THE JOURNAL OF EXPERIMENTAL ZOOLOGY 238:225–234 (1986)

Immunocompetence in *Hydra*: Epithelial Cells Recognize Self-Nonself and React Against It

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ABSTRACT The evolution of effective immunologic defense mechanisms in multicellular organisms involves the ability of host cells to distinguish betweeen "self" and "nonself" and to react appropriately to eliminate foreign tissue. By producing interspecies grafts we have obtained evidence that immunorecognition followed by incompatibility reactions occur in *Hydra*. Our results demonstrate that epithelial cells of *Hydra* recognize and phagocytose foreign hydra cells, indicating that they are the effector cells in the incompatibility reactions. This observation is consistent with the idea that immunocompetence appeared early in the evolution of multicellular organisms.

The study of immune reactions in primitive invertebrates is important from a phylogenetic point of view. It may also contribute to our understanding of the complex immunology of higher animals since the basic principles are probably similar. In order to compare invertebrate and vertebrate phyla the term "immunity" should be used in a broad sense. An animal may be said to possess immunity if it is capable of recognizing and protecting against nonself (Lackie, '80). Involvement of specific memory in primitive immune responses may or may not occur and can be viewed as an evolving characteristic (Hildemann, '74; Lackie, '80; Coombe et al., '84).

There is now well-documented evidence of immunocompetence in a variety of multicellular invertebrates ranging from sponges and coelenterates to echinoderms and protochordates (for review see Hildemann, '79).In colonial hydrozoans and anthozoans immunocompetence characterized by specific reactivity to nonself and subsequent cytotoxic reaction is common at the level of colony specificity (Theodor, '70; Francis, '73; Hildemann, '74; Buehrer and Tardent, '80; Buss et al., '84). Interclonal histoincompatibilities under predictable genetic control have been described in the colonial hydroid *Hydractinia* (Hauenschild, '54, '56; Müller, '64; Buss et al., '84). More recently, autoreactivity and self-tolerance were found to occur in *Hydractinia* (Buss et al., '85). A specific memory component has been demonstrated in an anthozoan coral (Hildemann et al., '77).

The underlying mechanisms, which are responsible for the incompatibility reactions as well as the cellular interactions at the site of contact, are not well understood in coelenterates. In particular, little is known about the nature of the effector cells involved in histoincompatibility and cytotoxic immune reactions in coelenterates (Lackie, '80). However, in one organism, *Hydractinia echinata*, Buss et al., ('84) have now shown that histoincompatibility reactions are due to accumulation and discharge of specialized nematocytes at the site of contact.

To examine cellular immune specificity in the freshwater polyp *Hydra* we have investigated the behavior of interspecies grafts. Using maceration techniques (David, '73; Bode et al., '73) we have identified epithelial cells as the effector cells in the immunological response.

MATERIALS AND METHODS

Strains

H. attenuata has been cultivated in the laboratory since 1966. The *H. oligactis* strain was obtained by Prof. P. Tardent, Zürich, in 1982.

Culture condition and maceration technique

The methods have been reported elsewhere (Bosch and David, '84).

Histological methods

To identify and count phagocytic vacuoles maceration preparations were stained by the Feulgen method as described previously

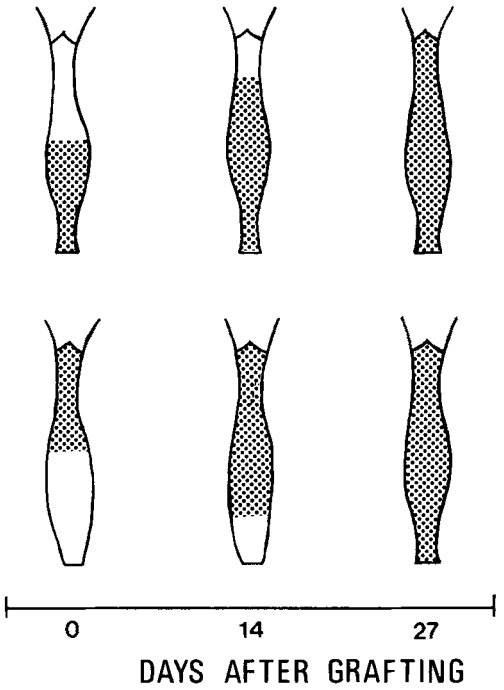

Fig. 1. Schematic drawing illustrating tissue displacement by *H. oligactis* in *att/oli* heterografts. *H. attenuata* (clear), *H. oligactis* (stippled).

