Original

Inhibition of mating pair formation by wheat germ agglutinin and wheat germ agglutinin-binding substances in *Paramecium caudatum* cells

Ariko FUKE[1,2]* and Katsuyuki SATO[1]

[1]Department of Biology, Naruto University of Education, Takashima, Naruto-cho, Naruto-shi, Tokushima, 772-8502, [2]Joint Graduate School in the Science of School Education, Hyogo University of Teacher Education, Shimokume, Yashiro-cho, Kato-gun, Hyogo, 637-1494, Japan

SUMMARY

In this study, we revealed that the lectin wheat germ agglutinin (WGA) and its specific binding sugars, N-acetylneuraminic acid (NeuAc) and N-acetylglucosamine (GlcNAc), inhibited mating pair formation in *Paramecium caudatum*. The concentrations that caused 50% inhibition (IC50) were 17 nM, 3 mM and 30 mM, respectively. Using FITC-WGA, it was shown fluorescence-cytochemically that WGA bound to the adhesion site between conjugated cells; this binding was inhibited by NeuAc but not GlcNAc. Moreover, the binding of FITC-WGA was maintained appreciably when its localization was changed in correspon-dence with progression of conjugation until exchange of gametic nuclei was completed at which point the binding between both cells disintegrated. These results strongly suggested that NeuAc plays a primary role in mating pair formation in *P. caudatum* as a specific WGA binding sugar.

*Corresponding author
TEL : +81-88-683-6663
E-mail: fuke02@abelia.ocn.ne.jp

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INTRODUCTION

Conjugation is the sexual process of ciliates such as *Paramecium caudatum*. During this process, cells form a mating pair through mutual recognition of complementary mating types. Conjugation in *P. caudatum* proceeds through the following steps: immediately after mixing cells of complementary mating types, cells adhere with each other through their cilia (mating reaction) (Takahashi et al., 1974; Kitamura and Hiwatashi, 1976). The cilia then degenerate at the ventral side of adherence and within about 60 minutes after onset of the mating reaction, they adhere anterior-ventrally along their surface (holdfast union). Then, within around 4 hours after beginning the mating reaction, cells adhere ventrally along their entire surface (paroral union). During this step, cells are united firmly as both cell membranes become partly fused (Vivier and André, 1961; Hiwatashi and Kitamura, 1985). Cells then exchange gametic nuclei derived from micronuclei through the oral region where the cell membranes have fused; this forms a fertilized nucleus. Thereafter, conjugated cells leave each other and a macro- and micronuclei are newly formed from the fertilized nucleus in each individual cell (Fujishima, 1988).

In this way, conjugation is a phenomenon, characterized by mating pair formation in which adherence between individual cells is mediated by their membranes. Attempts have been made to investigate the involvement of specific complex polysaccharides in the cell membrane during mating pair formation using lectins with considerable specificities in recognizing certain sugar residues in the complex. For example, Concanavalin A (Con A) does not have any inhibitory effect on mating pair formation of *Paramecium tetraurelia* (Pape et al., 1988), but it does effect other ciliates including *P. caudatum* (Casci and Hunaggel, 1988; Evelyn et al., 1995; Frisch and Loyter, 1977; Tsukii and Hiwatashi, 1978). Mating pair formations of *P. tetraurelia* and *P. primaurelia* were inhibited by another lectin, wheat germ agglutinin (WGA) (Pape et al., 1988; Delmonte et al., 1997). Moreover, in *P. tetraurelia*, Pape et al. (1988) observed specific labeling of fluorescein isothiocyanate-conjugated WGA (FITC-WGA) at the adhesion site between conjugated cells. These results strongly suggest that these lectins inhibit mating pair formation by recognizing and then binding specifically to the sugar of the complex polysaccharide at the adhesion site in these ciliates.

