Monoclonal Antibodies Directed against the Sexual Binding Site of *Chlamydomonas eugametos* Gametes

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Abstract. Monoclonal antibodies were raised against the mt^- sexual agglutinin of *Chlamydomonas eugametos* gametes. Those that blocked the agglutination site were selected. They were divided into two classes dependent upon whether they gave a weak (class A) or clear positive (class B) reaction with mt^- flagellar membranes in an ELISA and an indirect immunofluorescence test using glutaraldehyde-fixed mt^- gametes. Class A antibodies were shown to be specific for the agglutinin in an extract of mt^- gametes, based on results from immunoblotting, immunoprecipitation, affinity chromatography, and the absence of a reaction with nonagglutinable cells. Surprisingly, class A mAbs also recognized two mt^+ glycoproteins, one of which is the mt^+ agglutinin. Class B antibodies were shown to bind to several glycoproteins in both mt^- and mt^+ gametes, including the mt^- agglutinin. Fab fragments from class A mAbs blocked the sexual agglutination process, but those from class B did not, even though the parent antibody did. We conclude that the class A epitope lies in or close to the agglutination site of the mt^- agglutinin, whereas the class B epitope lies elsewhere on the molecule. We also conclude that the mt^- agglutinin is the only component on the mt^- flagellar surface directly involved in agglutination. Class A mAbs were found to elicit several reactions displayed by the mt^+ agglutinin. They bound to the mt^- agglutinin on gamete flagella and induced most of the reactions typical of sexual agglutination, with the exception of flagellar tip activation. None of these reactions was induced by Fab fragments. High concentrations of class A mAbs completely repressed the sexual competence of live mt^- gametes, but low concentrations stimulated cell fusion.

THE mutual recognition of sexually compatible *Chlamydomonas* gametes is the best studied cell recognition system in plant biology. Not only do we know much of the morphological aspects of recognition, but we also appreciate many of the physiological consequences whereby those gametes that have recognized a partner are activated to prepare themselves for cell fusion (Goodenough and Thorner, 1983; Snell, 1985; van den Ende, 1985). More importantly, the recognition factors, the agglutinins, have been identified and partially characterized for two different *Chlamydomonas* species (Musgrave et al., 1981; Adair, 1983; Saito and Matsuda, 1984; Collin-Osdoby et al., 1984; Collin-Osdoby and Adair, 1985; Klis et al., 1985; Samson et al., 1987), and thus a molecular analysis of the recognition process is in progress.

The two species that have commanded most attention are *Chlamydomonas reinhardtii* and *Chlamydomonas eugametos*. They are sexually incompatible and, as judged by their heterologous chloroplast DNA sequences, are only distantly related to each other (Lemieux and Lemieux, 1985). This is also expressed in significant differences in the mating processes of the two species. In this study we have worked solely with *C. eugametos*. If sexually compatible strains, referred to as mating type plus (mt^+) and mating type minus (mt^-), are mixed, they collide at random (Tomson et al., 1986) and adhere together via the mating type–specific agglutinins exposed on their flagellar surfaces. Adhesion not only holds potential partners in close proximity but triggers responses that are essential for fusion. For example, the gametes no longer swim normally; they remain relatively stationary, even though the flagella move vigorously. Under the microscope, the agglutinating clumps of cells seem to vibrate, a phenomenon we call sexually induced twitching. It can be artificially induced by adding the agglutinin from one mating type to gametes of the other mating type (Homan et al., 1980). Agglutination also induces a higher level of agglutinability because more active sites become exposed on the flagellar membrane (Demets et al., 1988). It does not produce the shedding of the cell wall, as in *C. reinhardtii* (Claes, 1971). Cell fusion can only occur at a restricted location on the cell surface where a mating structure (or papilla) protrudes through the cell wall covering the flagellar ridge (Mes-

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land, 1976). Because the papilla only just penetrates the cell wall, cell fusion can only occur if the flagellar ridges of partners are brought into direct apposition. This is achieved by two flagellar properties. First, agglutinins involved in the initial adhesions are transported to the flagellar tips (Musgrave et al., 1985; Homan et al., 1987*b*). This tipping of agglutination sites was first observed microscopically in *C. reinhardtii* and is a form of flagellar surface motility that has been well documented by Bloodgood (e.g. 1987). In this way, the tips of partner flagella become aligned and this allows the rest of the flagellar surface to become involved in adhesion via agglutinins that have not been tipped. Because each papilla protrudes from the cell body at a middle point situated between the bases of the two flagella, aligning the flagellar tips automatically aligns the papillae. When the flagella adhere over their entire length, the papillae tend to be brought into mutual contact. This is assured by a second property, viz., all four partner flagella become positioned around the mt^- cell body (Musgrave et al., 1985). This results in the cells lying vis-a-vis with papillar tips confronting each other. After fusion, the cells remain for many hours just connected by a narrow plasma bridge. About 24 h later, the cell wall is slowly digested so that the protoplasts can fuse completely (Musgrave et al., 1983). The agglutination process in *C. reinhardtii* is similar to that of *C. eugametos*, but due to the loss of the cell wall the whole papillar surface becomes exposed for recognition and fusion (Forest, 1983).

It should now be obvious that sexual agglutination is not just a means of cell recognition but a complete mechanism for promoting cell fusion. The whole process is directed by the agglutinins. They are extremely large ($\sim 1.3 \times 10^3$ kD) glycoproteins extrinsically bound to the flagellar membranes. They have been visualized as long linear molecules (290–340 nm, Crabbendam et al., 1987) and their equivalents in *C. reinhardtii* have been depicted as projecting from the flagellar surface, somewhat like the hairs of a "bottle cleaner" (Goodenough et al., 1985). To be able to monitor the presence and distribution of at least one of the agglutinins, we have been trying to raise monoclonal antibodies to the mt^- agglutinin of *C. eugametos*. In the hope that the antibody could also be useful in mapping the active site of the molecule, antibodies were selected that blocked the activity of the agglutinin. Several attempts to produce such antibodies failed but we have now developed a successful protocol that has produced several interesting antibodies. Attempts to produce similar antibodies against the *C. reinhardtii* agglutinins have already been described. Adair (1985) has characterized a number of mAbs that bind the mt^+ agglutinin of *C. reinhardtii*, but none of them seem to block agglutinability and they all recognized other additional flagellar components. More recently, Snell et al. (1986) have reported the isolation of a mAb that blocks the agglutination of mt^+ gametes and is capable of inducing sexual responses in *C. reinhardtii* but its usefulness is limited, for it did not label any flagellar components in situ using the immunofluorescence technique, nor in vitro on blots of components separated in sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE) gels. In contrast, all the mAbs described here label the mt^- agglutinin of *C. eugametos* in blots, block the biological activity of the isolated agglutinin, and label the flagellar surface of live gametes but not vegetative cells using the imunofluorescence test. In binding the mt^- agglutinin, one group of mAbs is able to induce nearly all the responses typical of sexual agglutination, including the formation of papillae.

