TIPPING AND MATING-STRUCTURE ACTIVATION INDUCED IN *CHLAMYDOMONAS* GAMETES BY FLAGELLAR MEMBRANE ANTISERA

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ABSTRACT

Antisera raised against vegetative and gametic flagella of *Chlamydomonas reinhardi* have been used to probe dynamic properties of the flagellar membranes. The antisera, which agglutinate cells via their flagella, associate with antigens that are present on both vegetative and gametic membranes and on membranes of both mating types (mt^+ and mt^-). Gametic cells respond to antibody presentation very differently from vegetative cells, mobilizing even high concentrations of antibody towards the flagellar tips; the possibility is discussed that such "tipping" ability reflects a differentiated gametic property relevant to sexual agglutinability. Gametic cells also respond to antibody agglutination by activating their mating structures, the mt^+ reaction involving a rapid polymerization of microfilaments. Several *impotent* mt^+ mutant strains that fail to agglutinate sexually are also activated by the antisera and procede to form zygotes with normal mt^- gametes. Fusion does not occur between activated cells of like mating type. Monovalent (Fab) preparations of the antibody fail to activate mt^+ gametes, suggesting that the cross-linking properties of the antisera are essential for their ability to mimic, or bypass, sexual agglutination.

KEY WORDS microfilament polymerization · membrane mobility · cell fusion · membrane mutants

During the sexual agglutination reaction of the eukaryotic protist *Chlamydomonas reinhardi*, gametes of mating-type plus (mt^+) recognize, and make contact via their flagellar tips with, gametes of mating-type minus (mt^-) (28, 32). The ability to agglutinate sexually is acquired during gametic differentiation: vegetative mt^+ and mt^- cells show no flagellar interactions with one another or with gametes. The reaction is, moreover, specific for both mating type and species: mt^+ gametes agglu-tinate only with mt^+ gametes, never with themselves or with gametes of other species of *Chlamydomonas*.

Sexual agglutination elicits three responses from gametic cells, all of which occur within 30 s: (*a*) Both mt^+ and mt^- cells release a wall-digesting substance, termed autolysin (6, 7, 18, 30, 35), which removes the glycoproteinaceous wall surrounding gametes so that cell fusion can proceed. (*b*) The mt^+ cell is induced to activate its mating structure, a process involving microfilament polymerization and membrane extrusion from a plaquelike structure beneath the anterior cell membrane (5, 11, 18, 38). (*c*) The mt^- cell is

induced to alter the configuration of the intramembranous particles in its mating structure in preparation for cell fusion (45). Both autolysin release and mating-structure activation can be induced by presenting gametes of one *mt* with isolated gametic flagella of opposite *mt* (7, 45). The ability to "signal" these responses, in other words, is clearly associated with the flagellar surface.

The present paper describes experiments that probe the nature of gametic agglutination and the sexual signal. The experiments utilize antisera raised against the flagellar surfaces of *Chlamydomonas* vegetative and gametic cells. None of the antisera prove to be diagnostic for mating-type or state-of-differentiation specificities: an antiserum raised against vegetative mt^+ flagella, for example, agglutinates gametic mt^- flagella quite as well as vegetative mt^+ flagella. We find, however, that gametic cells respond to antiserum presentation by mobilizing bound antibody to the flagellar tips, a response not mimicked by vegetative cells. We also find that antiserum-induced flagellar agglutination will elicit mating-structure activation both in wild-type gametes and in certain mutant strains that are incapable of sexual agglutination, thereby allowing the mutants to fuse with wild-type cells of opposite *mt*. Finally, we find that two mutant strains fuse poorly or not at all when agglutinated by antisera, apparently because of a defective signaling ability. Models are proposed to explain how antibody "tipping" may occur and how the antibody-induced signal may be generated. Preliminary reports of these results have been presented at recent meetings (13, 16).

MATERIALS AND METHODS

Strains and Culture Conditions

Clones of strain 137c, *mt* and *mt*, of *C. reinhardi* were utilized that possess high (near 100%) mating efficiencies (14). The mutant strains *bld-2* (*bald*; no flagella) (17), *imp-2, imp-5, imp-6, imp-7,* and *imp-8* (*impotent*; see references 2, 14, 15) and *gam-1* (gametogenesis-defective; see references 9, 10) were used in certain experiments.

Vegetative cultures were grown in Tris-acetate-phosphate (TAP) medium (20) as previously described. Gametes were taken from 1–2 wk-old TAP agar plates (23) and suspended in nitrogen-free high salt medium (N-free HSM) (36) for 1–2 h before use. Zygotes were plated and germinated by standard procedures (22). Zygote formation was in some cases monitored by direct examination of plates at the end of the matura-

tion period. In other cases, zygotes were distinguished from unmated cells by exposing plates to chloroform vapors to which zygotes alone are resistant (22), and then monitoring the formation of colonies. In other cases, zygotes were allowed to form in liquid culture and were recognized by the "pellicle" they produce (29).

Autolysin Preparation

Autolysin was prepared by mixing cells at a concentration of 5×10^7 cells/ml and allowing them to mate for 2 h. The suspension was then spun at 31,000 g for 15 min to remove cells. The supernate was frozen at $-85°C$ to kill any unpelleted cells.

Isolation of Flagella

Flagella were separated from cells by the pH-shock method as described by Witman et al (46). Cell bodies were pelleted at 3,000 g, and the supernate was layered over a 25% sucrose solution in 10 mM Tris, pH 7, and centrifuged for 15 min at 3,000 rpm in an HS swinging bucket rotor in a Sorvall RC-2 centrifuge (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.). The flagella in the interface were aspirated and then pelleted at 31,000 g. Gametic flagella were routinely checked for their retention of isoagglutinating activity following isolation.

