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Fibronectin Is Apparently Not Involved in Species-Specific Reaggregation of Cells From the Marine Sponge Geodia cydonium

J. Conrad, B. Diehl-Seifert, R. K. Zahn, G. Uhlenbruck, E. Zimmermann, and W. E. G. Müller

Institut für Physiologische Chemie, Universität, Duesbergweg, 6500 Mainz (J.C., B.D.-S., R.K.Z., W.E.G.M.), Medizinische Universitätsklinik, Abt. "Experimentelle Innere Medizin," Kerpener Str. 15, 5000 Köln (G.U.), and Physiologisches Institut 2, Universität, Robert-Koch-Straße 28, 4400 Münster, (E.Z.), Federal Republic of Germany

Experiments were carried out to test the hypothesis that fibronectin is involved in reaggregation of dissociated sponge cells. Cells from the siliceous sponge Geodia cydonium were extracted with urea to solubilize fibronectin from cells of higher multicellular organisms. The crude extract was further fractionated by DNA, heparin, and collagen affinity chromatography; they were termed Geodia fibronectinlike fractions. The fibronectinlike fractions contained a series of proteins with molecular weights different from that of the genuine fibronectin. The Geodia fibronectinlike fractions did not react with antiserum, produced against human fibronectin, under formation of a precipitin line. Using this antiserum the sponge cells could not be specifically labeled with FITC-anti-IgG antiserum. Radioimmunoprecipitation experiments revealed that the Geodia fractions contain—if at all—0.1% fibronectin or fibronectinlike protein at the most. In the crucial experiments it was shown that the Geodia fibronectinlike fractions, human fibronectin, and antifibronectin antiserum exerted no influence on adhesion of Geodia cells either in the absence or in the presence of the soluble aggregation factor. Based on these findings, we conclude that fibronectin is apparently not present on Geodia cells and does not play a role in aggregation of this biological system.

Key words: fibronectin, sponges, Geodia cydonium, aggregation, cell recognition

Studies investigating the molecular mechanisms of cell-cell recognition and adhesion led to the isolation and characterization of a series of factors involved in these cellular activities. These factors may be classified into intrinsic membrane factors and extrinsic "bridging" factors. [1]. Vertebrate cells are provided with both intrinsic membrane factors (eg, CAM protein [2], cognin [3]), which are either CA^{++}-dependent or CA^{++}-independent [4] and extrinsic "bridging" factors (galap-

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tins)[5]. In addition, most of the vertebrate cells have the cell surface glycoprotein, fibronectin [for review, see 6–8], which is thought to be involved in cell-substratum contact. Some evidence has been presented demonstrating that fibronectin mediates organization of such sites on the cell surface at which cell attachment occurs [9].

Marine sponges, which provided the first direct demonstration of differential cellular affinities [10] have been used as sources for the isolation [11, 12] and subsequent purification [13, 14] of the first extrinsic "bridging" factor termed "aggregation factor." The sponge aggregation factors, being some of the most interesting cell adhesion ligands thus far described, are established to be those molecules that contribute to intercellular adhesion [11–13, 15], species-specific cell recognition [11–13, 16, 17], host defense mechanisms against foreign invaders [18, 19], and controlled cell proliferation [12, 20]. First experimental studies on the freshwater sponge Ephydatia mulleri (=E mulleri), which is distributed in Europe preferentially in northern and eastern countries [21], revealed fibronectinlike protein to be present in sponge cell membranes and to a lesser content in the intercellular matrix [22]. Moreover, these authors report that reassociation of dissociated sponge cells is inhibited by antihuman fibronectin antiserum.

In this communication, we summarize a comprehensive study which aimed to elucidate the possible function of fibronectin, or molecules that are antigenically related to it, in control of cell-cell interactions using the marine sponge system Geodia cydonium. Using this system, we got no evidence that fibronectinlike protein is involved in Geodia cell association.