Materials and Methods

Cell Culture

Chlamydomonas eugametos, strains Utex 9 (mt^+) and Utex 10 (mt^-) from the Collection of Algae, University of Texas, Austin, TX, were cultured on agar as described by Mesland (1978). Vegetatively dividing mt^- cells were obtained as described by Tomson et al. (1985). The light-sensitive mt^- strain 5.39.4 (Kooijman et al., 1988) was used in some monoclonal antibody binding studies. This strain is nonagglutinable in the dark but fully agglutinable in the light.

Production of Monoclonal Antibodies

A BALB/c mouse was immunized intraperitoneally and subcutaneously with 3 μg mt^- agglutinin (PAS 1.2) together with 60 μg mt^- isoagglutinin, i.e., membrane vesicles in the culture medium that are rich in mt^- agglutinin but contain all the other flagellar glycoproteins (Musgrave et al., 1981). The mouse was immunized first in Freund's complete adjuvant and then approximately every 2 wk in Freund's incomplete adjuvant, except that the last immunization occurred without adjuvant. On day 127, a booster was given that contained twice the normal antigen quantity. The monoclonal antibodies described in this report result from a fusion with the code number 66. They are referred to as mAb 66.X, where X is the clone number.

The fusion of spleen cells from immunized mice with SP_2/0-Ag-14 mouse myeloma cells, the subcloning of positive hybridomas and the growth of hybridoma cell lines in mice for the production of ascites were performed as described previously (Kolk et al., 1984). Immunoglobulin subclasses were determined by double diffusion using subclass-specific goat antisera (Nordic Diagnostics, Tilburg, The Netherlands).

Isolation of Flagellar Membranes and Membrane Glycoproteins: Indirect Immunofluorescence and Enzyme-linked Immunoassay

All these methods and assays were performed as described by Homan et al. (1987*a*).

Mt^--Agglutinin Coupling to Sepharose AH

Sepharose AH (Pharmacia/LKB, Uppsala, Sweden) was prepared as described by the manufacturers. 1 ml packed gel was incubated with 1 ml mt^- agglutinin solution (23 μg, titer 2^9, purified by gel filtration as described by Musgrave et al., 1981), and 10 mg ethyl-3(3dimethylaminopropyl)-carbodiimide (Pierce Chemical Co., Rockford, IL) in 0.5 M sodium acetate buffer, pH 4.5, for 18 h at 4°C. The slurry was washed five times alternatively in 0.2 M acetate, 0.5 M NaCl, and in 0.2 M sodium carbonate, pH 8.3, 0.5 M NaCl. The spheres were stored in 10 mM Hepes, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.6 (Hepes buffer) containing NaN_3 at 4°C. Control Sepharose beads were treated in the same manner except for the omission of the mt^- agglutinin.

Agglutination Inhibition Test

10 μl suspension of agglutinin spheres (1:1 vol/vol suspension in Hepes buffer) was pipetted into the wells of a flat-bottomed microtiter plate (Costar Europe Ltd., Badhoevedorp, The Netherlands). 50 μl hybridoma culture supernatant was added to each well and incubated 45 min at room temperature. Spheres were washed twice for 10 min with 100 μl Hepes buffer, and two drops of mt^+ gamete suspension (1.3×10^7 cells/ml) were added. Agglutination of the cells to the spheres was monitored microscopically 1–5 min after adding the gametes.

The ability of the mAbs to inhibit the agglutinability of glutaraldehyde-fixed and live mt^- gametes was also tested. 100 μl gametes were mixed with different concentrations of antibody and at certain times thereafter, live mt^+ gametes were added to a sample under a microscope to assess their agglutinability. Because the antibody was able to isoagglutinate at least the mt^+ test gametes, the mixture had to be critically assessed immediately after mixing. With experience, the effect on sexual agglutination could be discriminated from other effects.

SDS-PAGE and Western Blotting

SDS-electrophoresis in 2.2–20% acrylamide gradient gels and Western blotting were performed as described earlier (Homan et al., 1987*a*). Gels were stained with periodic acid Schiff reagent (PAS)[1] according to Zacharius et al. (1969) to visualize the glycoproteins. This was followed by silver staining as described by Morrissey (1981) to visualize any other proteins present.

Two sources of molecular weight markers were used to calibrate the distribution of proteins in the gels. Firstly, those from Pharmacia and secondly, the proteins in erythrocyte ghosts, which can be used as markers because they have been so well characterized (Bennett, 1985).

Immunoprecipitation of Antigens Exposed on Intact mt^- Gametes

25 ml mt^- gametes (5×10^8) metabolically labeled with ^{35}S as described by Pijst et al. (1984) were incubated with protein A-purified mAb 66.3 (40 μg/ml) in the presence of 7.5 mg/ml colchicine (which inhibits the redistribution of agglutinin over the flagellar surface, see later in Discussion), for 30 min. Nonbound mAb was quickly washed away by centrifugation in 10 mM Hepes buffer, pH 7.6, and the gametes were extracted in 15 ml 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM phenyl methane sulphonyl chloride, 5 mg/ml aprotinine in 100 mM Tris buffer, pH 8.1, for 1 h at 4°C. The insoluble material was spun down at 50,000 *g* for 60 min. The supernatant was incubated with protein A–Sepharose (Pharmacia/LKB) (5×10^6 dpm/7.5 mg protein A–Sepharose) for 18 h at 4°C. After extensively washing the beads in 10 mM Tris buffer, pH 8.1, 150 mM NaCl, 0.2% Triton X-100, the bound material was dissolved in sample buffer and loaded onto a 2.2–20% gradient SDS-polyacrylamide gel.

