A new monoclonal antibody inhibiting mating agglutination in *Paramecium caudatum*

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(Communicated by Koichi HIWATASHI, M.J.A., Oct. 12, 2001)

Abstract: Mating reaction occurs between the cells of the odd and even complementary mating types in *Paramecium caudatum*. The molecules involved in this cell-to-cell sexual recognition called mating type substances are suggested to be proteins and exist on the ventral surface cilia of the cell. To obtain more varied epitopes, mating-reactive membrane vesicles reconstituted from odd mating type cilia of syngen 3 were used as antigen. A new monoclonal antibody XomO inhibiting the mating reactivity of both mating types was obtained. The unusual characteristic of XomO is the localization of the antigen, which particularly is recognized on basal portion, and not the whole length, of the ventral surface cilia. The antigen was suggested to be membrane protein and the detection of the antigen was correlated with the mating reactivity of the cells. These results suggest that the antigen of XomO is a substance involved in mating reaction under a dynamic-conformational change.

Key words: Mating-type substance; *Paramecium caudatum*; monoclonal antibody.

Introduction. In hypotrich ciliates, conjugation-inducing substances (called gamones) are secreted in the culture medium and their chemical structures have already been elucidated.^{1)–3)} Membrane bound conjugation-inducing substances, such as mating type substance in *Paramecium*, have not been identified so far. When the odd and even complementary mating type cells of *P. caudatum* are mixed together under appropriate conditions, they undergo massive agglutination, which eventually leads to conjugation. This agglutination, resulted from syngen-specific cell-to-cell sexual recognition, is referred to as the mating reaction.⁴⁾ Gilman^{5),6)} once reported 16 syngens (sibling species, groups of complementary mating types) in *P. caudatum*. Mating-type substances involved in the mating reaction, are protein and exist on the membrane of the ventral surface cilia of highly mating-reactive cells.^{7)–10)} Immunological methods are suggested to be a useful way to isolate and monitor the biosynthetic pathway of mating type substances. In such an approach, monoclonal antibodies specifically inhibiting mating reactivity of odd mating types of *P. caudatum* with no immobilization effect had been obtained,¹¹⁾ but the identification of the

antigen has not been successful.

To obtain varied epitopes, we prepared mating-reactive membrane vesicles reconstituted from mating-reactive cilia of O³ (odd mating type of syngen 3) as the antigen. A monoclonal antibody XomO, inhibiting mating reactivity of both odd and even mating types, was obtained. The antigen is a membrane protein and detection of the antigen was correlated with the mating reactivity of the cells. The unique localization of the antigen in the basal portion of the ventral surface cilia suggested that the antigen is a substance connected with the mating reaction under a dynamic change.

Materials and methods. *Strains and culture.* The main strains used in the study were Bky13, the odd mating type of syngen 3 (O³), and C103s7 (a selfing progeny of C103, kindly supplied by Dr. T. Watanabe, Tohoku University) belonging to the even mating type of syngen 3 (E³). Cells were cultured at 25 °C in the 2.5% fresh lettuce juice medium¹²⁾ diluted with modified Dryl's solution (called K-DS), prepared by substituting KH₂PO₄ for NaH₂PO₄.¹³⁾ They were incubated with *Klebsiella pneumoniae* one day before use. Several hundred cells were inoculated into 2 ml of culture medium and then double volumes of fresh medium were added on successive 4 days. One day after the last

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feeding, mating reactive cells were obtained. To obtain starved cells, mating-reactive cells (about 5×10^6 cells) were washed and transferred into the K-DS and 5 days later, very starved cells completely without mating reactivity were obtained. To obtain well-fed cells, about 5×10^5 cells were transferred into 500 ml fresh culture medium and 16 h later, the cells in log phase without mating reactivity were obtained.

Immature cells. Immature cells were obtained basically by the method of Takahashi *et al.*¹⁴⁾ Mating-reactive cells of Ugy 144 (O^3) and Kyk 5-2 (E^3) were mixed together and conjugating pairs were isolated into the Dryl's solution and then transferred into the fresh culture medium two days later. When a single-exconjugant clone divided about 16 fissions, the mating reactivity of these cells was tested to confirm that the macronuclear degeneration did not occur.¹⁵⁾ The cells without mating reactivity were used in dot-blot analysis as immature cells.