MATERIALS AND METHODS

Materials

Fibronectin (prepared from human plasma [23]) from BRL, Gaithersburg, Maryland; starch (S-2630), protein standards (phosphorylase a, bovine serum albumin, egg white albumin, cytochrome c), heparin, and collagen (from calf skin) from Sigma Chemicals, St. Louis; Labtrol from Serva, Heidelberg; ^{125}J-fibronectin from NEN, Dreieichenhain (sp. activity 2.98 μCi/μg); and Pansorbin (Staphylococcus aureus cells; 1 g/10 ml) from Calbiochem, Giessen.

The siliceous sponge Geodia cydonium Jam. (Tetractinellida) was collected in the vicinity of Rovinj, Yugoslavia.

Cells and Aggregation Factor

The dissociation of Geodia cydonium tissue into separate cells was performed in calcium- and magnesium-free artificial sea water [13]. For the experiments described, only aggregation-susceptible cells [24] were used. The cells were counted in a hemocytometer. The aggregation factor of Geodia cydonium was isolated and purified as described previously [25]. The purified factor with a protein content of 1.4 mg/ml had a specific aggregation-promoting activity of 6×10^6 aggregation units/mg protein; the definition of the aggregation units was given earlier [13].

Biological Assay

Reaggregation was assayed at 20°C in glass roller tubes, containing in a volume of 3 ml 75 $\pm$ 15×10^6 Geodia cells [13]. Primary aggregation of the cells was determined in the absence of the aggregation factor. Secondary aggregation was

studied in the presence of the soluble aggregation factor at a concentration of 100 μg protein/assay. The size of the aggregates was determined after 60 min [26]. In some experiments the cells were preincubated (30 min) with serum, fibronectin, or Geodia fibronectinlike fractions.

Isolation of Fibronectinlike Fractions

Fibronectin is known to possess binding sites for cell surfaces [27], collagens (gelatin) [28], heparin [29], and DNA [30]. Considering these properties, the methods were chosen for the purification of fibronectinlike fractions.

The procedures were carried out at 2°C. Ten grams of packed, chemically dissociated Geodia cells [13] was extracted (2 h) with 30 ml of a 10 mM Tris-HCl buffer (pH 8.5) containing 1 M urea [31]. For inhibition of proteases, either 2 mM phenylmethylsulfonyl fluoride [31] or a mixture of 1 mM diisopropyl fluorophosphate and 10 mM sodium tetrathionate [32] was used; in both cases, identical results were obtained both in the analytical and functional determinations. After centrifugation (30 min; 20,000g) the supernatant (=crude extract) was collected (28 ml) and subsequently dialyzed against 50 mM Tris-HCl (pH 7.2), containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A). The crude extract contained 0.42 mg protein/ml. In some cell systems this urea extraction results already in a homogeneous fibronectin preparation [27]. Extracts from other cell lines, which contained some other proteins as well, were purified further by specific affinity chromatographical methods; these methods were also applied for fractionation of the crude extract from Geodia. Ten-milliliter aliquots of each were taken for the affinity chromatography procedures; columns of 0.8×7 cm size were chosen, and the flow rate was adjusted to 5 ml/h. The affinity matrices were prepared according to published procedures: DNA-cellulose [33], collagen-Sepharose [34], and heparin-Sepharose [29].

After application of the crude extracts to the columns, equilibrated with buffer A, elution of the fibronectinlike fraction was performed under the described specific conditions: DNA-cellulose chromatography with buffer A containing 0.5 M NaCl [30], collagen-Sepharose chromatography with buffer A containing 1 M KBr [34], and heparin-Sepharose with buffer A containing 0.5 M NaCl [29].

The protein content in these fractions was determined to be 0.28 mg after DNA-cellulose chromatography, 0.22 mg after collagen-Sepharose, and 0.35 mg after heparin-Sepharose. Basing on protein concentration, the percentage of recovery after the affinity chromatographic procedures was as follows: DNA-cellulose chromatography, 96%; collagen-Sepharose; 92%; and heparin-Sepharose, 97%.