Affinity Purification of mAb 66.3 Antigen

Immobilization of mAb 66.3 onto protein A–Sepharose (Pharmacia/LKB) was performed as described by Schneider et al. (1982), using 20 mM dimethyl suberimidate (Pierce Chemical Co.) as cross-linker. The immunoabsorbant column was used as described in the same paper.

Purification of mAbs

Ascites was diluted with an equal volume of PBS and centrifuged for 30 min at 100,000 *g*. The supernatant was brought to 50% saturation with ammonium sulphate and stirred for 4 h at 4°C. After centrifugation for 30 min at 10,000 *g*, the pellet was dissolved in 50 mM Tris-HCl, pH 8.6, in 150 mM NaCl and dialysed extensively against the same buffer. This material was loaded onto a protein A–Sepharose column and the monoclonal antibodies were eluted with 50 mM acetate buffer, pH 4.3, in 150 mM NaCl, at a rate of 15 ml/h. The mAbs were dialyzed in the buffer in which they were to be used in further experiments, and concentrated by ultrafiltration (Amicon Diaflo ultrafilters PM10).

Labeling mAbs with a Fluorochrome

mAbs 66.3 and 66.6 were labeled with fluorescein isothiocyanate (FITC) according to the procedure of Mishell and Shiigi (1980).

Production of Monovalent Antibodies (Fabs)

For each of the mAbs 66.3 and 66.6 (both IgGs), optimal conditions for Fab formation were determined as monitored by SDS-PAGE using 12.5% acrylamide gels. The protein A–purified mAb in 0.1 M phosphate buffer, pH 7.5, and 4 mM EDTA was incubated with 10 mM 2-mercaptoethanol and mercuripapain (Sigma Chemical Co., St. Louis, MO, 1% of mAb wt/wt). The reaction was stopped with iodoacetamide (final concentration, 14 mM). Optimal conditions were 5 min at 4°C for mAb 66.3 and 30 min at 4°C for mAb 66.6. The binding activity of the Fab fragments was tested in an indirect immunofluorescence test using a mixture of mt^+ and mt^- gametes that had been fixed in 1.25% glutaraldehyde after 10 min agglutination, because such gametes expose a high level of antigens at their tips (Homan et al., 1987*b*). Nondegraded IgG, and fragments with a molecular weight >60 kD were separated from the mAbs on a Sepharose G-150 column in 0.1 M phosphate buffer, pH 7.5, in 20 mM NaCl. The purity of the Fab fragments was checked via HPLC using a Bio-Sil TSK-125 column and SDS-PAGE. Fabs were stored frozen at −70°C in the presence of 1% BSA.

Influence of mAbs and Fabs on Live Gametes

The antibodies were dialyzed against 10 mM Hepes buffer, pH 7.6. When added to gamete suspensions the cells reacted as if they were involved in sexual agglutination. The following aspects of this response were tested.

mAb-induced Isoagglutination and Twitching. Live gametes (10 μl, 1.3 × 10^5 cells) were mixed under a microscope with 10 μl mAb 66.3 (final concentration, 1–200 μg/ml) and assessed for the appearance of clumps of isoagglutinating cells, or the presence on the surface of the drop of immobilized cells that twitched violently in a manner resembling agglutination (Homan et al., 1980). In the latter case the cells were not bound to others in their vicinity.

mAb-induced Flagellar Tip Activation (FTA) and Papilla Formation. 100 μl (1.3 × 10^6 cells) gametes were treated for 60 min with various concentrations of mAb 66.3 and then fixed 10 min in 1.25% glutaraldehyde. To visualize FTA, the cells were fixed a further 60 min in 5% glutaraldehyde and then washed consecutively in 50, 80, and 96% ethanol. A 10-μl drop of the cells was then brought onto a cover slip on a warming plate such that the suspension spread and dried within a few seconds. The coverslip was then inverted and fixed to a microscope slide using nail varnish. The dry preparation was inspected under phase contrast optics.

To test for the presence of papillae, the cells were subjected to an indirect immunofluorescence test using mAb 44.2 as described by Musgrave et al. (1986). mAb 44.2 only binds to the flagellar and papillar surfaces, as it can not penetrate the cell wall. Papillae were then visible as fluorescent points between the bases of the flagella.

mAb-induced Redistribution of Flagellar Antigen. 100 μl gamete suspension (1.3 × 10^6 cells) was incubated for 10 min at room temperature with 5 μl hybridoma culture suspension or FITC-labeled mAb 66.3 (final concentration, 5 μg/ml). Gametes were fixed in 1.25% glutaraldehyde and washed in PBS followed by incubation with FITC-labeled goat anti-mouse IgG (H and L chain, Tago Inc., Burlingame, CA). In some experiments, incubation with Fab fragments was followed by an incubation with 5 μl rabbit anti-mouse serum before fixation of the cells. In these cases the antibody was visualized with a FITC-labeled goat anti-rabbit IgG (Nordic Diagnostics, Tilburg, The Netherlands). The cells were photographed under a Zeiss photomicroscope fitted with an epifluorescence attachment.

mAb Effects on Mating Efficiency. 100 μl mt^- gametes (1.3 × 10^6 cells) were incubated with different concentrations of mAb 66.3 or its Fab fragments. At certain time intervals they were mixed with 10 μl mt^+ gametes (3 × 10^6 cells). After 40 min the cells were fixed in 1.25% glutaraldehyde and the number of vis-a-vis pairs was assessed using a hemocytometer. The mating efficiency was then calculated as the percentage mt^- cells that had fused.