Preparation of Antisera

Norwegian White rabbits were bled to obtain preimmune serum. They were then injected with flagella that were isolated and purified from $\sim3 \times 10^9$ mt^+ or mt^- gametic or vegetative cells and suspended in 1 ml of Freund's complete adjuvant. For subsequent injections, equivalent numbers of flagella were suspended in Freund's incomplete adjuvant. A total of four weekly injections produced high titers of flagellar agglutinating activity, at which time the rabbits were exsanguinated by cardiac puncture.

Indirect Immunofluorescence

A suspension of antibody-agglutinated gametes was washed several times by gentle centrifugation and mixed, at a 1:20 dilution, with goat anti-rabbit immunoglobulin conjugated with fluorescein (courtesy of Dr. V. L. Sato). Cells were examined with a fluorescence microscope (courtesy of Dr. S. C. R. Elgin).

Preparation of Fab

An aliquot of αG^+ (antiserum directed against mt^+ gametic flagella) was crudely purified by precipitation with 20% Na_2SO_4 as described in Weir (39). Twice-crystallized papain (Worthington Biochemical Corp., Freehold, N. J.) was activated by dissolving the enzyme to a concentration of 0.1 mg/ml in 1.0 mM ethylenediaminetetraacetate, 0.6 mM 2-mercaptoethanol, 5 mM

cysteine-HCl, and incubating for 30 min at 22°C. To 1 ml of the activated enzyme solution was added 1 ml of the partially purified αG^+, and digestion proceeded for 16 h at 37°C, after which the solution was chilled. The monovalent nature of the resultant Fab preparation was judged by its failure to agglutinate cells at a 1:40 dilution. That the preparation retained its ability to bind the flagellar surface was shown by presenting *imp-5* gametic cells with a 1:40 dilution of papain digest and then with goat-anti-rabbit antiserum. Extensive agglutination was observed, whereas the goat antiserum had no agglutinating effect on untreated cells, or on cells treated with preimmune serum. Papain digestion of gametic cells for 10 min has no effect on their sexual or antibody-mediated agglutinability (4).

Electron Microscopy

Techniques for thin-section, freeze-cleave, negative-staining, and scanning electron microscopy are found in previous publications from this laboratory (2, 18, 40) and in legends to the Figures.

Estimation of Mating-Structure Activation

Mating-structure activation was assessed in thin-sectioned material, scoring mt^+ structures as being activated (microfilaments present) or unactivated (microfilaments absent) after exposure to antisera or concanavalin A for 10-15 min. This assay proved to be far more sensitive than examining whole cells by Normarski optics, scanning electron microscopy (SEM), or negative-stain transmission electron microscopy (TEM), but several of its features should be noted. First, since the small mating structure is only infrequently included in thin section, each assay necessitates examining some 500-1,000 cell sections. Second, sectioning angles can create "false negatives": if an activated mating structure is cut tangentially, the central, microfilament-filled tube may not be included and the image will be scored as "unactivated." At least 10 mating structures were counted for each sample to assure that central sections were well represented. Samples containing no activated mating structures were unambiguous. For experiments in which activation had been induced, the presence of activated structures was also unambiguous. The "percent activation" data, however, are necessarily influenced by sectioning angle and have only limited quantitative value.

RESULTS

General Properties of the Antisera

Four types of antisera were analyzed: αV^+ and αV^- (antisera raised against vegetative mt^+ and mt^- flagella, respectively), and αG^+ and αG^- (antisera raised against gametic mt^+ and mt^- flagella, respectively). All four antisera proved

effective, by light microscopy, in causing flagellar isoagglutination using mt^+ vegetative, mt^- vegetative, mt^+ gametic, and mt^- gametic cells; they also agglutinate the flagella of *imp* mutants and of young zygotes, neither of which can agglutinate sexually (2, 12). Agglutination is observed after the serum is diluted more than a thousand-fold in N-free HSM, and persists after the complement pathway is inactivated by heating the antisera at 56°C for 1 h. Preimmune serum elicited no agglutination response from any of the cell types.

Characterization of the antisera has been performed by a variety of immunological procedures, the most sensitive being to subject isolated flagella to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, incubate the gels with antisera, and detect the presence of antigen-antibody complexes by incubating the gel with ^{125}I-labeled protein A (reference 21 and Adair, Jurivich, and Goodenough, *J. Cell Biol.* **79**:281-285). Such experiments reveal that each antiserum carries major antibody species directed against the major glycopolypeptide family of the *C. reinhardi* flagellar membrane (2, 33, 46), plus at least 10 minor antibody species directed against minor glycopolypeptides. In the face of this heterogeneity, the biological effects of the antisera are necessarily reported without precise information as to the particular surface antigen(s) responsible for particular effects. All of the effects, however, are elicited by all four antiserum types tested. Therefore, if any subtle antigenic differences distinguish the two mating types or the vegetative vs. gametic state, these differences are not endowing the antisera with the properties we are monitoring.

Microscopy of Gametic Antibody Agglutination

By light microscopy, both wild-type and *imp* gametes are found to associate almost exclusively at their flagellar tips in the presence of antibody, forming clumps of cells that are indistinguishable by cursory inspection from true mating mixtures. Closer inspection reveals, however, that antibody-agglutinated gametes carry aggregates of material associated with the distal ends of their flagella; with time, these aggregates increase in size until the flagella appear totally weighted-down at their tips. Scanning and negatively-stained images of such tip aggregates are shown

in Figs. 1 and 2, and a thin-section image is shown in Fig. 3. In both Figs. 2 and 3, the aggregates are seen to include vesicles (V) derived from the flagellar membrane, plus a dense material which we assume to be antibody. Supporting this assumption are observations on antibody-agglutinated gametes treated with fluoresceinated goat anti-rabbit antiserum and examined with a fluorescence microscope: the tip aggregates are fluorescent (and are nonfluorescent in preimmune serum controls).