Antiserum Preparation

Fibronectin from human plasma was used to immunize female New Zealand rabbits. Four injections, each containing 500 μg of protein, suspended in 2 ml of Freund's complete adjuvant (Difco), were given intradermally on days 1, 14, and 28. The antisera were collected 7 days after the booster.

Radioimmunoassay

Immunoprecipitation was performed in a 50 mM Tris-HCl buffer (pH 7.4, containing 100 mM NaCl and 2 mg/ml bovine serum albumin). Assay mixtures were prepared by sequential addition of 80 μl of the standard unlabeled fibronectin or of

the Geodia sample to be assayed; 20 μl of a 1:512 diluted antifibronectin antiserum; 20 μl of ^{125}J-fibronectin, containing 32,000 cpm. This mixture was incubated for 60 min at 22°C. Then 10 μl of a 10% suspension of Pansorbin was added and the mixture was incubated for a further 30 min. After addition of 1 ml 50 mM Tris buffer (see above), the tubes were centrifuged (12,000g, 3 min). The resulting pellet was determined in a gamma counter. The potentially crossreactive Geodia material was added at a number of those concentrations, which are within the linear range of the assay, determined with human fibronectin.

Other Methods

Protein (standard: Labtrol) was determined according to Lowry et al [35]. Neutral carbohydrates (standard: starch) were determined according to the method of Dubois et al [36]; lipids, according to Hinsberg et al [37]; and hexuronic acid (standard: D-glucuronic acid), according to Avigad [38]. SDS/polyacrylamide (10%) gel electrophoresis was carried out according to Weber and Osborn [39]; the gels were stained with Coomassie brilliant blue. For the determination of the apparent molecular weight, the following protein standards were used: phosphorylase a, 98,000; bovine serum albumin, 68,000; eggwhite albumin, 43,000; and cytochrome c, 12,500. The Ouchterlony-microdiffusion procedures were performed as described earlier [17]. Immunofluorescence studies were performed essentially as described by Ali et al

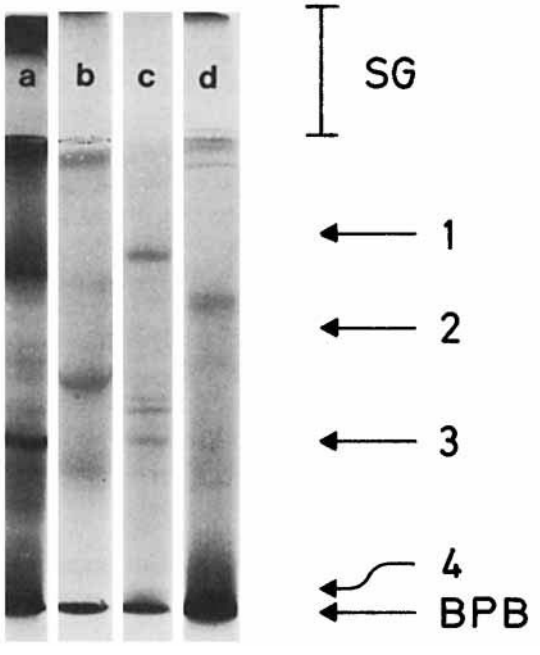

Fig. 1. SDS/polyacrylamide gel electrophoresis of fibronectinlike fractions. a) Crude extract; b) DNA-cellulose fraction; c)collagen-Sepharose fraction; and d) heparin-Sepharose fraction. All proteins were reduced with 0.1 M dithiothreitol, boiled, electrophoresed, and stained with Coomassie blue after electrophoresis. The arrows mark the positions of the following protein standards, which were run in parallel gels: 1) phosphorylase a (M_r=98,000); 2) bovine serum albumin (M_r=68,000); 3) egg white albumin (M_r=43,000); and 4) cytochrome c (M_r=12,500). SG, stacking gel; BPB, bromphenol blue.

[40]: The cells were fixed with formaldehyde (neutralized with calcium carbonate), incubated with either antifibronectin antiserum or control serum, and finally stained with FITC-conjugated goat antirabbit IgG antibodies. As a positive control—eg, cells which contain cell surface-bound fibronectin—primary rabbit kidney cells [41] were used.