Since these works indicate that inhibition by lectin might be species specific to some extent, and since no attempts have been made to investigate the inhibitory effect of WGA on mating pair formation in *P. caudatum* cell, we investigated the effect of WGA. The effects of various concentrations of WGA-specific binding sugars, N-acetylneuraminic acid (NeuAc) and N-acetylglucosamine (GlcNAc), were examined quantitatively to identify the sugar that plays a primary role in mating pair formation of *P. caudatum*. Moreover, we followed the changes in localization of the specific WGA binding site with progress of conjugation using FITC-WGA.

MATERIALS AND METHODS

Cell culture and preparation of cell suspension

KNZ2 (mating type E) and KNZ5 (mating type O) strains of *P. caudatum* were provided by Mikami, Environment Education Center, Miyagi University of Education, and cultured at 25±1°C by adding fresh medium everyday for 4 days. The medium was prepared by growing *Klebsiella pneumoniae* in fresh lettuce juice medium (pH6.8) for 1 day at 25±1°C. Cells were collected by manual centrifugation then precipitated cells were washed twice in Dryl's solution (Dryl, 1959) and incubated at 25±1°C for 1 hour after cell density was adjusted to 2500 cells/ml with Dryl's solution.

Effect of WGA and its specific sugars on mating pair formation and calculations of the rate of mating pair formation

WGA, NeuAc and GlcNAc, purchased from Sigma (St Louis, MO, USA), were stored at –20°C then dissolved in phosphate buffered saline (PBS) (pH 7.0) immediately before use at concentrations of 28 μM, 25 mM and 1 M, respectively. For individual inhibition tests of the lectin and its specific sugars, upon mixing equal volumes of suspensions of cells of complementary mating types in a depression glass slide, the WGA, NeuAc and GlcNAc-PBS solutions were added at maximum final concentrations of 83 nM, 5 mM and 100 mM, respectively.

Four hours after mixing, both the total number of cells and number of pairs were counted in the cell mixture after fixing with a saturated picric acid solution (Nacalai Chemcal Co. Ltd., Kyoto, Japan). Fixation was performed in a petri dish using a drop of cell suspension mixed with saturated picric acid. As control experiments, the lectin or conjugated sugars were omitted from the incubation medium. Each inhibition and control experiment was run at least in triplicate. The rate of mating pair formation was calculated by the following

equation from the average of three experiments: rate of mating pair formation (%) ={(Total number of mating pairs) / (Total number of cells/2)}×100.

Cytochemical staining of conjugated cells with FITC-WGA

Equal volumes of cell suspension with complementary mating types were mixed in a small Erlenmeyer flask then incubated at 25±1°C. At each prescribed conjugation step, aliquots of cell suspension were centrifuged manually to collect conjugated cells, which were then suspended in Dryl's solution. FITC-WGA (Sigma) dissolved in PBS (pH 7.0) at 1 mg/ml was added to each suspension at a final concentration of 30 µg/ml then incubated at 25±1°C for 15 minutes. After centrifuging at 600 ×*g* for 1 minute, precipitated cells were suspended and incubated in Dryl's solution at 25±1°C for 10 minutes. After a second round of centrifugation at 600×*g* for 1 minute, cells were then suspended and fixed in 4% paraformaldehyde (Wako Pure Chemical Indus-tries, Ltd., Osaka, Japan) dissolved in PBS (pH 7.0) at room temperature for 15 minutes. Cells were then washed twice in PBS (pH 7.0) by incubation at room temperature for 5 minutes combined with manual centrifugation. To confirm the conjugating step, cell nuclei were stained with 0.0001%(W/V) 4,6-diamino-2-phenylindole (DAPI) (Wako Pure Chemical Industries, Ltd.). The localization of FITC-WGA was observed under a fluorescence microscope (OPTIPHOT-2, Nikon, Tokyo, Japan) under conditions of B excitation (excitation at 450-490 nm).

Inhibition of WGA binding to the adhesion site in the presence of NeuAc or GlcNAc

Before adding to the conjugated cells (4 hours after cell mixing), 30 µg/ml FITC-WGA was pre-incubated with 4 mM NeuAc or 100 mM GlcNAc in PBS (pH 7.0) at 25°C for 20 minutes. Cells were incubated in WGA-NeuAc or WGA-GlcNAc mixtures at 25°C for 15 minutes then washed, fixed and observed as described above. Attempts were also made to determine whether cells could maintain their integrity under the maximum concentration of GlcNAc (200 mM).