Gametic tip agglutination could result from high intrinsic antigen concentrations at the tips of gametic flagella; alternatively, gametes might mobilize antigen toward the tips once antibody is bound. Two observations favor the second possibility. First, it is found that if glutaraldehyde-fixed gametic "corpses" are presented with antisera, the corpses proceed to agglutinate by their flagella, but agglutination now occurs all along the flagellar lengths and not just at the tips. Second, if gametic flagella are detached from cells and then presented with antisera, the flagella make agglutinating contacts at all positions, showing no detectable preference for the tips. It appears, therefore, that gametes may actively mobilize bound antibody towards the distal ends of their flagella by a process we can designate as "tipping."

Freeze-fracture electron microscopy of antibody-agglutinated gametic flagella (Fig. 4) fails to reveal any change in the random distribution of intramembranous particles (3, 26).

Microscopy of Vegetative Antibody Agglutination

When vegetative-cell flagella are isolated and presented with a wide range of antiserum concentrations, contacts are made at all positions along the flagellar lengths, exactly as with gametic flagella. Intact vegetative cells, on the other hand, respond to antiserum presentation very differently than intact gametic cells, differences that are summarized in Table I. Vegetative cells are found to shed their flagella instantly unless the antiserum is diluted at least 100-fold. Moreover, at the lower antiserum concentrations, no membrane vesiculation or antibody accumulation is observed at the tips (Fig. 5). Instead, the flagella make apparently random contacts throughout their lengths, so that it is common to see the tip of one flagellum aggluti-

nated near the base or towards the middle of another; it is also common to find two vegetative flagella agglutinated in the middle or along their entire lengths. Since gametes tip antibody normally at these dilute concentrations, it would appear that vegetative cells are incapable of antibody "tipping."

## Antibody Activation of mt^+ Gametes

When wild-type mt^+ gametes are presented with any of the four antiserum types, the resultant flagellar agglutination elicits an apparently normal mating-structure activation: a fertilization tubule, filled with microfilaments, extends from the doublet zone of the mt^+ mating structure. To determine whether or not this response was caused by antibody interactions with sexual agglutinations of the wild-type mt^+ flagella, the experiment was repeated with impotent mt^+ strains (imp-2, imp-5, and imp-6) that fail to agglutinate sexually (2, 14, 15). Excellent mating-structure activation was again observed (Figs. 6 and 7).

Table II summarizes the results of scoring such mating-structure activation in thin section; details of the assay are given in Materials and Methods. Experiments A and B of Table II reveal that preimmune serum does not cause mating-structure-activation of either wild-type (WT) or imp-5 gametes. In contrast, αG^+ is highly active, at 1:50 dilutions, on the imp-2, imp-5, and imp-6 strains, causing extensive activation within 10 min (Table II, C–E). Activation also occurs at higher antiserum concentrations (Table II, F) but since deflagellation often occurs at such elevated levels, lower concentrations were chosen for most studies.

The strain imp-7 shows a unique response. Despite the fact that imp-7 gametes agglutinate strongly in the presence of αG^+ antisera at a 1:50 dilution, mating-structure activation is not stimulated (Table II, G). A limited response occurs at a 1:25 dilution (Table II, H), and an excellent response is observed when the serum is diluted by only 1:1.5 (Table II, I).

The αG^- antiserum directed against mt^- gametic flagella is fully as effective at promoting mt^+ mating-structure activation as is the αG^+ (Table II, J).

In a normal mating or in an activation mediated by isolated mt^- flagella, mt^+ fertilization tubules are very constant in diameter ($\sim$0.2 μm)
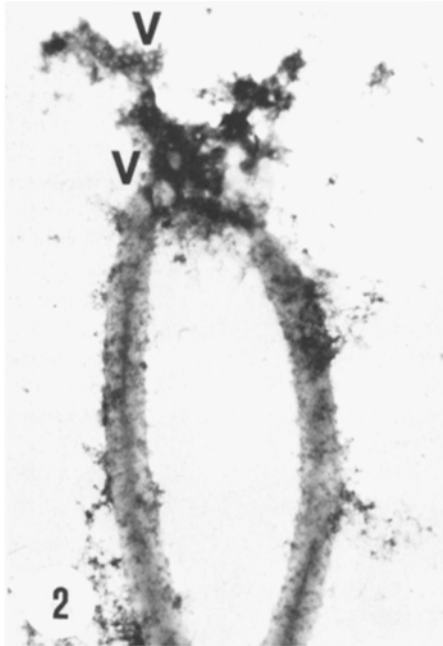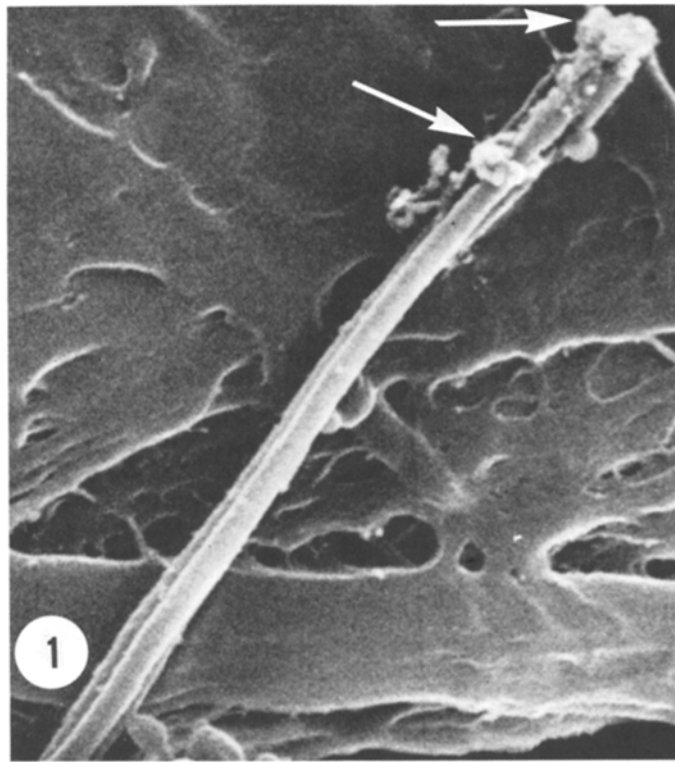
and in length (~1 μm). In contrast, the fertilization tubules generated under the aegis of antisera are often seen to extend for several microns. These elongated structures are typically narrow, and contorted or wavy at the distal ends.

