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Sponge cell culture: a comparative evaluation of adhesion to a native tissue extract and other culture substrates

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Abstract. Hexactinellids are deep water sponges that possess syncytial rather than cellular tissues. In order to investigate the syncytial character of the tissue of these unusual sponges, primary cultures were developed using a substrate of acellular tissue extract (ATE) that promotes the adhesion and spreading of sponge tissues. Primary cultures of the hexactinellid sponge *Rhabdocalyptus dawsoni*, grown on this substrate, form thinly spread, multinucleate, confluent tissue masses which exhibit active cytoplasmic streaming. Sponge tissue adhered equally well to commercial substrates of concanavalin A and poly-l-lysine, but did not adhere to chicken collagen. Acellular tissue extracts prepared from demosponges, which are known to be cellular, also promoted adhesion and spreading of cells from those sponges. Scanning electron microscopy showed adherent *Rhabdocalyptus* tissue to have an uninterrupted, smooth membrane covering the entire culture, unlike primary cultures of the cellular demosponge, *Haliclona* sp., which consisted of numerous individual cells. Tissue from freshly collected sponges adhered preferentially to ATE from a conspecific. However, after continued wounding, tissue adhered indescriminately to any substrate. The tissue extract congealed if added to sea water or 10 mM $CaCl_2$, forming a white, cloudy solid, which could be fixed and sectioned for transmission electron microscopy. Thin sections of the congealed extract showed it to contain membranes but no visible collagen fibrils.

Keywords: Sponge, cell adhesion, hexactinellid, syncytium, cell culture, concanavalin A

Introduction

No continuous cell lines from marine invertebrates have been established to date, in part because little is understood of the nutritional requirements of these cells and because of complications caused by microbial contaminants in the sea water. None the less, primary cell cultures have been developed with great success from a variety of marine invertebrates to study specific processes e.g. starfish follicle cells (Mita et al., 1988), jellyfish neurons (Przysiezniak & Spencer, 1989), tunicate haemocytes (Rinkevich & Rabinowitz, 1993), cells from bivalve gills (Auzouz et al., 1993), and abalone myocytes (Naganuma et al., 1994).

In the past, primary cell cultures of sponges have been invaluable for the study of histocompatability responses and cell adhesion molecules (Wilson, 1907; Curtis, 1962; Humphreys, 1963; Moscona, 1968). However, recent interest in the vast number of novel biometabolites that sponges produce has rekindled the desire to develop continuous sponge cell cultures. Efforts in that direction have shown that long-term culture of

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sponge cells in suspension may be possible (Pomponi & Willoughby, 1994). However, adhered and spread cell cultures are better suited for studies of cell types and cytological organization (Gaino et al., 1985a; Gaino et al., 1993), information that is still needed for this group of animals.

In particular, the use of primary cell cultures has been invaluable in studying the cytological organization of hexactinellid sponges. The inaccessibility of hexactinellid sponges, which inhabit deep water, as well as difficulties with fixation, has meant that ultrastructural investigations of their tissues were only recently conducted (Mackie & Singla, 1983; Boury-Esnault & de Vos, 1988; Reiswig, 1979; Reiswig, 1991; Reiswig & Mehl, 1991; Reiswig & Mehl, 1994; Boury-Esnault & Vacelet, 1994). These studies have supported earlier reports that claimed the tissue was syncytial rather than cellular (Ijima, 1901, 1904; Reiswig, 1979). With the addition of electrophysiological evidence showing that the syncytial network is capable of propagating electrical impulses (Mackie, 1979; Lawn et al., 1981; Mackie et al., 1983), it was proposed that because of their vastly different tissue organization, hexactinellids be separated from other sponges at the subphylum level (Reiswig & Mackie, 1983). However, because many tissues once thought to be syncytial have subsequently been found to be cellular, an adhered tissue preparation was sought in which aspects of tissue morphology, and in particular the question of syncytiality, could be studied in culture.

A central problem in culturing sponge cells for the purpose of studying cell morphology is the tendency for dissociated sponge cells to form opaque, multicellular aggregates. Some researchers have avoided this difficulty by using asexually produced propagules (gemmules) from fresh water sponges, which can be made to hatch and grow within a coverslip chamber (Weissenfels, 1980; 1992), or by explanting tissue from freshwater or marine sponges (Bond & Harris, 1988; Wachtmann & Stockem, 1992). Although hexactinellids lack propagules, explants can be made (Wyeth et al., 1996). However, because these sponges have spicules up to several centimeters long, the preparations tend to be too thick for optimal viewing of the tissue structure. An alternative is to provide dissociated cells with a highly adhesive substrate. Gaino et al. (1985a, 1985b, 1993) have shown that dissociated cells from calcareous sponges will adhere to mammalian extracellular matrix extracts and to synthetic substrates, although extended culture was not attempted. Cultures which do remain adhered, generally form multicellular aggregates (Shimizu & Yoshizato, 1993) which eventually detach, again forming opaque spheres.

In order to obtain adhered tissue cultures from hexactinellids, an acellular tissue extract (ATE) was prepared from the hexactinellid sponge *Rhabdocalyptus dawsoni*. When dried onto glass coverslips or plastic petri dishes, the extract was found to promote both adhesion and spreading of dissociated *Rhabdocalyptus* tissue for periods up to two weeks. This paper describes the preparation of the sponge acellular tissue extract and discusses characteristics of the adhesion it promotes in comparison with adhesion of sponge tissue to other natural and commercial substrates. Evidence of the syncytial nature of the adhered preparation is presented elsewhere (Leys, 1995).

Materials and methods

Specimen collection

Specimens of *R. dawsoni* were collected by SCUBA from 30–40 m depth in Barkley Sound and Saanich Inlet, British Columbia, Canada and kept in the flow-through sea water tanks at the Bamfield Marine Station and in the recirculating sea water system at the University of Victoria. Specimens of *Haliclona* sp., *Ophlitaspongia pennata*, and *Halichondria* sp. were collected intertidally at Clover Point, Victoria, British Columbia, Canada for use in adhesion assays.

