Proc. R. Soc. Lond. B **266**, 465–477 (1986)
Printed in Great Britain

Allogeneic cell interactions during graft rejection in *Callyspongia diffusa* (Porifera, Demospongia); a study with monoclonal antibodies

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(*Communicated by D. C. Smith, Sec. R.S. – Received* 13 *June* 1985)

[Plates 1–5]

Many aspects of the cellular immune system in the marine sponge *Callyspongia diffusa*, have been defined by using artificially transplanted allogeneic tissues. Rejections show specificity of 'non-self' recognition, cytotoxic effector responses and short-term immunological memory. Histological investigations reveal a generalized mesohyl migration to the graft zone where archaeocytes line up at the allogeneic interface. Monoclonal antibodies (mAbs) raised to sponge cells have shown that little or no allogeneic cell mixing occurs at the graft interface and that certain mesohyl cell types do not appear to be directly involved in graft rejections. However, all mesohyl cell types are present in autograft fusion zones and in inflammatory responses to injury. The involvement of only some of the mesohyl cell types in graft rejections suggests specific interactions of an effector 'immunocyte'.

Introduction

There is increasing evidence to suggest that cell-mediated immunity evolved with the invertebrates before the advent of humoral immunity with vertebrate evolution. Many invertebrates show highly discriminating cellular immune systems (Hildemann *et al.* 1979*a*; Manning 1980) with (i) specific 'non-self' recognition; (ii) reactions to non-self by various effector mechanisms; and (iii) inducible memory as measured by an accelerated secondary response (Hildemann *et al.* 1980). Tissue transplantation, often a natural occurrence in habitats of sedentary marine invertebrates, has been used to access the immune reactivity of *Callyspongia diffusa*, an Indo-Pacific sponge.

Morphological analysis of allografts in *C. diffusa* shows that the rejection response begins with an infiltration of mesohyl cells into the area of 'non-self' contact. This progresses to the formation of tissue bridges (cellular extensions that span the graft zone to contact the allogeneic tissues) and ends in necrotic reactions

This work was supported by National Institutes of Health grants AI 15075 and AI 19470.

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that result in allogeneic separation (Johnston & Hildemann 1983). Rejection kinetics reveal an inducible short-term alloimmune memory (Hildemann *et al.* 1979*b*, 1980*b*; Bigger *et al.* 1980) with alterations in rejection kinetics resulting from variations in histoincompatibilities (Hildemann *et al.* 1980*b*) and temperature (Johnston *et al.* 1981). Recently, the cell types within normal tissues of *C. diffusa* have been histologically defined (Smith 1985) allowing a more detailed description of internal cytological changes in tissue organization during rejections (Smith & Hildemann 1986). In addition, morphological changes associated with autograft fusion and inflammatory responses to injury have been reported (Smith & Hildemann 1986).

Sponge immunocyte identification is of interest to comparative immunologists since characterizing these cells will eventually allow investigations into the mechanisms of non-self recognition and effector cytotoxicity in this group of invertebrates. However, morphological analyses of the rejection zones have not yielded definitive identification of specific immunocytes. No enrichment of a specific cell type was observed in rejection zones of *C. diffusa* (Johnston & Hildemann 1983), and when differential counts of the cells at the graft zone were compared with counts in normal mesohyl, no significant differences in proportions of cell types were found (Smith & Hildemann 1986). Both archaeocytes and cells containing membrane-bound vacuoles, or spherulous cells, were observed in the general region of the graft zone (Johnston & Hildemann 1983; Smith & Hildemann 1986), however, allogeneic cell interfaces were composed of archaeocytes that had lined up in 'fronts' against each other (Smith & Hildemann 1986).

Monoclonal antibodies (mAbs) have been used extensively in dissecting mammalian immune systems. Differences in cell surface markers have enabled identification of many lymphocyte subpopulations and immunofluorescence techniques have allowed lymphoid tissues to be defined according to the types of lymphocytes localized within. Now that the morphological interactions that occur at sites of active rejection or wound healing have been described in *C. diffusa* (Johnston & Hildemann 1983; Smith & Hildemann 1986), mAbs have been produced to identify sponge cell types and to locate them within sectioned tissue by immunofluorescence techniques.

Two mAbs presented here identify specific cell types, and a third identifies the individual sponge that was used as the antigen. Results show little or no allogeneic cell mixing to occur at the allograft interface. In addition, neutrophilic spherulous cells and an archaeocyte subpopulation (which includes sclerocytes, spicule-producing cells) migrate into the rejection zone but are not located at the allogeneic interface. In contrast, both of these cell types are found in areas of autograft fusion and both respond (as do other mesohyl cells) to tissue damage. Cellular responses in sponges to different challenges of self-integrity suggest fundamental variations in specific versus non-specific responses.

Materials and methods

Sponges

Callyspongia diffusa is a branching sponge found on shallow reef crest habitats in Hawaii and the Indo-Pacific. Some of the sponges used previously (Smith & Hildemann 1986) were also used in the present study. All sponges were maintained in the running seawater system at the Hawaii Institute of Marine Biology (University of Hawaii) located on Coconut Island in Kaneohe Bay, Oahu, Hawaii.

Monoclonal antibody production

Cell suspensions were obtained by soaking sponge tissue in three changes of cold calcium- and magnesium-free seawater (c.m.f.s.w.) (Curtis 1979), squeezing with tweezers, straining through nylon mesh (60 μm), and washing twice in cold c.m.f.s.w. Balb/c mice (6-week-old females) were injected intraperitoneally with 10^6 unfractionated sponge cells, and boosted on days 8 and 17. Spleens were removed from two mice on day 19 or 20, dissociated and fused to an equal number of myeloma cells (P3Ag8.653 or S194) by using polyethylene glycol (Galfrè & Milstein 1981). A total of five fusions was done, each with cells from an individual sponge as the antigen. Hybrids were selected with HAT (hypoxanthine, aminopterin, thymidine) in RPMI 1640 (Irvine Scientific) or Dulbecco's modified Eagles' medium with high glucose (DMEM) (Gibco), 20 % (by volume) foetal calf serum (Irvine Scientific), epithelial cell growth supplement (Collaborative Research) or syngeneic mouse peritoneal macrophage filler cells.

Preparation of microtitre plates

Supernatants from wells with growth were assayed by using radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) on whole fixed sponge cells. Immulon II (Dynatech) microtitre plates for ELISA or flexible (Dynatech) plates for RIA were pretreated for 1 h at 37 °C with 50 μl per well of 0.1 % (by mass) poly-L-lysine in 0.15 M phosphate buffered saline (PBS) (pH = 7.4). The plates were washed with PBS, 50 μl of filtered seawater (f.s.w.) was placed in each well, followed by 50 μl of live cells in c.m.f.s.w. (2×10^5 to 2×10^6 cells per well). The cells were allowed to settle for 1.5 h at 4 °C before being gently fixed by immersing the entire plate in cold glutaraldehyde (0.25 % (by volume) in f.s.w.) for 5–10 min.

The fixative was removed from the plates by inverting and shaking, the plates were washed four times in PBS, blocked with 50–100 μl of 1 % (by mass) bovine serum albumin (BSA) in PBS, and could be stored at 4 °C for at least eight months in sealed containers. Plates were prepared by using either a mixture of cells from several sponges or cells from an individual sponge.