Modification of the mt^- Agglutinin

The mt^- agglutinin was modified to test the effect on the binding of mAb 66.3 after SDS-PAGE and Western blotting. In each case 0.5 μg agglutinin in 25 μl 10 mM phosphate buffer, pH 7.5, in 10 mM $CaCl_2$ was treated as follows: (*a*) It was incubated with either 2 mg/ml pronase (Sigma Chemical Co.), 2 mg/ml trypsin (Merck, Darmstadt, FRG), 0.25 mg/ml thermolysin (Sigma Chemical Co.) or subtilisin C or BPN (Sigma Chemical Co.) for 24 h at 37°C under toluene with and without the addition of 0.5% SDS. The reaction was stopped by the addition of 25 μl double strength sample buffer followed by electrophoresis in SDS-polyacrylamide gels. (*b*) It was incubated at 37°C for 2 h in 100 mM KOH. The reaction was stopped by neutralizing the sample and adding sample buffer. (*c*) It was dialyzed in 100 mM acetate buffer, pH 4.5, and incubated with 10 mM sodium periodate at 4°C in the dark for 20 min. Excess periodate was inactivated with ethylene glycol for 1 h at room temperature in the dark, followed by the addition of excess sodium borohydride in 65 mM acetate buffer, pH 8.5, for 2.5 h. Excess borohydride was then destroyed by acidification to pH 5 using acetic acid. The sample was concentrated in an Amicon micro-concentrator and dissolved in double strength sample buffer.

Results

When we first started raising mAbs specific for the mt^- agglutinin, we screened for hybridomas producing mating type–specific antibodies. Although many were found (Ho-

1. *Abbreviations used in this paper*: FTA, flagellar tip activation; PAS, periodic acid Schiff staining.

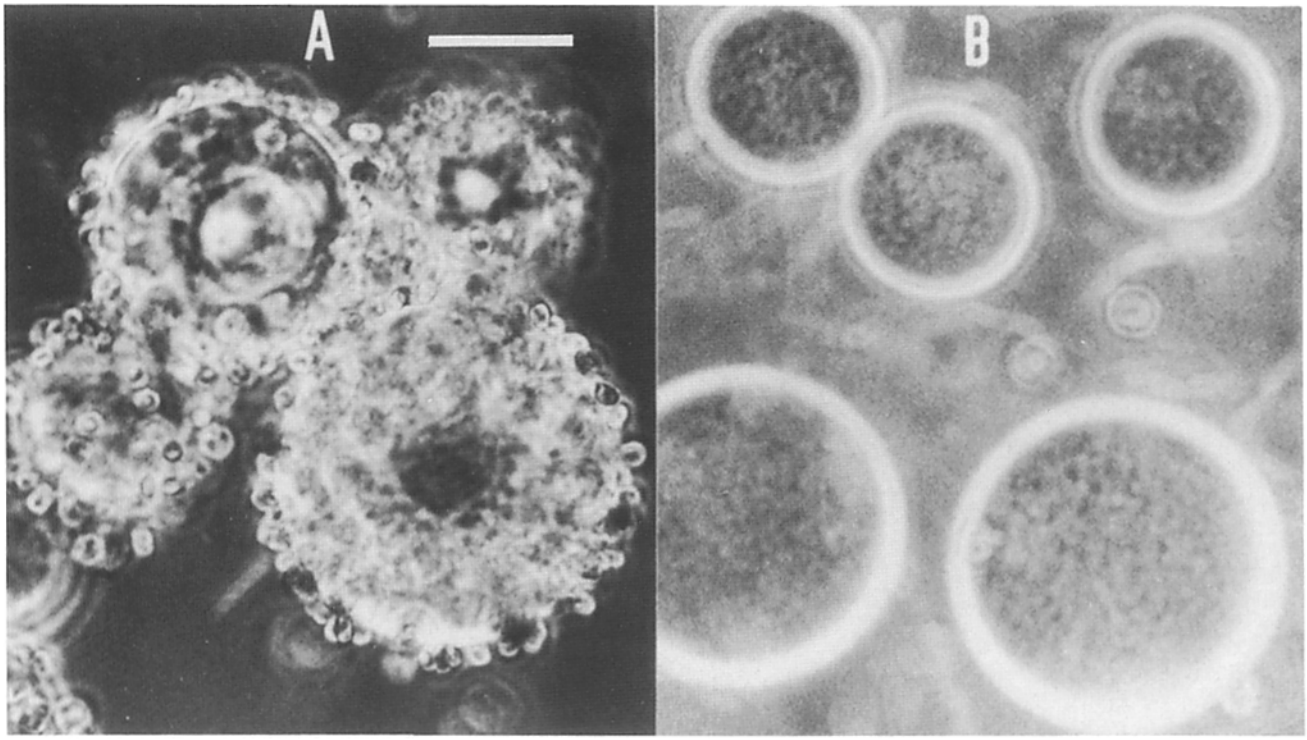

Figure 1. Inhibition of mt^- agglutinin activity by antibodies. Agglutinin bound to Sepharose AH beads was mixed with a noninhibiting (*A*) and an inhibiting (*B*) antiserum for 45 min, after which mt^+ gametes were added. Bar, 50 μm.

man et al., 1987*a*), none of them recognized the mt^- agglutinin in immunoblots. Consequently, a more stringent test was designed, in which the antibodies were screened for their ability to block mt^- agglutinability. The agglutinin was coupled to Sepharose AH beads and the ability to inhibit the adhesion of mt^+ gametes to these beads was visually assessed under the microscope. The effectiveness of the test is illustrated in Fig. 1. A mouse was immunized approximately every 2 wk with partially purified mt^- agglutinin mixed with mt^- isoagglutinin vesicles (Homan et al., 1980). Just

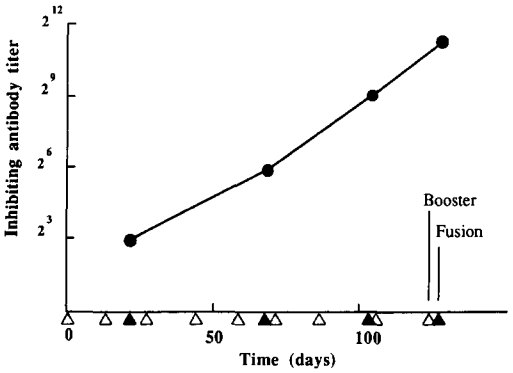

Figure 2. Inhibition titer of the serum from mouse 66 during the period of immunization. The titer is the highest binary dilution that just inhibits the binding of mt^- agglutinin to mt^+ gametes in the test illustrated in Fig. 1. The mouse was immunized on those days indicated by open triangles and sera were tapped on days indicated by closed triangles. On day 127, the mouse was killed and the spleen cells isolated for fusion with the hybridoma cell line.