Preparation of Substrates

Natural. Blades of the red alga *Callyophyllis* sp., commonly found attached to the base of *R. dawsoni*, were used whole or the blade was crushed and the resulting extract was diluted in distilled water, pipetted onto coverslips, and allowed to air dry. Fragments of clam or gastropod shell and pieces of rock from the habitat of *R. dawsoni* were used whole.

Dissociated sponge cell substrate, consisting of whole cells, portions of flagellated chambers, and spicule debris was made from *R. dawsoni* by forcing 3–4 cleaned pieces of sponge, each approximately 1 cm^3, through 100 μm Nitex mesh. The dissociated tissue was pipetted onto coverslips and allowed to dry overnight.

ATE, containing no whole cells or tissues, was made from *R. dawsoni*, *Haliclona* sp., *Halichondria* sp., and *O. pennata* using distilled water to lyse the cell membranes. The procedure involved briefly rinsing 3–4 pieces of sponge, again approximately 1 cm^3, in calcium- and magnesium-free sea water (CMFSW) to help dissociate cell-cell junctions, and then soaking the pieces four times, for 2 h each, in 20 times the volume of distilled water at 4°C. After the final soaking, the tissue was mechanically dissociated in one times the volume of distilled water, causing the release of a cloudy suspension, which was confirmed by light microscopy to be acellular. This suspension was diluted approximately ten fold with cold distilled water and stored at 4°C with 0.03% sodium azide. Fifty microlitres of the acellular tissue extract was pipetted onto coverslips and allowed to air dry overnight.

Commercial. Concanavalin A (from *Canavalia ensiformis*, Sigma) was dissolved in phosphate buffered saline (PBS) and diluted in double distilled water

(DDW) to make 100 $\mu g \cdot ml^{-1}$ and 20 $\mu g \cdot ml^{-1}$. Poly-l-lysine (Sigma) was diluted in DDW to 500 $\mu g \cdot ml^{-1}$ and 10 $\mu g \cdot ml^{-1}$. Type II collagen from chicken (Sigma) was used at a concentration of 2 $\mu g \cdot ml^{-1}$ in DDW to determine the behaviour of hexactinellid sponge tissue on a commercially available extracellular matrix tissue culture substrate. Fifty microlitres of each of the commercial substrates were pipetted onto glass coverslips and allowed to air dry overnight.

Preparation of primary tissue cultures

Sponges from collections made throughout the year varied greatly in their ability to produce well adhered aggregates. Consequently, all sponges were first tested for their ability to adhere to the ATE prior to starting an adhesion assay with different substrates.

Whole sponge tissue was cleaned of debris, and five pieces, approximately 1 cm^3 each, were squeezed through 100 µm Nitex mesh into a beaker to make 3.0–5.0 ml of dissociated tissue, and diluted to 200 ml with sea water. The dissociated tissue was poured into petri dishes containing one or more coverslips coated with the ATE, and dishes were either floated on the surface of 10°C sea water or kept in an incubator at 10°C overnight. After 12–24 h, if the tissue had adhered, the coverslips could be removed and placed in dishes with clean sea water without dislodging the cultures from the coverslips.

Adhesion assays

Substrates. Approximately 2 ml of the dissociated sponge tissue was poured into each 1.8 mm plastic petri dish, each containing one of the above substrates. Dishes were kept in an incubator at 10°C for 6–9 h. After the allotted time, the coverslips, pieces of shell, rock, or algae, were removed, rinsed gently in a beaker of sea water, and placed in a petri dish of clean sea water for observation. Any tendency of the sponge tissue to adhere to the substrates was noted.

Characteristics of the ATE. In order to shed light on the particular proteins of importance in the ATE, adhesion assays were carried out with ATE that had been subjected to the following treatments: 1. Centrifugation at $6000 \times g$, only the supernatant was dried on coverslips; 2. Dialysis through a 6000 MW membrane, only the fraction <6000 MW was dried on coverslips; 3. Treatment with Trypsin (0.2% and 2%) at 4°C overnight followed by 30 min at 37°C; 4. Treatment with Pronase E (0.2% and 2%) as in (3); 5. Treatment with collagenase (0.2% and 2%) as in (3); 6. Treatment with hyaluronidase (0.02% and 0.2%) as in (3). Finally, ATE was made using artificial sea water to determine if the adhesion factor was in the sea water. All adhesion assays with treated ATE were conducted with five sponges which had only been tested once previously for their ability to adhere to ATE. Tissue from each sponge was plated on all substrates. After 12 h incubation at 10°C, coverslips were rinsed gently and placed in a dish of clean sea water for observation. A description of the tissue morphology on each substrate was made, whether tissue had adhered or not. In control experiments, tissue was plated on uncoated coverslips, DW-coated coverslips, and coverslips with normal ATE.