RIAs and ELISAs

RIAs were performed according to A. Benedict (unpublished) with his reagents. Wells were incubated with about 50 μl of hybridoma supernatant, washed in PBS, incubated with 50 μl of rabbit antiserum to mouse immunoglobulins (RαMIg), washed, and finally incubated with 50 μl of iodinated protein A. Wells were cut

apart and counted in a gamma counter. ELISAs used RαMIg labelled with β-galactosidase (RαMIg-βgal) (Bethesda Research Labs) and were run according to the enclosed technical sheet.

Cloning of hybridomas

Hybridomas producing mAbs to *C. diffusa* cells were expanded and cloned by limit dilution with epithelial cell growth supplement or macrophages, or both, to maximize hybridoma growth rate. Because the supernatant titre of one cloned hybridoma (1CH) was too low to produce adequate immunofluoresence, 50 ml were concentrated by lyophilization, rehydrated to 10 % of the original volume and dialysed against PBS.

Tissue processing

Procedures for allografting, autografting and injury induction were performed according to the methods of Smith & Hildemann (1986). Tissues were processed according to the procedures of Sainte-Marie (1961) for indirect immunofluorescence analysis. Sponges were simultaneously fixed and partly dehydrated in two changes of cold 95 % (by volume) ethanol for 1 h and overnight, respectively. Tissues were then run through four changes of cold absolute ethanol and three changes of cold xylene for 1–2 h each. The final xylene step was allowed to come to room temperature before beginning paraplast infiltration (Monoject Scientific). Specimens were passed through four changes of paraplast at 56–60 °C for 1–2 h each, the final bath being placed in a vacuum oven at *ca.* 103 kPa. Finally, specimens were embedded in prewarmed cubical plastic moulds (Peel-A-Way Scientific) filled with molten paraplast. After cooling, the blocks were stored at −20 °C. Blocks were cut according to the methods of Smith & Hildemann (1986). Sections were stored at −20 °C until processed for immunofluorescence or for routine staining by Harris' haematoxylin and eosin (H & E).

Immunofluorescence of tissue sections

Sections were deparaffinized by slightly modifying the procedures of Sainte-Marie (1961). It was found that 2 or 3 min were necessary for each step in the rehydration series rather than placing the slides in cold baths of xylene and ethanol for 10–15 s. The sections were brought to room temperature, washed in PBS, blocked with 0.1 % (by mass) BSA and 1 % (by volume) normal rabbit immunoglobulins (NRIg) (Accurate Chemicals) in PBS, and incubated for 30–120 min in mAb supernatant (neat or concentrated). The secondary Ab, RαMIg conjugated to fluorescein isothiocyanate (RαMIg-FITC) (Accurate Chemicals) was incubated for 30–60 min. For colour photography, some sections were briefly incubated in 75 μM propidium iodide (Sigma) in PBS for nuclear counter staining.

Immunofluorescence of cell suspensions

Cell suspensions were also stained with mAbs. Cells were washed in two changes of cold c.m.f.s.w. and spun into microscope slides using cytobucket trunnion carriers (International Equipment). They were fixed in 95 % (by volume) ethanol,

air dried, and processed for immunofluorescence according to the above procedures for sections. Some cells were fixed in suspension with 95% (by volume) ethanol, washed twice in PBS, incubated for 30 min in 0.1% (by mass) BSA and 1% (by volume) NRIg in PBS, washed, incubated in mAb supernatant for 30–120 min, washed, and finally incubated in RαMIg-FITC for 30–60 min. Suspensions of live cells were stained with mAbs following the same procedures except they were kept at 4 °C. All cells and sections were prepared in wet mounts for observation.

Immunofluorescence controls

Controls on both cells and sections used irrelevant mouse mAbs as the primary Ab, or the primary mAb was omitted. All controls were negative; neither mouse nor rabbit Abs cross reacted or non-specifically bound to sponge cells. All slides were photographed on Tri-X or Ektachrome 400 film with an Olympus or Zeiss fluorescence microscope.

Results

A total of 85 parental hybridomas were positive for *C. diffusa* by RIA or ELISA. Of those, 10 were analysed on dissociated sponge cells and 25 on normal *C. diffusa* tissue to assess their staining specificities (table 1). Three were chosen for further analysis on sections of allografts, autografts, and inflammatory responses to injury.

4CH identified the sponge used for immunization

Screening of parental hybridomas from the last two fusions was done by RIA on cells from five individual specimens of *C. diffusa* (including the specific sponge used for immunization for that fusion (denoted the D sponge)), and one specimen of *Toxadocia violacea* (another sponge species found slightly deeper on patch reefs in Kaneohe Bay than *C. diffusa*). Cells from individual sponges were placed in different plates. Most of the supernatants positive for *C. diffusa* were also positive for *T. violacea* (85%). Some positive for *C. diffusa* were negative for *T. violacea* (13%), and one (2%), 4CH, was positive only for the D sponge.

Allografts between the D sponge and two other sponges collected near Coconut Island showed massive cell infiltrate in the graft zone at 24 h, although tissue bridges did not form (figure 1, plate 1). When sections were stained with 4CH, most of the cells in the D sponge were positive (see table 1) while the sponge to which it had been paired was negative and only revealed the red propidium iodide counter stain (figure 2). The most striking feature in the 4CH staining pattern was the lining up of allogeneic cells on either side of the graft interface without obvious mixing (figures 2 and 3). Some 4CH positive (4CH+) material (not whole cells) was noted in the negative animal (figure 3). This may have been 4CH+ cell debris phagocytosed by the 4CH negative (4CH−) archaeocytes. Non-mixing of the allogeneic cells was maintained after the separation of allogeneic tissues (by 48 h). Cells positive for 4CH were not present in the 4CH− sponge.

Neither autografts nor damaged specimens were prepared for analysis for 4CH on the D sponge.

TABLE 1. IMMUNOFLUORESCENT STAINING PROPERTIES OF SOME mAb ON NORMAL *CALLYSPONGIA* TISSUE†

	immunofluorescent staining on *Callyspongia* tissue							results of ELISA or RIA on *Toxadocia*
	general mesohyl cells	archaeocytes	spherulous cells: acidophilic	spherulous cells: neutrophilic	pinacocytes	choanocytes: collar	choanocytes: body	
clone								
1A11.4G10	+‡	.	.	.	+++	+/−	+/−	+/−
1E9	.	−	−	−	−	+/−	−	+
1F1.C4	.	−	−	−	−	+/−	−	+/−
1CH§	.	+	−	−	−	−	−	n.d.¶
1G12.E7.F4	.	+/−	−	−	−	−	+/−	+/−
2F10.D11.H12	+/−	.	.	.	+/−	+/−	+/−	+
2CH‖	.	−	−	+++	−	−	−	+/−
3D5.E11	.	−	−	−	−	+	−	−
3D7.E1	+/−	.	.	.	++	−	−	−
3E2.F8	+/−	.	.	.	+	−	+/−	−
3F9.D9	.	+/−	−	−	−	+	−	+
3F9.D1 and C4	.	−	−	−	−	+/−	−	+
3G2.F9	+	.	.	.	+	+	+	+
4C4.H10	+/−	.	.	.	+/−	+/−	+/−	−
4CH††	.	+	−	?	+	−	−	n.d.
4B8	.	+	−	−	+	+	+	n.d.
5B2.1E11.A1	+	.	.	.	+++	+	+	

† 15 supernatants that were positive by ELISA or RIA were negative by immunofluorescence. These are not included in the table.