RESULTS

Properties of Geodia Fibronectinlike Fractions

Analyses of the fibronectinlike fractions (both the crude extract and the further purified fractions, obtained after DNA-cellulose, collagen-Sepharose, and heparin-Sepharose affinity chromatography) by SDS/polyacrylamide gel electrophoresis revealed banding patterns which are shown in Figure 1. It is obvious that all of the fractions, obtained by the procedures for the preparation of homogeneous fibronectin, are far from being pure. The apparent molecular weight of the major bands in the Geodia fractions were as follows: crude extract (Fig. 1a)—150,000, 84,000, 82,000, 42,000, and 36,000; DNA-cellulose fraction (Fig. 1b)—150,000, 105,000, 52,000, and 38,000; collagen-Sepharose fraction (Fig. 1c)—88,000, 52,000, 48,000, and 42,000; heparin-Sepharose fraction (Fig. 1d)—150,000, 140,000, 72,000, 55,000, and 36,000. This means that, after separation of the urea crude extract from Geodia on affinity matrices under conditions at which homogeneous fibronectin is obtained from serum and cells of higher eukaryotes, only mixtures of proteins with different molecular weights were isolated. Moreover, the molecular weights of the proteins visualized in the three Geodia fibronectinlike fractions that were collected after the affinity chromatographical separation were totally noncorresponding.

The ultraviolet absorption spectra of the Geodia fibronectinlike fractions were compared with that of human fibronectin (measured at pH 8.2 in buffer A). Human fibronectin showed a maximum at 278 nm and a minimum at 258 nm; the absorbance ratio at 278:258 nm was 1.48. The comparative values for the Geodia fractions were as follows: crude extract—maximum at 272 nm, minimum at 253 nm, 272:253 nm = 1.09; DNA-cellulose fraction—276 nm, 259 nm, 276:259 nm = 1.05; collagen-Sepharose fraction—276 nm, 251 nm, 276:251 nm = 1.13; and heparin-Sepharose—278 nm, 248 nm, 278:248 nm = 1.39.

Chemical analyses of the Geodia fibronectinlike fractions revealed that they consist mainly of protein. However, in all fractions considerable amounts of neutral carbohydrates, lipid, and hexuronic acid are also present (Table I).

TABLE I. Analyses of the Geodia Fibronectinlike Fractions

	Components (% (w/w))			
	Protein	Neutral carbohydrates	Lipid	Hexuronic acid
Crude extract	65.7	5.7	11.4	7.2
DNA-cellulose	83.9	2.1	3.1	2.9
Collagen-Sepharose	71.2	12.3	4.9	0.4
Heparin-Sepharose	76.0	2.4	1.8	0.7

Results are calculated as a percentage of dry weight minus ash. The samples contained between 5% and 11% ash as analyzed after exhaustive dialysis against distilled water.

Reaction of the Geodia Fractions With Antifibronectin Antiserum

In Ouchterlony analysis, the antiserum raised against human fibronectin produced strong precipitation lines against human fibronectin down to a concentration of 10 μg/ml (Fig. 2). However, the Geodia urea-crude extract as well as the DNA-cellulose fraction, the collagen-Sepharose fraction, and the heparin-Sepharose fraction (at a concentration higher than 0.22 mg protein/ml), prepared from the crude extract, were found not to precipitate with antifibronectin antiserum.

Radioimmunological Determination

For a further tracing of fibronectin or fibronectinlike material in Geodia extracts, a highly sensitive radioimmunoassay was applied. The assay for fibronectin could measure concentration lower than 0.2 ng of antigen/assay (Fig. 3). The assay was linear over an antigen concentration range of 0.2–3.2 ng/assay. Addition of Geodia extracts (both crude extract and fractions obtained after affinity chromatography purification procedures) did not cross-react immunologically with human fibronectin, even when very large amounts of the Geodia material were used (Fig. 3). These results show that the Geodia fractions contain—if at all—0.1% fibronectin or fibronectinlike protein in maximum.

