecognition? Many of these organisms form colonies of numerous individuals which are genetically identical and physically linked. Moreover, many invertebrates such as sponges, corals, bryozoans and tunicates are sedentary or encrusting organisms and undergo metamorphosis before or immediately after attaching to the substrate. This life cycle allows co-settlement of genetically different but conspecific larvae and fusion of allogeneic individuals. Following allogeneic contact, multipotent stem cells of one colony or individual may invade the body of another colony, get to the gonads, differentiate into gametes, and exclude the host from reproduction. This risk of “germline parasitism” may have provided the evolutionary pressure under which allorecognition has evolved in invertebrates (Buss,

1982; Buss, 1987; Stoner and Weismann, 1996; Magor et al., 1999). The ability for allorecognition is the only and indispensable system which protects colonial invertebrates from somatic fusion of genetically different individuals, allowing the animal to maintain its genetic homogeneity and, hence, individuality. Consistent with this hypothesis, colonial cnidarians such as the marine hydroid *Hydractinia echinata* have developed efficient and genetically based detection and defence mechanisms against allogeneic cells (Buss, 1982; Mokady and Buss, 1996). These “simple” organisms, therefore, may occupy a key position for understanding the evolution of the self/nonself recognition system.

No evidence for a discriminative recognition system in the solitary freshwater polyp *Hydra*

Little is known about the molecules involved in allorecognition in *Hydractinia*. Since we study cell-cell interactions in the related hydrozoan *Hydra* using a wide spectrum of molecular techniques, we were inter-

ested whether we could find evidence for its ability to recognize and remove allogeneic cells. If so, this organism could be used to gain insight in the molecular mechanisms controlling a phylogenetically old recognition system. We tested the discriminative recognition potential of *Hydra* by bringing tissue of conspecific but genetically distinct strains of *H. vulgaris* in close contact and monitoring the phagocytic activity in the contact zone. Despite considerable genetic distance between the strains, no rejection or increase of phagocytosis could be observed in any of the allografts (Fig. 4). This was in contrast to our previous assumption (Bosch and David, 1986) and raises the interesting question why *Hydra* unlike, for example, *Hydractinia* appears to lack a discriminative recognition system and the ability to remove allogeneic cells. The answer may be found in differences in the life cycles. While in many cnidarians including *Hydractinia* natural transplantation is a frequently occurring event, for *Hydra* polyps direct cell-cell contact between allogeneic or xenogeneic tissue and entering of “predatory” cells of one individual into the body of another individual is a rather artificial event. Thus, due to reduced selection pressure to maintain an elaborated discriminative recognition system, *Hydra* may have lost or substantially reduced its allorecognition abilities secondarily. Analogous to animals that live in caves and have lost their vision ability, it seems conceivable that animals that never experience allogeneic contact have lost the allorecognition system. Since allorecognition systems are widely spread among colonial *Hydrozoa* and also common in other cnidarian classes, their existence, however, was probably one of the primary features of the phylum. This is consistent with the view (Collins, 2000) that the freshwater polyp *Hydra* is highly derived and member of the most evolved group within the class Hydrozoa.

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