To demonstrate that mt^+ activation response is not caused by some nonspecific serum component(s) that happen to be present in the αG^+ and αG^- but not in the preimmune antisera, each serum was absorbed against living gametes until all detectable flagellar agglutination activity was
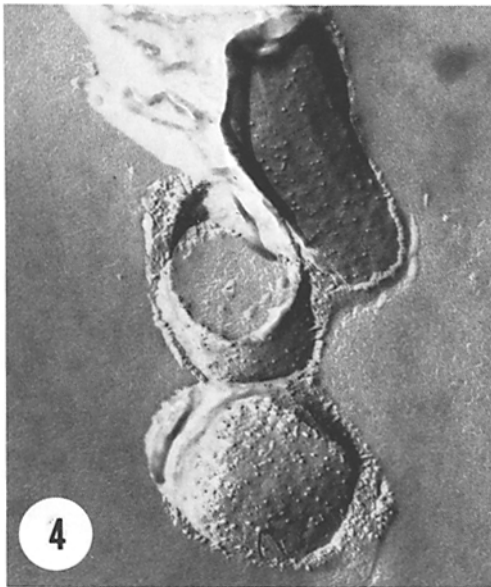

FIGURE 4 Freeze-fracture replica of three wild-type mt^- gametic flagella agglutinated in the presence of αG^+. Particles on protoplasmic (P) faces are distributed in the same random fashion as unmated gametes (2, 33). × 54,000.

eliminated; the absorbed sera were then presented to $imp-5$ gametes. As indicated in Table II, K and L, the absorbed sera no longer elicit mating-structure activation.

The specificity of the response was further demonstrated by presenting wild-type and $imp-5$ mt^+ gametes with concanavalin A (Con A). The lectin mediates an mt-nonspecific flagellar isoagglutination response (24, 42) that appears, by light microscopy, to mimic antisera agglutination. The lectin fails, however, to cause mating-structure activation even after 15 min of vigorous agglutination (Table II, M and N).

Finally, αG^+ antiserum was subjected to a crude purification for immunoglobulins and was treated with papain to produce monovalent (Fab fragment) antibody. The monovalent antibody could be shown to bind to the flagella (see Materials and Methods) but it failed to agglutinate the cells, and it failed to induce any mating-structure activation (Table II, O).

Antibody Activation of mt⁻ Gametes

Mating-structure activation in mt^- gametes is not readily visualized in thin section but can be detected in freeze-cleave replicas (45). Fig. 8 shows an mt^- mating structure activated in the presence of a 1:20 dilution of αG^+ antiserum: the central particles are clustered and prominent compared to control samples.

Antisera Stimulation of Cell-Wall Lysis

A quantitative study of antisera-stimulated cell wall lysis in gametes of one mt was not performed, but general impressions can be recorded. The antisera clearly stimulated wall lysis in most mixtures; they clearly did not in some. High

FIGURE 1 Gametic $imp-5$ mt^+ flagella isoagglutinated by αG^+ antibody, as seen by scanning electron microscopy. Cells were incubated for 10 min in the presence of a 1:40 dilution of an αG^+ preparation purified by Na$_2$SO$_4$ precipitation. Aggregated material (arrows) associated exclusively with the flagellar tips and not the rest of the flagella. Fibers of Whatman no. 50 filter paper are evident in background. × 17,000.

FIGURE 2 Gametic wild-type mt^+ flagella isoagglutinated by αV^- antiserum, as seen by negative stain electron microscopy. Cells were incubated for 10 min in the presence of a 1:20 αV^+ dilution, applied to Formvar-coated copper grids, and directly stained with uranyl acetate without prior fixation. Vesicles (V) embedded in a dense material are evident at the tips of the agglutinated flagella. Granular background material is precipitated uranyl acetate, which has a very different appearance from the material at the tip. × 22,000.

FIGURE 3 Flagella of $imp-5$ mt^+ gametes agglutinating in the presence of a 1:40 dilution of αG^-. Vesicles (V) of flagellar membrane associate with the dense material that surrounds the flagellar surfaces. × 62,000.

TABLE I

Response of Gametic and Vegetative Cells to Antisera Presented at Various Dilutions as Monitored by Light Microscopy

Serum: Cell suspension volumes	Gametes	Vegetative cells
1:1–1:5	Huge clumps, immobilized. Flagella often shed after 10–15 min	Flagella shed instantly
1:10–1:100	Vigorous clumping with agglutination via flagellar tips	Flagella shed instantly
1:100–1:1000	Moderate agglutination via flagellar tips	Agglutination via flagella: sides and base as well as tips are involved Generally immobilized

antisera concentrations definitely disallow wall lysis. Since strong antibody binding to cell walls is observed by indirect immunofluorescent microscopy, it is possible that cell-wall dissolution is impeded at high antibody concentrations. Lysis was not, however, invariably observed at low antisera concentrations, an observation for which no explanation can be offered. Additional studies on antiserum induction of cell-wall lysis are reported by Claes (7).