Indirect Immunofluorescence Studies

By using a further technique, the indirect immunofluorescence staining of cells, efforts were undertaken to detect fibronectin on the surface of sponge cells. The method applied in the present study and adopted from Ali et al [40] was appropriate to detect fibronectin on primary rabbit kidney cells. Cells incubated with antifibronectin antiserum (1:80 dilution) were shown to be surrounded with a complex network of fibronectin over the surface of the cells (Fig. 4a). Rabbit kidney cells, which had been pretreated with control serum (serum from unimmunized rabbits), were not stained with FITC-labeled goat antirabbit IgG antibodies. By applying this procedure to Geodia cells, no staining with antirabbit IgG was achieved after preincubation with antifibronectin antiserum at the following dilutions: 1:2, 1:20, 1:40, 1:80, 1:160, and 1:320 (Fig. 4b).

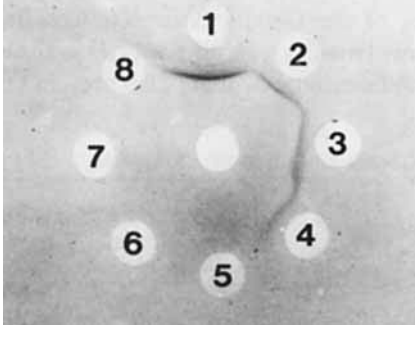

Fig. 2. Specificity of the antifibronectin antiserum. Substances are arranged clockwise from 12 o'clock on. Human fibronectin: 0.1 mg/ml (1), 0.05 mg/ml (2), 0.02 mg/ml (3), and 0.01 mg/ml (4); Geodia crude extract 0.4 mg/ml (5); Geodia DNA-cellulose fraction 0.28 mg/ml (6); Geodia collagen-Sepharose fraction 0.22 mg/ml (7); and Geodia heparin-Sepharose fraction 0.35 mg/ml (8). In the center well is antifibronectin antiserum.

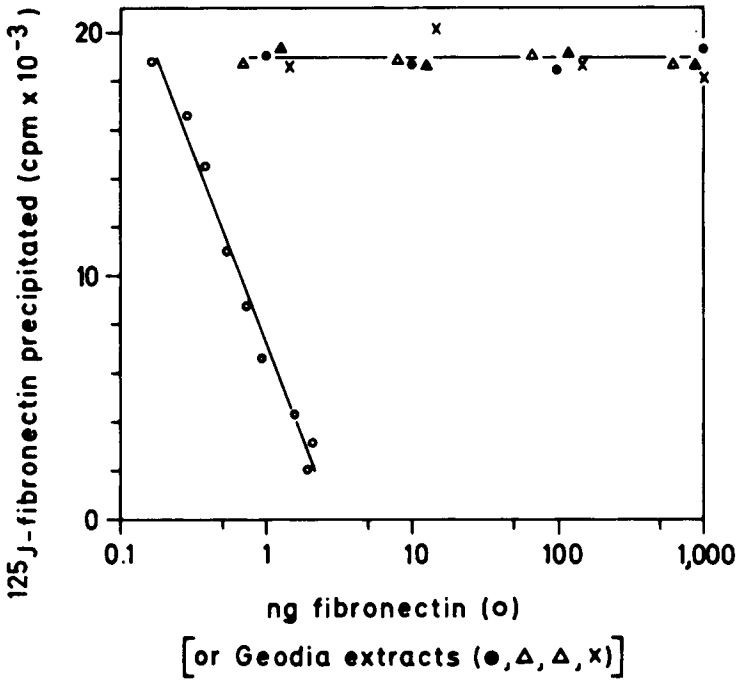

Fig. 3. Radioimmunoassay for fibronectin (human), using antifibronectin and ^{125}J-fibronectin probe. Procedural details are described in Methods. Unlabeled fibronectin (human) (○) was added in the amounts indicated. Addition of increasing amounts of Geodia crude extract (●), Geodia DNA-cellulose fraction (×), Geodia collagen-Sepharose fraction (▲), and Geodia heparin-Sepharose fraction (△).