Preparation of the antigen. Cilia detached from cells were obtained by the method used in the previous report.¹¹⁾ About 5×10^7 packed cells with high mating reactivity were treated for 15 min with an ice-cold Triton X-100 solution containing 0.008% (v/v) Triton X-100, 20 mM KCl, 10 mM of tripotassium salt of EDTA and a 10 mM MOPS buffer, pH 7.0 (adjusted by KOH). The cells were centrifuged at 600 *g* for 2 min and then transferred into an ice-cold deciliation solution of 20 mM $CaCl_2$ in 10 mM Tris, pH 7.4. Twenty minutes later, a suspension of cilia was obtained by slow centrifugation at 600 *g* for 2 min and then filtered through a sheet of filter paper (Advantac Co., Ltd., Tokyo, Japan). The filtrate was centrifuged at 10,000 *g* for 30 min. The precipitated cilia were washed by the same centrifugation twice in K-DS. The cilia were treated with the SM 1200 solution containing 0.5% sucrose monolaurate (Wako Chemicals, Osaka, Japan), 10 mM Tris, pH 7.4, 10 mM EDTA pH 7.0 (adjusted by KOH), and 10 μ g/ml Leupeptin hemisulfate monohydrate (Wako Chemicals, Osaka, Japan) for 30 min on ice. Then, they were centrifuged at 12,000 *g* for 30 min. The supernatants were centrifuged again in 100,000 *g* for 1 h at 4 °C. The supernatant was diluted with 10 mM Tris buffer, pH 7.2, containing 10 μ g/ml Leupeptin hemisulfate monohydrate and set overnight at 4 °C. The diluted solution was centrifuged again at 100,000 *g* for 2 h at 4 °C. The mating reactivity of the pellets (membrane vesicles) was tested and only those with mating reactivity were used as the antigens.

Production of monoclonal antibodies. One hun-

dred μ l of the antigens (0.1-0.2 mg) described in *Preparation of the antigen* were injected into spleen of the BALB/c mice 5 times in 2-week intervals. Three days after the last boost, the spleen cells were fused with p3-X63-Ag8-653 mouse myeloma cells by polyethylene glycol (1500; Boehringer Mannheim, Germany) according to the method described by Galfer and Milstein.¹⁶⁾ Culture fluids from the hybridoma cells were subjected to screening. Fifty μ l of the highly mating-reactive cell suspension of Bky13 was incubated with the same volume of culture fluid of the hybridomas for 20 min after adding 50 μ l of 12.5 mM phosphated buffer (pH 7.2), and then highly mating-reactive C103s7 cells were added. The hybridomas secreting the antibodies which inhibited mating reactivity of Bky13 cells without immobilization effect were cloned by limiting dilution.

Determination of inhibition of mating reactivity. Fifty μ l of the highly reactive cell suspension was incubated with the same volume of culture fluid of hybridomas diluted with 12.5 mM phosphate buffer, pH 7.2, and then complementary cells (the tester) were added. Mating reactivity was observed 10-15 min after adding the testers. The degree of inhibition of the mating reactivity by antibodies was estimated by the size of the clumps or agglutinates compared with that of when the cells without the antibody treatment were mixed with the complementary mating type. Culture fluid of myeloma cells was used as the control of the monoclonal antibodies.

Dot-blot Method. Fifty μ l samples of cells in each stage were applied on the ImmobilonTM Transfer membranes (Millipore, U.S.A.) with a Millipore dot-blot system. The membranes were blocked with 3% skim milk (Wako Chemicals, Osaka, Japan) in TBS (150 mM NaCl in 10 mM Tris, pH 7.2) for 2 h at room temperature. The membranes were incubated with the supernatants of the hybridoma culture containing monoclonal antibody for 2 h at room temperature and then washed with TBS-T solution (0.5% (v/v) Tween-80 in TBS) 3 times and twice with TBS. The membranes were incubated with HRP-conjugated goat anti-mouse IgG for 2 h at room temperature and then washed as indicated in the previous steps. Dot-blot signals on the membranes were detected by addition of substrates using Konica immunostain HRP-1000 (Konica, Osaka, Japan). To obtain the samples of the surface antigen, the packed cilia were treated with the salt alcohol solution containing 0.45% NaCl, 15% EtOH and 10 mM Tris buffer, pH 7.2, stirring for 6 h at 4 °C after Preer and Preer.¹⁷⁾ This solution was centrifuged at 12,000 *g* for 30 min at 4 °C. The supernatant