(Bosch and David, '84). Developing nematoblasts in the body column were identified by staining whole mounts with lead nitrate-thioacetic acid (David and Challoner, '74).

^{3}*H-thymidine labelling*

Me-^{3}H-thymidine (specific activity 44 Ci/ml; Amersham Buchler, Braunschweig) was injected into the gastric cavity at a concentration of 50–100 μCi/ml (David and Campbell, '72). Continuously labeling was achieved by repeated injections at 12-hour intervals.

Transplantation procedure

Heterografts were produced by stringing hydra halves onto a nylon fishline (Rubin and Bode, '82). Sleeves of polyethylene tubing were then strung onto both ends of the fishline to maintain the cut surfaces in close contact. After 1–2 hours the grafts were taken off the fishline. The grafts were inspected daily for position of the graft junction.

Nematocyst analysis

To examine the nematocyst composition in the tentacles three to five tentacles were cut off each heterograft, transferred to a microscope slide, and gently flattened under a coverslip. The nematocysts were analyzed by using interference microscopy. Measurements of the lengths of desmonemes were done by using a ocular micrometer.

RESULTS

Stability of interspecies grafts

Grafting success depends on the species combination and decreases as species diversity increases (Bibb and Campbell, '73). In agreement with previous reports (Campbell and Bibb, '70) investigations in our laboratory indicate that most interspecies combinations are not permanently stable. However, one pair of species, *H. attenuata* and *H. oligactis*, appears to tolerate parabiosis.

To test whether permanently stable heterografts could be produced distal halves of *H. oligactis* were grafted to proximal halves of *H. attenuata* and vice versa. Both species can be recognized due to differrent carotenoid pigments in the endoderm. *H. attenuata* is pink-orange and *H. oligactis* is yellow-orange in color (Wanek and Campbell, '82). Within 24 hours the graft junction identified by the different pigmentation was perfectly healed in the 455 *H. attenuata/H. oligactis* (*att/oli*) heterografts made thus far.

When culturing these heterografts we observed in all cases continuous tissue displacement of *H. attenuata* from the heterograft. Complete displacement required several weeks. Displacement was observed regardless of whether *H. attenuata* was the apical or the basal partner in the heterograft (Fig. 1).

Cell cycle length during parabiosis

To determine if displacement involved growth inhibition of *H. attenuata* we measured the epithelial cell cycle length of both species during parabiosis by continuous labeling with ^{3}H-thymidine. Grafts were cultivated for 7 days and then "continuously" labeled with ^{3}H-thymidine. After varying periods of labeling heterografts were separated at the boundary between the two partners and the labeling index of epithelial cells in each partner determined by maceration and autoradiography. The results indicate that 90% of the epithelial cells of both partners are labeled after 3 days (Fig. 2). From the labeling kinetics an epithelial cell cycle length of 3.8 days was calculated for both partners in the heterografts. In addition the

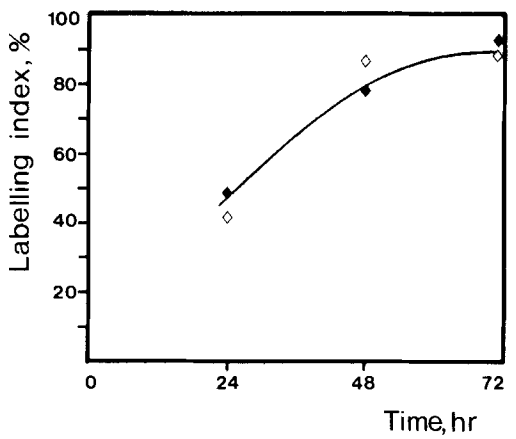

Fig. 2. ^{3}H-thymidine-labeling of epithelial cells in *att/oli* heterografts. Heterografts were cultivated for 7 days. Then the heterografts were "continuously" labeled with ^{3}H-thymidine for 3 days (see Materials and Methods). Each day ten heterografts were separated at the boundary between the two partners and the labeling index in each partner was determined by maceration and autoradiography. *H. attenuata* (solid diamond), *H. oligactis* (open diamond).

