Part II

A new monoclonal antibody XomO inhibiting mating reactivity in *Paramecium caudatum*

Introduction

Monoclonal antibodies used in Part I was suggested to be raised against O mating-type substances of *P. caudatum*. Using these antibodies made it possible to visualize the cellular localization of the mating-type substances of *P. caudatum* and examine quantitatively the mating reactivity of the cells for the first time. All the efforts to identify the antigen molecules of these monoclonal antibodies, such as native-PAGE followed by western blotting, gel filtration, immuno-precipitation had been done, but identification of antigen molecules had not succeeded. In order to identify the mating-type substances, I started to obtain new monoclonal antibodies. Mating-reactive O^3 cilia were used to immunize mouse and monoclonal antibodies (i. e. OmA, OmB, OmC, OmD, and OmE) were obtained (Azuma et al, 1996). To obtain more varied epitopes, I used mating-reactive membrane vesicles constituted from O^3 cilia as antigen. Monoclonal antibody XomO inhibiting the mating reactivity of both mating types of *P. caudatum*, syngen 3, was obtained.

Materials and Methods

Strains and culture. The same with those described in the **Materials and Methods of Part I.**

Immature cells. Immature cells were obtained basically by the method of Takahashi et al. (1985). 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. Two fissions after the separation of the conjugation pair, each exconjugant was re-isolated, and the exconjugant clones were established. When the number of the single-exconjugant clone was up to about 10^5 cells (exconjugant cell divided about 16 times), the mating reactivity of these cells was tested to confirm that the macronuclear degeneration did not happened (Mikami and Hiwatashi 1975). The cells without mating reactivity were immature cells (named KU in Tables 1 and 4) and were used in dot-blot analysis.

Preparation of the antigen. Cilia detached from cells were obtained by the same method described in **Materials and Methods of Part I.** The detached and packed cilia were treated with a SM

1200 solution containing 0.5% sucrose monolaurate (Wako Chemicals, Japan), 10 mM Tris, pH 7.4, 10 mM EDTA pH 7.0 (adjusted by KOH), and 10 μg/ml Leupeptin hemisulfate monohydrate (Wako Chemicals, 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, pH7.2, containing 10μg/ml Leupeptin hemisulfate monohydrate and set overnight at 4 °C. The diluted solution was centrifuged again at 100,000 *g* for 2 hours at 4 °C. The mating reactivity of the pellets (membrane vesicles) was tested with E^3 (C103s7) cells and only those with mating reactivity were used as the antigens.

Production of monoclonal antibodies. One hundred μl of the antigens (0.1-0.2mg) described as in **Preparation of the antigen** were injected into spleen of the BALB/c mouse 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) according to the method described by Galfer and Milstein (1981). Culture fluids from the hybridoma cells were subjected to screening. Fifty μl of the highly mating-reactive cell suspension of Bky13 was incubated with the same volume of culture fluid of

hybridomas for 20 min after adding 50 μl of 12.5 mM phosphate buffer (pH7.2), 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 on mating reactivity. Fifty μl of the highly reactive cell suspension was incubated for 10 min with the same volume of culture fluid of hybridomas and its diluted series with 12.5 mM phosphate buffer, pH 7.2, and then complementary mating-type cells were added. Mating reactivity was observed 10-15 min after adding the testers. The degree of inhibition on the mating reactivity by antibodies was estimated by the size of the clumps or agglutinates compared with that of when the cells without treatment by antibodies were mixed with the complementary mating type. Culture fluid of myeloma cells was used as the control to the monoclonal antibodies.

