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Aggregation of Sponge Cells: Immunological Characterization of the Species-Specific Geodia Aggregation Factor

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Antibodies were raised against the purified aggregation factor from Geodia cydonium in order to clarify its function during cell aggregation in the homologous and heterologous system. These antibodies inhibited only cell aggregation in the homologous Geodia system and were inactive in the heterologous Tethya lyncurium system. These findings directly indicated that the species-specific reaggregation of sponge cells was initiated by the soluble aggregation factor as already assumed in earlier studies. The amount of neutralizing antibodies was determined by a precipitation reaction with the antigen in capillaries and by microdiffusion. By using the latter technique we got evidence that the Geodia aggregation factor contained a component that was antigenetically related to a galactose-specific lectin present in Geodia cydonium.

Key words: cell aggregation, aggregation factor, sponges, glycoproteins

Sponges are the first and oldest multicellular animals and have existed for more than 1 billion years. During the evolution from the unicellular to the multicellular organisms, the sponges have developed two essential mechanisms: first, a tissue-specific recognition system localized on the cell surface [1–3] and second, a host defense mechanism against foreign invaders [4, 5]. The marine sponges have proved to be the classic experimental model for the elucidation of the molecular basis for these two mechanisms. The species-specific reaggregation of dissociated sponge cells is mediated by large, soluble glycoproteins termed aggregation factors; they have been purified from a series of sponge species [2, 6–8]. For some of these aggregation factors the species specificity has been established [2, 3, 9]. First biochemical results revealed that species-

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specific intercellular recognition and adhesion occur between the soluble aggregation factor and the cell membrane-bound aggregation receptor [10, 11]. The results obtained in the Microciona [12], Geodia [13, 14], and Suberites systems [15] suggested that specific saccharide moieties (eg, glucuronic acid) linked to one component are recognized in a species-specific manner by lectin-like binding sites present at the second component. In addition, it is suggested [16, 17] that in the Microciona system a specific Ca^{++}-dependent interaction between two aggregation factor monomers, which are functionally univalent, is necessary. These basic findings alone are not sufficient as a rigorous evidence that the two isolated ligands (aggregation factor and aggregation receptor) are actually involved in the species-specific reaggregation as discussed earlier [18]. It could well be (1) that the external ligands isolated and tested only modulate a different "natural" adhesion system; or (2) that a second molecular recognition system exists besides the aggregation factor–aggregation receptor system.

We have approached the problem of species specificity of the reaggregation of dissociated cells from Geodia cydonium by immunochemical studies. A procedure for preparation of specific antibodies against the purified Geodia aggregation factor is described. In addition, methods to determine the immunological and biological properties of the antibodies in the homologous and heterologous system are given. In earlier studies [19, 20] it was reported that antibodies against sponge cell extracts and crude sponge aggregation factor [21] influence reaggregation in the homologous system. In addition, our studies now provide evidence that the aggregation factor contains a component which antigenetically cross-reacts with a galactose-specific lectin [22], isolated and purified from the same species.

MATERIALS AND METHODS

Animals

The following sponges were collected in the vicinity of Rovinj, Yugoslavia: Tetraxonida; Homosclerophora: Geodia cydonium (Jam.), Ancorina cerebrum (Schm.); Astromonaxonellina: Tethya lyncurium (L.); Cornacuspongida; Protorhabdina: Mycale massa (Schm.), Hemimycale columella (Bow.); Poikilorhabdina: Tedania anhelans (Lieberk.), Crella rosea (Top.), Clathria coralloides (Schm.); Phtinorhabdina: Axinella cannabina (Esp.), Hymeniacidon sanguinea (Top.), Pellina semitubulosa (Schm.); Aporhabdina: Dysidea tupha (Vac.), Ircinia muscarum (Schm.), Hippospongia communis (Lam.), Verongia aerophoba (Schm.).

Cells

The dissociation of sponge tissue into separate cells was performed in calcium- and magnesium-free artificial sea water containing trypsin [2]. Counting of the cells was performed in a haemocytometer.