Table 1 The rate of mating pair formation of *P. caudatum* cells under various concentrations of WGA, NeuAc and GlcNAc.

WGA (nM)	Rate of mating pair formation (%)	NeuAc (mM)	Rate of mating pair formation (%)	GlcNAc. (mM)	Rate of mating pair formation (%)
0	68	0	58	0	59
14	39	1	54	20	37
28	19	2	42	40	20
42	1.4	3	28	60	6.2
56	2.2	4	12	80	1.2
68	n.d.*	5	0.9	100	1.0

*: Not determined

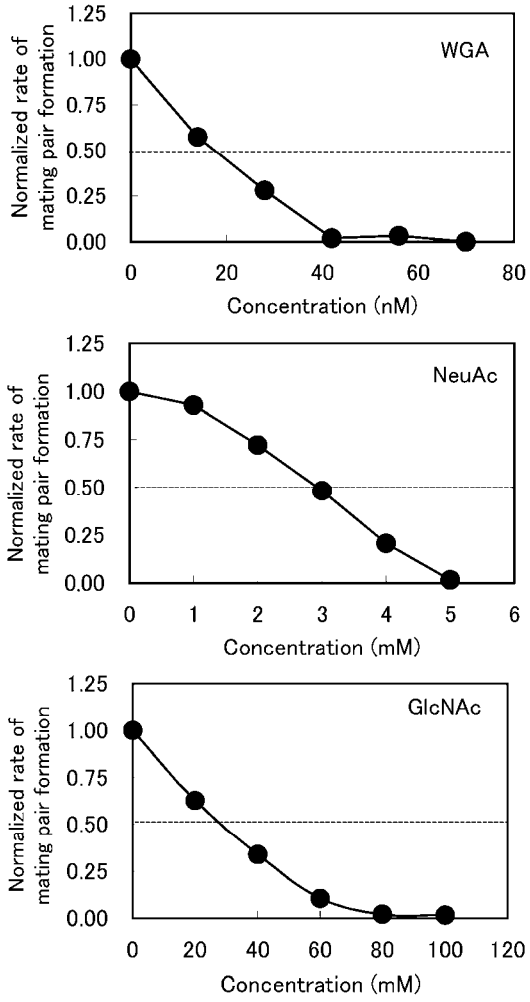

Fig. 1. The relationship between the concentrations of WGA, NeuAc and GlcNAc, and normalized rates of mating pair formation. These normalized late averaged of three experiments.

RESULTS

Inhibition of mating pair formation by WGA and conjugated sugars

The mating pair formation of *P. caudatum* was investigated at various concentrations of WGA, NeuAc and GlcNAc, as summarized in Table 1. Although 68% of cells formed mating pairs in the absence of WGA, the rate in the presence of WGA largely decreased with increasing concentration. A less than 5% rate was observed with more than 42 nM of WGA. In both the inhibition tests in the absence of NeuAc and GlcNAc, around 60% of cells engaged in mating pair formation. But, as in the case of WGA, rates of mating pair formation decreased in the presence of each sugars at increasing concentrations. By dividing by the control rate, all values were normalized and plotted against the concentration of lectin and its specific sugars (Fig. 1). From these relationships, the value representing 50% inhibition (IC50) of mating pair formation was estimated empirically at around 17 nM, 3 mM and 30 mM for WGA, NeuAc and GlcNAc, respectively.