‡ Staining patterns were recorded either as positive (+) or weakly positive (+/−) for all mesohyl cells, or as +, +/− or negative (−) for archaeocytes and spherulous cells.

§ Several clones of this hybridoma gave identical, but weak staining on tissue.

¶ Not determined.

‖ Two clones gave identical staining patterns.

†† Two clones secreting this mAb gave identical staining on pinacocytes and some of the mesohyl cells only on the individual *C. diffusa* (identified as the D sponge) that was used in the immunization for that fusion.

2CH identified neutrophilic spherulous cells

2CH supernatants tested positive for all sponges by RIA and ELISA. On dissociated cells and normal tissue, 2CH identified neutrophilic spherulous cells that were scattered throughout the mesohyl and were occasionally located on pinacoderms. The staining pattern outlined the cytoplasmic spherules within the cells while the plasma membrane was negative (figures 4 and 5, plate 2). Viable cells did not stain with this mAb. Whether the 2CH+ cells included all of the neutrophilic spherulous cells or only a subset of this cell type was not determined.

2CH+ cells in allografts

During early time periods of allograft responses (3–24 h), 2CH+ cells were present on the exopinacoderm, in the mesohyl (figure 6), and with the cells that migrated into the graft zone (figure 7). By 24 h, mesohyl cells, arriving via tissue tracts containing 2CH+ cells (figure 8), began to accumulate and push the choanocyte chambers from the graft zone while the local aquiferous canals were rerouted or filled in. Tissue bridges appeared between 24 and 48 h, and contained fewer than normal 2CH+ cells (figure 9). By 72 h, non-mixing allogeneic cell interfaces were discernable in some of the specimens. The cells in these areas and their immediate vicinity were negative for 2CH (figure 10). After the tissue bridges broke down and graft separation occurred (72–120 h), 2CH+ cells were found within the remnants of the tissue bridges and on reforming exopinacoderm (figure 11, plate 3).

2CH+ cells in autografts

When autografts were analysed at 3, 12, 24 and 48 h, cells positive for 2CH were distributed in the graft zone as in normal tissues (figure 12). No cellular migrations or rearrangements were apparent.

2CH+ cells in inflammatory responses

In the early time periods of inflammatory responses to injury (1–6 h), 2CH+ cells were located in both normal and injured tissues. They were present in the mesohyl tracts oriented towards the injured area, within the accumulating inflammatory infiltrate at the injury edge, and on new exopinacoderm converted from injury exposed endopinacoderm (figure 13). Much of the inflammatory infiltrate had dissipated by 24 h, yet the 2CH+ cells were present on the 'neo-exopinacoderm' that formed over the damaged choanosome. Injury repair was complete by 48 h with normal distributions of all cell types. At no point during inflammatory responses were there areas observed where 2CH+ cells were concentrated or depleted.

1CH defined a subpopulation of archaeocytes including sclerocytes

On live and fixed cells, 1CH stained the plasma membrane of cells with large nucleolate nuclei characteristic of archaeocytes. In sections, these cells were widely distributed throughout normal mesohyl tissues and were less common than the 2CH+ cells. Staining on live and fixed cell suspensions revealed many of the 1CH+

cells to contain spicule precursors (figures 14 and 15, plate 4). Therefore, by definition, some of these cells were sclerocytes (spicule-secreting cells). Whether 1CH identified only sclerocytes (some without nascent spicules) or if archaeocytes without skeleton-secreting function were included was not determined.

1CH+ cells in allografts

In allografts, 1CH+ cells had similar distribution patterns as that seen for 2CH+ cells. In the early stages of graft rejection (1–24 h), before discernable tissue bridges formed, these cells were found within the mesohyl infiltrate that accumulated in the graft zone. Once the tissue bridges formed (beginning at about 48 h), 1CH+ cells were present within these structures (figure 16), however, none were located within one or two cell lengths of the graft interface that was in contact with the allogeneic cells (figure 17). Once tissue separation occurred (72–120 h), these cells were present within the remnant tissue bridges and cell accumulations, and were noted on the reforming exopinacoderm (figure 18).

1CH+ cells in autografts

1CH+ cells had similar tissue distributions in autografts as that found for 2CH+ cells. They were present on the fusing exopinacoderms and within the nearby mesohyl (figure 19, plate 5). Their distribution did not alter during autograft fusion.

Description of plates 1 and 2

Figure 1. Allograft. A 24 h allograft between the D sponge and another individual *C. diffusa* shows a massive cellular infiltration. The graft interface is not discernable. H & E; scale bar, 60 μm.

Figure 2. Allograft. The section shown in figure 1 has been stained with 4CH mAb. The D sponge stains positive while the other sponge (4CH−) shows the red propidium iodide counter staining. The staining pattern reveals the cellular interface between the allogeneic sponges. Scale bar, 60 μm.

Figure 3. Allograft. The cells at the graft interface show little allogeneic mixing. Some 4CH+ debris can be seen in the 4CH− animal. Scale bar, 30 μm.

Figure 4. 2CH stains the spherules in the neutrophilic spherulous cells. Scale bar, 10 μm.

Figure 5. Nomarski optics show the spherules in the neutrophilic spherulous cells shown in figure 4. Some cells within this field are 2CH−. Scale bar, 10 μm.

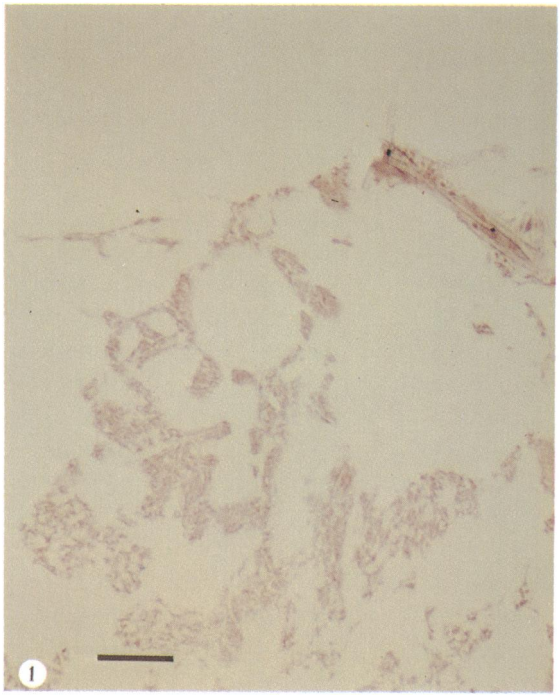
Figure 6. Allograft. An early allograft shows exopinacoderm contacts (between the open arrows) with 2CH+ cells on the exopinacoderms and within the nearby mesohyl. s.s., subdermal space; m., mesohyl; s., spicule. Scale bar, 60 μm.