before each immunization, a blood sample was taken from the mouse and tested for its ability to inhibit agglutinin activity. A dramatic increase in titer with time was observed (Fig. 2). After a booster on day 127, 3 d before the fusion was performed, the inhibition titer was 2^{11}. Even with this high level of activity, <2% of the ~1,000 culture supernatants from the hybridomas contained inhibiting antibodies. Half of the positives reacted well with extracts of both mt^- and mt^+ flagellar membranes in an ELISA test, and with both glutaraldehyde-fixed mt^- and mt^+ gametes in an indirect immunofluorescence test. The other supernatants did not react convincingly in either test, with either mating type. Nine clones were eventually isolated. Their characteristics are summarized in Table I. They were divided into two classes (A and B) based on their negative or positive reaction in the assays just mentioned. It must be emphasized, that for the sake of comparison, class A mAbs are registered as nonreactive, however, in the immunofluorescence test, most of the mt^- flagella were weakly labeled. Furthermore, class A mAbs (as well as class B mAbs) clearly labeled the flagellar tips of gametes that had been agglutinating for a few minutes. In contrast, class B mAbs always clearly labeled the flagella and cell walls of mt^- and mt^+ gametes.

Antigenicity of High Molecular Mass Glycoproteins

To determine the number of proteins in *C. eugametos* cells that are recognized by the mAbs, proteins were extracted and then separated in SDS-polyacrylamide electrophoresis gels, blotted onto nitrocellulose and the binding of the different mAbs visualized. Only the results from using mAb 66.3 (class A, IgG_3) and mAb 66.6 (class B, IgG_3) will be presented, for they are representative of their classes. Three

Table I. Characteristics of Culture Supernatants from Fusion 66

Clone No.	Ig class	Inhibition titer	ELISA*		IIF‡		Class
			mt⁻	*mt*⁺	*mt*⁻	*mt*⁺	
1	§	2^{12}	+	+	+	+	B
3	IgG_3	2^{10}	−	−	−	−	A
5	IgM	2^{9}	+	+	+	+	B
6	IgG_3	2^{9}	+	+	+	+	B
7	IgM	2^{8}	−	−	−	−	A
8	IgM	2^{9}	+	+	+	+	B
9	IgM	2^{11}	+	+	+	+	B
10	IgG_3	2^{12}	−	−	−	−	A
11	IgG_3	2^{8}	−	−	−	−	A

* Extracts of flagellar membrane vesicles were used in the ELISA.
‡ IIF, indirect immunofluorescence assay using glutaraldehyde-fixed gametes.
§ No detectable reaction was found with any of the class-specific sera used.

sources of *Chlamydomonas* proteins were used, isolated *mt*⁻ gamete flagella, and 1% Triton extracts of intact *mt*⁻ and *mt*⁺ gametes. The immunoblots are illustrated in Fig. 3. Considering the reaction with *mt*⁻ material first, mAb 66.3 did not bind to any flagellar components and only a single band in the region of the agglutinin band in the extract of whole cells. This implied that mAb 66.3 could be specific for the *mt*⁻ agglutinin. Its lack of reactivity with an equivalent agglutinin band in the flagellar preparation was not surprising because it is a minor flagellar component, particularly compared with the quantities found on the outside of the cell bodies (Pijst et al., 1983; Kooijman et al., 1986). mAb 66.3 also labeled a band in the *mt*⁺ extract in the region of the *mt*⁺ agglutinin. In Fig. 3 (*mt*⁺-cell-66.3) it appears as one band but it can sometimes be distinguished as two that migrate close to each other. To check whether one of the bands was indeed the *mt*⁺ agglutinin, it was purified as described by Samson et al. (1987), subjected to SDS-PAGE, blotted onto nitrocellulose and immunostained. The *mt*⁺ agglutinin was indeed clearly labeled (Fig. 3, extreme right). The mAb 66.6 bound to several other glycoproteins present in both *mt*⁻ and *mt*⁺ samples (Fig. 3), of which the cell wall proteins (230 and 400 kD) and a glycoprotein of 125 kD (referred to as PAS-6, see Musgrave et al., 1981) were the prominent species. Although hardly visible on the blot (*mt*⁻, cell, 66.6), a band migrating with the *mt*⁻ agglutinin was usually faintly labeled. The reactivity with the wall components was to be expected because in an indirect immunofluorescence test, the walls were always labeled, as mentioned before.

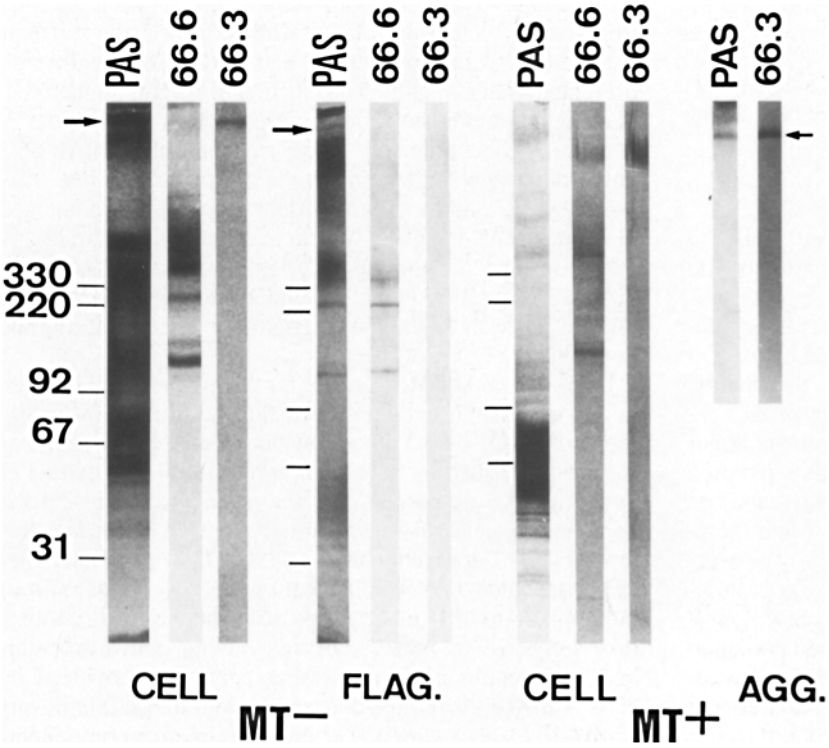

Figure 3. SDS-polyacrylamide gels and immunoblots of *C. eugametos* glycoproteins labeled with mAbs 66.3 and 66.6. *mt*⁻ gamete flagella and 1% Triton X-100 extracts of *mt*⁻ and *mt*⁺ intact gametes, as well as a sample of purified *mt*⁺ agglutinin, were separated in a 2.2–20% polyacrylamide gradient gel. The glycoproteins were stained with periodic acid Schiff reagent (PAS) or all the proteins were blotted onto nitrocellulose and immunostained using mAb 66.3 as a representative of class A mAbs, or mAb 66.6 as a representative of class B. The positions of molecular mass markers are indicated (kD × 10^{-3}) together with the position of the *mt*⁻ and *mt*⁺ agglutinins (*arrows*). The *mt*⁺ agglutinin was subjected to electrophoresis in a minigel, which explains why the gel and blot on the right are relatively small.

