MATING-REACTIVE MEMBRANE VESICLES FROM CILIA OF *PARAMECIUM CAUDATUM*

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Conjugation of *Paramecium* is normally induced by interaction between cells of complementary mating types (10). When these cells meet under appropriate conditions, they stick together in an agglutinate. This agglutinative adhesion is called the mating reaction (11) and it is known that the union of the cells in this reaction occurs between the cilia of complementary mating types. When the cilia detached from mating-reactive cells of one mating type and the mating-reactive living cells of the opposite type are brought together, the former adhere to the latter (1, 8, 9). Furthermore, Takahashi et al. (12) have recently shown the agglutination of cilia detached from complementary mating types. These facts strongly support the previous conclusion that cilia are essential for the mating reaction and suggest that the cilia of mating-reactive paramecia are likely to bind the mating type substances (or mating substances).

This article deals with isolation of small membrane vesicles with mating reactivity from cilia of *Paramecium*. Electron microscope observation of the vesicles and analysis of their components by polyacrylamide gel electrophoresis are also presented.

MATERIALS AND METHODS

The stocks used were Ksy 1 and KoK 1 of mating type V, and d-N14a and d-12b of VI in syngen 3 of *Paramecium caudatum*. The culture medium was 1.25% (wt/vol) lettuce juice medium (5) inoculated with *Klebsiella aerogenes* usually 1 day before use. Cilia were isolated by the $MnCl_2$ method (3) with slight modifications (50 mM $MnCl_2$ was used instead of 10 mM).

Cilia obtained by the above method were treated with a solution containing 2 M urea, 0.1 mM Na_2-EDTA, and 5 mM Tris-HCl, pH 7.3, for 60 min at 4°C. Concentration of the total ciliary proteins in this suspension was about 0.25 mg/ml. The cilia thus treated were precipitated by centrifugation at 9,000 *g* for 10 min and the supernate was percolated through 0.45 μm Membranfilter (Sartorius SM11306) to remove remaining cilia fragments. Then the filtrate was dialysed against 3 liters

of 10 mM Tris-HCl, pH 7.3, overnight with a change of the same buffer. The dialysate was centrifuged at 105,000 *g* for 60 min and the pellet was called the urea-EDTA particles (Fig. 1).

To determine mating activity, a geometric series of 50% dilutions of the urea-EDTA particles was prepared. Two drops (about 30 μl) of samples from each dilution were dispensed with a micropipet into 0.4 ml of a suspension of mating-reactive cells of the opposite mating type (tester) (cell density, about 2,000 cells/ml). Observation was made 5–10 min after mixing. The unit of mating activity was indicated as the reciprocal of the lowest concentration (milligrams/milliliter) of proteins that can induce more than 10 clumps in about 800 mating-reactive tester cells suspended in 0.4 ml of the stationary phase culture medium. The amount of protein was determined by the method of Lowry et al. (6). Estimations of mating activities of the intact cilia and those treated with the urea-EDTA solution (the treated cilia) were also performed by the same method.

For electron microscopy, a small drop of the suspension of the urea-EDTA particles was placed onto a 150-mesh copper grid covered with a carbon-coated collodion film, negatively stained with 2% uranyl acetate