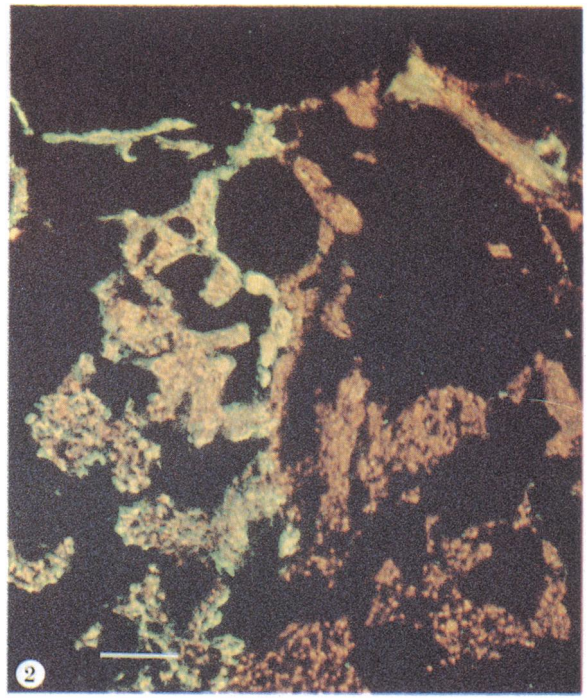
Figure 7. Allograft. 2CH+ cells can be seen within the graft zone in cellular infiltrate. The approximate graft interface is indicated by the open arrows. Scale bar, 60 μm.

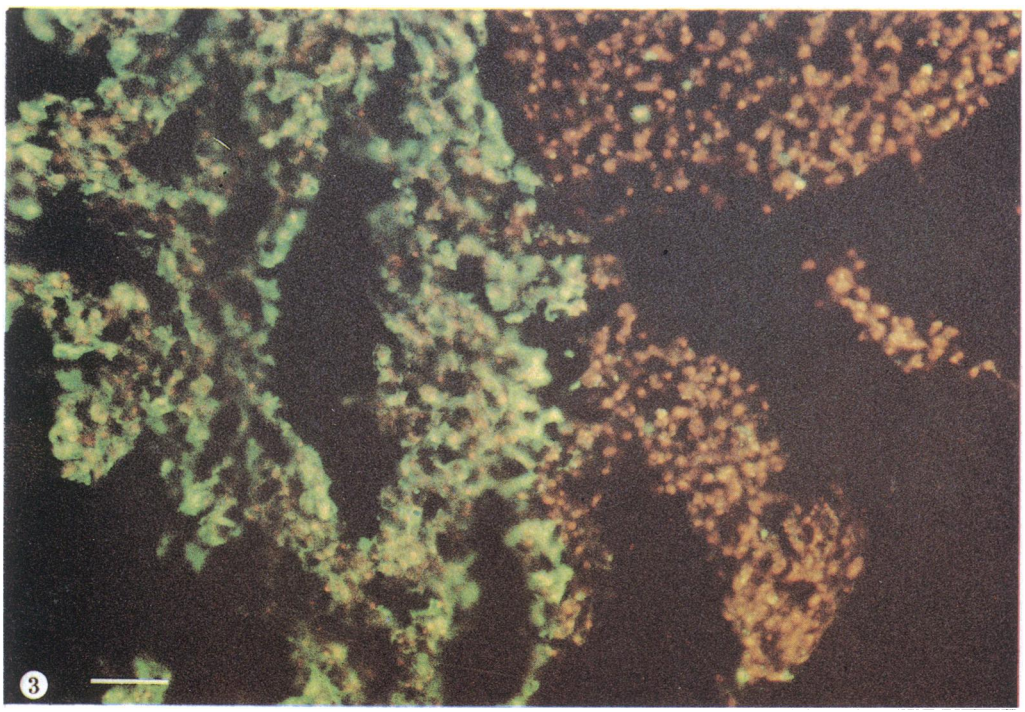
Figure 8. A mesohyl tract near the graft interface contains 2CH+ cells. Scale bar, 60 μm.

Figure 9. Allograft. A tissue bridge located at the graft interface shows 2CH+ cells within the bridge and in the surrounding area. Scale bar, 60 μm.

Figure 10. Allograft. The allogeneic interface is denoted by the contact points of the paired triangles. The cells in the immediate vicinity of the interface are 2CH−. Scale bar, 40 μm.







FIGURES 1–3. For description see opposite.

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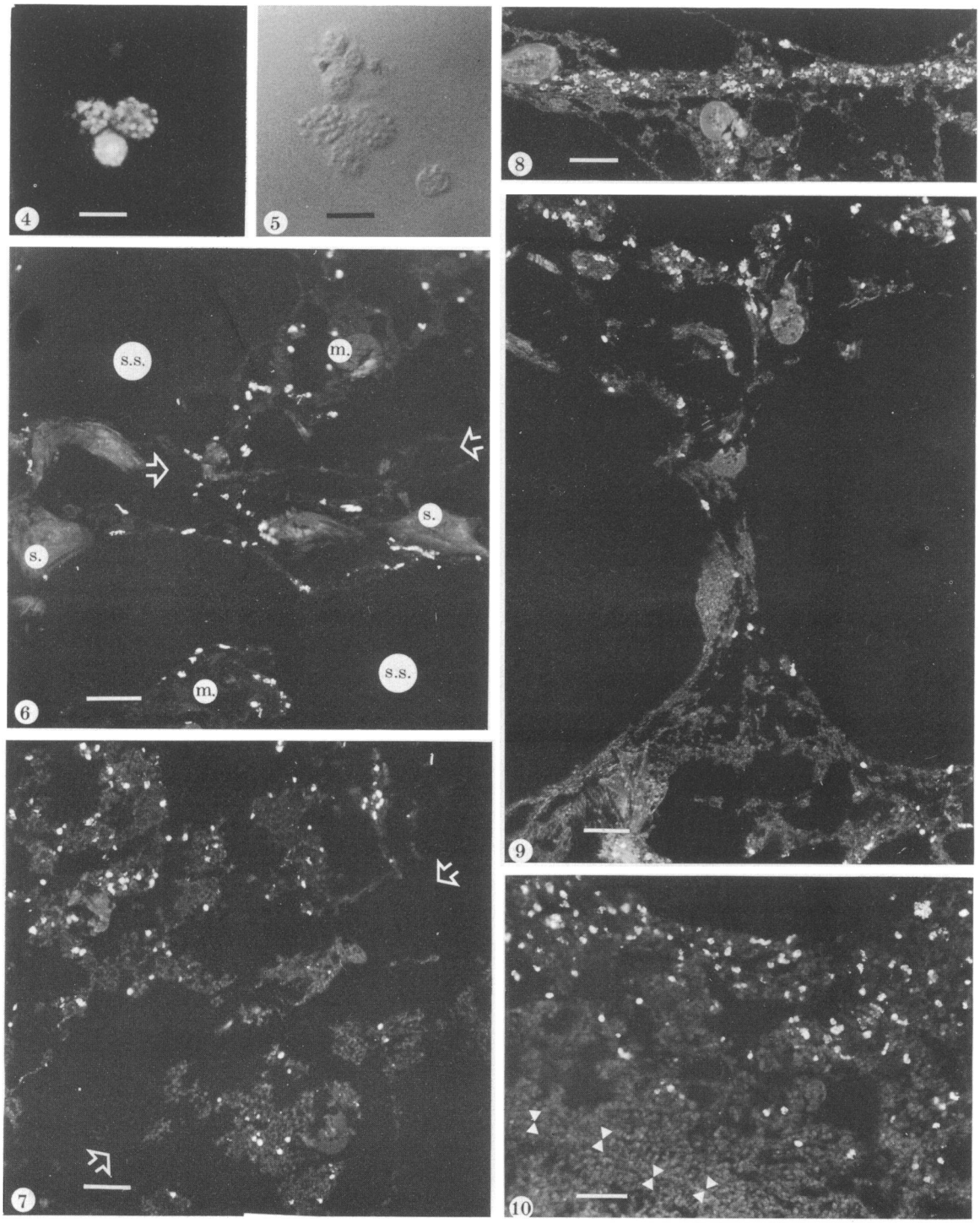
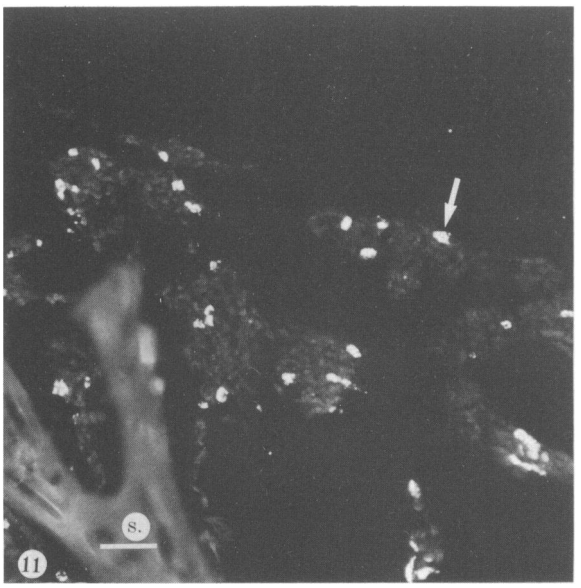