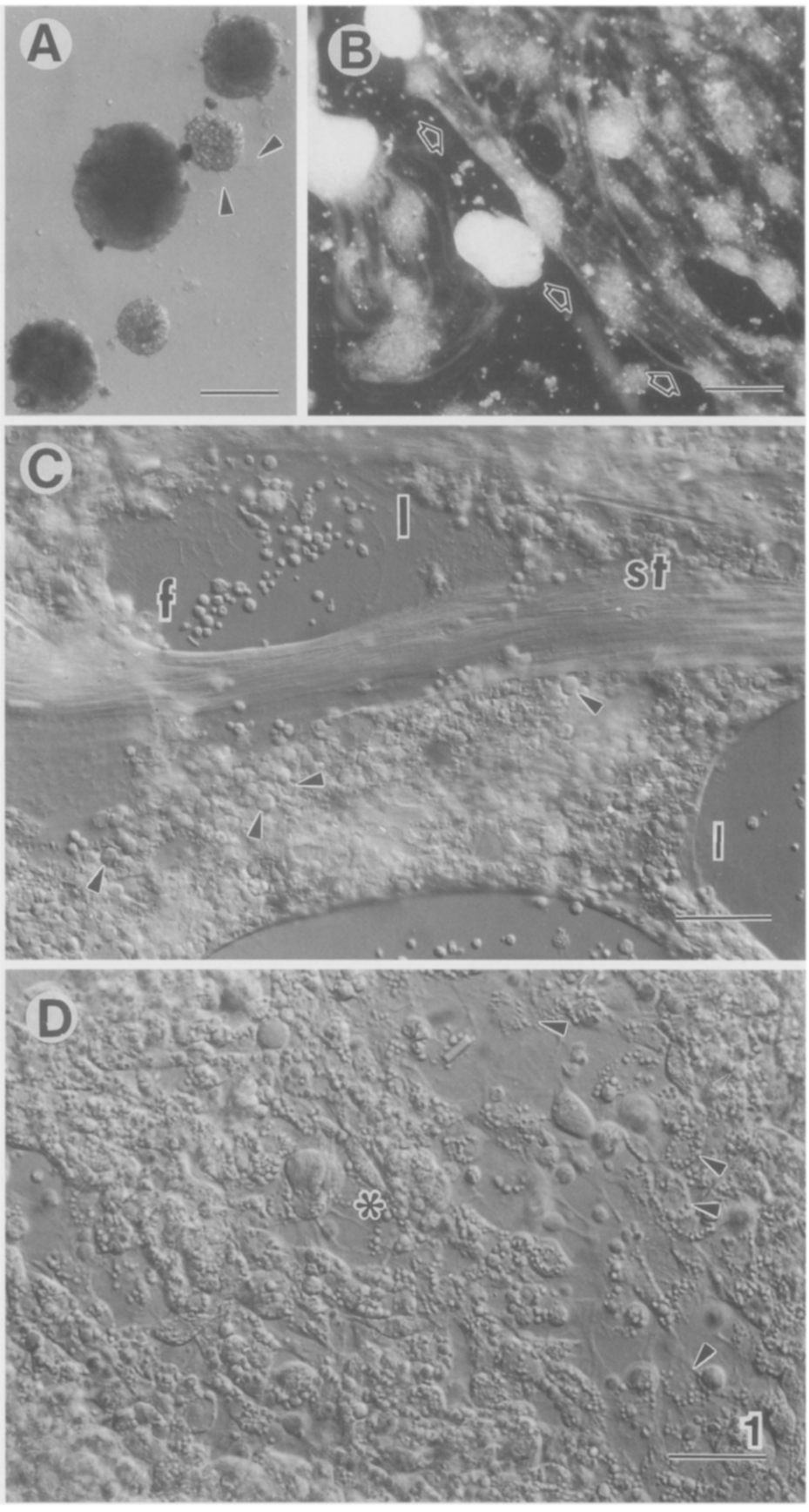
Wounding. To determine the effect of continued wounding of sponges on tissue adhesion, pieces were cut from one sponge at 12 h intervals and plated in three 1.8 mm petri dishes containing a coverslip coated with ATE, Con A (20–100 $\mu g \cdot ml^{-1}$), or type II collagen (2 $\mu g \cdot ml^{-1}$). Control coverslips were coated with 2% PBS. After 12 h at 10°C, coverslips were gently rinsed in clean sea water and placed in a fresh dish of clean sea water for observation. Percent adhesion was calculated by estimating the amount of area of the coverslip covered by adherent tissue using a numerical scale of adhesion and tissue confluence. Adhesion of confluent tissue to the entire substrate coated area of the coverslip was interpreted as 50% adhesion. A rating of 100% indicated adhesion and spreading of the tissue beyond the coated area to cover the entire coverslip. The percent adhesion in the three dishes was averaged to give the mean percent adhesion. Because tissue from different sponges did not adhere equally well, adhesion experiments could only be conducted with those sponges that showed an ability to adhere during the first screening. Consequently, Figure 2 represents the results of adhesion by tissue from two individuals. This experiment was repeated with four more individuals from subsequent collections, and gave identical results.

Electron microscopy

The difficulty of preserving hexactinellid tissue has called for unusual techniques of tissue fixation in the past (see Reiswig, 1979; Mackie & Singla, 1983; Reiswig & Mehl, 1991). Therefore, methods which were found to best preserve the cytoskeleton for both scanning and transmission electron microscopy in a previous study (Leys, 1995) were also used in this study.

For scanning electron microscopy (SEM) of adhered aggregated tissue from *Rhabdocalyptus*, coverslips with adherent tissue were transferred to calcium-free sea water (CFSW) for 30 min prior to fixation to prevent the rapid depolymerization of the cytoskeleton when fixatives were added. Preparations were fixed in a cocktail containing 2% glutaraldehyde, 1% OsO_4, 0.45 M sodium acetate buffer at pH 6.4, 10% sucrose, and 5 µM EGTA final concentration, for 2 h on ice. Coverslip preparations were dehydrated through a graded ethanol series, critical point dried in CO_2, mounted on stubs with silver conducting paint, coated with gold in an Edwards S150B sputter coater, and examined in a JEOL JSM-35 scanning electron microscope.

For transmission electron microscopy (TEM), prep-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arations adhered to 5 cm diameter plastic petri dishes or plastic coverslips (Fisher Scientific) were acclimated to CFSW for 30 min and fixed as above. Fixed tissue was treated with 4% hydrofluoric acid overnight to remove silica, dehydrated in ethanol, and embedded in Epon. For cross sections the embedded material was taken off the coverslip or petri dish and re-embedded in Epon. Thin sections were cut on a Reichert UM2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Hitachi 7000 electron microscope.

Acellular tissue extract was prepared for TEM as follows. A white solid which formed upon addition of substrate to 10 mM $CaCl_2$ or normal sea water (NSW) was fixed in 1% OsO_4, 6.25% glutaraldehyde, and 10% sucrose in 0.2 M s-collidine buffer (pH 7.4) on ice for 2 h. The material was dehydrated in a graded ethanol series and embedded in Epon. Thin sections were cut on a Reichert UM2 ultramicrotome, stained with uranyl acetate and lead citrate, and viewed with a Hitachi 7000 electron microscope.