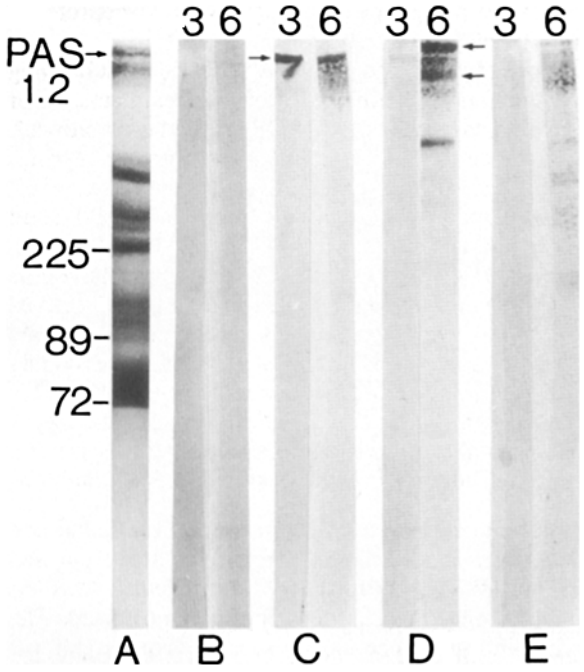

Figure 4. Immunostaining of fractionated high molecular weight proteins extracted from *mt*⁻ gametes, using mAb 66.3 and 66.6. *mt*⁻ gametes were extracted in 3 M guanidine thiocyanate and the extract (lane *A*) was fractionated by gel filtration over Sephacryl S-500. Fractions were pooled to provide a void volume fraction (lane *B*); a first active fraction (lane *C*) containing most of the *mt*⁻ agglutinin (titer 2^6); a second active fraction (lane *D*), which although still active (titer 2^2) was seen to contain the flagellar glycoproteins designated PAS-1.1 and PAS-1.3 (Musgrave et al., 1981); and lastly a nonactive fraction containing a variety of glycoproteins eluting before the total volume (lane *E*). The pooled fractions were concentrated, subjected to SDS-PAGE and blotted onto nitrocellulose. Strips from each blot were then stained with mAbs 66.3 and 66.6, which resulted in the patterns shown here. The positions of the *mt*⁻ agglutinin (PAS-1.2, lanes *A* and *C*) and the two glycoproteins PAS-1.1 and PAS-1.3 (lane *D*) are indicated by arrows. Some molecular mass markers are also included. Note that lane *A* is PAS stained. The arrowhead form of the band stained by mAb 66.3 in lane *C* is due to the corner of the gel being folded back during blotting.

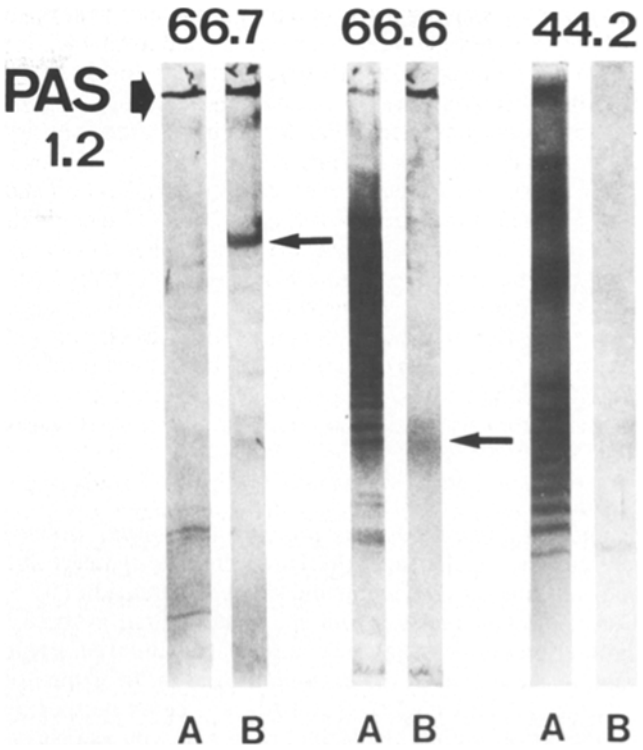

Figure 5. Affinity chromatography of *C. eugametos* proteins over a column of mAb 66.3. A 1% Triton X-100 extract (lanes *A*) of *mt*⁻ gametes was eluted over mAb 66.3 bound to protein A-Sepharose. Bound material was eluted in glycine buffer pH 2.3 (lanes *B*). The complete extract and the bound fraction were then subjected to SDS-PAGE, blotted onto nitrocellulose, and strips of each fraction were immunolabeled with mAb 66.7, mAb 66.6, and mAb 44.2.