Of direct relevance here is the fact that mt^+ mating-structure activation was frequently observed in gametes that retained their walls, with mating structures literally bumping into the intact walls. These occurrences make it seem unlikely that the sera exert their effects by interacting directly with the membrane overlying the mating structure, and argue for an interaction with the flagellar surface. A more direct demonstration of the need for flagella was made by treating flagella-less $bld-2$ mt^+ gametes with autolysin to remove their walls and exposing such cells to antiserum. Mating-structure activation was in this case monitored by subjecting fixed cells to negative staining and looking for protuberant mating structures extending from the round $bld-2$ cells. Whereas such protuberances are readily detected in $imp-5$ controls, no activation of the $bld-2$ mating structure is elicited by antiserum.

Antisera-Mediated Cell Fusion

To ascertain whether antisera induce various combinations of gametic cells to fuse with one another, cells were treated for 1 h with autolysin to remove their cell walls so that failure to shed walls could not bias the outcome. They were then presented with antisera for specific lengths of time. The extent of cell fusion was monitored either by counting fused cells by light microscopy or by analyzing the extent of zygote formation.

Table III summarizes results of these experiments. No appreciable cell fusion results when gametes of a single mt agglutinate with one another, and the few quadriflagellate cells (QFC's) that do appear by light miocroscopy are not reflected in a population of chloroform-resistant zygotes (Table III, A and B). This discrepancy may signify that rare fusion events do occur between cells of the same mt but that such fused cells do not undérgo a normal zygotic maturation.

Mixtures of $imp-2$ or $imp-5$ mt^+ gametes with wild-type mt^- cells, on the other hand, proceed to fuse with high efficiency in the presence of αG^+ antisera (Table III, C–I) and also in the presence of αG^+ that was heated for 1 h at 56°C to inactivate the complement pathway (Table III, J). The resultant QFC's, moreover, mature into chloroform-resistant zygotes (Table III) that carry both imp and wild-type genes (15). Light microscopy of the fusion process reveals that it proceeds exactly as in a normal mating: agglutinating cells make contact by their anterior ends, form a cytoplasmic bridge, and then "jackknife" to form quadriflagellate cells (11).

The experiments summarized in Table III, K–M, reveal that, as with mating-structure activation (Table II), αG^- is as effective as αG^+ in promoting zygotic cell fusion. The αV^+ antiserum is also fully effective in promoting cell fusion except perhaps at a 1:500 dilution (Table III, N–R). Finally, Con A is shown in Table III, S, to have no ability to promote zygote formation, even with autolysinized cells; indeed, the lectin appears to inhibit the formation of the few zygotes that "leak through" in an $imp-5$ mt^+ mating (reference 14 and Table III, E).

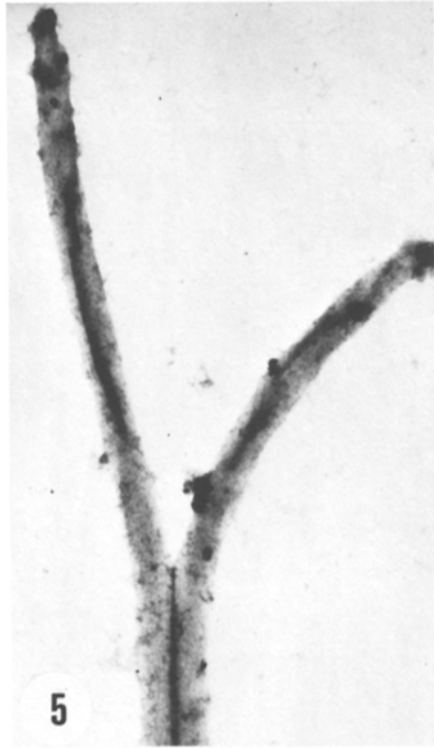Since short exposure to papain has no effect on the zygote-forming ability of wild-type cells (4), it

FIGURE 5 Vegetative wild-type mt^+ flagella incubated in the presence of a 1:65 dilution of αV^+ antiserum. Most of the cells lost their flagella but this pair remained attached to the cell body. Sample was prepared for negative staining as described for Fig. 2. No vesicles or dense aggregates are accumulated at the tips. Punctate densities appear along the flagellar length that are not seen in control samples presented with preimmune serum. The flagella associate medially, but it is not known whether this represents an antibody adhesion since similar lateral associates are occasionally encountered in controls. × 20,000.

became possible to ask whether the papain-digested Fab preparation of αG^+ was capable of promoting cell fusion between imp-5 mt^+ and mt^- gametes. No zygotes were found (Table III, T) in concordance with the inability of such Fab preparations to stimulate mating-structure activation (Table II, O).

The imp-7 strain once again presents an exceptional case: despite extensive antisera-induced agglutination, cell fusion is very rarely observed in imp-7 mt^+ × $+mt^-$ mixtures at ordinary antisera dilutions. Zygote formation remains poor even when the very high antiserum concentrations necessary for imp-7 activation (Table II, I) are used, perhaps because the $+mt^-$ cells become

unresponsive at such concentrations. As a result, only a few zygotes have to date been recovered from repeated antiserum-mediated imp-7 crosses (15).

DISCUSSION

The Gametic Tipping Response

None of the antisera we have raised exhibits any mt or state-of-differentiation specificity by the assay systems thus far employed; similarly nonspecific antisera have been reported for C. moewusii (43), Pandorina (8), and for C. reinhardi (W. Snell, personal communication). The one significant difference detected between vegetative and gametic (but not mt^+ and mt^-) cells in the present study lies in their response to all the antisera: whereas gametic flagella remain associated with cell bodies even when heavily loaded with bound antibody, vegetative flagella instantly detach from the cells unless the antisera are presented in extremely dilute form. Gametes, moreover, tend to agglutinate via antibody by their flagellar tips whereas vegetative cells agglutinate throughout their flagellar lengths. These two observations can be coupled by proposing that vegetative flagella snap off as soon as antibody associates with the transition-zone region at the flagellar base, which acts as a natural "break point" (26), while gametes are able to "tip" the antibody before it has time to so associate. We should note in this regard that Bloodgood (3) has described a saltatory, bidirectional movement of particles (e.g., polystyrene beads) that associate with the C. reinhardi flagellar surface, a motion that occurs in both vegetative and gametic cells. The relationship, if any, between this phenomenon and gametic tipping is presently being investigated in our laboratory.