FIGURES 4–10. For description see p. 472.



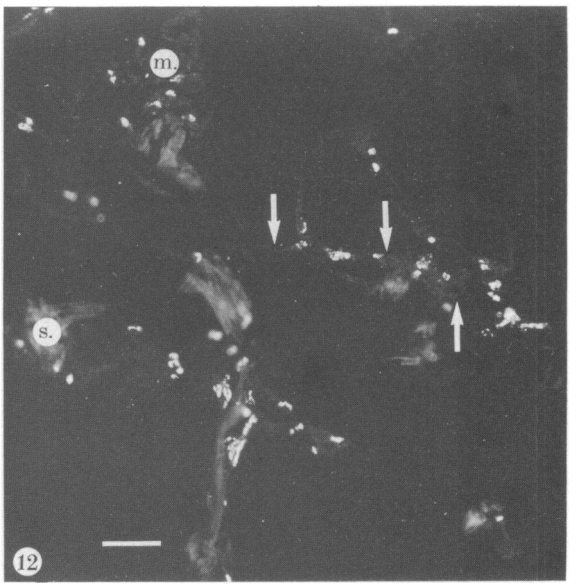
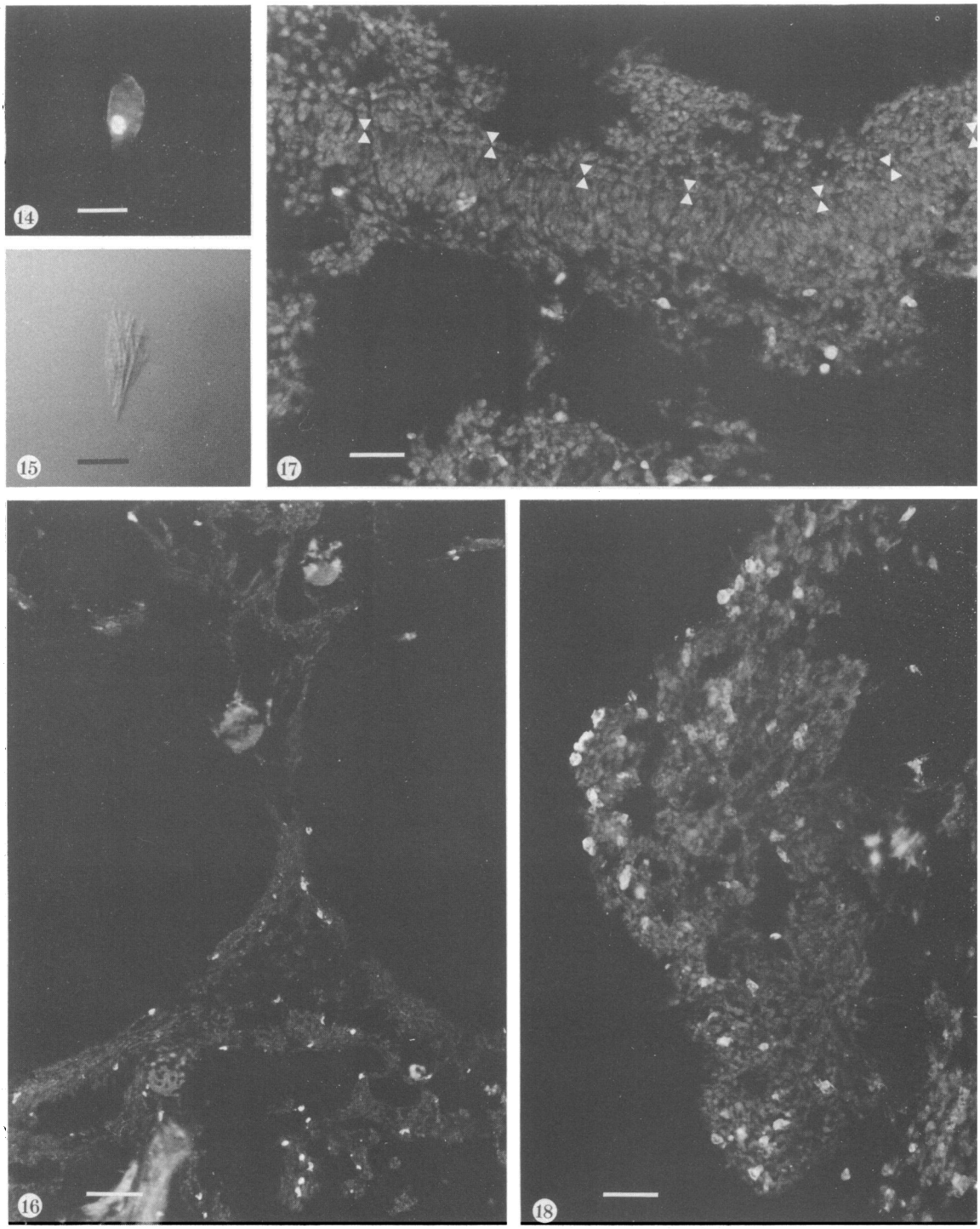


FIGURE 11. Allograft. Rejection cytotoxicity destroys the cells in the graft zone. 2CH + cells can be seen within the infiltrate with one 2CH + cell on the reforming exopinacoderm (arrow). s., Spicule. Scale bar, 30 μm.

FIGURE 12. Allograft. An early stage autograft shows 2CH + cells on the joined exopinacoderms (arrows) and in the nearby mesohyl. m., Mesohyl. Scale bar, 60 μm.