Cytochemical staining of conjugated cells with FITC-WGA

Using FITC-WGA, changes in the localization of WGA binding sites were indicated clearly with progression of conjugation, as shown in the fluorescent photomicrographs in Fig. 2. Before mixing, somatic *P. caudatum* cells were not labeled by FITC-WGA except for in the paroral region (Fig. 2A). Two hours after cells of complementary mating types were mixed, fluorescence of FITC-WGA could be detected only in the anterior-ventral part where cells adhered (Fig. 2B). With the progress of conjugation, marked fluorescence was observed clearly in the ventral part where cells were united firmly (Fig. 2C). The fluorescence was maintained while the cells were united (Figs. 2C-G); however, when the bond between the cells disintegrated, fluorescence was detected only in a small fraction of the ventral part where the cells had previously been united, and in the paroral region (Figs. 2H and I).

Inhibition of WGA binding to the adhesion site by NeuAc and GlcNAc

To define the specific WGA binding sugar at

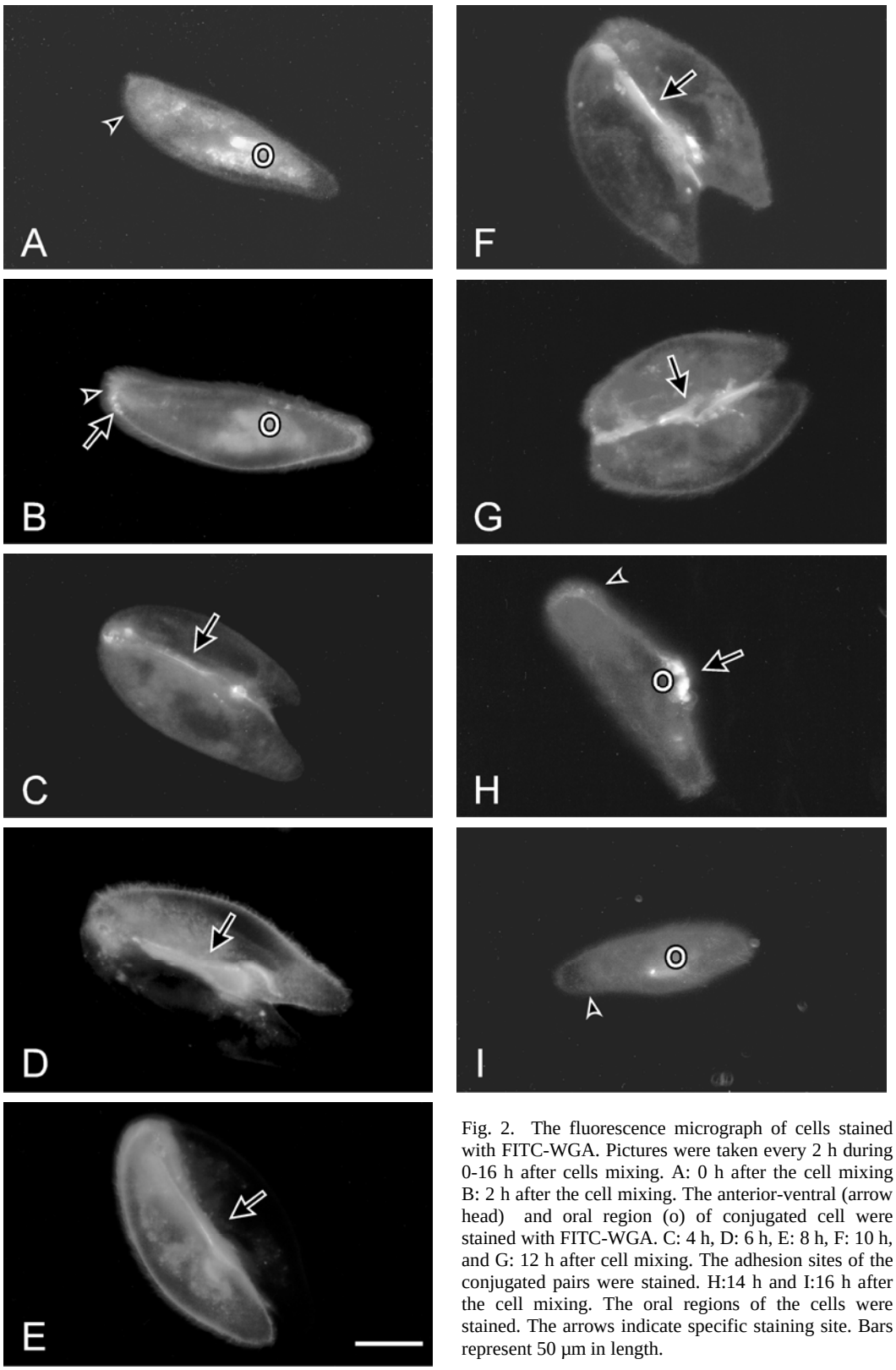

Fig. 2. The fluorescence micrograph of cells stained with FITC-WGA. Pictures were taken every 2 h during 0-16 h after cells mixing. A: 0 h after the cell mixing B: 2 h after the cell mixing. The anterior-ventral (arrow head) and oral region (o) of conjugated cell were stained with FITC-WGA. C: 4 h, D: 6 h, E: 8 h, F: 10 h, and G: 12 h