Results

Because of the difficulties involved in collecting hexactinellid sponges, which generally inhabit depths of greater than 25 m and have a patchy distribution, sponges were collected sporadically throughout the year. It was found that sponges which had been held in sea water aquaria for many weeks did not produce aggregates or adhere to any of the substrates; only freshly collected specimens were capable of producing aggregates. Furthermore, not all animals collected from one dive adhered equally well in culture. Tissue from some sponges adhered very well, tissue from others adhered only tenuously, and tissue from yet others did not adhere at all. Dissociated tissue from most animals collected during the winter months of November to April tended to form poor aggregates and to adhere poorly to all substrates. Generally, a greater number of the sponges collected during the summer months of May to October produced strongly adherent and well spread aggregates. These observations are summarized in Table 1.

Table 1 Percent of sponges which produced well-adhered aggregates from those collected throughout the year

Month	Number of sponges collected	Percentage of sponges which produced adherent cultures		
		A*	B	C
January	35	0	5.7	2.8
	10	10		
	14	0		
	15	33		
	12	0	8	
	11	10		
February	28	10	10	
March	25	12	12	8
April	3	0		
May	20	30		35
	15	0		
	15	27	47	20
	10	60	70	
June	10	50		
	10	10		
July	11	18		
August	12	58		
September	10	10		
	12	33		
October	3	33		
	3	33		
	10	100		
December	10	0	40	

* Time of plating. A: immediately after collection, B: 2–5 days after collection, C: 7–14 days after collection. Sponges were not plated more than once if used for other experiments

Characteristics of adhesion

Dissociated sponge tissue plated on a coverslip without a substrate generally did not adhere, but formed detached opaque aggregates of tissue (Fig 1A). In some instances, these spherical aggregates made temporary attachments to the substrate, but always detached shortly thereafter. Of the natural substrates used, neither the algal extract nor whole cell extract promoted adhesion of dissociated sponge tissue, nor did tissue show any particular association with the pieces of whole alga, shell, or rock (Table 2). In dishes with these substrates, the dissociated sponge tissue formed small (< 1 mm diameter) spherical aggregates in the petri dish.

In contrast, *Rhabdocalyptus* tissue adhered to and spread on substrates coated with ATE from a conspecific. Although in initial experiments sponge tissue did not adhere to commercial tissue culture substrates, later experiments showed that *Rhabdocalyptus* tissue did in fact adhere to and spread to form confluent cultures on substrates coated with poly-l-lysine and Con A. The sponge tissue adhered in the form of very small spheres, but did not spread or form confluent cultures on type II collagen-coated substrates. The ATE and Con A-coated

Fig. 1 Primary cultures of sponge tissue. **A**: Opaque, detached aggregates from *R. dawsoni* plated without extract coated substrate. Note that some spherical aggregates will form temporary attachments to the coverslip (arrowheads). Scale bar: 1 mm. **B**: A low magnification micrograph of adherent and spread tissue from *R. dawsoni* adhered to ATE-coated coverslips. At this magnification the tissue gives the appearance of swirling tracks of cytoplasm. Tissue has adhered preferentially to the coated area (open arrows indicate where the extract ceases), but also adheres in patches to the uncoated parts of the coverslip. Scale bar: 1 mm. **C**: An adherent culture of *R. dawsoni* shown at high magnification. A stream (st) of moving cytoplasm appears blurred in the micrograph. Spheres may be individual archaeocytes (arrowheads) or vesicles. Giant lamellipodia (l) and filopodia (f) anchor the entire tissue mass to the coverslip. Nomarski optics. **D**: Primary culture of tissue from the demosponge *Haliclona* sp.. Individual cells in which vesicles surround the nucleus can be identified where the culture is thin (arrowheads). In other areas, such as that indicated by the asterisk, the culture may be several cell layers thick. Scale bar, C and D: 20 μm.