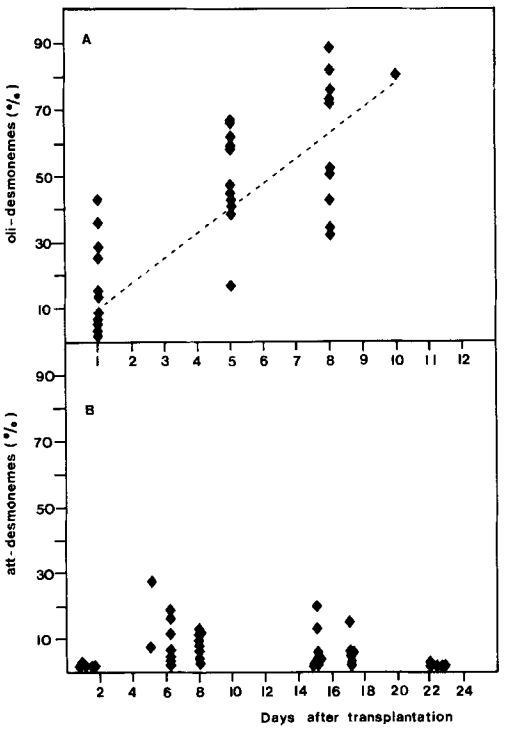

Fig. 3. A. Displacement of *H. attenuata* desmonemes in tentacles of *att/oli* heterografts. At each time point two to four tentacles were analyzed. After each analysis all tentacles were removed to permit regeneration of new tentacles. Thus the nematocyt content of tentacles reflects the relative numbers of *H. oligactis/H. attenuata* nematocytes produced between two time points. Each value represents the results from an single animal. B. Occurrence of *H. attenuata* desmonemes in tentacles of *oli/att* heterografts (same procedure as in A).

interstitial cell population of both partners was completely labeled by this procedure (data not shown). Thus, displacement is not due to different cell cycle lengths of the two species during parabiosis.

Fate of the interstitial cell lineage in heterografts

To study the fate of cells of the interstitial cell lineage in heterografts we investigated the behavior of nematocytes in the tentacles of interspecies grafts. Desmonemes of *H. oligactis* and *H. attenuata* differ significantly in the length of their capsules (Lee and Campbell, '79). Thus it is possible to follow the fate of this cell population in the tentacles of heterografts. The results in Figure 3A indicate that *H. oligactis* desmonemes displace *H. attenuata* desmonemes at roughly the same rate as tissue displacement occurs. After 10 days about 80% of the desmonemes in tentacles of *att/oli* heterografts are of the *H. oligactis* type. In *oli/att* heterografts, where the tentacle cells are of the *H. oligactis* type, only few *H. attenuata* desmonemes were found (Fig. 3B) even at early times when half the animal consists of *H. attenuata* tissue.

To test whether displacement depended on the continued presence of *H. oligactis* tissue, *att/oli* heterografts were separated after 10 days (when most desmonemes were of the *H. oligactis* type) and the *H. attenuata* half cultured further. Figure 4A indicates that *H. oligactis* desmonemes disappeared from the tentacles over a period of about 12 days in the absence of *H. oligactis* tissue.

The observed displacement of *H. attenuata* desmonemes (Fig. 3A) and its dependence on the presence of *H. oligactis* tissue in heterografts (Fig. 4A) is surprising in view of the well-known migration capacity of interstitial cells in *Hydra* tissue (Heimfeld and Bode, '84). Due to migration one would expect intestitial cells in both species to be homogeneously mixed after several days of parabiosis. Indeed, we could demonstrate extensive interstitial cell migration between *H. oligactis* and *H. attenuata* tissue in heterografts. In *att/oli* heterografts, in which *H. oligactis* was labeled with ^{3}H-thymidine, 17% of the interstitial cells in the apical *H. attenuata* half were labeled 2 days after grafting.