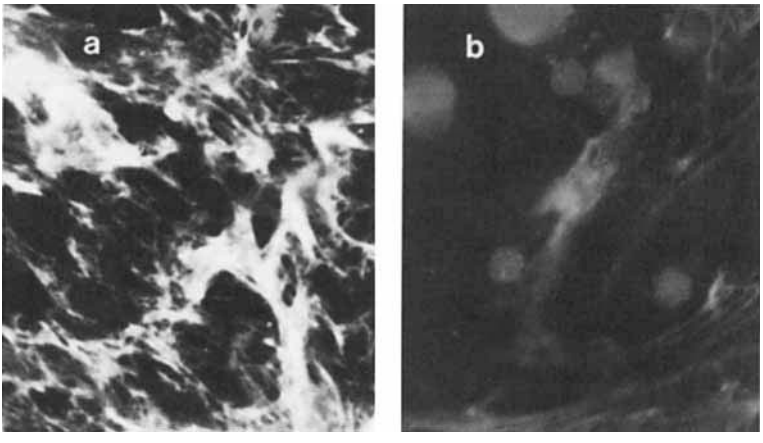

Fig. 4. Immunofluorescent staining of primary rabbit kidney cells (a) and sponge cells (b). Magnification: 750×.

Influence of Human Fibronectin and of Geodia Fibronectinlike Fractions on Reaggregation

In previous reports [6, 8] it was shown unequivocally that transformed cells show an increased adhesion after treatment with fibronectin. In analogous experiments, the influence of human fibronectin and of Geodia fibronectinlike fractions on sponge cell aggregation was determined. As summarized in Table II, human fibronectin and Geodia fibronectinlike fractions do not exert any influence on primary or secondary aggregation. In the absence of the aggregation factor, primary aggregates of a size of 82 μm are formed, whereas in the presence of 100 μg of purified aggregation factor/assay, secondary aggregates of a diameter of approximately 1,700 μm were assembled.

Influence of Antiserum to Fibronectin on Reaggregation

In a last approach to find a hint about the presence of fibronectin on the surface of Geodia cells, the influence of antiserum to fibronectin on aggregation of sponge cells was determined. As shown in Table II, the antiserum as well as the control serum had an influence neither on the size of "primary aggregates" nor on the one of the "secondary aggregates."

DISCUSSION

Four approaches were chosen to clarify whether fibronectin is involved in reaggregation of sponge cells, mediated by the soluble aggregation factor. In the first part of the study, macromolecular fractions were isolated and purified from Geodia cells following those procedures, which had been successfully applied for the isolation of fibronectin from vertebrate cells; these fractions were therefore termed Geodia fibronectinlike fractions. The sponge cells were extracted with urea [42] in the presence of protease inhibitor [31]; the extract was subsequently processed by affinity chromatography using heparin [29], DNA [33], and collagen [34] as ligand. Analysis

TABLE II. Influence of Human Fibronectin, Geodia Fibronectinlike Fractions, and Antifibronectin Antiserum on Sponge Cell Aggregation

Pretreatment of the cells	Diameter of aggregates (μm)	
	Without aggregation factor	In the presence of aggregation factor
None	85 ± 11	1,750 ± 228
Fibronectin (human); 10 μg/ml	83 ± 10	1,700 ± 220
Geodia fibronectin-like fraction		
Crude extract; 40 μg/ml	87 ± 10	1,670 ± 204
DNA-cellulose; 28 μg/ml	86 ± 12	1,720 ± 258
Collagen-Sepharose; 22 μg/ml	81 ± 11	1,650 ± 201
Heparin-Sepharose; 35 μg/ml	80 ± 9	1,780 ± 249
Antifibronectin antiserum; 20 μl/ml	79 ± 12	1,660 ± 198
Control serum; 20 μl/ml	78 ± 12	1,670 ± 200