Dot-blot analysis. The protocol of dot-blot analysis was the same with that described in **Dot-blot method of Materials and Methods in Part I.** In the experiments to determine the relationship between the detection of antigen with XomO and the mating reactivity of the cells, monoclonal antibody OmE

thoroughly described in the **Introduction of the Part I** was used as positive control to indicate whether mating-type substances were contained in the samples or not. In the experiment of determining whether the antigen molecules of antibody XomO are membrane protein or not, the monoclonal antibody XomM whose antigen is immobilization antigen was used to indicate the localization of the immobilization antigen in the supernatant and pellet, and monoclonal antibody mentioned previously was used as positive control. To obtain the samples of immobilization 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 (Preer and Preer 1959). This solution was centrifuged at 12,000 *g* for 30 min at 4 °C. The supernatant contained mainly the immobilization antigen. Both the supernatant and the 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 phosphate buffer, pH 7.2, at room temperature and washed 3 times with 25 K·PB (25 mM KCl in 10 mM phosphate buffer, pH 7.2). The other steps were same with

those described in **Indirect immunofluorescence of Materials and Methods of Part I.**

Results

Inhibition of the mating reactivity of both complementary mating types in *P. caudatum*. After screening, the monoclonal antibody XomO inhibiting mating reactivity of O^3 and E^3 was obtained. The inhibition profiles of the XomO are shown in Table 3. Tester cells of Bky 13 (O^3) and C103s7 (E^3) were incubated for 10 min in the supernatants of hybridoma culture, containing antibody XomO, and the myeloma culture used as controls. Incubation with the culture supernatant of myeloma had no effect on the mating reactivity of both mating-type cells. Inhibiting effects on the mating reactivity of both mating types without ciliary immobilization were observed on dilution 16 on O^3 type and 8 on E^3 type, respectively.

Cellular localization of antigen recognized by the XomO. Next, the localization of the antigen of antibody XomO in O^3 and E^3 cells was determined by indirect immunofluorescence (Fig. 6). The ventral surface cilia (VSC) of both O^3 and E^3 cells were specifically labeled (Fig. 6 B, D). The most noticeable thing was that the antigen was specifically localized on the root side, but not whole length of cilia (Fig. 6 F, G). No antigen was detected from 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 on the mating reactivity of both mating-type cells indicated that the antigen was involved in the mating reaction. Thus, I determined whether the detection of the antigen was correlated with the mating reactivity of the cells (Fig. 7). In order to show whether the mating-type substances are contained in the samples, monoclonal antibody OmE that is raised against O mating-type substances (described in **Introduction of Part I**) was used as a positive control. Same with monoclonal antibody OmE, strong dot-blot antigen signals of antibody XomO were detected only from the mating-reactive O^3 and E^3 cells. Although almost no antigen was detected in the starved O^3 and E^3 cells that are without mating reactivity completely, faint signals of the antigens were detected in the well-fed cells in logarithmic growth phase. No antigen was detected from the immature cells that are non-mating competency.

The antigen of antibody XomO is membrane-bound. There is a thick cover of immobilization antigen outside of the cilia. The mating-type substances are suggested to be tightly

membrane-bound protein (Kitamura 1988). The packed cilia were treated with salt alcohol solution to remove immobilization antigen from them and the sample of immobilization antigen was obtained. The antigens of antibody XomM, whose antigen is immobilization antigen, were detected from both the supernatant and pellet (Fig. 8, lane XomM) and suggest that not all immobilization antigens were removed from the cilia by this treatment. Monoclonal antibody OmE, which is suggested to rise against O mating-type substances as described in Materials and Methods in Part II, was used as a positive control. Same with antibody OmE, little antigen of XomO was detected from the sample of immobilization antigen (Fig. 8. lanes OmE and XomO). This result suggested that the antigen of antibody XomO is membrane-bound.

Inhibiting effect on mating reactivity is syngen 3-specific. To determine whether the antigen molecules of monoclonal antibody XomO are mating-type substance or not, it is important to test its syngenic specificity of the inhibiting effect on mating reactivity in *P. caudatum*. Therefore, I tested the inhibiting effect of antibody XomO among 5 syngens of *P. caudatum* (Table 4). In syngen 3 besides G3 and Bky13, I determined the inhibitory effect on mating reactivity of XomO among several

strains belonging to O^3 or E^3 with totally different genetic background. Antibody XomO inhibited the mating reactivity of all tested O^3 and E^3 strains without immobilization effect. About syngen 1, syngen 4, syngen 6, and syngen 12, inhibiting effect on the mating reactivity and ciliary immobilization were not observed. These results indicated that the inhibiting effect of antibody XomO might be syngen 3- specific.