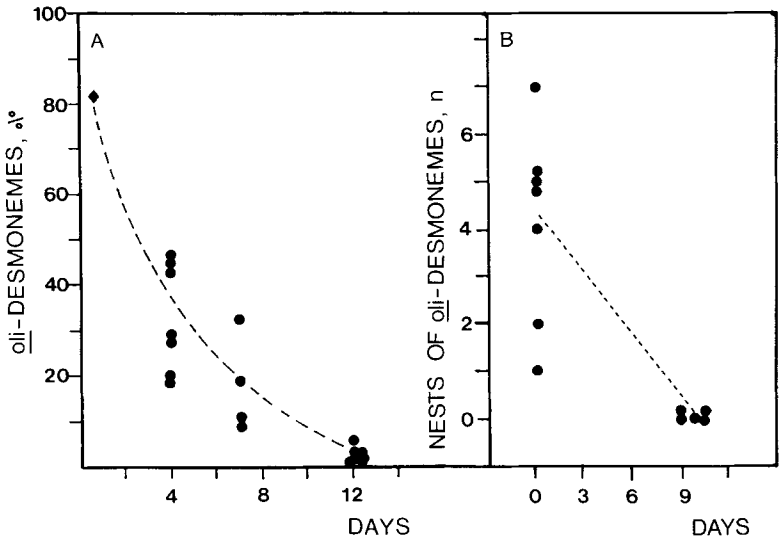

Fig. 4. Displacement of *H. oligactis* desmonemes from *H. attenuata* tissue. Each value represents the results from an single animal. A. After 10 days of parabiosis *att/oli* heterografts were separated at the graft boundary, the apical *H. attenuata* portions were cultured, and the tentacles were analyzed for presence of *H. oligactis* desmonemes. Solid diamond, value from Figure 3A. B. After 2 days of parabiosis *att/oli* heterografts were separated at the graft boundary and the *H. attenuata* portions were cultured. These animals were stained with lead nitrate thioacetic acid on day 0 and day 8 to identify nests of differentiating *H. oligactis* desmonemes.

In *oli/att* heterografts, in which *H. attenuata* was labeled with ^{3}H-thymidine, 9% of the interstitial cells in the apical in the apical (H. oligactis half were labeled 2 days after grafting. The extent of this interstitial cell migration is similar to that reported for *H. attenuata/H. attenuata* homografts (Heimfeld and Bode, '84).

To test whether migrating interstitial cells can differentiate nematocytes in the foreign tissue we used a histochemical procedure which specifically stains the nematocyst capsules of differentiating nematoblasts (David and Challoner, '74). The capsules of *H. oligactis* desmonemes and holotrichous isorhizas are clearly distinguishable from those of *H. attenuata* (Lee and Campbell, '79). Figure 5 shows a nest of *H. oligactis* desmonemes in the *H. attenuata* portion of a heterograft. An average of four such nests were found in the *H. attenuata* half of each heterograft beginning 2–3 days after grafting (Fig. 6). Nests of holotrichous isorhizas of the *H. oligactis* type were also observed although they were less frequent. In contrast, no *H. attenuata* desmoneme nematoblasts were found in the *H. oligactis* portion of *oli/att* heterografts (Fig. 6).

The migration of *H. oligactis* interstitial cells into *H. attenuata* tissue and the differentiation capacity of *H. oligactis* nematoblasts in *H. attenuata* tissue suggest that a mixture of both *H. attenuata* and *H. oligactis* interstitial cells occurs in *att/oli* heterografts. To test the stability of this mixed population of interstitial cells we isolated the *H. attenuata* portion of heterografts, cultured them for several days, and stained them for differentiating nematoblasts. The results in Figure 4B indicate that nests of *H. oligactis* desmonemes disappear from isolated *H. attenuata* tissue within 9 days. Since both *H. oligactis* nematocytes (Fig. 4A) and *H. oligactis* nematoblasts (Fig. 4B) are displaced following removal of *H. oligactis* tissue, we conclude that mixed populations of *H. oligactis* and *H. attenuata* interstitial cells are not stable.

Evidence for phagocytosis

An active phagocytic system has long been known to be present in coelenterates (Metschnikoff, '05) and recent studies in *Hydra* have demonstrated phagocytosis by epithelial cells under certain conditions (Campbell, '76; Fujisawa and David, '84; Bosch and David, '84). Hence we investigated whether displacement of one species in heterografts was due to phagocytosis of that species by the other. Phagocytosis was estimated quantitatively by counting the number of epi-