The ability of gametes to tip antibody would clearly be facilitated if, during gametogenesis, the lipid and/or fatty-acid composition of the flagellar membrane were to change so that the membrane became more "fluid" (27). Postulated changes in the overall chemical composition of the flagellar membrane do not in themselves, however, readily explain the one-way movement of the antibody, nor do they explain why antibody is not mobilized towards the tips of isolated gametic flagella. Some sort of process that requires an intact flagellum therefore seems to be involved (cf. reference 12).

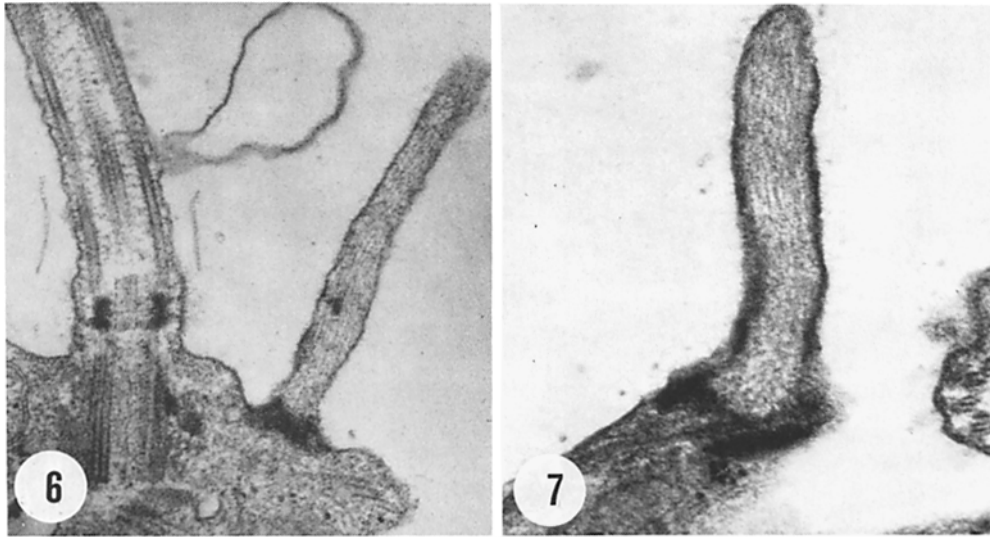One possible tipping mechanism is suggested by the observation that the aggregated material

FIGURE 6 Activation of an *imp-5* *mt*⁺ mating structure induced by a 1:50 dilution of αG⁻. No *mt*⁻ gametes were present in the culture. × 54,000.

FIGURE 7 Activation of an *imp-2* *mt*⁺ gamete induced by a 1:50 dilution of αG⁺, showing the dense array of microfilaments in the extended tubule. No *mt*⁻ gametes were present in the culture. × 62,000.

TABLE II

Effect of Antisera and Concanavalin A on Mating Structure Activation in mt⁺ Gametes

Exp	Strain	Treatment	Dilution (serum:cell suspension) or concentration	No. activated mating structures	No. unactivated mating structures	% Activated
A	WT	Preimmune serum	1:1	0	15	0
B	imp-5	Preimmune serum	1:40	0	17	0
C	imp-2	αG⁺	1:50	29	8	78
D	imp-5	αG⁺	1:50	20	9	73
E	imp-6	αG⁺	1:50	10	12	45
F	WT	αG⁺	1:1	9	11	45
G	imp-7	αG⁺	1:50	0	12	0
H	imp-7	αG⁺	1:25	5	18	25
I	imp-7	αG⁺	1:1.5	11	2	84
J	imp-5	αG⁻	1:40	12	5	71
K	imp-5	αG⁺ adsorbed *mt*⁻	1:20	0	15	0
L	imp-5	αG⁻ adsorbed *mt*⁺	1:20	0	10	0
M	WT	Con A	0.05%	0	10	0
N	imp-5	Con A	1.0%	0	16	0
O	imp-5	αG⁺, Fab preparation	1:40	0	19	0

at the gametic flagellar tips contains numerous small membrane vesicles that bleb off the flagellar ends. While it seems more likely that such vesiculation is simply a consequence of the tipping process, it is conceivable that ligands bound to the gametic flagellar surface are moved distally as a consequence of tip vesiculation.

A second possibility is that tipping is effected, at least in part, by structural components of the flagellar matrix (25) that make contact with both the membrane and the axoneme (12) and mediate motion of surface-associated macromolecules in a unidirectional fashion. In this regard, several analogies can be drawn between flagellar tipping and the "capping" response to bound antibody by mammalian lymphocytes (see reference 31 for a
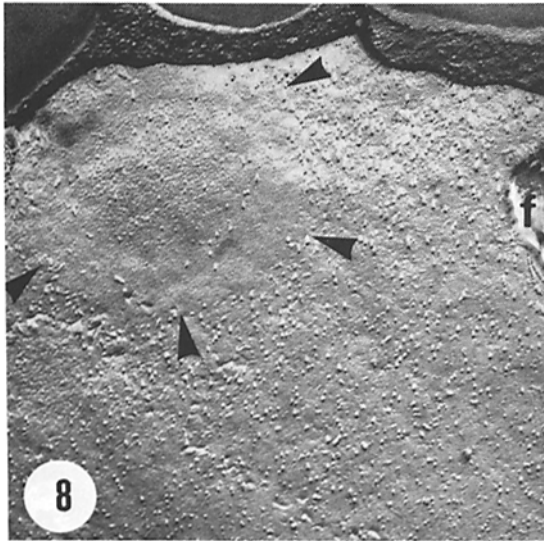
FIGURE 8 Activation of a wild-type mt^- mating structure (delimited by arrowheads) induced by a 1:50 dilution of αG^+ and visualized in the P face of a freeze-fracture replica. No mt^+ gametes were present in the culture. Flagellum at $f. \times 54,000$.

recent review), during which surface ligands are rapidly concentrated in the pericentriolar region of the cell.