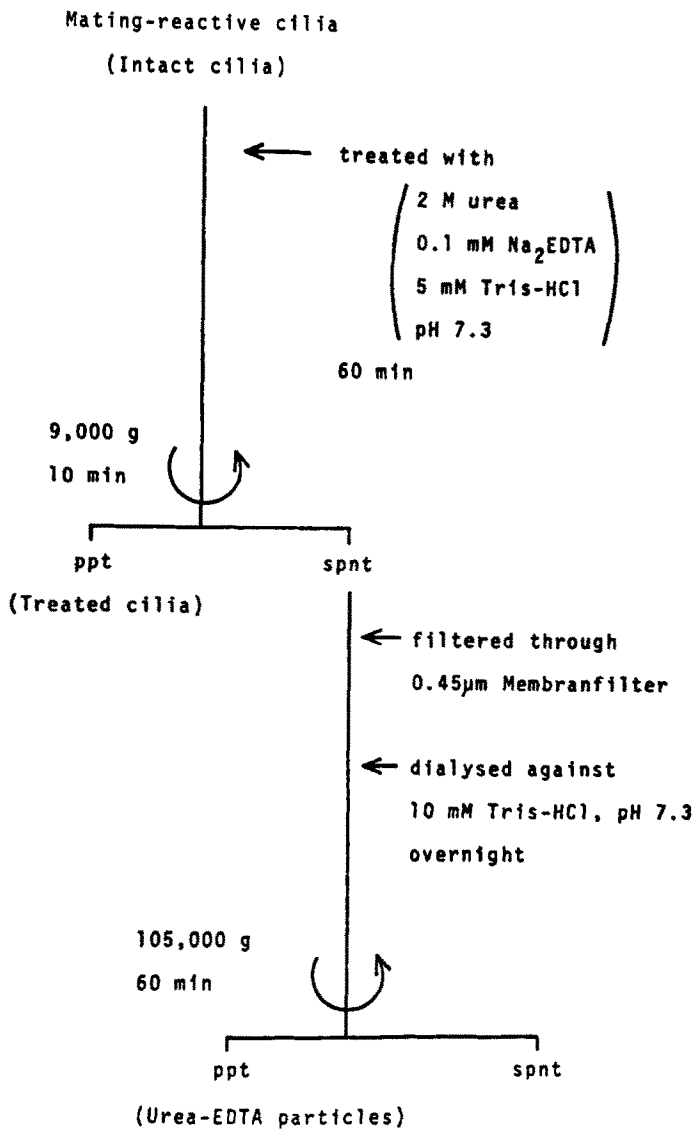

FIGURE 1 Schematic presentation of the procedure of isolating the urea-EDTA particles of *Paramecium caudatum*. For explanation, see text.

TABLE I

Distribution of Proteins after Extraction of Cilia of Paramecium caudatum by 2 M Urea and 0.1 mM Na_2EDTA

Stock	Mating type	Number of experiments	Percent of proteins recovered*		
			Treated cilia	Urea-EDTA particles	Soluble fraction
d-N14a	VI	2	14.0 ± 2.1	10.4 ± 0.2	36.5 ± 0.2
Ksy 1	V	5	16.9 ± 2.5	4.5 ± 1.1	25.2 ± 5.7

* Mean values of different experiments. 26.9 ± 6.4 were absorbed by the 0.45 μm Membranfilter.

TABLE II

*Specific Activities of Intact Cilia (IC), Urea-EDTA-Treated Cilia (TC), and Urea-EDTA Particles (UP)**

Stock (mating type)	Fraction	Concentration in end point‡	Units§
		μg/ml	
d-N14a	IC	13	77
(VI)	TC	75	13
	UP	12	83
Ksy 1	IC	11	91
(V)	TC	90	11
	UP	7	140
Kok 1	IC	6.5	150
(V)	TC	31	32
	UP	6.5	150

* For experimental details, see Fig. 1.

‡ Concentration in end point is the lowest concentration of proteins inducing cells of opposite mating type to form large clumps.

§ Units of mating reactivity, see Materials and Methods.

and observed under the electron microscope (Hitachi HS9 75 kV).

SDS polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (13) with some modifications. Gels of 5% acrylamide plus 0.134% *N,N'*-methylenebis-acrylamide were polymerized with 0.75 mg/ml ammonium persulfate and 0.15% *N,N,N',N'*-tetramethyl ethylenediamine as catalyst in in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% SDS. Gels of 75 mm were prepared in glass tubes of 80-mm length and 5 mm ID. The respective samples were solubilized in 10 mM phosphate buffer, pH 7.2, with 1% SDS, 1% 2-mercaptoethanol (2-ME), and 8 M urea for 3 h at 35°C. Then denatured and reduced samples were dialysed overnight against 10 mM phosphate containing 0.1% SDS, 0.05 M iodoacetate, and 8 M urea at 25°C. Electrophoresis was run at 6 mA per column for 230 min. After electrophoresis the gels were stained with Coomassie brilliant blue according to the method of Fairbanks et al. (2).

RESULTS

10–20 mg (as protein) of mating-reactive cilia were prepared from a 16-liter culture of paramecia by the modified $MnCl_2$ method. The urea-EDTA particles were obtained from the cilia by the procedure mentioned above. The distribution of ciliary proteins after extraction of the cilia with 2 M urea and 0.1 mM EDTA is shown in Table I. Differences were recognized between the distribution patterns of stocks Ksy 1 and d-N14a. The urea-EDTA particles obtained were 4.5% and 10.4% of total ciliary proteins, respectively. Only 50% of the proteins were recovered and the remaining 50% were lost. Among this loss of 50%, about 27% were those absorbed by the 0.45 μm Membranfilter.

When the particles thus obtained were added to mating-reactive cells of the complementary mating type, strong agglutination occurred. The agglutinated cells then underwent self pairing formation, and more than 90% of them performed normal conjugation processes, including meiosis and macronuclear breakdown. When the particles were added to mating-reactive cells of the same mating type, neither agglutination of the cells nor induction of conjugation occurred.