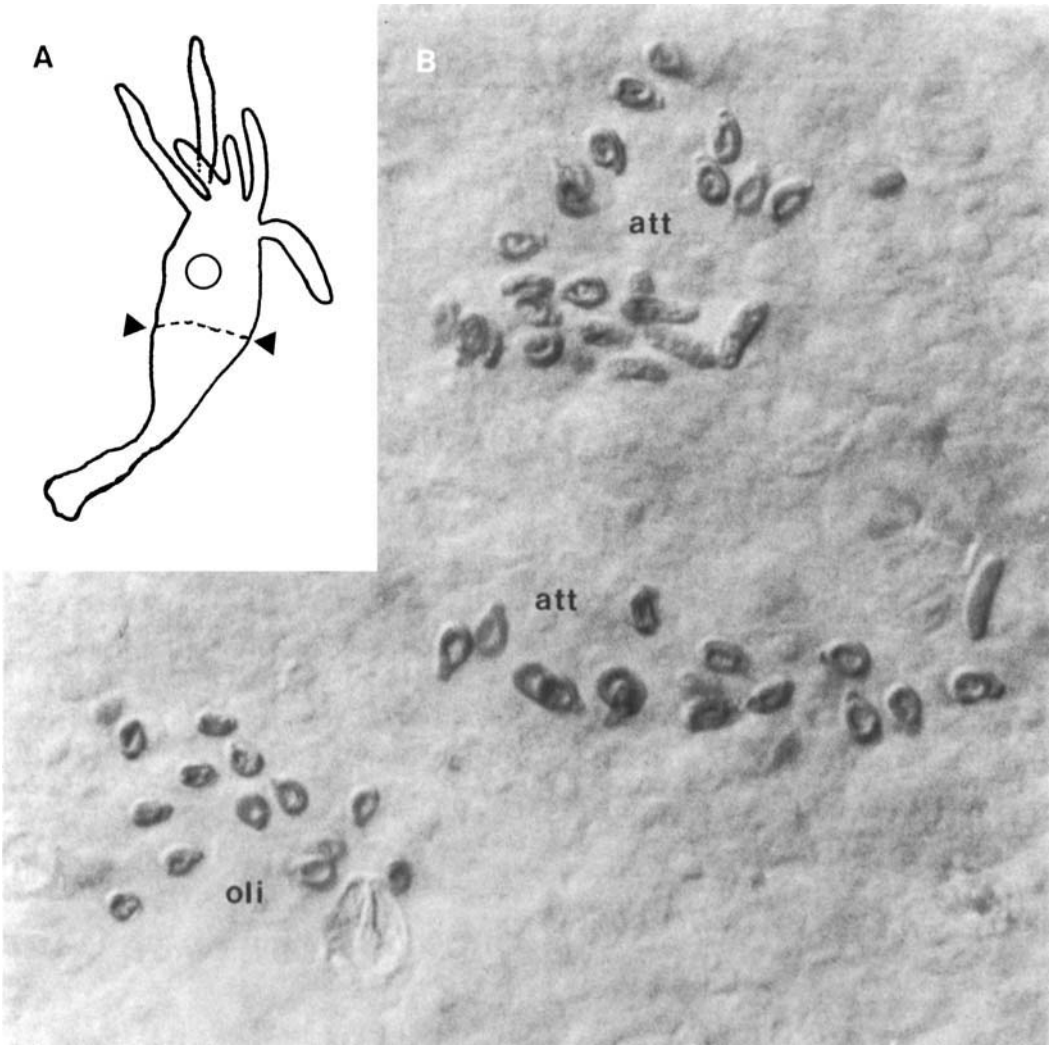

Fig. 5. Nests of differentiating desmoneme nematoblasts in the *H. attenuata* portion of an *att/oli* heterograft stained with thioacetic acid and lead nitrate. A. Camera lucida drawing of the heterograft detailed in B indicating the boundary between the two partners (arrowheads) and the localization of the detailed view (circle). B. Detailed view of the *H. attenuata* region of an *att/oli* heterograft 3 days after transplantation showing one nest of *H. oligactis* desmonemes and two nests of *H. attenuata* desmonemes. ×1,500.

thelial cells containing phagocytic vacuoles in macerated, Feulgen-stained preparations of tissue from heterografts.

Figure 7a shows an epithelial cell containing a phagocytized cell in a vacuole. In heterografts two to three times more epithelial cells containing such phagocytic vacuoles were found at the graft site than in other body regions (Fig. 8). Such cells were found both in endoderm and ectoderm. By comparison, homografts between individuals of the same species did not contain more phagocytized cells than ungrafted control animals. The somewhat enhanced levels of phagocytosis in the basal half of *att/oli* heterografts appear to be due to migration of *H. attenuata* interstitial cell into *H. oligactis* tissue and to the more effective phagocytosis of *H. attenuata* cells by *H. oligactis* (see Discussion).

Recognition and phagocytosis of foreign cells

To investigate whether phagocytosis involved cells of the opposite species, one partner in heterografts was prelabeled with ^{3}H-