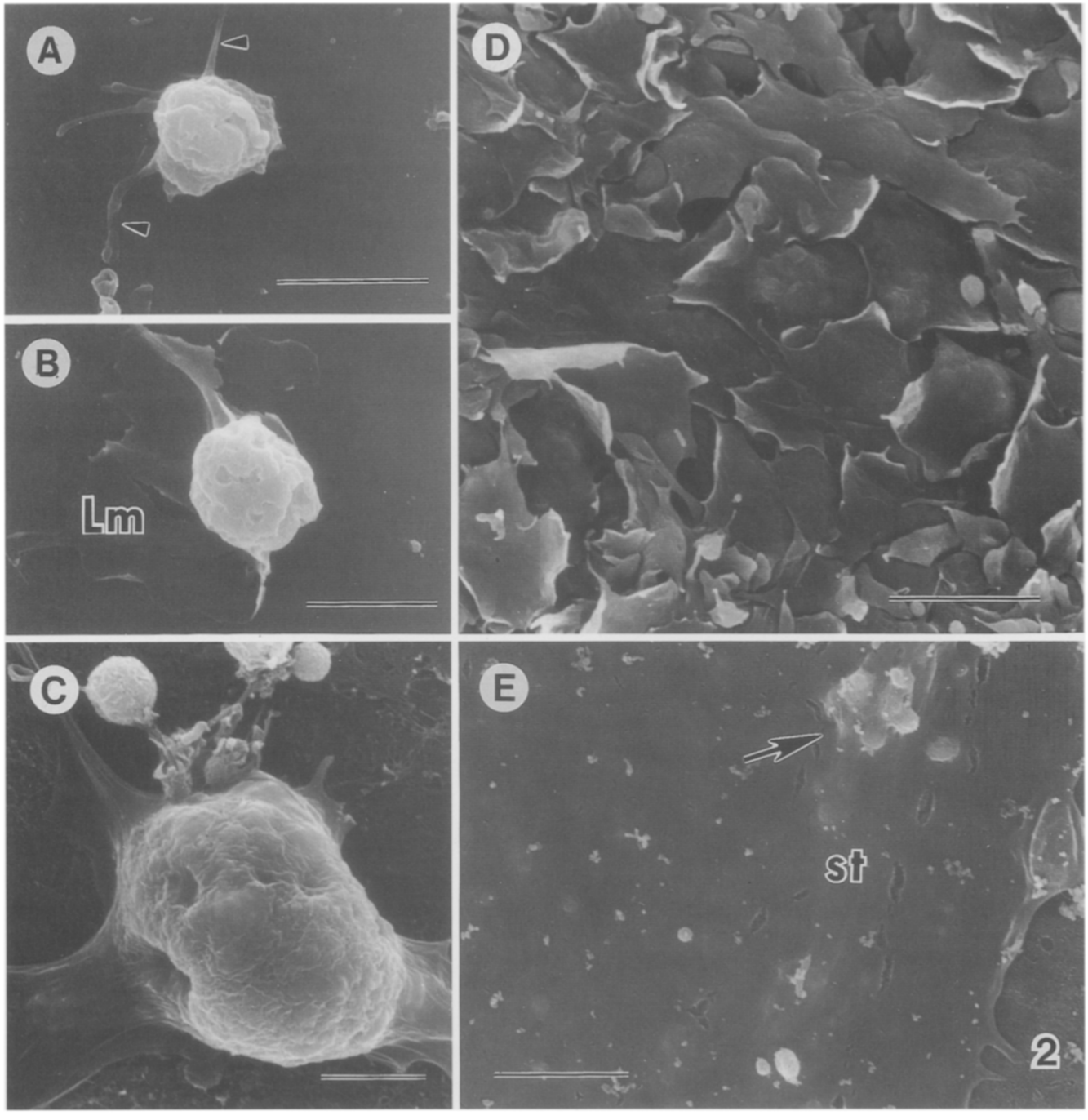

Fig. 2 Adherent tissue from syncytial and cellular sponges viewed by scanning electron microscopy. **A**: Dissociated cells from *Haliclona* sp. immediately after plating are approximately half the size of dissociated tissue pieces from *R. dawsoni*. **B**: *Haliclona* cells generally extended long filopodia (arrowheads) while most tissue pieces from *R. dawsoni* spread a broad, skirt-like lammelipodium (Lm). Scale bars A: 5 μm; B: 10 μm. **C**: 3 h after plating tissue from *R. dawsoni*, pieces were considerably larger and had a smooth surface membrane. Bar: 10 μm. **D**: 12 h after plating *Haliclona* sp. tissue, individual cells were clearly identifiable in the adherent tissue. Scale bar: 20 μm. **E**: 12 h after plating tissue from *R. dawsoni*, cultures had spread to form a thin layer covered by a continuous membrane, only broken in places by desiccation caused by the preparation procedures. Former streams (st) of cytoplasm can be identified by their bulky appearance (arrow). Scale bar: 10 μm.

substrates promoted adhesion and spreading of the tissue on and away from the coated area, while tissue plated on poly-l-lysine-coated substrates generally did not spread further than the coated area. When dissociated *Rhabdocalyptus* tissue was plated on the ATE, the boundary of the dried extract was always evident from the morphology of adherent tissue (marked by the open arrows in Fig. 1B).

This was not true for tissue plated on Con A or poly-l-lysine-coated substrates. Although the tissue preferred the area coated with ATE and clearly showed the boundaries of the extract, small patches of tissue also

Table 2 Adhesion of *Rhabdocalyptus dawsoni* tissue to natural and commercial substrates

Substrates tested	Adhesion
Algal extract	–
Whole algae	–
Clam shell	–
Rock pieces	–
Dissociated sponge cells	–
Acellular tissue extract	+ +
Concanavalin A (20–100 μg/ml)[c]	+ +
Poly-l-lysine (10–500 μg/ml)[c]	+ +

(key: – no adhesion; + + adhesion and spreading; [c]concentration dependent)

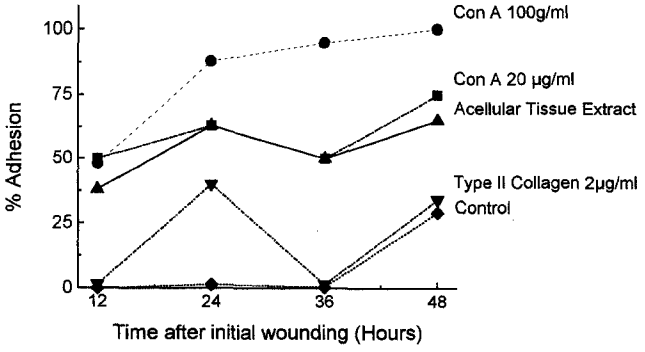

Fig. 3 The effect of repeated wounding on adhesion by *Rhabdocalyptus* tissue. Percent adhesion was estimated as described in the text. Concanavalin A was very effective at causing both adhesion and spreading of sponge tissue in a concentration dependent manner. Tissue removed from the same individual every 12 h eventually adhered even to control coverslips coated with 2% PBS. Circles: Con A 100 μg/ml; squares: Con A 20 μg/ml; upright triangles: *Rhabdocalyptus* ATE; inverted triangles: Type II collagen 2 μg/ml; diamonds, control 2% PBS.

adhered to adjacent, uncoated coverslip. At higher magnification some cell-like round objects could be seen that may be archaeocytes, which are distinct cells within the syncytial mass in intact *Rhabdocalyptus*, and large cytoplasmic vesicles (Fig 1C). However, the adherent tissue appeared fused and possessed giant lamellipodial and filopodial projections from its edges. Furthermore, vast streams of cytoplasm flowed uninterrupted throughout the tissue mass at rates just greater than 2 $\mu m \cdot s^{-1}$, appearing blurred in Figure 1C. Thin sections through fixed adhered tissue cultures showed that nuclei were abundant in former streams, and were apparently randomly distributed throughout the adherent preparations. No membranes were found separating adjacent nuclei.