Because the mAbs had been selected for their ability to block the activity of the *mt*⁻ agglutinin, it was attractive to believe that the labeled high molecular mass band in extracts of *mt*⁻ material (Fig. 3, *mt*⁻-cell-66.3) was the agglutinin, and therefore, that the class A mAbs could be used as agglutinin probes. To test this possibility more thoroughly, we fractionated a guanidine thiocyanate extract of *mt*⁻ gametes over a Sephacryl S 500 column to separate the larger glycoproteins from each other. The nature of this separation has been illustrated before (Musgrave et al., 1981), and has the advantage that the *mt*⁻ agglutinin elutes first and therefore occurs in a nearly pure form in a few fractions. The presence of the agglutinin (PAS 1.2) was confirmed by its biological activity. All the fractions were subjected to SDS-PAGE to discover their constitution, and some were then pooled to provide samples of (*a*) void volume proteins; (*b*) the first active fractions containing the *mt*⁻ agglutinin; (*c*) subsequent active fractions that were also seen to contain the flagellar glycoproteins PAS-1.1 and PAS-1.3 (Musgrave et al., 1981); (*d*) several major flagellar glycoproteins eluting before the total volume. These fractions were again subjected to electrophoresis, blotted onto nitrocellulose and tested for their antigenicity with mAbs 66.3 and 66.6. The results are presented in Fig. 4, *A–E*. The labeling of several high molecular mass components is now more obvious than in Fig. 3, because of their relatively higher concentration. mAb 66.3 only bound to a single band in the biologically active fractions (lane *C* and to a lesser extent lane *D*) that contained the typical PAS-1.2 band. We therefore assume that class A mAbs are specific for the *mt*⁻ agglutinin in *mt*⁻ gametes. mAb 66.6 again bound to several glycoproteins including PAS-1.2, as well as the bands PAS-1.1 and 1.3, that migrate in the same region of the gel.

If class A mAbs are specific for the *mt*⁻ agglutinin, then one should be able to demonstrate this by affinity chromatography using mAb 66.3 covalently bound to protein A–Sepharose, as described by Schneider et al. (1982). An extract of *mt*⁻ gametes was passed over the affinity column and the nonbound material washed through with excess buffer, followed by 0.1% deoxycholate and 0.1% SDS. The bound material was eluted in a glycine buffer, pH 2.3. A sample of the original extract and a sample of the bound material were then subjected to SDS-PAGE and blotted onto nitrocellulose. Any components possessing epitopes recognized by class A mAbs, were then detected by immunostaining using mAb 66.7 (class A, IgM). The presence of other components

was tested by staining a separate part of the blot with mAb 66.6 for cell wall and other components, and mAb 44.2 for the presence of major flagellar glycoproteins (Homan et al., 1987*a*) that contain epitopes not present on the agglutinins. The results are presented in Fig. 5. The bound fraction (Fig. 5, *B* lanes) can be seen to contain only three labeled bands (see arrows). The one consistently labeled by mAbs 66.7 and 66.6 migrated at the position of PAS-1.2, the *mt*⁻ agglutinin. Of the other two bands, one was specifically labeled by group A mAbs (66.7, lane *B*) and the other by group B mAbs (66.6, lane *B*), but they were not normally encountered in extracts, for example they were not present in the original extract used here (e.g., lane *A*, 66.7). They may be breakdown products of PAS-1.2 that contain the group A and group B epitopes, respectively. This result also confirms that class B mAbs recognize PAS-1.2.

In a similar manner, an immunoprecipitation study of the specificity of mAb 66.3 was performed. In this case, we tested for the presence of antigens exposed on the flagella of live gametes. Cells were radioactively labeled during both their vegetative and gamete phases by cultivating them in $^{35}SO_4$, such that most of the proteins in the extract were radioactive; PAS-1.2 is never more than weakly labeled under these conditions (Pijst et al., 1984). These radioactive live gametes were then treated 30 min with mAb 66.3 in the presence of colchicine (7.5 mg/ml, to prevent the transport of surface components, see Discussion) after which they were extensively washed and extracted into 1% Triton X-100. Material in solution that was bound to mAb 66.3 was precipitated with protein A–Sepharose; the Sepharose beads were thoroughly washed, and all bound material, including the antibodies, were taken up into sample buffer for SDS-PAGE. When the gel was PAS-stained only an agglutinin band was visible (not shown). The gel was then dried and any radioactive proteins were detected in an autoradiogram. While the original extract produced a mass of bands on the autoradiogram, the bound material produced no convincing bands (not shown). Though this is a negative result, it illustrates that mAb 66.3 did not precipitate any of the many components that had incorporated ^{35}S. It did precipitate the agglutinin, but this was not sufficiently radioactive to be detected in the autoradiogram. Nonetheless, we may safely conclude that class A mAbs specifically bind the active form of the agglutinin on the flagella of *mt*⁻ gametes. This is confirmed by the fact that nonagglutinable *mt*⁻ vegetative cells or gametes of light-sensitive strains in the dark (Kooijman et al., 1986), did not respond to mAb 66.3 as did gametes (see later). Similarly, when these nonagglutinating *mt*⁻ cells were fixed, they could not be shown to bind mAb 66.3 in the indirect immunofluorescence assay (Kooijman et al., 1987), and as such could be distinguished from *mt*⁻ gametes, because some gamete flagella invariably fluoresced weakly. In addition, the relatively nonagglutinable *mt*⁻ flagella of fused pairs, in general, did not bind mAb 66.3. This represents a marked change from the labeling of the same gamete flagella just before cell fusion, when they readily bound mAb A antibodies, especially at the flagella tips (Homan et al., 1987*b*). When extracts of vegetatively dividing *mt*⁻ cells were subjected to SDS-PAGE and blotted onto nitrocellulose, mAb 66.3 did not immunostain any high molecular weight components.

We conclude that class A mAbs (mAb 66.3) specifically recognize the *mt*⁻ agglutinin in *mt*⁻ cells, whereas class B mAbs recognize an epitope shared by several glycoproteins, including the *mt*⁻ agglutinin.

The nature of the antigenic sites was tested by subjecting partially purified agglutinin to various treatments, after which it was analyzed by SDS-PAGE, blotted onto nitrocellulose and immunostained. Treatment with pronase, trypsin, thermolysin, or subtilisin or 100°C for 5 min had no effect on the binding of any of the mAbs. In contrast, mild alkali destroyed all antigenicity towards all the mAbs. This treatment disrupts O-glycosidic links with serine and threonine (Sharon, 1975). Surprisingly then, treatment with 10 mM periodate, which opens up the ring structure of many sugars containing vicinal hydroxyl groups, did not affect the binding of class A mAbs, but abolished that of class B mAbs. This suggests that both classes of mAbs recognize sugar-containing epitopes but that the class A epitope contains sugars that are insensitive to periodate, at least under the conditions tested.