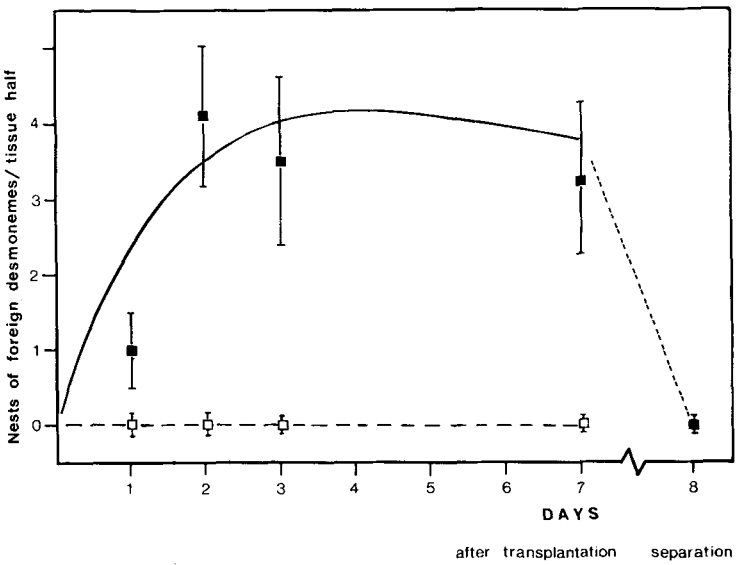

Fig. 6. Number of nests of differentiating desmoneme nematoblasts in heterografts stained with thioacetic acid and lead nitrate at various times after transplantation. Solid squares, nests of *H. oligactis* desmonemes in the *H. attenuata* portion of *att/oli* heterografts. Hallow squares, nests of *H. attenuata* desmonemes in the the *H. oligactis* portion of *oli/att* heterografts. Symbols represent mean (± S.D.) of five to seven animals.

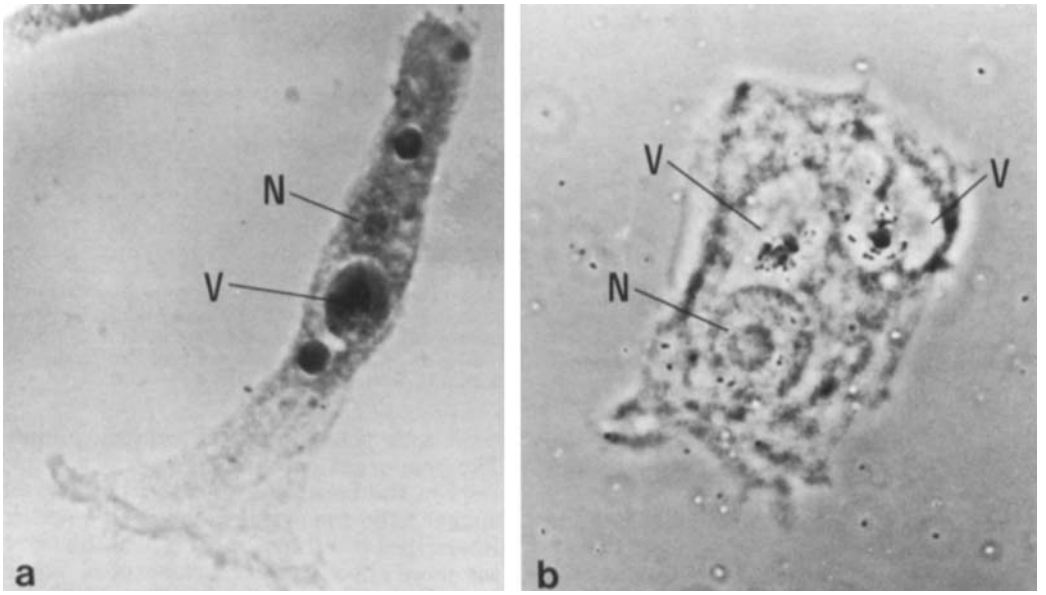

Fig. 7. a. Epithelial cell containing phagocytozed cells. Stained by the Feulgen method with Light Green counterstain (V = vacuole, N = nucleus), ×1,370. Such phagocytic vacuoles were found in ectodermal and in endodermal epithelial cells at the graft region. They have been described in detail previously (Bosch and David, '84). b. Autoradiograph of an epithelial cell containing a ^{3}H-thymidine-labeled vacuole (V = vacuole, N = nucleus) ×2,370. From the graft junction of an *att/oli* heterograft in which the *H. attenuata* partner was labeled with ^{3}H-thymidine.