Dissociated cells from the demosponge *Haliclona* sp. adhered to ATE prepared from a conspecific and spread to form a confluent culture which in some places was several cell layers thick and in others appeared to be a monolayer of cells (Fig. 1D). No streaming was found in these cultures although crawling of individual cells was readily observable.

Scanning electron microscopy of adherent tissue showed that dissociated cells from *Haliclona* sp. were approximately half the size of dissociated hexactinellid tissue pieces immediately after plating on ATE, and possessed filopodia but few lamellipodial projections (Fig. 2A). Dissociated tissue from *Rhabdocalyptus* adhered almost instantly upon plating, and spread a broad skirt-like lamellipodium (Fig. 2B). Such pieces contained between two and five nuclei when viewed by thin section transmission electron microscopy. After 3 h pieces of hexactinellid tissue had grown substantially (Fig. 2C) and, 12–24 h later, tissue masses had formed a confluent network coating the entire substrate (Fig. 2E). No cell boundaries could be seen, the surface of the entire aggregate was covered by a smooth, uninterrupted membrane and large filopodia were abundant at the edges of tissue masses. In contrast, 24 hour-old cultures from *Haliclona* sp. formed a monolayer of cells or multicellular aggregates in which individual cells could always be identified (Fig. 2D).

Wounding

Although dissociated tissue normally did not adhere to plain coverslips, repeated wounding of an individual sponge by removing tissue for dissociation caused the tissue to adhere even to uncoated coverslips (Fig. 3). Wounding of the tissue seemed to improve responsiveness to higher concentrations of Con A more rapidly than to lower concentrations. After 3–4 periods of repeated wounding over 48 h, tissue from some animals readily adhered to control coverslips coated with either 2% PBS or DDW (Fig. 3). Thorough washing of the dissociated tissue prior to plating, by rinsing it with artificial sea water three times, each time pelleting the tissue and discarding the water, did not reduce adhesion to plain coverslips, 2% PBS, or DDW controls during the period of peak adhesion.

Characteristics of the tissue extract

The ATE formed a white, cloudy, buoyant solid when added to 10 mM $CaCl_2$ or NSW (Fig. 4A). The ATE did not coagulate in calcium-free sea water with 50 mM EGTA. Thin section transmission electron microscopy of the calcium-congealed material showed no collagen fibrils but rather profiles of membranous vesicles (Fig. 4B). Thin sections of tissue adhered to the ATE showed that the tissue formed a thin layer enclosed on top and bottom by a continuous membrane, and contained archaeocytes, enucleate vesicles, and other organelles (Fig. 4C). The ATE layer appeared as a thin electron-dense material (Fig. 4C inset).

Tissue adhered preferentially to the supernatant of extract centrifuged at $6000 \times g$, and to the fraction dialyzed through a 6000 MW dialysis membrane, compared to control coverslips. Enzymatic treatment of the