after cell mixing. The adhesion sites of the conjugated pairs were stained. H:14 h and I:16 h after the cell mixing. The oral regions of the cells were stained. The arrows indicate specific staining site. Bars represent 50 μm in length.

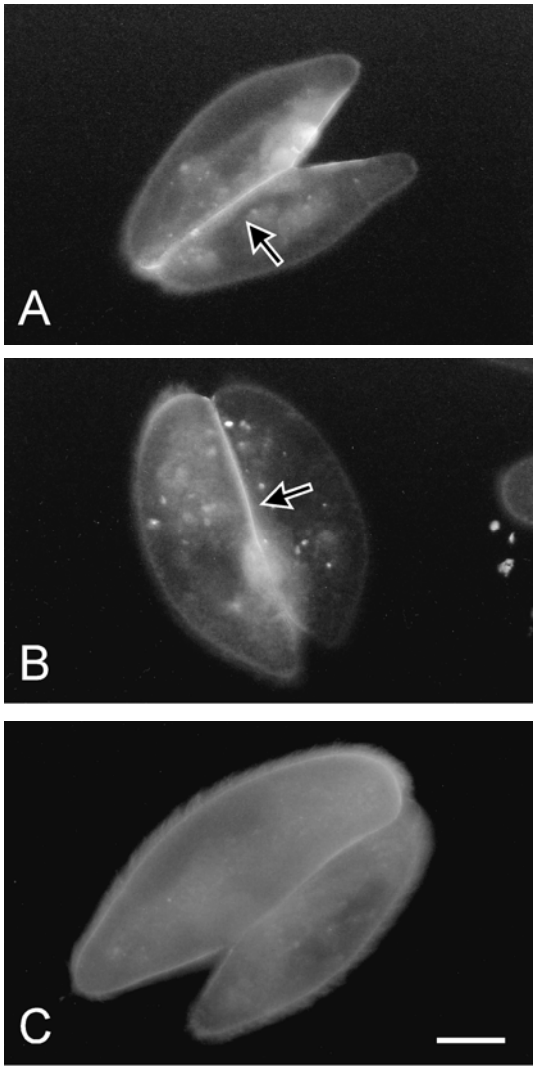

Fig. 3. Inhibition of FITC-WGA staining by NeuAc and GlcNAc. A: Conjugated cells (4 h after cell mixing) treated with 30 μg/ml FITC-WGA. B:Conjugated cells treated with 30 μg/ml FITC-WGA saturated with 100 mM GlcNAc. The adhesion sites of the conjugated cells were stained. The arrows indicate specific staining site. C: Conjugated cells treated with 30 μg/ml FITC-WGA saturated with 4 mM NeuAc. Staining of FITC-WGA in the adhesion site was not detectable. Bars represent 50 μm in length.

the adhesion site of conjugated cells, cells were incubated in FITC-WGA-NeuAc or FITC-WGA-GlcNAc. The final concentrations of FITC-WGA, NeuAc and GlcNAc were 30 μg/ml, 4 mM and 100 mM, respectively. NeuAc but not GlcNAc (Fig. 3B) inhibited cytochemical staining of FITC-WGA in the adhesion site between cells (Fig. 3C). Even in the presence of a high concentration of GlcNAc (200 mM), the inhibition of staining was not observed. Sometimes the surfaces of the cell bodies as well as the cilia of the conjugating cells were stained by FITC-WGA. Since, however, this staining was shown to be independent of the presence or absence of sugars, it appears to be non-specific.