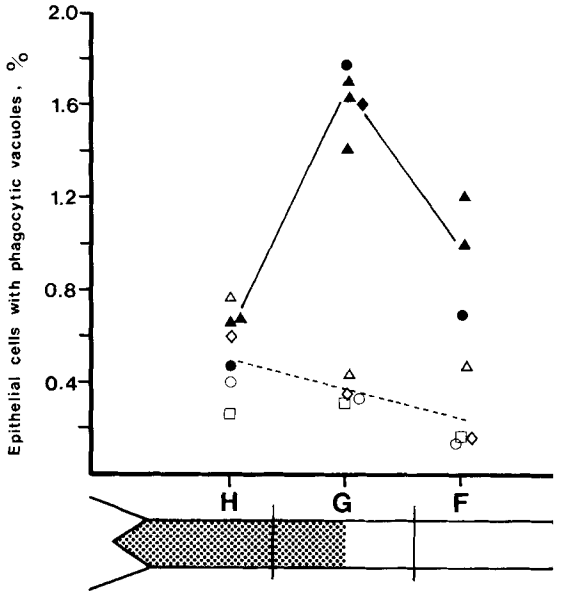

Fig. 8. Frequency of epithelial cells containing phagocytic vacuoles in different body regions of heterografts. After several days of parabiosis eight to twelve heterografts were cut in three parts (H = head region, G = graft region, F = foot region), macerated, and stained by the Feulgen method with Light Green counterstain. The percentage of epithelial cells containing phagocytic vacuoles was scored. Heterografts used:

(●), *H. attenuata/H. oligactis* 1 day after transplantation. (▲), *H. attenuata/H. oligactis* 3 days after transplantation. (♦), *H. attenuata/H. attenuata* 3 days after transplantation. Controls: (○), *H. attenuata/H. attenuata* 3 days after transplantation. (△), *H. oligactis/H. oligactis* 3 days after transplantation. (□), *H. attenuata*, (◇), *H. oligactis*, standard animals.

thymidine by repeated injections into the gastric cavity at 12-hour intervals for 2 days. This procedure labels about 80% of the epithelial cells and 100% of the interstitial cells. Apical halves of labeled *H. attenuata* or *H. oligactis* were then grafted to unlabeled proximal halves of the opposite species. After 3 days of parabiosis heterografts were cut into head, graft, and foot regions, macerated, and autoradiographed. Under these conditions 1–2% of epithelial cells in the graft region were observed to contain labeled vacuoles (Fig. 7b), indicating that cells of the opposite species were phagocytized. Labeled vacuoles were found irrespective of which partner was labeled in the heterografts. Hence, both *H. attenuata* and *H. oligactis* epithelial cells are capable of phagocytizing cells of the opposite species. Since cells are not phagocytized in homografts (Fig. 8) we conclude that epithelial cells are capable of recognizing nonself and reacting to it.

Involvement of a memory component?

In anthozoan cnidaria an enhanced response to second-set grafts has been reported in the scleractinian coral (Hildemann et al., '77) but not in gorgonian corals (Theodor, '70) nor in sea anemones (Lubbock, '80). In an attempt to demonstrate memory in the hydra immune response, we looked for an enhanced response to second-set heterografts. Primary grafts were separated after 2–4 days and the isolated partners cultured for further 2–6 days. Thereafter the same partners were grafted together again, cultured for several days, and the level of phagocytosis was measured. We found no evidence for a more vigorous response (i.e., tissue rejection) nor an increase in the number of epithelial cells containing phagocytic vacuoles.

DISCUSSION

Self/nonself recognition and the elimination of foreign cells

Our results show that in grafts between *H. attenuata* and *H. oligactis* there is continuous displacement of *H. attenuata* epithelial cells (Fig. 1). Figure 2 demonstrates that displacement of *H. attenuata* by *H. oligactis* is not due to alterations in the rate of epithelial proliferation. The present study provides evidence that displacement is accompanied by phagocytosis of cells by both partners in heterografts (Fig. 7b). Since *H. oligactis* always displaces *H. attenuata* (Fig. 1) the simplest interpretation of these results is that *H. oligactis* is more efficient in phagocytizing *H. attenuata* than vice versa.

Displacement also affects cells of the interstitial cell linage in heterografts. Figures 4B and 6 show that displacement of *H. oligactis* desmoneme nematoblasts occurs in the isolated *H. attenuata* half of *att/oli* heterografts. Displacement of *H. attenuata* desmoneme nematoblasts could not be directly demonstrated since such nematoblasts were not found in the *H. oligactis* portion of heterografts (Fig. 6). Nevertheless several results strongly support the idea that cells of the *H. attenuata* interstitial cell lineage are also displaced in heterografts. Our data show that a considerable number of *H. attenuata* interstitial cells (9%) migrate into the *H. oligactis* half of *oli/att* heterografts. Thus it appears likely that our failure to observe differentiating *H. attenuata* desmonemes in the *H. oligactis* half of heterografts is due to the very effective phagocytosis of these cells by *H. oligactis* tissue. Such an explanation is consistent with the increased number of epithe-

lial cells containing phagocytic vacuoles in the *H. oligactis* half of *att/oli* heterografts (Fig. 8). Furthermore, in *oli/att* heterografts, in which *H. attenuata* was labeled with ^{3}H-thymidine, epithelial cells containing labeled vacuoles were observed in the *H. oligactis* portion at positions well removed from the graft junction (data not shown). Such an increased rate of phagocytosis is probably also responsible for the absence of *H. attenuata* desmonemes in the tentacles of *oli/att* heterografts (Fig. 3B).