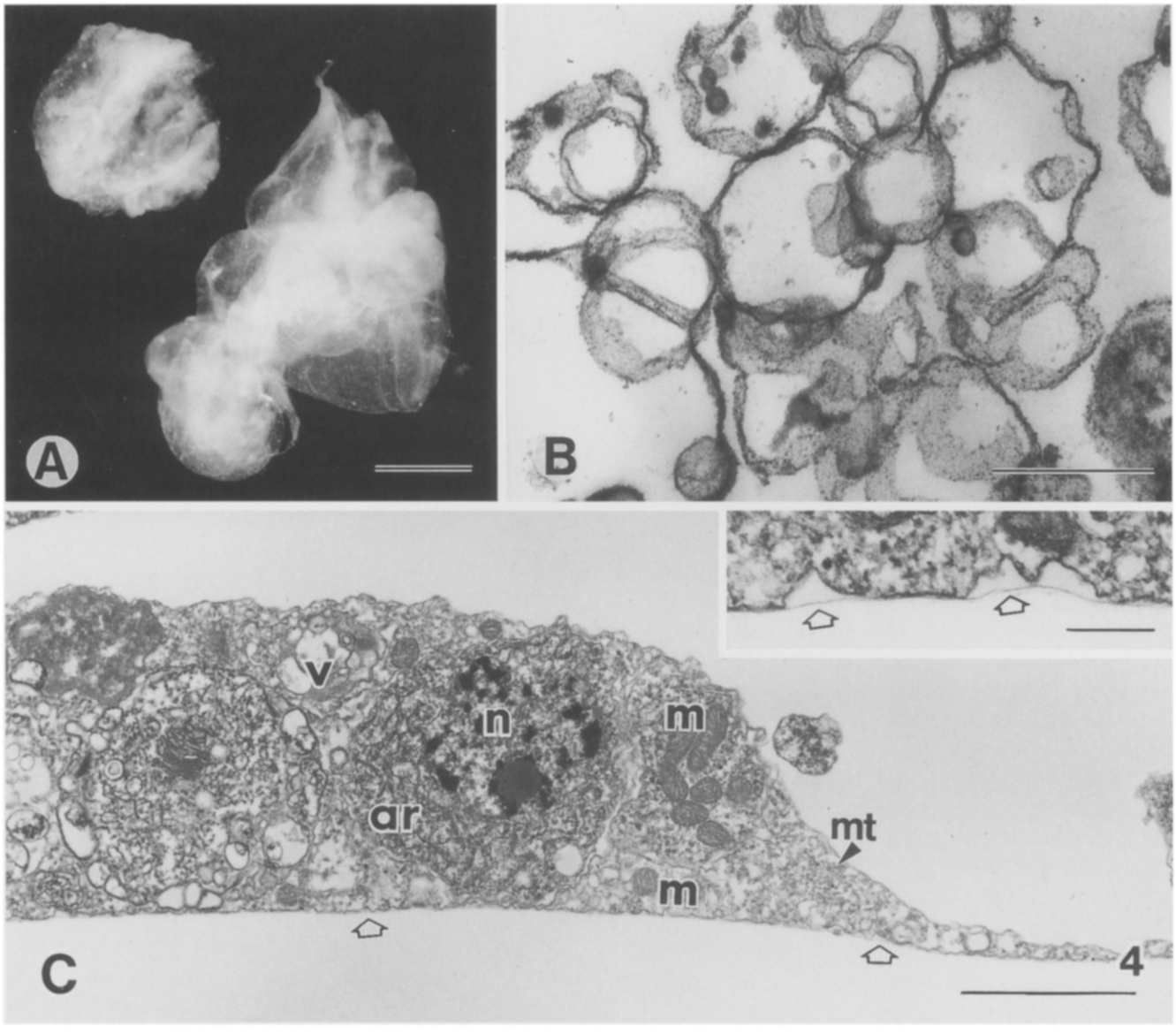

Fig. 4 Acellular tissue extract from *Rhabdocalyptus dawsoni*. **A**: The congealed, buoyant solid which forms when the acellular tissue extract, which is otherwise a transparent liquid, is pipetted into sea water. Scale bar: 2 mm. **B**: A thin section through the calcium-congealed acellular tissue extract from (A) reveals profiles of membranes but no collagen. Scale bar: 0.5 μm. **C**: A cross section of tissue from *R. dawsoni* adhered to the extract approximately 18 h after plating shows a former stream of cytoplasm, which can be identified by the presence of microtubule bundles (mt). Open arrows indicate the substrate (magnified in inset). Archaeocytes (ar), nucleus (n), vesicle (v), mitochondria (m). Scale bar: 2 μm; inset: 1 μm.

ATE (Table 3) had little effect on tissue adhesion but substantially altered tissue spreading and the formation of cytoplasmic streams. Just as adhesion improved rapidly with wounding, it waned quickly 3–4 weeks after collecting the animals. At this time, tissues of most sponges lost responsiveness first to plain uncoated substrates and then to coated substrates.

Species-specific adhesion

Dissociated tissue from newly collected specimens of *Haliclona* sp., *Halichondria* sp., *O. pennata*, and *R. dawsoni*, plated on freshly prepared substrates, each showing a higher degree of adhesion and spreading on ATE from a conspecific (Fig. 5). *R. dawsoni*, *Halichondria* sp. and *Haliclona* sp. adhered most specifically to their own ATE, while *O. pennata* adhered best to its own extract but also to a lesser extent to the ATE from *Halichondria* sp.. All sponge extracts congealed in normal sea water, but to differing degrees. Sponge biomass affected the amount of congealable extract obtainable. Thin encrusting sponges, such as *Haliclona* sp. and *O. pennata*, were more difficult to derive extract from than either large encrusting sponges, such as *Halichondria* sp., or large non-encrusting sponges such

as *R. dawsoni*. When dried on coverslips, some extracts appeared transparent, and others, such as *Halichondria* sp. ATE, left a clearly visible opaque ring at the periphery.

Discussion

While dissociated sponge cells may adhere temporarily to uncoated substrates upon initial plating (Gaino et al., 1985a), I have demonstrated that an acellular tissue extract from a conspecific promotes prolonged adhesion and spreading of sponge cells. Adhesion and spreading of tissue from the demosponge *Haliclona* sp. on a substrate coated with ATE from a conspecific, produced cultures reminiscent of many mammalian cell cultures in which individual cells are recognizable. In contrast, hexactinellid tissue from *R. dawsoni* adhered and spread on the ATE in a manner that revealed its syncytial tissue organization. Hexactinellid tissue cultures possessed a smooth continuous membrane covering what appeared to be a single, multinucleate, giant cell, which may be many centimeters in diameter. A detailed description of adherent *Rhabdocalyptus* cultures is given elsewhere (Leys, 1995).

The giant syncytial networks formed by adherent hexactinellid tissue are a fascinating preparation for study, but they are very awkward for analyzing adhesion in a quantitative assay for several reasons. Firstly, although *Rhabdocalyptus* is perhaps the most accessible of hexactinellids, it is nonetheless not very abundant and lives at such depths that animals are hard to collect in great numbers. Since only freshly collected animals produce well-adhered tissue cultures, often it was only possible to have fewer than 10 individuals available for an assay. Secondly, because tissue from different individuals varies greatly in responsiveness to coated substrates (see Table 1), it was often not possible to use all individuals collected during one dive, for an assay. Thirdly, although some sponges produced healthy aggregates during the winter months, tissue from most sponges adhered and aggregated poorly at this time. Field observations showed that during this period, sponges are flaccid and in poor condition, often to such an extent that a number of the animals die (Leys, unpublished). Variability between individuals in adhesion of tissues in

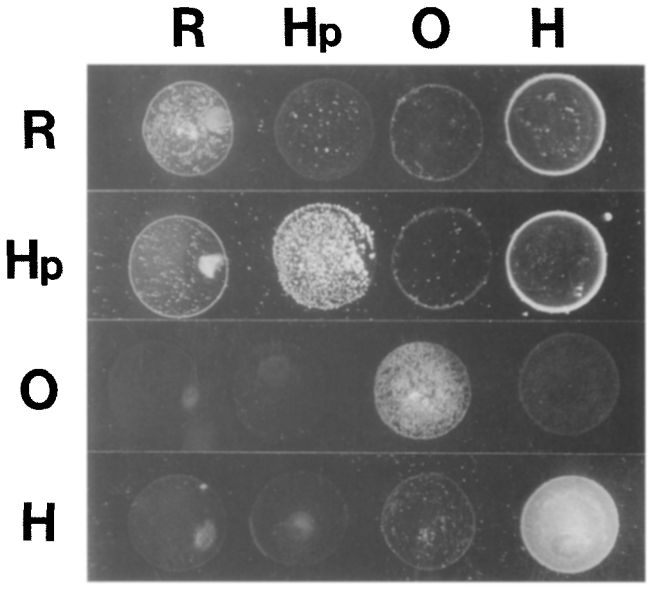

Fig. 5 Preferential adhesion of sponges to acellular tissue extract from a conspecific. Fifty microlitres of each ATE was dried on 50 mm-long coverslips. ATE left to right along top: *R. dawsoni* (R), *Haliclona* sp. (Hp), *O. pennata* (O), and *Halichondria* sp. (H). Dissociated tissue from each sponge was plated on one of the coverslips, top to bottom: (R) *R. dawsoni*, (Hp) *Haliclona* sp., (O) *O. pennata*, (H) *Halichondria*. The presence of white, almost particulate material, indicates adhesion of tissue. ATE from large, bulky sponges, such as *Halichondria*, left a clearly visible opaque ring marking the periphery of the dried extract.