Isolation of Aggregation Factors

The aggregation factor of Geodia cydonium was isolated and purified as described previously [11]. After absorption on sheep red blood cells, the factor with a protein content of 1.4 mg/ml had a specific aggregation-promoting ac-

tivity of 6×10^6 aggregation units (AU)/mg protein. The activity of the aggregation factor was determined as described earlier [2], using the formula AU = nPA/nSA where nPA is the number of primary aggregates in the assay without aggregation factor and nSA is the number of secondary aggregates in the assay with the factor. Under standardized assay conditions nPA is a constant ($= 6.7 \pm 2.6 \times 10^5$). Thus, an aggregation factor with an activity of 3.35×10^3 causes 200 secondary aggregates in the assay. The aggregation factor preparation used had been preabsorbed on sheep red blood cells (see above) and did not contain any measurable lectin contamination. Lectin activity was measured in a hemagglutination assay [22] with a lower sensitivity limit of 5 ng of homogeneous Geodia lectin per assay (110 μl). A contamination of the Geodia aggregation factor with components from Tethya lyncurium is also very unlikely, because the two sponges were collected in two different habitats.

The aggregation factors of the other sponge species were isolated from 10 g tissue each. The cut material was extracted in 20 ml of calcium- and magnesium-free artificial sea water containing 20 mM EDTA [2]. After centrifugation (10,000g, 10 min, 2°C), 4 ml of the supernatant was fractionated by gel filtration on Sephadex G-50. A 1 × 15 cm column equilibrated with calcium- and magnesium-free artificial sea water [2] was used. The fractions within the V_e/V_0 range between 1.0 and 1.3 were pooled. The different, fractionated sponge extracts were characterized as follows: Ancorina cerebrum 1.9 mg protein/ml, 2.1×10^5 aggregation units (AU)/ml; Tethya lyncucium 5.7 mg/ml, 13×10^5 AU/ml; Mycale massa 0.2 mg/ml, 0.4×10^5 AU/ml; Hemimycale columella 1.1 mg/ml, 4.7×10^5 AU/ml; Tedania anhelans 0.8 mg/ml, 0.9×10^5 AU/ml; Crella rosea 1.3 mg/ml, 14×10^5 AU/ml; Clathria coralloides 0.8 mg/ml, 3.9×10^5 AU/ml; Axinella cannabina 1.4 mg/ml, 8.4×10^5 AU/ml; Hymeniacidon sanguinea 1.0 mg/ml, 9.3×10^5 AU/ml; Pellina semitubulosa 2.8 mg/ml, 1.8×10^5 AU/ml; Dysidea tupha 2.8 mg/ml, 0.7×10^5 AU/ml; Ircinia muscarum 2.1 mg/ml, 0.6×10^5 AU/ml; Hippospongia communis 2.2 mg/ml, 1.9×10^5 AU/ml; and Verongia aerophoba 1.9 mg/ml, 2.6×10^5 AU/ml.

Reaggregation Assay

Reaggregation was assayed in glass roller tubes, containing a volume of 3 ml $75 \pm 15 \times 10^6$ cells [23]. The homotypic aggregation factor was added at a concentration of 100 μg protein per assay. The size of the aggregates was determined after 60 min [23]. The aggregation activity was expressed in aggregation units (AU) as described [2].

Lectin

The Geodia lectin was isolated and purified from the extracellular material according to Vaith et al [22]; the preparation was chromatographically pure.

Preparation of Antibodies

Antisera to the pure Geodia aggregation factor were produced in female New Zealand rabbits (weighing about 3.5 kg). The first injection was made with antigen (2.8 mg of aggregation factor) suspended in an equal volume of Freund's complete adjuvant (Difco) and given into the toe pads. Additional injections (without adjuvant) were made after 2, 4, 6, and 8 weeks subcutane-

ously over the limbs of the animals. Blood was taken from the ear veins 1 week after each immunization. The sera (termed antiaggregation factor) were obtained from clotted samples. Antiserum was raised in two animals. Precipitating and neutralizing antibodies were first detected in animals at week 3; the titers of all the sera varied between 5 and 12 μg aggregation factor precipitated per assay.