FIGURE 13. Inflammation. 2CH + cells are located within the inflammatory infiltrate and upon the reforming exopinacoderm. Arrows indicate the edge of the injury. s., Spicule. Scale bar, 40 μm.



Figures 14–18. For description see opposite.

Description of plate 4

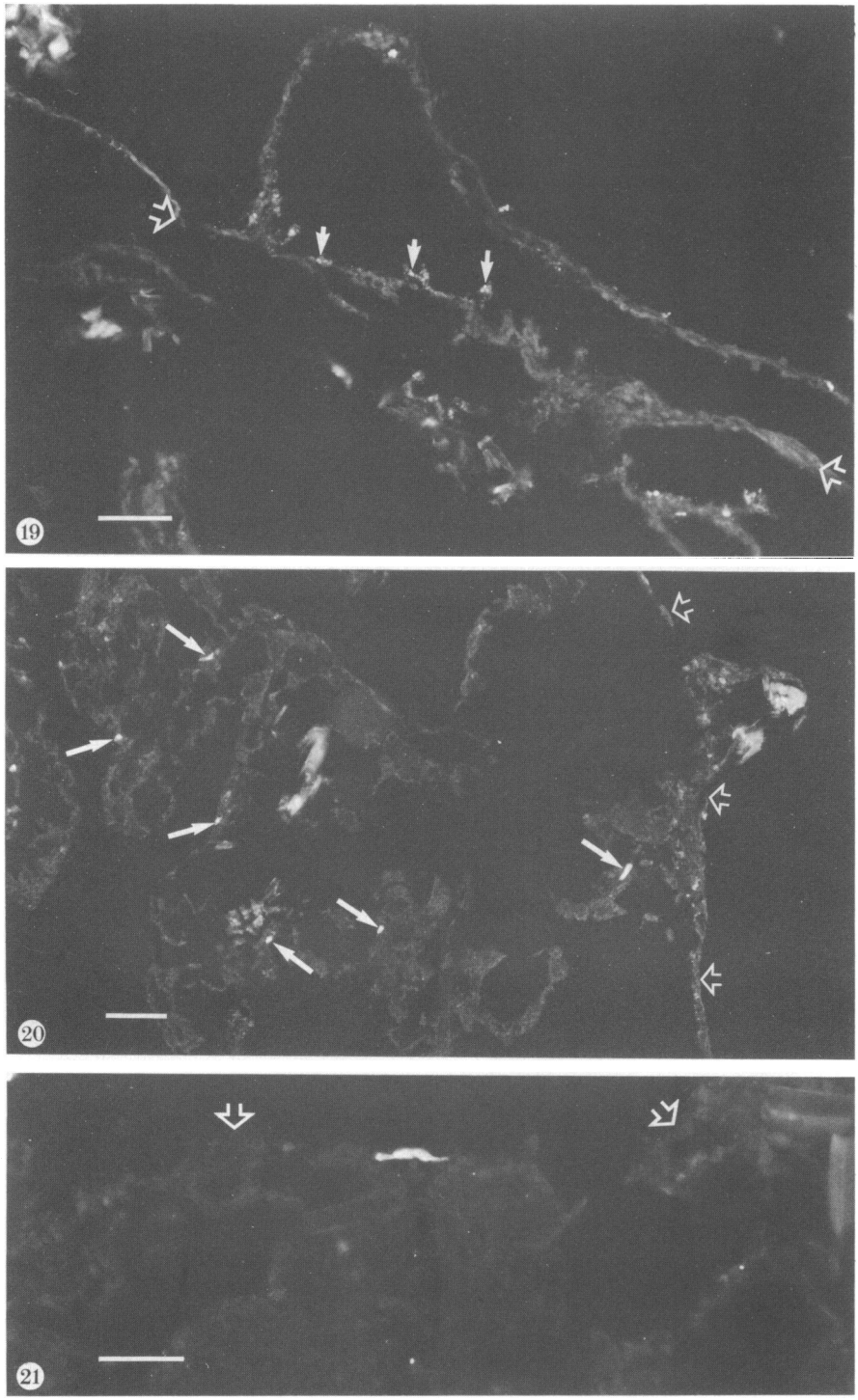
Figure 14. 4CH stains the plasma membrane of a subpopulation of archaeocytes. The nucleus is counter stained with propidium iodide. Scale bar, 10 μm.

Figure 15. By Nomarski optics, spicule precursors can be seen within the 4CH+ cell shown in figure 14. Scale bar, 10 μm.

Figure 16. Allograft. 4CH+ cells are located within a tissue bridge and within the nearby tissues. (This is a serial section of the tissue bridge shown in figure 9.) Scale bar, 60 μm.

Figure 17. Allograft. The allogeneic interface is denoted by the contact points of the paired triangles. The 4CH+ cells are not located closer than one or two cell lengths from this boundary. Scale bar, 30 μm.

Figure 18. Allograft. After cytotoxic reactions separate allogeneic tissues, 4CH+ cells can be seen within the remnant cell accumulations and on the reforming exopinacoderm. Scale bar, 30 μm.



FIGURES 19–21. For description see opposite.

1CH+ cells in inflammatory responses

In the inflammatory response to injury, 1CH+ cells were occasionally noted in mesohyl tracts and a few were present within the inflammatory infiltrate (figure 20). Normally, they were located away from the edge of the damaged tissue, but on occasion, they were situated on the reforming exopinacoderm (figure 21). These cells did not show areas of concentration or depletion during inflammatory responses.

Discussion

Some of the details of allograft rejection between parabiotic pairs of *C. diffusa* have been pieced together over the last several years. The specificity of the non-self recognition phase of the response has been inferred from accelerated secondary rejection times (specific alloimmune memory) (Hildemann *et al.* 1980*b*; Johnston *et al.* 1981). To investigate the specificity of the cytotoxic effector phase of graft rejection, Bigger *et al.* (1981) substituted different allogeneic or autogeneic sponge pieces into rejection sites after sensitization but before the onset of cytotoxicity. They were able to demonstrate that the effector phase of a primary rejection response was non-specific; naive second set and naive third party substitutions rejected at the same rates. Furthermore, 80 % of the sensitized pieces that received autogeneic substitutions showed no rejection necrosis. The remaining 20 % showed necrosis at the graft site before fusing compatibly with the autogeneic piece. This necrosis was assumed to be due to allogeneic cell or tissue carry over.

Bigger *et al.* (1981) speculated that the cytotoxic mechanism in *C. diffusa* involved cell migration and mixing in the graft zone which was followed by effector cell activation and direct cytotoxicity. In light of the surprising finding that little or no mixing of allogeneic cells occurs at the graft interface, cell mixing with direct cytotoxicity cannot be the basis of the response in this species. Buscema & Van de Vyver (1984*a*) have also suggested that allogeneic cell mixing with direct cell lysis and phagocytosis occurs in allograft interfaces of *Axinella polypoides*. Although this may be true for this species, they did not have the benefits of a mAb to aid in identifying and following cells from the paired sponges. Time-lapse photographic analysis of barrier formation in *Ephydatia fluviatilis*, a freshwater sponge, shows that no allogeneic cells cross the graft interface before barrier

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Description of plate 5

Figure 19. Autograft. In an early autograft, 4CH+ cells can be seen on the fused exopinacoderms (arrows). The line of fusion is indicated by the open arrows. Scale bar, 40 μm.