Table I. Inhibition of the mating reactivity in *Paramecium caudatum*, syngen 3, by monoclonal antibody XomO

Dilution (1/X)	XomO		Myeloma (control)	
	O ³	E ³	O ³	E ³
4	–	–	+	+
8	–	–	+	+
16	–	±	+	+
32	±	+	+	+
64	+	+	+	+

Cells of O³ (Bky13) and E³ (C103s7) were incubated for 20 min in diluted supernatant of the hybridoma culture containing the antibody XomO, and then tester cells of the complementary mating types were added. – indicates no mating reaction; weak mating reaction and strong mating reaction are indicated by ± and +, respectively. The marks of –, ±, and + in every point indicate the results of three independent experiments. The supernatant of myeloma culture and its dilution series were used as controls.

contained mainly the surface antigens. Both the supernatant and pellet were used in dot-blot analysis.

Indirect immunofluorescence. To know the localization of antigens by indirect immunofluorescence, cells were incubated for 20 min with the antibody XomO before the fixation. The cells were fixed for 45 min with 2% Paraformalin solution containing 25 mM KCl, 10 mM phosphated buffer, pH 7.2, at room temperature and washed 3 times with 25 K·PB (25 mM KCl in 10 mM phosphated buffer, pH 7.2). Then, the cells were incubated with fluorescein-conjugated goat anti-mouse IgG (CPL, U.S.A., 1 : 100 dilution) for 2 h at room temperature. The treated cells were mounted with 90% glycerol with 1% p-phenylene diamine and observed with a confocal laser-scanning microscope (CLSM) (BioRad MRC-500).

Results. *Inhibition of the mating reactivity of both complementary mating types in P. caudatum.* After screening, monoclonal antibody XomO inhibiting mating reactivity of O³ and E³ was obtained. The inhibition profiles of the XomO are shown in Table I. Tester cells of Bky 13 (O³) and C103s7 (E³) were incubated for 20 min in the supernatant of the hybridoma culture containing antibody XomO, and in that of myeloma culture as controls. Inhibiting effects on the mating reactivities of O³ and E³ without ciliary immobilization were observed on dilutions of 16 and 8, respectively. Incubation with culture of the myeloma has no effect on the mating reactivity of both mating type cells. Moreover, XomO did not inhibit the mating reaction of both mating types of syn-

gen 1 (data not shown).

Cellular localization of antigen recognized by the XomO. We determined the localization of the antigen of XomO in O³ and E³ cells by indirect immunofluorescence (Fig. 1). The ventral surface cilia of both mating type cells were specifically labeled (Fig. 1B, D). The most noticeable thing is that the antigen was localized specifically on the basal portion (1/3 to 1/2 from the base) of the cilia (Fig. 1F, G). No antigen was detected in the immature cells that are non-mating-competent (date not shown).

Correlation between the detection of the antigen and mating reactivity of the cells. Inhibiting effect of the antigen on the mating reactivity of both mating type cells indicated that the antigen is involved in the mating reaction. Thus, we determined whether the detection of the antigen was associated with the mating reactivity of the cells (Fig. 2). The monoclonal antibody OmE, which suggests to be raised against the mating type substances of O³, was used as the positive control. Strong dot-blot signals of the antigen by using XomO were detected only from the mating-reactive O³ and E³ cells as the same as when OmE was used (Fig. 2). Although almost no antigen was detected from the starved O³ and E³ cells that are completely without mating reactivity, faint signals of the antigen was detected from the well-fed cells in logarithmic growth phase (Fig. 2). No antigen was detected from the immature cells that are non-mating competency (Fig. 2).

The antigen of antibody XomO is a membrane protein. The mating-type substances are suggested to be tightly bound membrane protein.¹⁰⁾ We treated the packed cilia with salt alcohol solution to obtain the sample of surface antigen from cilia as usual.¹⁷⁾ The antigen of XomM, which is an immobilization antigen, was detected from both the supernatant and pellet (Fig. 3), and suggests that not all surface antigens were removed from the cilia by this treatment. In the same with the antigen of antibody OmE, which is a membrane protein,¹⁸⁾ little antigen of XomO was detected from the sample of supernatant (Fig. 3. lanes OmE and XomO).