Immunological Procedures

Microdiffusion was carried out on glass microscope slides coated with 1% agarose in phosphate-buffered saline. Undiluted serum was used in all cases. The precipitin lines were stained with Amido black [22].

Antibody precipitation reaction was carried out according to the procedure described by Palmiter et al [24]. The reaction mixture contained 30 μl of 10% sodium deoxycholate in 10% Triton X-100, 20–120 μl of phosphate-buffered saline, 50 μl of antiaggregation factor, and 0–100 μl of aggregation factor in a final volume of 200 μl. Controls were included and contained control serum instead of antiaggregation factor. After standing at room temperature for 60 min aliquots of 100 μl were centrifuged in 1 × 75 mm capillaries for 2 min in a Micro-Haematocrit centrifuge (Hawksley-London) at 8,500g. The amount of precipitate formed in the capillaries was expressed in μl (1 μl corresponds to 1.3 mm of precipitate).

Analytical Methods

Protein concentration was determined spectrophotometrically at 230, 260, and 280 nm [25].

RESULTS

Reaction of Geodia Aggregation Factor With Antiaggregation Factor

Because of the high molecular weight of the Geodia aggregation factor (approximately 1.3×10^8 daltons [26]), we have chosen the antibody precipitation technique in capillaries to determine the specificity and the titer of the antiaggregation factor preparation. As shown in Figure 1, the antibodies prepared

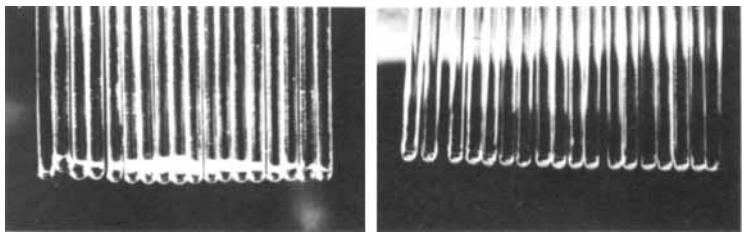

Fig. 1. Antiaggregation factor precipitation reaction. Left: Different quantities of the purified aggregation factor from Geodia (assay from the left to the right: 0, 0, 1, 1, 2, 3, 3, 5, 5, 10, 15, 20, 30, 40, 110, 120, 0, or 0 μg protein) were precipitated with a constant volume (50 μl) of antibodies, raised against Geodia aggregation factor. The precipitate was collected in capillaries (outer diameter: 1.3 mm) by centrifugation (see Methods). Right: Precipitation reaction with Tethya aggregation factor; the same quantities as in the assays with Geodia aggregation factor were used.

against Geodia aggregation factor precipitated only the Geodia aggregation factor (left figure); no precipitation was observed during the reaction with Tethya aggregation factor (right figure). In a control series, using serum from a rabbit that had not been immunized, it was clarified that no unspecific precipitation of Geodia aggregation factor occurred. Quantitative evaluation of the amount of aggregation factor precipitated by the antiaggregation factor revealed that, in the range between 1 and 5.5 μg of the factor/assay, the volume of the precipitates formed in the capillaries increased linearly (Fig. 2). Beyond 5.5 μg of factor, increasing quantities of aggregation factor resulted in smaller amounts of precipitate. In a control series using Tethya aggregation factor, precipitates of a volume smaller than 0.2 μl were formed during the precipitation reaction (Fig. 2).

Neutralization of Aggregation Factor Activity

As summarized in Figure 3, serum for an unimmunized rabbit exerted no effect on the time kinetics of the aggregation factor-mediated reaggregation of dissociated Geodia cells, compared to the control assay. However, addition of 100 μl antiserum against the Geodia aggregation factor caused a significant inhibition of the reaggregation. Addition of larger amounts of aggregation factor (200 μl antiserum/assay and higher) resulted in an abolition of the inhibitory influence on the cell aggregation (data not shown).