The specific activities of the urea-EDTA particles were compared with those of intact cilia and of treated cilia (Table II). In all stocks employed, the urea-EDTA particles showed the highest activity to induce large clumps in the cells of the complementary mating type, and the treated cilia showed the lowest. The number of the tester cells used in this assay was about 800 in 0.4 ml. The lowest protein concentration of the particles to induce mating reaction was about 10 μg/ml.

When the particles of stock d-N14a were preserved in 10 mM Tris-HCl, pH 7.3, at 4°C, their mating reactivity did not decrease for 3 wk. Similarly, the particles from other stocks also

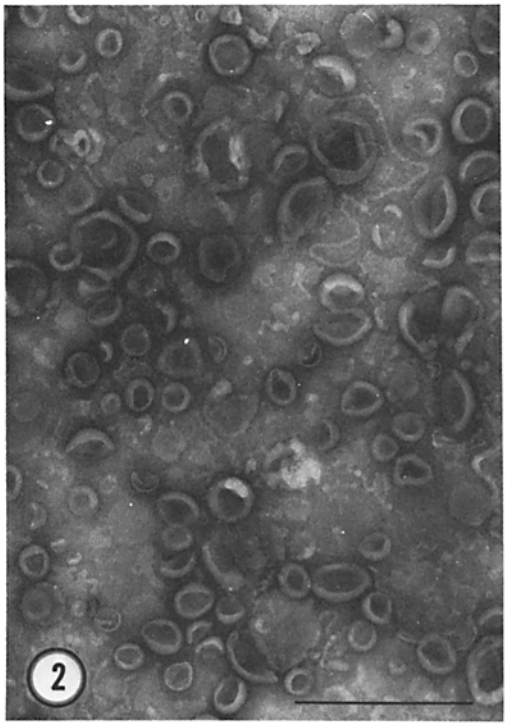

FIGURE 2 Electron micrograph of negatively stained preparation of the urea-EDTA particles from stock d-12b, mating type VI. Calibration bar is 0.5 μm.

retained their reactivity for more than 1 wk at least.

The urea-EDTA particles were negatively stained with uranyl acetate and observed under the electron microscope. The electron micrographs showed that the particles consist chiefly of vesicles with a diameter of about 100–150 nm (Fig. 2).

Intact cilia and the urea-EDTA particles of stocks KoK 1 (mating type V) and d-N14a (VI) were electrophoresed on polyacrylamide disc gels. About 30 bands, including three predominant groups, were detected in intact cilia preparations of both stocks (Fig. 3 A, B). On the other hand, the urea-EDTA particles from both complementary mating types showed as many as 20 bands (Fig. 3 C, D). Some of the latter bands were included in the pattern of intact cilia. The results show that the particles are still very complex in their composition of proteins. No great difference in the patterns between different mating types was observed.

DISCUSSION

The urea-EDTA particles here reported have a strong specific activity not only to agglutinate mating-reactive cells of only the opposite mating type but also to induce self conjugation in them. The particles can be prepared from cells of both complementary mating types, V and VI in syngen 3 of *P. caudatum*. Since the activity to induce neither mating agglutination nor conjugation was detected in the supernatant fluid from a 105,000 *g* centrifugation of the urea-EDTA extract, the molecules involved in the mating activity appear to be tightly bound to the urea-EDTA particles.

Electron microscope observation revealed that the urea-EDTA particles are a sort of membrane vesicle with a diameter of 100–150 nm. Recently, McLean et al. (7) revealed that the isoagglutinating substances in *Chlamydomonas moewusii* are really membrane vesicles. Although the particles from *Paramecium* here obtained are similar membrane vesicles, they are not so easily released into

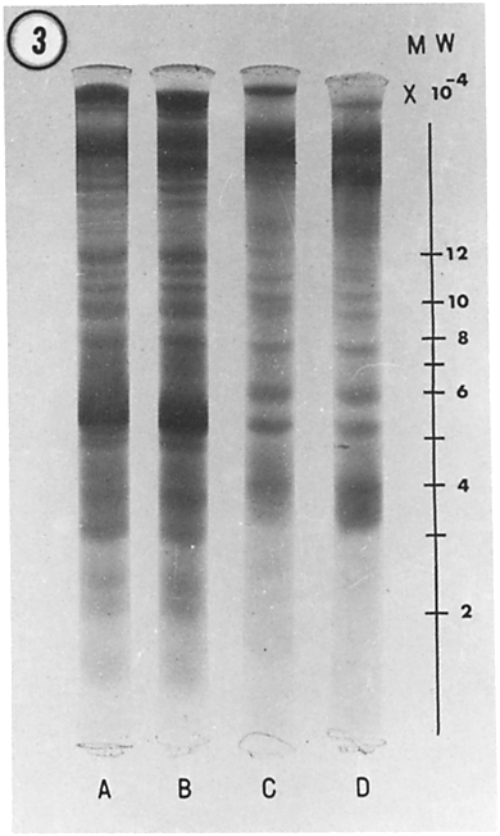

FIGURE 3 Disc electrophoretic patterns of intact cilia and the urea-EDTA particles. A and B, intact cilia of stocks Kok 1 (mating type V) and d-N14a (mating type VI), respectively; C and D, urea-EDTA particles of stocks Kok 1 and d-N14a, respectively.