Discussion. *Antigen correlated with mating reaction.* Using mating-reactive membrane vesicles reconstituted from the cilia to immunize the mouse, we obtained monoclonal antibody XomO. The antibody XomO inhibited the mating reactivity of O³ and E³ cells. The inhibition of mating reactivity by XomO, which was not as effective as those monoclonal antibodies reported by Azuma *et al.* (1996), and this may partially result from the localization of the antigen only on the basal portion of

Fig. 1. Cellular localization of the antigen detected by monoclonal antibody, XomO, in *Paramecium caudatum*. Laser-scanning confocal microscopic images (B, D) and difference interference contrast images (A, C) of the same cells (A and B, C and D) are shown, respectively. (A-P) Anterior-posterior axis. To show the localization of the antigen in a cilium, central section images of the O³ cell (E, F) and the merged image (G) are presented. The antigens of XomO were detected from root (basal portion) of ventral surface cilia of both mating type cells. Bar = 20 μ m in all images. Arrow indicates the same cilium.

the ventral surface cilia. But the detection of the antigen was associated with the mating reactivity of the cells (Fig. 2). These results clearly indicate the antigen of XomO is a substance associated with mating reaction. The most noticeable thing is that the antigen was only detected on the basal portion and not on tail portion of the cilium. What is happening on the antigen on a cilium? A conformational change of the molecules might occur and XomO cannot recognize the substances on the tail any more.

The antigen might be mating-type substance.
About the mating-type substances, a precursor-product

hypothesis was first suggested for *P. aurelia* by Butzel¹⁹⁾: the odd mating-type substances are the basic substances, and the activity of the *Mt* locus changes the odd mating-type substances into the even mating-type substances. This hypothesis explained the fact that the unidirectional mating-type change of selfing stocks occurs naturally only in even mating types.^{20)–22)} The determination and inheritance of mating types in *P. caudatum* have been extensively analyzed and revealed that three gene (i.e. *Mt*; *MA*; *MB*) are involved in them.^{23),24)} *Mt* is epistatic to *MA* and *MB* and determines the syngen-specificity of even mating types. *MA*

Fig. 2. The detection of the antigen was correlated with the mating reactivity of cells. Each line was incubated with the supernatants of the hybridoma cultures containing monoclonal antibody XomO and OmE, and the supernatant of culture medium of myeloma, respectively. Antibody OmE, which is raised against odd mating-type substance of O³ was used as a positive control. The supernatant of the myeloma culture was used as a negative control.

and MB are expressed only in the recessive homozygote at *mt* locus and determine the odd mating-type specificity of each syngen. However, the relationship between the three genes and the odd and even mating-type substances is not known. In *P. aurelia*, a protein in the ciliary membrane is absent in the log phase, increases in the early stationary phase and decreases in the starved cells.²⁵⁾ However, no difference in protein pattern has been observed between mating reactive odd and even cilia in *P. aurelia*²⁵⁾ and in *P. caudatum*.^{10),26)} Furthermore, we found that the E³ cytoplasm contains mating-reactive O³ mating-type substances.¹⁸⁾ Odd mating-type substances might be precursor molecules of even mating-type substances and the achievement of the mating reactivity of even mating-type substance might be exposing the different mating-reactive domain of the same molecule. The antigen of XomO is a membrane protein and the detection of the antigen is closely related to the mating reactivity of the cells. The notable characteristic of antibody XomO is that the antigen is only localized on the root (basal portion) of the ventral surface cilia (Fig. 1F, G). Thus, it is reasonable to suppose that antibody XomO is recognizing the mating-type substances, which are being transferred to the end of the cilia. The mating-type substances on the end of the cilia may have different conformations from those on the basal portion and cannot be recognized by XomO any more.

In *Euplotes octocarinatus*, gamones are secreted

Fig. 3. The antigen of monoclonal antibody XomO is a membrane protein. The supernatants of the myeloma culture or OmE were used as negative or positive control. XomM is the antibody against immobilization antigen. Dot-blot results show that surface antigens are contained in both the supernatant and pellet (XomM), while membrane proteins contained only in the pellet (OmE and XomO). Abbreviations of sup and ppt mean supernatant and pellet, respectively.

via the cortical ampules.²⁷⁾ But no research shows from where the mating-type substances are transferred to the cilia although the localization is postulated to be on ventral surface cilia.⁷⁾ If the antigen of XomO is mating-type substance, use of this antibody can help us to visual the transporting pathway of membrane protein, such as mating-type substances. Thus, oral cortical region may be a place for transporting various proteins including mating-type substances.