Figure 20. Inflammation. A few 4CH+ cells can be seen within the inflammatory infiltrate (arrows). Open arrows indicate the edge of the injury. Scale bar, 60 μm.

Figure 21. Inflammation. A 4CH+ cell is located on the edge of the reforming exopincoderm. Injury edge is indicated by the open arrows. Scale bar, 20 μm.

deposition occurs (Van de Vyver & de Vos 1979). Allogeneic non-mixing has been assumed in other barrier-forming sponges before secretion of the barrier material (Van de Vyver & Barbieux 1983; Buscema & Van de Vyver 1983, 1984*b*). The mechanism for preventing allogeneic cell mixing in either barrier-forming sponges or cytotoxic responders is intriguing but unknown.

In *C. diffusa*, necrosis in the graft zone commonly reaches 2–5 mm and can even result in the death of an entire parabiont (Hildemann *et al.* 1980*b*). Because two unmixed populations of effector immunocytes can induce such necrosis, the underlying mechanism must be based on the activity of short-range cytotoxic factors. Bigger *et al.* (1981) previously suggested this as an alternative hypothesis of necrosis in *C. diffusa*, and it must now be seriously considered.

Cytotoxic factors, induced by direct allogeneic contact, could act in a variety of ways. They could be non-specifically lytic to any non-self cell or tissue or they could be directed towards self in the form of self-destruct signals. Either of these would result in the death and necrosis of cells in the graft zone. Speed or magnitude of a rejection response (resulting from differences in histoincompatibilities or from presensitization) and rejection directionality (uni- or bilateral necrosis) could be directly related to the amount of cytotoxic factors released from the effector cells that accumulate at the graft interface.

The magnitude of rejection responses in sponges has been postulated to be due to the degree of histoincompatibility based on genetic and molecular differences (Hildemann *et al.* 1980*b*; Buscema & Van de Vyver 1984*b*; Smith & Hildemann 1984). Therefore, the extent of the response exhibited during rejections may depend on the magnitude of non-self recognition. A theoretical sequence of cellular interactions in *C. diffusa* is presented to connect the observable effector phase with the inferred specific recognition. The numbers of putative recognizing cells responding to the antigeneic differences between allogeneic animals would determine the concentration of chemotactic mediators released from the points of allogeneic contact, and would determine the numbers of effector cells that would migrate towards these points. The numbers of accumulated effector cells would determine the concentration of cytotoxic factors released into the graft zone, which then would establish the numbers of cells killed and the magnitude of necrosis. To follow this line of reasoning, unilateral rejections would only be possible if the cytotoxic factors had a concentration threshold for observable activity. If this concentration was not reached by one of the parabionts, a unilateral response would ensue. In addition, each individual sponge could conceivably have different levels of 'susceptibility' to the cytotoxic factor. Finally, from the autograft substitution data of Bigger *et al.* (1981), one must entertain the possibility of (i) a regulatory mechanism to turn off the secretion of the cytotoxic factors upon removal of allogeneic contact; or (ii) an 'immunity' to cytotoxic factors released by self. With this second possibility the cytotoxic factors could not be a self-destruct signal.

The second point brought forth in the present study shows that (some?) neutrophilic spherulous cells (1CH+) and an archaeocyte–sclerocyte subpopulation (2CH+) are not found at the allogeneic interface and are perhaps only indirectly involved in the effector phase of rejection. However, both cell types are found in mesohyl cell tracts, within the cell infiltrate responding to allogeneic contact and

injury, and both are present on reforming exopinacoderm after injury or rejection cytotoxicity have removed or destroyed this structure. The locations of 1CH+ and 2CH+ cells may be related to the spicule-secretion function of sclerocytes and the postulated spongin-secretion function of neutrophilic spherulous cells (Smith 1985). The presence of 'skeleton-building' cells at the allogeneic interface would seem unnecessary, whereas their involvement in reconstruction after tissue destruction (from either injury or cytotoxic rejection) would be expected. The change in the composition of cell types at rejecting allogeneic interfaces implies that alloimmune responses in *C. diffusa* may be based on specific cellular interactions within and between individuals of this sponge species during allogeneic contact.

The cells that line up along the allogeneic interface in *C. diffusa* are an archaeocyte subpopulation (1CH+ cells, with archaeocyte morphology, are not present). These cells are relatively large, amoeboid phagocytes that are similar to phagocytes found in all metazoans (for review, see Anderson 1981). Smith (1985) have suggested that archaeocytes may be a complex of subpopulations that function in digestion, skeleton secretion, phagocytosis and lysis of microbes. Increasing evidence indicates that an archaeocyte subset is the effector immunocyte in *C. diffusa* allograft rejections. Similarly, mammalian macrophages are a complex of cell types with different subpopulations varying substantially in their abilities to phagocytose and lyse invasive microorganisms, act as accessory cells for activating lymphocytes (T cell presentation) or as effector cells responding to factors released from T cells in delayed-type hypersensitivity (DTH) reactions (Norman & Sorkin 1982). Preliminary speculation leads to comparisons of macrophages and archaeocytes as non-specific effector cells and suggests macrophages to be a very old defence system in mammals (Varesio *et al.* 1980).

The cytology of graft rejection in sponges is in its infancy, but the *in vivo* cytology of allograft rejection in mammals is also poorly understood (for recent reviews, see Moller 1984). The cell types infiltrating into kidney and heart allografts include T helper cells (T_H), cytotoxic T lymphocytes (CTLs), T cells involved in delayed-type hypersensitivity (T_{DTH}), and macrophages (Christmas & MacPherson 1982*a*, *b*, *c*; Lowrey & Gurley 1983; Lowrey *et al.* 1983; Gurley *et al.* 1983). Although Christmas & MacPherson (1983*a*, *b*, *c*) have suggested that the non-specific effects of macrophages may be the chief effector mechanism in allograft rejections in the rat, rejection in mammals may simultaneously involve T_H, T_{DTH}, CTLs, macrophages (with and without Fc-bound Ab), neutrophils, NK cells, Ab-dependent cellular cytotoxicity, Ab with complement and a variety of lymphokines and monokines (Forbes & Guttmann 1984; Hall & Dorsch 1984; Hancock 1984). Because the complexity of the mammalian immune system makes it exceedingly difficult to understand *in vivo*, some investigators have begun to search for a less complex model system of allograft rejection. The *C. diffusa* immune system shows specific non-self recognition coupled to non-specific effector cytotoxicity and seems to be at least superficially similar to DTH which has specific non-self recognition by T cells that activate non-specific macrophage effectors. Future developments in sponge cell separation and *in vitro* cytotoxicity assays should further clarify the cell types and mechanisms involved in sponge immune

responses. Investigations of simpler immune systems that perhaps only involve a single rejection mechanism have potential for shedding light on immune complexities of more advanced animals.

Dr Albert Benedict and Ms Carla Mizumoto at the University of Hawaii gratiously offered laboratory space and technical excellence which allowed this study to succeed. Expert technical assistance was also contributed by Douglas Doyle and Aimee Nguyen at U.C.L.A. The manuscript was critically read by Dr E. L. Cooper. Special thanks is offered to Dr L. Muscatine for his help and guidance. This work was supported by National Institutes of Health grants AI 15075 and AI 19470.