Discussion

Antigen of antibody XomO is related with mating reaction. Using mating-reactive membrane vesicles constituted from the cilia to immunize the mouse, I obtained monoclonal antibody XomO. Antibody XomO inhibited the mating reactivity of O^3 and E^3 cells. Although the inhibition of mating reactivity by XomO, which was not as effective as those monoclonal antibodies reported by Azuma et al. (1996), may partially result from the localization of the antigen only on the root side of the ventral surface cilia. But the detection of the antigen was associated with the mating reactivity of the cells (Fig. 7). These results clearly indicate the antigen of XomO is a substance related with mating reaction.

Antigens may be mating-type substances. The inhibiting effect of antibody XomO is non-mating-type specific, that is, antibody XomO inhibited the mating reactivity of both O^3 and E^3. Therefore, it is difficult to rule out the possibility that there is a common factor that involved in the mating reaction of *P. caudatum*. It will be discussed later in this part. However, one characteristic of mating reaction in *P. caudatum* is syngen-specificity, that is, mating-type substances are

syngen-specific too. According to syngen 3-specific inhibition on mating reactivity of antibody XomO, the antigen molecules are possible to be mating-type substances. Both of O^3 and E^3 cells are recognized by antibody XomO suggested that there are common domains between O and E mating-type substances.

I found mating-reactive O^3 mating-type substances exist in E^3 cytoplasm (see **Results of Part I**). This result suggested that O mating-type substances are precursor molecules of E mating-type substances. Therefore, it is reasonable to deduce that there is a common domain between O and E mating-type substances, and it is possible to obtain an antibody as XomO inhibiting the mating reactivity of both complementary mating types.

The notable characteristic of antibody XomO is that the antigen is localized only on the root side of ventral surface cilia (Fig. 6 F, G). What does this specific localization mean? Since the antigen of antibody XomO detected from mating-reactive cells are much more than those in starved or well-fed cells in logarithmic growth phase, it is hard to deduce that the antigen of XomO is a molecule involved in the catabolic process on a cilium in the mating-reactive cells. Moreover, mating-type substances detected by antibody OmA in Part I are localized all through a cilium of ventral surface cilia. If the antigens of XomO are

mating-type substance, XomO cannot recognize those on the tail side of a cilium. What is the difference between mating-type substances on the root side and those on tail side of a cilium? One possibility is to suppose that mating-type substances at the end of the cilia may have different conformations from those on the root side and cannot be recognized by antibody XomO any more.

In *Euplotes octocarinatus*, gamones are secreted via the cortical ampules (Kusch and Heckmann 1988). 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 (Hiwatashi 1961). If the antigen of XomO was mating-type substance, using this antibody can help us to visualize the transporting pathway of membrane protein, such as mating-type substances. Iwamoto and Fujishima (1955) once reported antigen specifically localized on ventral surface cilia but nothing with mating reactivity of *P. caudatum*. Thus, oral cortical region will be a place for transporting verified protein including mating-type substances.

Some molecules besides mating-type substances might be involved in mating reaction. It was not the first time to obtain antibodies inhibiting mating reactivity without mating-type

specificity, antibodies blocking mating reaction without species specificity had been reported although the localization of the antigen was not reported (Hiwatashi and Takahashi 1967, Barnett and Steers 1980). Antibodies inhibiting the mating reaction without ciliary immobilization were also obtained whose antigen localized all over the cells (data not shown). If the antigens of these antibodies were not mating-type substances themselves, there must be some other factors involved in the mating reaction.