Table 3 Effect of enzymatic treatment of the acellular tissue extract on adhesion and spreading by dissociated *Rhabdocalyptus* tissue(+/− = yes/no)

Enzyme treatment*	Adhesion (+/−)	Aggregate morphology	Streaming (+/−)	Spreading (+/−)	Confluence (+/−)
Trypsin					
2%	+	Large, round	−	−	−
0.2%	+	Small, round	+	−	−
Pronase E					
2%	+	Flat, adhered	−	−	−
0.2%	+	Small, round	−	+	−
Collagenase					
2%	+	Flat, adhered	−	+	−
0.2%	+	Flat, adhered	+	+	−
Hyaluronidase					
0.2%	+	Flat, adhered	+	+	+
0.02%	+	Flat, adhered	+	+	+
Control (overnight at 4°C)	+	Flat, adhered	+	+	+
Control (30 min at 37°C)	+	Flat, adhered	+	+	+

* overnight at 4°C, 30 min at 37°C

culture appears to be characteristic of other marine invertebrates (Przysiezniak, personal comm.). Considering the seasonal trends in adhesion ability observed here, this may reflect varying states of the health of individuals in the population, throughout the year. However, at all times of the year, some sponges nonetheless produced strongly adherent cultures on the ATE.

Acellular tissue extract

Previous attempts to examine adhesiveness of invertebrate cells to substrates have used a variety of matrix components (Day & Lenhoff, 1981; Gaino et al., 1993). In these studies, responsiveness to different substrates was determined by whether the cells adhered or not, the morphology of pseudopodia, and the extent of spreading. Based on the variation in sponge cell responsiveness to substrates in this study, it appears that cells may respond differently depending on the individual, on the season, and on previous wounding. Furthermore, use of a native extract rather than mammalian and artificial matrix components is likely to promote a response closer to that in vivo.

Electron microscopy of the ATE prepared from *Rhabdocalyptus* showed membranes to be the predominant component, suggesting that the adhesion factor is a membrane-associated molecule rather than an extracellular matrix (ECM) component such as collagen, and that adhesion involves a cell-cell adhesion factor. Furthermore, because a membranous solid formed when the ATE was added to a medium containing calcium, it is likely that calcium-dependent cell adhesion proteins are present in the ATE. In marine sponges, calcium-dependent and species-specific cell adhesion may involve any or all of protein-protein, protein-carbohydrate, and carbohydrate-carbohydrate interactions (Müller, 1982; Coombe et al., 1987; Müller et al., 1988; Parish et al., 1991, Misevic & Burger, 1993). A fibronectin-like protein has been shown to be present in demosponges (Labat-Robert et al., 1981), but its role in cell adhesion is not well understood (Conrad et al., 1982).

All the demosponges used in this experiment and *Rhabdocalyptus* showed a higher degree of adhesion to substrate from a conspecific, suggesting that adhesion to the ATE works in a similar manner to cell-cell aggregation involving recognition of specific groups of carbohydrates in the ATE. Family-specific adhesion by jellyfish cells to mesoglea has also been documented for hydrozoans, and is thought to involve the carbohydrate groups of ECM-glycoproteins which are associated with collagen-like fibres (Schmid & Bally, 1988; Schmid et al., 1991). It will be interesting to confirm in further experiments whether other hexactinellid extracts promote family- or species-specific adhesion.

Considering the role of carbohydrate-protein interactions in sponge cell aggregation, and the presence of sugar groups on most cell membranes, it is not surprising that Con A, a plant lectin which recognizes α-d-glucose and α-d-mannose groups, should promote adhesion of sponge tissues when used as a substrate. The spreading promoted by Con A could imply that these particular sugars are important in ECM-cell signalling in *Rhabdocalyptus*. However, because the outline of area coated with the ATE can be identified by the morphology of spread tissue (Fig. 1B), a feature not apparent on Con A-coated substrates, components of the ATE appear to be providing signals not present in the plant lectin.

Adhesion is enhanced after wounding

Repeated wounding of the sponge tissue, by snipping off pieces for dissociation, was found to enhance the adhesion of dissociated tissue from that animal even to uncoated substrates. Vigorous washing of dissociated tissue prior to plating did not prevent wounded tissue from attaching to uncoated substrates, suggesting that the adhesion factor is not soluble. There have been many reports of culture media being conditioned by wounded tissue (reviewed by Hay, 1981), but it is not known what factor is responsible for the activation of cells. Some evidence suggests that the polysaccharide hyaluronan abounds in healing tissues where it is important for cell migration (Laurent & Fraser, 1992). The lack of effect on adhesion or spreading by treatment of the ATE with hyaluronidase may be a result of using too low a concentration of hyaluronidase, or might indicate that this polysaccharide is not important in adhesion and spreading in hexactinellids. Other enzymatic treatment did interfere with spreading, suggesting that there is a role for collagen and other ECM proteins in spreading in hexactinellids. Infrequently I have seen bundles of collagen between the tissue and the coverslip and within pockets of the tissue in thin sections of adhered preparations. However, since scanning electron micrographs of aggregates fixed at 1 h after plating the tissue showed no collagen, it is likely that collagen is produced after the dissociated tissue has already adhered and spread. The actual mechanism by which adhesiveness to substrates increases with wounding is not clear and merits further investigation.

This study has demonstrated that the preparation of an acellular tissue extract which promotes the adhesion and spreading of sponge tissue as primary cultures is uncomplicated. With further analysis and purification, it is possible that similar extracts from other marine invertebrates might prove to be useful in primary culture of marine invertebrate tissues, and may even help in developing continuous cell lines from these animals.

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