Precipitation Cross-Reaction

Agar gel diffusion experiments showed that control serum from unimmunized animals did not react with Geodia aggregation factor and Geodia lectin. Also, no precipitin line was observed between lectin and aggregation factor. In the experiment shown in Figure 4 (left), the antibody well contained antibodies against the Geodia aggregation factor. This antiserum reacted with the aggregation factor and the lectin under formation of strong precipitin lines.

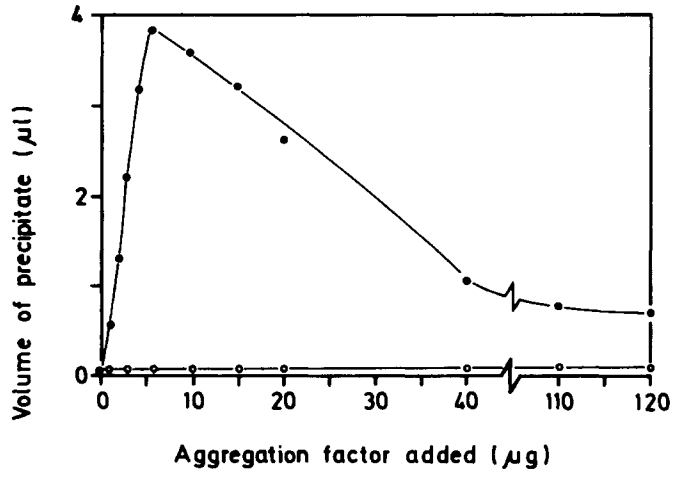

Fig. 2. Precipitation of Geodia aggregation factor (•——•) and Tethya aggregation factor (○——○) by antibodies against Geodia aggregation factor. The antibody precipitation reaction was performed as described under Methods.

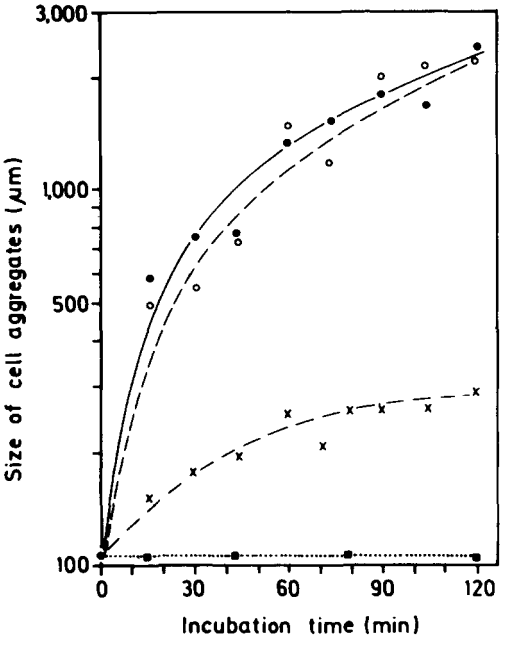

Fig. 3. Effect of antibodies against the Geodia aggregation factor on the aggregation factor-mediated reaggregation of Geodia cells. The standard aggregation assay was composed as described in Methods and contained Geodia aggregation factor at a concentration of 2×10^4 aggregation units/ml. Control assay without addition of rabbit serum (•——•). Assay, containing 100 μl of serum from an unimmunized animal (○ - ○), or 100 μl (x - • - x) of serum, containing antibodies against Geodia aggregation factor. Size of the aggregates in the absence of aggregation factor (□ ••••••••• □). The diameter of the aggregates was determined after different incubation periods. Results are from five parallel determinations; the mean values presented have a maximum variation of 25%.