The possible relevance of tipping ability to the gametic state is strengthened by Bergman's (1) studies of Con A binding to *Chlamydomonas* isolated flagella. Both vegetative and gametic flagella prove to bind equivalent amounts of [³H]Con A in vitro, but only gametic cells are agglutinated by the lectin (2, 24), agglutination taking place at the flagellar tips. Since Con A and the antibodies appear to bind to some of the same flagellar surface glycopolypeptides (our unpublished observations), it is not unexpected to find that both bound reagents are handled in a similar fashion by gametic cells.

If these observations are extended to include sexual agglutination, it is conceivable that the enhanced tipping ability of gametic flagella reflects an important feature of the gametic state. The gametic flagellum might, for example, be capable of concentrating rare agglutinin species into the flagellar tip upon contact with gametes of opposite mt (cf. reference 28). Tipping ability appears, moreover, to be correlated with the ability to send a "signal-to-mate" to the cell body: the $gam-1 \, mt^-$ mutant (10) is found to be conditionally defective both in signal generation and in anti-

body and sexual tipping (9). More generally, gametogenesis may well involve the acquisition of the ability to rearrange existing vegetative surface molecules into agglutinative patches, a concept that is explored more fully in later sections.

Antiserum Activation of Microfilament Polymerization

In mt^+ cells, mating-structure activation entails the rapid polymerization of microfilaments from a dense, Z-band-like structure (5, 11, 18, 38), a response highly reminiscent of the acrosome reaction in echinoderm sperm (37). The observations reported here reveal that this same response can be triggered by antibody agglutination. It should be stressed that the fertilization tubules so generated are often abnormal in appearance, being narrow and contorted in their distal regions. Similarly contorted fertilization tubules are often elicited from mt^+ gametes by glutaraldehyde-fixed mt^- "corpses" (C. Forest, unpublished observations). Isolated mt^- flagella, on the other hand, stimulate the extension of apparently normal fertilization tubules (45). It would appear, therefore, that a fully effective signal requires the presence of a native mt^- membrane, with fixed membranes or antiserum being only imperfect mimics.

One other case has been reported in which antisera elicit rapid microfilament polymerization: when B lymphocytes are presented with anti-IgG, the capping response that ensues involves the polymerization of microfilaments (31). The lymphocyte response is not elicited by Con A, in parallel with present observations, and appears to be mediated in some fashion by Ca^{++}. It is not known, in the case of lymphocytes, whether the response is a result of antibody binding at any position on the cell surface or whether only certain localized surface antigens signal the ensuing events. It is clear in the case of *C. reinhardi*, however, that the site of the stimulus—the distal half of the flagellum—is many microns away from the site of the mating-structure activation. Therefore, some component(s) of the flagella and cell body must spatially transduce the agglutination reaction into an activation response (19, 35). It seems likely that the same transduction mechanism operates during sexual and antiserum-induced agglutination, although this has not yet been demonstrated directly.

TABLE III

Effect of Flagellar-Directed Antisera on Cell Fusion and Zygote Formation

Exp	Gametic cells	Serum added (dilution)	% Quadriflagellates*	Chloroform-resistant colonies
A	mt+ alone	αG+ (1:1)	1	none
B	mt- alone	αG- (1:1)	5	none
C	imp-2 mt+ × +mt-	none	4	a few
D	imp-2 mt+ × +mt-	αG+ (1:1)	36	many
E	imp-5 mt+ × +mt-	none	1-2	a few
F	imp-5 mt+ × +mt-	αG+ (1:1)	38	many
G	imp-5 mt+ × +mt-	αG+ (1:5)	43	-‡
H	imp-5 mt+ × +mt-	αG+ (1:250)	43	many
I	imp-5 mt+ × +mt-	αG+ (1:500)	—	many
J	imp-5 mt+ × +mt-	αG+ (1:50) (heat 1 h at 56°C)	44	—
K	imp-5 mt+ × +mt-	αG- (1:1)	6	—
L	imp-5 mt+ × +mt-	αG- (1:10)	43	—
M	imp-5 mt+ × +mt-	αG- (1:50)	35	—
N	imp-5 mt+ × +mt-	αV+ (1:1)	5	—
O	imp-5 mt+ × +mt-	αV+ (1:10)	41	—
P	imp-5 mt+ × +mt-	αV+ (1:50)	42	—
Q	imp-5 mt+ × +mt-	αV+ (1:100)	—	many
R	imp-5 mt+ × +mt-	αV+ (1:500)	—	fewer than Q
S	imp-5 mt+ × +mt-	Con A (1%)	—	none
T	imp-5 mt+ × +mt-	αG+ Fab Prep. (1:40)	—	none

* Calculated as 2(QFCs)/[2 (no. QFCs) + (no. BFC)] where QFC = quadriflagellated cell, BFC = biflagellated cell.

‡ Dash indicates no observations made.

The mt Specificity of the Fusion Process

The experiments with antiserum-activation of one mating type reveal an important feature of gametic cell fusion in C. reinhardi: the fusion process itself appears to require the specific participation of both mt+ and mt- mating-structure membranes. Thus when antisera are given to mt+ alone, a fertilization tubule is generated from each cell and tubule-to-tubule and tubule-to-cell appositions must occur frequently. Cell fusion rarely, however, results from such contacts (Table III, A), nor from contacts between mt- cells (Table III, B).