References

Anderson, R. S. 1981 Comparative aspects of the structure and function of invertebrate and vertebrate leucocytes. In *Invertebrate blood cells 2* (ed. N. A. Ratcliffe & A. F. Rowley), pp. 629–641. New York: Academic Press.

Bigger, C. H., Hildemann, W. H., Jokiel, P. L. & Johnston, I. S. 1981 Afferent sensitization and efferent cytotoxicity in allogeneic tissue responses of the marine sponge *Callyspongia diffusa*. *Transplantation* **31**, 461–464.

Bigger, C. H., Hildemann, W. H. & Johnston, I. S. 1982 Characterization of alloimmune memory in a sponge. *J. Immun.* **129**, 1570–1572.

Buscema, M. & Van de Vyver, G. 1983 Variability of allograft rejection processes in *Axinella verrucosa*. *Devl. comp. Immunol.* **7**, 613–616.

Buscema, M. & Van de Vyver, G. 1984*a* Cellular aspects of alloimmune reactions in sponges of the genus *Axinella*. I. *Axinella polypoides*. *J. exp. Zool.* **229**, 7–17.

Buscema, M. & Van de Vyver, G. 1984*b* Cellular aspects of alloimmune reactions in sponges of the genus *Axinella*. II. *Axinella verrucosa* and *Axinella damicornis*. *J. exp. Zool.* **229**, 19–32.

Christmas, S. E. & MacPherson, G. G. 1982*a* The role of mononuclear phagocytes in cardiac allograft rejection in the rat. I. Ultrastructural and cytochemical features. *Cell. Immunol.* **69**, 248–270.

Christmas, S. E. & MacPherson, G. G. 1982*b* The role of mononuclear phagocytes in cardiac allograft rejection in the rat. II. Characterization of mononuclear phagocytes extracted from rat cardiac allografts. *Cell. Immunol.* **69**, 271–280.

Christmas, S. E. & MacPherson, G. G. 1982*c* The role of mononuclear phagocytes in cardiac allograft rejection in the rat. III. The effect of cells extracted from rat cardiac allografts upon beating heart cell cultures. *Cell. Immunol.* **69**, 281–290.

Curtis, A. S. G. 1979 Individuality and graft rejection in sponges or a cellular basis for individuality in sponges. In *Biology and systematics of colonial organisms* (ed. G. Larwood & B. R. Rosen), pp. 39–48. Systematics Association Special vol. no. 11. New York: Academic Press.

Forbes, R. D. C. & Guttmann, R. D. 1984 Pathogenetic studies of cardiac allograft rejection using inbred rat models. *Immunol. Rev.* **77**, 5–29.

Galfrè, G. & Milstein, C. 1981 Preparation of monoclonal antibodies: strategies and procedures. *Meth. Enzymol.* **73**, 3–46.

Gurley, K. E., Lowrey, R. P. & Forbes, R. D. C. 1983 Immune mechanisms in organ allograft rejection. II. T helper cells, DTH, and rejection of renal allografts. *Transplantation* **36**, 401–405.

Hall, B. M. & Dorsch, S. E. 1984 Cells mediating allograft rejection. *Immunol. Rev.* **77**, 31–59.

Hancock, W. W. 1984 Analysis of intragraft effector mechanisms associated with human renal allograft rejection; immunohistological studies with monoclonal antibodies. *Immunol. Rev.* **77**, 61–84.

Hildemann, W. H., Bigger, C. H. & Johnston, I. S. 1979*a* Histocompatibility reactions and allogeneic polymorphism among invertebrates. *Transplantn. Proc.* **11**, 1136–1142.

Hildemann, W. H., Johnston, I. S. & Jokiel, P. L. 1979*b* Immunocompetence in the lowest metazoan phylum: transplantation immunity in the sponge *Callyspongia diffusa*. *Science, Wash.* **204**, 420–422.

Hildemann, W. H., Bigger, C. H., Jokiel, P. L. & Johnston, I. S. 1980*a* Characteristics of immune memory in invertebrates. In *Phylogeny of immunological memory* (ed. M. J. Manning), pp. 9–14. Amsterdam: Elsevier–North-Holland Biomedical Press.

Hildemann, W. H., Bigger, C. H., Johnston, I. S. & Jokiel, P. L. 1980*b* Characteristics of transplantation immunity in the sponge *Callyspongia diffusa*. *Transplantation* **30**, 362–367.

Johnston, I. S., Jokiel, P. L., Bigger, C. H. & Hildemann, W. H. 1981 The influence of temperature on the kinetics of allograft reactions in a tropical sponge and a reef coral. *Biol. Bull. mar. biol. Lab., Woods Hole* **160**, 280–291.

Johnston, I. S. & Hildemann, W. H. 1983 Morphological correlates of intraspecific grafting in the marine demosponge *Callyspongia diffusa*. *Mar. Biol.* **74**, 25–33.

Lowry, R. P., Gurley, K. E. & Forbes, R. D. C. 1983 Immune mechanisms in organ allograft rejection. I. Delayed-type hypersensitivity and lymphocytotoxicity in heart graft rejection. *Transplantation* **36**, 391–401.

Lowry, R. P. & Gurley, K. E. 1983 Immune mechanisms in organ allograft rejection. III. Cellular and humoral immunity in rejection of organ allografts transplanted across MHC subregion disparity $RT_1.B$ ($RT_1.D$). *Transplantation* **36**, 405–411.

Manning, M. (ed.) 1980 *Phylogeny of immunological memory*. Amsterdam: Elsevier–North-Holland Biomedical Press.

Möller, G. (ed.) 1984 *Intragraft rejection mechanisms. Immunol. Rev.* vol. 155. Copenhagen: Munksgaard International.

Norman, S. J. & Sorkin, E. (eds) 1982 *Macrophages and natural killer cells; regulation and function. Adv. exp. med. Biol.* vol. 155. New York: Plenum Press.

Sainte-Marie, G. 1962 A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* **10**, 250–256.

Smith, L. C. 1985 Cellular defense systems in sponges. Ph.D. dissertation, University of California, Los Angeles.

Smith, L. C. & Hildemann, W. H. 1984 Alloimmune memory is absent in *Hymeniacidon sinapium*, a marine sponge. *J. Immun.* **133**, 2351–2355.

Smith, L. C. & Hildemann, W. H. 1986 Allograft rejection, autograft fusion and inflammatory responses to injury in *Callyspongia diffusa* (Porifera; Demospongia). *Proc. R. Soc. Lond.* B **226**, 445–464.

Van de Vyver, G. & de Vos, L. 1979 Structure of a non merging front between two fresh water sponges *Ephydatia fluviatilis* belonging to different strains. In *Biology des Spongiares* (ed. C. Levi & N. Boury-Esnault), pp. 233–237. Colloques du C.N.R.S. no. 291. Paris: Editions du C.N.R.S.

Van de Vyver, G. & Barbieux, B. 1983 Cellular aspects of allograft rejection in marine sponges of the genus *Polymastia*. *J. exp. Zool.* **227**, 1–7.

Varesio, L., Landolfo, S., Giovarelli, M. & Forni, G. 1980 The macrophage as the social interconnection within the immune system. *Devl. comp. Immunol.* **4**, 11–19.