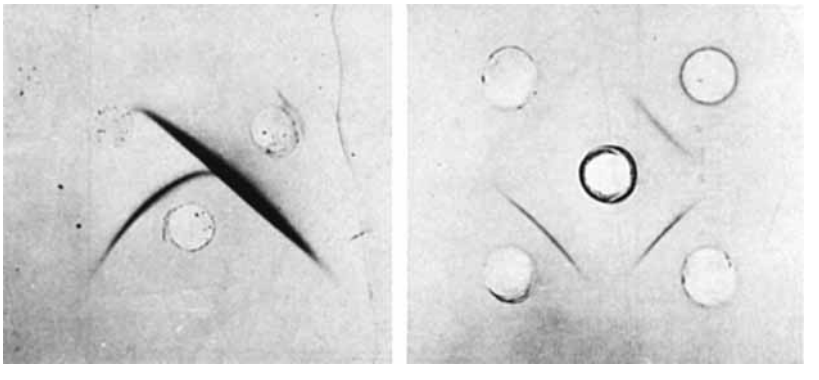

Fig. 4. Agar gel diffusion experiments with aggregation factors and Geodia lectin. Left: Reaction of Geodia lectin (0.5 mg protein/ml) (left) and Geodia aggregation factor (1.4 mg protein/ml) (right) with antiserum against the Geodia aggregation factor (below). Right: Reaction of Tethya aggregation factor with Geodia antiaggregation factor (center). The Tethya aggregation factor was assayed in the following concentrations: 0.2 mg protein/ml (upper left well), 0.6 mg/ml (upper right), 1.9 mg/ml (lower right), and 5.7 mg/ml (lower left).

Usually a spur to the lectin-containing well was observed, indicating a partial identity between a component of the aggregation factor that diffused into the gel and the lectin.

Specificity of Anti-Geodia Aggregation Factor

The influence of antiserum against the Geodia aggregation factor was studied in the heterologous system of Tethya lyncurium (Fig. 5). Chemically dissociated cells from this sponge form only small aggregates [2] of 50–80 μm in the absence of any aggregation factor. Addition of homologous aggregation factor to Tethya cells resulted in the assembly of large aggregates with a diameter of 1,500 μm after an incubation period of 90 min. This activity of the Tethya aggregation factor was found to be not influenced by control antiserum or by antiserum against Geodia aggregation factor. In a control assay using Geodia aggregation factor, earlier experiments were confirmed [9], which showed that the Geodia factor reacts species-specifically in the heterologous Tethya system.

As a further proof for the specificity of the antiserum against the Geodia aggregation factor, immunological precipitation reactions were performed. As mentioned above, the antiaggregation factor reacted in the capillary precipitation assay with the Geodia aggregation factor but not with the Tethya aggregation factor (Figs. 1 and 2). It was therefore surprising that, in the microdiffu-

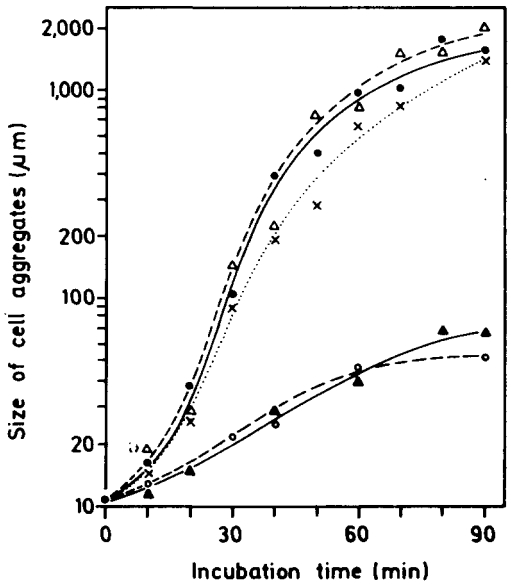

Fig. 5. Influence of antiserum against Geodia aggregation factor on the activity of the species-specific aggregation factor from Tethya lyncurium. Tethya cells were incubated in the standard reaggregation assay in the absence of aggregation factor (○ - - ○) or in the presence of Tethya aggregation factor (60 μl; 8 × 10^4 AU) (●——●), Tethya aggregation factor (8 × 10^4 AU) plus 100 μl of serum from unimmunized rabbits (△ - - - △), Tethya aggregation factor (8 × 10^4 AU) plus 100 μl of Geodia antiaggregation factor (x ····· x) or Geodia aggregation factor (10 μl; 8.4 × 10^4 AU) (▲——▲). Results are from five parallel determinations; the mean values presented have a maximum variation of 15%.