The single cell suspension was incubated in calcium- and magnesium-containing seawater [13], in either the absence or presence of the soluble aggregation factor as described in Methods. The diameter of the primary aggregates (formed in the absence of the soluble aggregation factor) and of the secondary aggregates (in the presence of the aggregation factor) was determined after 60 min. Results are from 10 parallel determinations; the mean values (± SD) are presented. The cells were pretreated in the presence of the indicated components for 30 min at 20°C in calcium- and magnesium-free seawater [13].

of the fractions obtained from the sponge revealed that they basically differ from each other by their molecular weights and their chemical composition. It is known that fibronectin is degraded by a variety of proteolytic enzymes resulting in the formation of low molecular fragments (M_r 150,000–140,000, 40,000, and 32,000 [43, 44]) from the high molecular weight form (M_r 210,000–230,000 [42]). In none of the isolated sponge fractions were proteins of those apparent molecular weights detected as predominant bands on $NaDodSO_4$/polyacrylamide gels.

To support the conclusion reached—that in none of the fibronectinlike sponge fractions is the genuine fibronectin (or its digestion products) present—immunodiffusion analysis using rabbit antiserum to human plasma fibronectin was performed. This antiserum did not react with the Geodia fibronectinlike fractions under formation of a precipitin line. This negative result was confirmed by indirect immunofluorescence studies using antifibronectin antiserum and Geodia cells. Also, this approach led to the observation that Geodia cells, incubated with antifibronectin antiserum, are not stained specifically with FITC-labeled goat antirabbit IgG antiserum. Using a highly sensitive radioimmunoassay, no evidence for the existence of fibronectin in Geodia extracts was obtained.

After reaching this point, one can argue against the use of antiserum produced against a heterologeous fibronectin in the Geodia system. The fourth characteristic of fibronectin was therefore taken as a parameter in the search for the existence of fibronectin in the sponge system: the property of fibronectin to increase adhesion of the cells [6, 8, 9]. Following this line, neither the Geodia fibronectinlike fractions (both the crude urea extract and the purified affinity fractions) caused an aggregation promoting effect by themselves, nor had they any influence on the primary or secondary aggregation of Geodia cells. Knowing this, it was not surprising that antifibronectin antiserum was also without effect on the Geodia aggregation system.

Taking all this together we reach the following conclusions: First, apparently no fractions can be isolated and purified from Geodia cells that have the physicochemical properties of fibronectin isolated from higher multicellular animals, with respect to the molecular weight and the affinity to collagen, DNA, and heparin. Second, human fibronectin and antiserum raised against it were found to be without any effect on the Geodia reaggregation system. Third and most important, none of the Geodia fibronectinlike fractions exert any effect on Geodia cell-cell interaction either in the absence or in the presence of the purified Geodia aggregation factor.

The comprehensive experimental results given in the present communication are in opposition to those reported by Labat-Robert et al [22] using the Ephydatia mulleri and the Tethya sp. sponge systems. It is difficult to explain the discrepancy, because basic experimental details have not been included in the published communication; eg, description of the aggregation medium, of the aggregation conditions, and of the number of cells added to the assays. Moreover, it has not been mentioned to what extent a soluble aggregation factor is involved in the aggregation events described, or whether the dissociated cells used for the studies were obtained from genetically identical or allogeneically distinguished specimens. Cells from the related species E fluviatilis have been shown to comprise strain-specific recognition properties [45]. The most plausible explanation for the differing results may be seen in the described observations [22] that fibronectinlike protein is synthesized only during distinct phases of sponge morphogenesis. In our view, it might well be that fibronectinlike protein exists in some sponge species. However, the attempted generalization that this protein plays an important role as the recognition site of the aggregation factor seems to be premature.

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NOTE ADDED IN PROOF

With the radioimmunoassay described we could detect in the glycoconjugates, isolated from Geodia cydonium according to S. Junqua, M. Lemonnier and L. Robert [32], a fibronectin-like protein at a concentration of 20 μg/mg protein.