Thus, our results demonstrate that cells of both the epithelial and interstitial cell lineages are recognized and eliminated by nonself tissue. It seems likely that this recognition is mediated by cell-surface molecules and that cells of both lineages express these recognition signals. Such recognition signals are formally equivalent to the histocompatibility antigens present on cells of higher organisms.

Previous studies concerning the compatibility of heterografts

Our results confirm and extend previous examinations of *att/oli* heterografts. Issayev ('24), Goetsch ('24), and Kolenkine ('58, '71) all observed a gradual disappearance of *H. attenuata* tissue (epithelial cells) in grafts between *H. attenuata* and *H. oligactis*. In addition Goetsch ('24) demonstrated displacement of cells of the interstitial cell lineage based on identification of nematoblasts in whole mounts. Although displacement of *H. attenuata* nematocytes was complete in most cases, one graft appeared to contain some *H. attenuata* nematocytes 2 months after transplantation. Goetsch suggested that this animal might contain a stable mixture of interstitial cells of both species. Our results provide no evidence for a stable mixture of interstitial cells in heterografts (Figs. 3, 4) and we view the one case reported by Goetsch ('24) as an example in which complete displacement of *H. attenuata* was delayed. Thus our results are in good agreement with previous observations of *att/oli* heterografts.

Recent observations of Lee and Campbell ('79) on interstitial cell/epithelial cell chimeras of *H. oligactis* and *H. attenuata* do not appear to be consistent with our results. These authors repopulated epithelial *H. attenuata* with interstitial cells of *H. oligactis*. Although these chimeras could be maintained for up to six months they showed severe behavioral defects probably due to abnormal interstitial cell differentiation and to cell incompatibilities between the two species. The fact that *H. oligactis* interstitial cells were not displaced from these chimeras is surprising in view of our own results. Although we do not have an explanation it seems possible that *H. attenuata* interstitial cells or their products are necessary for displacement of *H. oligactis* cells and that therefore in epithelial *H. attenuata* no displacement can occur.

Epithelial cells as effector cells of the immune response

Tissue recognition and histocompatibility reactions in hydra are mediated by epithelial cells (Fig. 7). The observation that epithelial cells are directly involved in the histocompatibility reaction is surprising since in other phyla specific phagocytic cells, rather than epithelial cells, are involved in immune responses. However, it should be noted that ectodermal as well as endodermal epithelial cells in hydra have previously been shown to be active phagocytes (Campbell, '76; Fujisawa and David, '84; Bosch and David, '84). Furthermore, recent work in a variety of organisms indicates that epithelial cells can phagocytose neighboring cells under certain conditions (e.g., hepatocytes, Bursch et al., '84). This process, which has been named "apoptosis" by Wyllie et al. ('80), appears to be widespread in both vertebrate and invertebrate organisms. Since phylogenetically old organisms like *Hydra* eliminate "nonself" by apoptosis, this kind of cell death may represent any evolutionary relic of an early immune response.

Possible significance of self/nonself recognition in Hydra

The role of self/nonself recognition in hydra is not immediately obvious since hydras exist as solitary polyps and are normally not confronted with foreign tissue as in our experimental situation. Hence it is interesting to ask what role such a recognition system may play in the natural habitat. Buss ('82) has suggested that systems of self/nonself recognition are required by organisms which proliferate asexually by budding ("ramet formation"). In such organisms one cell lineage must proliferate continuously during organismal growth and remain totipotent to form germ cells during sexual phases of the life cycle. Such organisms are susceptible to "infection" with variant cell lines which may

compete with the totipotent host cell lineage for positions in the germline ("somatic cell parasitism"; Buss, '82; Buss and Green, '85). Thus an efficient self/nonself recognition system may have evolved in such organisms in order to combat overgrowth by somatic variants. Since gametes in *Hydra* arise from interstitial cells which proliferate continuously during growth and budding of the organism (Littlefield, '85; Bosch and David, in preparation), hydra would appear to require such a self/nonself recognition system in particular for the interstitial cell lineage. Our results demonstrate that just such a mechanism exists in hydra.

ACKNOWLEDGMENTS

We thank P. Tardent for sharing unpublished observations and T. Holstein for helpful discussion. The research was supported by the Deutsche Forschungsgemeinschaft (grant Da 163/1-1). T.C.G.B. is recipient of a postgraduate studentship of the University of Munich.

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