sion assay, a precipitin line was formed between the Tethya aggregation factor preparation and the antiaggregation factor (Fig. 4, right). From earlier studies, however, it is known [27] that Tethya cell extracts contain a high molecular weight glycoprotein (155,000 daltons) that interacts strongly with Geodia lectin. Therefore, at the moment, we assume that this glycoprotein might be present in the Tethya aggregation factor preparation used for the experiments and reacted with the antibodies. Without further data this supposition seems to be possible, especially in the light of the above-mentioned property of the antibodies to recognize not only determinants on the Geodia aggregation factor but also those on the Geodia lectin.

As in the experiments with the Tethya aggregation factor, the species specificity of the antiserum raised against Geodia aggregation factor was tested against the other 14 heterologous aggregation factors. The characteristics of these heterologous factors are summarized under Methods. Both in the microdiffusion assay and in the capillary precipitation assay, no precipitation was observed if these aggregation factors were tested against anti-Geodia aggregation factor. The antiserum also had no influence on the biological activity of the aggregation factors other than that, isolated from Geodia cydonium.

DISCUSSION

In this paper we present the first direct evidence that it is indeed the previously isolated Geodia cydonium aggregation factor that is involved in the initial adhesion of dissociated sponge cells, by applying antibodies raised against purified aggregation factor. The aggregation factor used for the production of antibodies in rabbits was contaminated neither with Geodia lectin nor with material from the sponge Tethya lyncurium. Eight antisera with almost identical titers were obtained from two animals. The aggregation factor-mediated reaggregation was found to be specifically inhibited by antibodies against the homologous aggregation factor. The inhibitory effect of antiaggregation factor could be neutralized by an excess of aggregation factor. This finding again indicated an interaction between the antibodies and the aggregation factor. The antibodies raised against the Geodia aggregation factor neutralized its functional activity only in the homologous system. These antibodies were found to be inactive in the heterologous Tethya lyncurium system. Microdiffusion as well as antibody precipitation studies revealed these antibodies to form complexes, with one exception (discussed under Results), only with the Geodia aggregation factor; no precipitation of the anti-Geodia aggregation factor was obtained with 13 other heterologous aggregation factor preparations. From these results two important consequences can be drawn: 1) Only one single extracellular molecular mechanism controls reaggregation during at least the time of observation chosen for the present study, and 2) the formation of cell–cell bonds is due to the one species-specific ligand (the species-specific aggregation factor).

Interesting was the finding that the antiaggregation factor preparation formed a precipitation with purified Geodia lectin in the immunodiffusion assay. In early studies we showed that the Geodia lectin and the Geodia aggregation factor are two different functional molecules [22, 9]; the Geodia lectin does not cause aggregation or agglutination of Geodia cells, and the purified Geodia aggregation factor is not associated with hemagglutinating activity. We

therefore have to conclude that a component that is associated with the aggregation factor is antigenetically related to the homologous lectin.

In a future report the antibodies against the Geodia aggregation factor and the Geodia lectin will be used to elucidate whether the aggregation factor is processed to the lectin (or vice versa) by, for example, the action of a protease. Whereas the Microciona aggregation factor does not exert hydrolytic activity [7], results with the Geodia aggregation factor indicate the presence of a protease (unpublished results). With these tools at hand, it should be also possible to clarify whether the Geodia aggregation factor consists of two types of binding sites (first, between the aggregation factor and the aggregation receptor, and second, between two aggregation factor molecules as proposed for the Microciona system [10, 16]); or only one type of recognition site (between the aggregation factor and the aggregation receptor).

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