the medium as those of *Chlamydomonas*. Moreover, particles could not be obtained in a mating-reactive form simply by sonication (two 5-A bursts for 10 s each).

When the urea-EDTA particles were subjected to the same sonication scheme, mating reactivity was completely lost. Electrophoretic patterns of SDS polyacrylamide gel show that the particles possess as many as 20 bands, indicating that the particles are chemically very complex. Unfortunately, we could not show a clear mating type difference between polypeptide patterns of the particles from complementary mating types. Perhaps the amount of mating type substances may be under the limit to be detected by this method or the molecular weights of the two mating substances may closely resemble each other. Otherwise, the mating type difference may be due to a difference of specific polysaccharides attached to proteins. Preliminary experiments indicate that the particles contain at least four kinds of glycoproteins.

Although the specific activity of the mating particles was as high as that of intact cilia, it was not so high as we expected. The principal cause of this level of activity would be that the urea-EDTA particles which are obtained from the cilia preparation contained some nonmating-reactive particles because the cilia on the dorsal surface of the cell are known to have no mating reactivity (4).

The mating particles obtained by the urea-EDTA extraction are a complex, water-insoluble system. Nevertheless, they are the simplest system so far obtained which can induce the whole process of conjugation including the initial mating reaction.

SUMMARY

Membrane vesicles with a high mating reactivity were obtained from cilia of *Paramecium caudatum* by treatment with a solution containing 2 M urea and 0.1 mM Na_2-EDTA. All processes of conjugation were induced in cells of the complementary mating type by approximately 10 μg/ml proteins of the vesicles. Electron microscope observation showed that the membrane vesicles have a diameter of 100–150 nm. Electrophoretic analysis on SDS polyacrylamide gel revealed no significant difference in polypeptide patterns of the particles from the two complementary mating types.

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REFERENCES

1. COHEN, L. W., and R. W. SIEGEL. 1963. The mating-type substances of *Paramecium bursaria*. *Genet. Res. Cb.* **4**:143–150.
2. FAIRBANKS, G., T. L. STECK, and D. F. H. WALLACH. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. **10**:2606–2617.
3. FUKUSHI, T., and K. HIWATASHI. 1970. Preparation of mating reactive cilia from *Paramecium caudatum* by $MnCl_2$. *J. Protozool.* **17**(*Suppl.*):21.
4. HIWATASHI, K. 1961. Locality of mating reactivity on the surface of *Paramecium caudatum*. *Sci. Rep. Tohoku Univ. Fourth Ser. (Biol.)* **27**:93–99.
5. HIWATASHI, K. 1968. Determination and inheritance of mating type in *Paramecium caudatum*. *Genetics*. **58**:373–386.
6. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
7. MCLEAN, R. J., C. J. LAURENDI, and R. M. BROWN, JR. 1974. The relationship of gamone to the mating reaction in *Chlamydomonas moewusii*. *Proc. Natl. Acad. Sci. U. S. A.* **71**:2610–2613.
8. METZ, C. B. 1954. Mating substances and the physiology of fertilization in ciliates. *In* Sex in Microorganisms. D. H. Wenrich, editor. American Association for the Advancement of Science, Washington, D. C. 283–334.
9. MIYAKE, A. 1964. Induction of conjugation by cell-free preparations in *Paramecium multimicronucleatum*. *Science (Wash. D. C.)*. **146**:1583–1585.
10. SONNEBORN, T. M. 1937. Sex, sex inheritance and sex determination in *Paramecium aurelia*. *Proc. Natl. Acad. Sci. U. S. A.* **23**:378–385.
11. SONNEBORN, T. M. 1939. *Paramecium aurelia:* mating types and groups; lethal interactions; determination and inheritance. *Am. Naturalist*. **73**:390–413.
12. TAKAHASHI, M., N. TAKEUCHI, AND HIWATASHI. 1974. Mating agglutination of cilia detached from complementary mating types of *Paramecium*. *Exp. Cell. Res.* **87**:415–417.
13. WEBER, K., and M. OSBORN. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406–4412.