DISCUSSION

It was previously reported that WGA inhibits mating pair formation in *P. tetraurelia* and *P. primaurelia* (Pape et al., 1988, Delmonte et al., 1997). In this study, we indicated quantitatively that WGA also inhibits mating pair formation in *P. caudatum* cells at an IC50 of 17 nM (Table 1 and Fig. 1). We also showed fluorescence cytochemically that WGA bound specifically to the adhesion site between conjugated cells (Figs. 2C-G). Moreover, FITC-WGA staining in the adhesion site could not be detected after the bond between the conjugated cells disintegrated (Figs. 2H and I). WGA localization changed in correspondence with progression of conjugation (Fig. 2). These results suggest that mating pair formation in *P. caudatum* cells is inhibited by WGA through specific binding to sugar in the complex polysaccharide that is crucial to mating pair formation.

Tsukii and Hiwatashi (1978) reported the inhibition of mating pair formation in *P. caudatum* cells in the presence of higher effective concentrations of Con A (480 nM) than required for complete inhibition by WGA (40 nM). This might sug-

gest that either Con A and WGA specific binding sugars are responsible for mating pair formation in *P. caudatum* or that Con A non-specifically binds to WGA-specific binding sugar. Since they did not show the IC50 of Con A, we could not compare our data with theirs. It appears necessary to conduct the inhibition test under various concentrations of Con A and through cytochemical staining of FITC-Con A as shown in this study.

Both WGA specific binding sugars, NeuAc and GlcNAc, also showed considerable inhibitory effects on mating pair formation in *P. caudatum* cells (Table 1). NeuAc showed a lower order of magnitude of IC50 than GlcNAc (Fig. 1). Moreover, NeuAc but not GlcNAc blocked WGA binding to the adhesion site between conjugated cells (Figs. 3B and C). These results suggest that NeuAc bound specifically and then masked the sugar recognition site of WGA on the cell membrane that was crucial for cell adherence during mating pair formation. Inhibition of mating pair formation seems to be caused not by toxicity of WGA, NeuAc and GlcNAc, because *P. caudatum* cells remained alive for 24 hours with all tested concentrations of lectin and sugars. It is therefore necessary to elucidate the details of the molecular mechanism of mating pair formation, in particular, the involvement of the complex polysaccharide that contains NeuAc as a residue.

In *P. tetraurelia*, FITC-WGA staining in the adhesion site was found at 4 and 7 h after initiation of conjugation, as shown here for *P. caudatum*. Lüthe and Plattner (1986) reported, however, that WGA binding substances were already localized in the expected adhesion site before starting conjugation in *P. tetraurelia* cells. Moreover, Pape et al. (1988) described that staining of the adhesion site between *P. tetraurelia* cells by FITC-WGA was not blocked by 200 mM NeuAc or neuraminidase treatment of the cell, but by 200 mM GlcNAc. These are not coincidental with the results of this study; WGA binding sites could not be visualized by FITC-WGA cytochemically until adhesion of the cells started and NeuAc was primarily the specific binding sugar to WGA. The crucial complex poly-saccharide and its behavior in the conjugation steps therefore appear to differ among species of *Paramecium*.

It could be concluded from the results of this study that a specific WGA binding complex polysaccharide is involved in cell adhesion during mating pair formation in *P. caudatum* cells. Furthermore, WGA, NeuAc, and GlcNAc inhibited the progression of conjugation; nuclear events were stopped at an early stage (data not shown). WGA binding substances therefore seem to be necessary for progress of conjugation as well as cell adhesion.

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