This report is not the first time to obtain an antibody inhibiting mating reactivity without mating-type specificity, although their localization of the antigens have not been reported.^{28),29)} If the antigens of these antibodies were not mating-type substances themselves, there must be some other factors involved in the mating reaction.

Acknowledgements. This work was supported, in part, by Grants-in-Aid for Exploratory Research (No. 10878132) from the Ministry of Education, Sports and Culture of Japan.

References

- 1) Miyake, A. (1996) In *The Ciliates: Cells as Organisms* (eds. Hausmann, K., and Bradbury, P. C.). G. Fischer Verlag, Stuttgart, pp. 243-290.
- 2) Brunen-Nieweler, C., Weiligmann, D. W., Hansen, B., Kuhmann, H. W., Mollenbeck, M., and Heckmann, K. (1998) *Europ. J. Protistol.* **34**, 124-132.
- 3) Ortenzi, C., Alimenti, C., Vallesi, A., Di Pretoro, B., La Terza,

- A., and Luporini, P. (2000) *Mol. Biol. Cell* **11**, 1445-1455.
- 4) Sonneborn, T. M. (1937) *Proc. Natl. Acad. Sci. U.S.A.* **23**, 378-385.
- 5) Gilman, L. C. (1954) *J. Protozool.* **1**, s6.
- 6) Gilman, L. C. (1962) *J. Protozool.* **9**, s13.
- 7) Hiwatashi, K. (1961) *Sci. Rep. Tohoku Univ. Ser. (Biol)* **27**, 93-99.
- 8) Cohen, L. W., and Seiegel, R. W. (1963) *Genet. Res.* **4**, 143-150.
- 9) Takahashi, M., Takeuchi, N., and Hiwatashi, K. (1974) *Exp. Cell Res.* **87**, 414-417.
- 10) Kitamura, A. (1988) In *Paramecium* (ed. Görtz, H.-D.). Springer-Verlag, Berlin, pp. 85-96.
- 11) Azuma, Y., Kaku, E., Maruo, F., and Takahashi, M. (1996) *Europ. J. Protistol.* **32**(Suppl. 1), 4-9.
- 12) Dryl, S. (1959) *J. Protozool.* **6**, s25.
- 13) Hiwatashi, K. (1968) *Genetics* **58**, 373-386.
- 14) Mikami, K., and Hiwatashi, K. (1975) *J. Protozool.* **22**, 536-540.
- 15) Takahashi, M., Haga, N., Hennessey, T., Hinrichsen, D. R., and Hara, R. (1985) *Genet. Res. Camb.* **46**, 1-10.
- 16) Galfer, G., and Milstein, C. (1981) *Meth. Enzymol.* **73**, 3-46.
- 17) Preer, J. R., Jr., and Preer, L. B. (1959) *J. Protozool.* **6**, 88-100.
- 18) Xu, X., Kumakura, M., Kaku, E., and Takahashi, M. (2001) *J. Euk. Microbiol.* (in press).
- 19) Butzel, H. M., Jr. (1955) *Genetics* **40**, 21-330.
- 20) Taub, S. R. (1966) *J. Exp. Zool.* **163**, 141-150.
- 21) Hiwatashi, K., and Myohara, K. (1976) *Genet. Res.* **27**, 135-141.
- 22) Myohara, K., and Hiwatashi, K. (1978) *Genetics* **90**, 227-241.
- 23) Tsukii, Y., and Hiwatashi, K. (1983) *Genetics* **104**, 41-62.
- 24) Tsukii, Y. (1988) In *Paramecium* (ed. Görtz, H.-D.). Springer-Verlag, Berlin, pp. 59-69.
- 25) Addutte, A., Ramanathan, R., Lewis, R. M., Dute, R. R., Ling, K.-Y., Kung, C., and Nelson, D. L. (1980) *J. Cell Biol.* **84**, 717-738.
- 26) Hori, M., and Fujishima, M. (1992) *Acta Protozoologica* **31**, 151-157.
- 27) Kusch, J., and Kechmann, K. (1988) *Europ. J. Protistol.* **23**, 273-378.
- 28) Hiwatashi, K., and Takahashi, M. (1967) *Sci. Rep. Tohoku Univ. Ser. (Biol.)* **33**, 281-290.
- 29) Barnet, A., and Steers, E., Jr. (1980) *J. Protozool.* **27**, 103-107.