These observations rule out certain models for the mechanism of zygotic fusion in C. reinhardi. One might imagine that specificity were created solely by the flagellar agglutination reaction: once an mt+ and mt- pair were locked into their mating configuration the outgrowing fertilization tubule bearing some generalized fusogenic substance might fuse with the first substrate it contacted. Instead, the fusion process appears to require that the tip of the mt+ fertilization tubule and the activated mt- structure at the cell membrane interact in an mt-specific fashion. Both sites bear a group of large intramembraneous particles (45) which may possibly mediate the mt-specific recognition and/or fusion events.

Antiserum Activation of Mutant Strains

Two classes of nonagglutinating imp mutations have been defined by the present study. The imp-2, imp-5, imp-6, and imp-8 strains, which we can denote as Class A, have lost the capacity to agglutinate sexually but remain intact in their ability to signal in the presence of divalent antibody. Strains of Class B, represented here by imp-7, are impaired in both sexual agglutination and in receptivity to antiserum stimulation, although remaining fully agglutinable by antisera and by Con A (2). When gametes of the Class A strain imp-5 mt+ are treated with either trypsin or pronase, the cells continue to agglutinate via the antibody molecules because at least some of the surface antigens are highly resistant to proteases (4). Such cells can no longer be stimulated by the antisera to fuse with + mt- gametes; however, neither are they stimulated to activate their mating structures (our unpublished experiments): they are converted, functionally, into imp-7 mt+

phenocopies. Thus it is proposed that the mt^+ flagellar surface carries at least three components: (a) an agglutinin, sensitive to proteolysis (34, 40, 41) and disfunctional in all 5 imp strains; (b) a signaling component, also sensitive to proteolysis, which is defective in the imp-7 strain and stimulated by antiserum-flagellar interactions in Class A strains; and (c) proteolysis-resistant antigens that are capable of stimulating signaling activity in Class A strains upon interaction with antisera.

The imp-7 mutation is closely linked to the imp-2, imp-5, and imp-6 mutations which define a locus called sag-1 (for sexual agglutination); all are unlinked to imp-8, which marks the sag-2 locus (15). If the four sag-1 mutations prove to lie within a single gene, then the product of this gene would appear to be involved in setting up and/or participating in both sexual agglutination and sexual signaling, with the imp-7 mutation rendering both features of the gene product defective and the imp-2, imp-5, and imp-6 mutations affecting the agglutination feature alone. If sag-1 instead proves to be a cluster of genes, the agglutination and the signaling activities may well reside in different gene products which, by definition, interact in some fashion during the mating reaction.

The postulate that distinct agglutinin and signal activities function during the mating reaction predicts that Class C mutations should exist which abolish sexual signaling but not sexual agglutination. The temperature-sensitive mutation gam-1 mt^- (10) appears to produce just such a phenotype: as reported in detail elsewhere (9), gam-1 gametes can agglutinate, but not fuse, at restrictive temperatures and cannot be induced to fuse by antiserum.

The Mechanism of Antiserum Activation

The fact that αV^+, αG^+, and αG^- are all comparable in their ability to elicit mating-structure activation and imp cell fusions argues strongly that the active antibody species of each serum has been raised against flagellar surface antigens present on all C. reinhardi cell types. That these antigens are distinct from the agglutinin/signal (A/S) component of the flagellar membrane is demonstrated both by the persistence of antiserum agglutination when sexual agglutinability is lost, and by the insensitivity of the antigens to a wide range of proteolytic enzymes that destroy the A/S component (4).

Two possibilities can therefore be entertained regarding the functional role of these ubiquitous antigens in the mating reaction. The first possibility, which endows the antigens with an active role in the mating process, supposes that sexual agglutination allows some interaction between the postulated signaling component and the antigens such that an effective signal is generated to the cell. In other words, the antigens would have an essential function in signal generation, and the antisera would be able to elicit that function by interacting with the antigens in Class A, but not Class B or Class C, strains. This model is rendered less attractive, but is not ruled out, by the failure of monovalent antibody to elicit activation.

The alternate possibility is that the antigens normally play no direct role in the mating interaction but that their cross linking has the effect of mimicking an essential feature of normal sexual agglutination. In the paragraphs below, this possibility is explored conceptually by presenting first a model for normal agglutination and then a proposal for how divalent antibody could stimulate this process.

The strong "tipping" response of gametic flagellar membranes provides a basis for proposing that the agglutinins and signal components are spatially dispersed throughout the flagellar membranes of unmated gametes but that, when mt^+ and mt^- gametic flagella make contact in a normal mating reaction, interacting agglutinins move together to form "patches" (cf. reference 31) at the distal ends of the flagella, such patches creating the strong adhesiveness generated between mating pairs. Assuming, as argued above, that the agglutinin and signal components are physically integrated in some way, such a patching would also bring signal components together, and their interaction in the patch might generate a mating-structure-activation signal to the two cell bodies. Once zygotic cell fusion is achieved, the patches would disperse and the cells would disagglutinate.

In the context of this model, then, the antisera could be visualized as creating localized "cages" of antigen/antibody complexes, both in the plane of the membrane and between interacting membranes, which move to the flagellar tips and leave behind an antigen-depleted membrane. The dispersed A/S components would tend to move towards one another in this "vacated" membrane, creating localized patches of A/S components which would proceed to carry out a signal-generating interaction in the same fashion as

postulated for a normal mating. The ability of divalent antibody to "short-circuit" the Class A but not the Class B or Class C mutations is explained by this proposal, as is the failure of monovalent Fab fragments to exert any short-circuiting activity.

The cross-linking model does not in itself explain why Con A fails to elicit mating-structure activation. It is possible that the lectin exerts inhibitory effects on the activation process, as is found in certain capping responses (31). Alternatively, it is possible that cross-linking the lectin receptors has different effects on the membrane than antibody-antigen cross linking. A detailed study of the Con A-binding and antibody-binding components of the flagellar surface is presently underway in this laboratory, the results of which should allow us to analyze these possibilities in an experimental fashion.

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