Inhibition of mt⁻ Agglutinability

All the mAbs characterized here were selected for their ability to inactivate isolated *mt*⁻ agglutinin bound to Sepharose. However, their ability to inactivate glutaraldehyde-fixed *mt*⁻ gametes seemed to depend on the class to which they belonged. Thus none of the class A mAbs inhibited fixed gametes, although all of the class B mAbs did. There is a trivial explanation for this peculiar finding that stems from the fact that the agglutinins are minor components, and therefore sparsely distributed over the flagellar membrane. It is as follows: class A mAbs bind the *mt*⁻ agglutinin on gamete flagella and block the sexual site, but only one of the antigen-binding sites on the antibody is used, leaving the other free to bind antigens on *mt*⁺ tester gametes. Thus sexual agglutination is prevented, but is replaced by antibody-induced agglutination. This hypothesis was confirmed by the capability of class A mAbs to induce the isoagglutination of fixed *mt*⁻ gametes with live *mt*⁻ gametes. Isoagglutination was abolished when a small quantity of *mt*⁻ agglutinin was added. Reasoning further, class B mAbs effectively block the agglutinability of fixed gametes because they never have a free binding site, for they can react with several other flagellar glycoproteins. As proof of this explanation, we made Fab fragments from each class of mAbs. As suspected, Fabs of class A mAbs completely blocked the agglutinability of fixed *mt*⁻ gametes and isolated *mt*⁻ agglutinin coupled to Sepharose (for a summary of mAb and Fab properties, see Table III). It is important to realize that such a fragment is effective because it indicates that the antigenic site is close to, or part of, the sexual site. In contrast, Fabs of class B mAbs did not inhibit *mt*⁻ agglutinability, even though the intact antibody did. One can predict therefore that the class B epitope lies outside, yet close to the sexual site. The intact class B mAb is now seen as blocking *mt*⁻ agglutinability because it sterically hinders access to the sexual site.

Class A and B mAbs were also able to prevent the agglutination of live *mt*⁻ gametes, although only concentrations above 20 μg/ml were effective (Table II).

Although mAb 66.3 has been shown to bind the *mt*⁺ agglutinin, it did not inhibit the activity of fixed or living *mt*⁺ gametes. The antigenic epitope may therefore be at a distance from the sexual binding site.

Table II. Biological Responses Induced by mAb 66.3 in Live Gametes

mAb conc.	Inhibition sex agglutination		mAb isoagglutination		Twitch effect		FTA		Tip trans*	Papillae
	mt⁻	*mt⁺*	*mt⁻*	*mt⁺*	*mt⁻*	*mt⁺*	*mt⁻*	*mt⁺*	*mt⁻*	*mt⁻*
μg/ml										
0	−	−	−	−	−	−	−	−	−	−
1	−	−	−	+	−	+	+−	+−	+	−
2	−	−	−	++	−	+	+−	+−	+	−
5	−	−	−	++	+−	+	+−	+−	++	−
10	−	−	−	++	+	+	+−	+−	++	−
20	−	−	−	+	+	+	+−	+−	++	−
50	+−	−	−	+−	+	+	+−	+−	++	+
100	+	−	−	−	+	+	+−	+−	++	+
200	+	−	−	−	+	+	+−	+−	++	+

(−) No effect. (+−) Effect by some cells. (+) Effect by many cells.
* Transport of agglutinin to the flagellar tips.

Biological Effects of Class A mAbs

Assuming that class A mAbs bind the *mt⁻* agglutination site, and in this respect resemble the *mt⁺* agglutinin, it is of interest to know whether they can induce the responses typical of sexual agglutination. If so, this would imply that the agglutinins function as both ligand and receptor during agglutination, and that there is no need to postulate the presence of other molecules responsible for invoking the responses needed for cell fusion. We tested the effect of different concentrations of class A mAbs (mostly mAb 66.3) on *mt⁺* and *mt⁻* gametes for antibody-induced agglutination, a change in flagellar movement, gamete activation (exposure of more agglutination sites), changes in the morphology of the flagellar tips, transport of mAbs to the flagellar tips, formation of papillae, and the general effect on gamete cell fusion.

Antibody-induced Isoagglutination

Because the class A mAbs are at least divalent, we expected them to isoagglutinate both *mt⁻* and *mt⁺* living gametes. Surprisingly, that was not the case. *mt⁻* gametes were not isoagglutinated by mAb 66.3, whereas *mt⁺* gametes were strongly isoagglutinated (Table II). This peculiar fact is not consistent with the report that fixed *mt⁻* gametes treated with mAb 66.3 isoagglutinated both *mt⁻* and *mt⁺* live gametes (previous section). Perhaps the antigenic sites on fixed *mt⁻* gametes are more exposed than on live *mt⁻* gametes, whereby cross-linking (isoagglutination) is made easier. When a multimeric mAb was tested, i.e., mAb 66.7, which is an IgM (class A), then *mt⁻* gametes were weakly isoagglutinated.

Twitching

One of the characteristics of sexual agglutination is a change in flagellar movement, whereby gametes twitch and vibrate. This is best seen when isolated *mt⁻* agglutinin is added to *mt⁺* gametes, the cells congregate in loose films at the surface of the medium and, without agglutinating, twitch violently (Homan et al., 1980). This twitching response was also induced by mAb 66.3 (Tables II and III) in *mt⁻* as well as in *mt⁺* gametes. The *mt⁺* response was less obvious because the cells tended to isoagglutinate and sediment to the substrate, but at high concentrations twitching was clearly visible, probably because the antigenic sites were saturated.

Flagellar Tip Activation

During sexual agglutination, the flagellar tip morphology is known to change. This was discovered for *C. reinhardtii* (Mesland et al., 1980) but is more conspicuous in *C. eugametos* because the central pair of microtubules normally project much further than the peripheral tubules, producing an obviously pointed end (Elzenga et al., 1982). As a result, flagellar tip activation (FTA) can be assessed under the light microscope as a change from pointed to dome-shaped flagellar tips (Fig. 6, *A* and *B*, respectively). The effect of mAb 66.3 was peculiar in that the pointed tip was maintained even though the subapical region became swollen (Fig. 6 *C*). The effect was variable in that the flagellar tips of some cell lines became more rounded than others, but for the *C. eugametos* strains defined here, the mAb-induced FTA was always different to that induced during sexual agglutination.

Induction of Antigenicity and Tipping

The most dramatic result of treating live gametes with mAb 66.3 is the exposure of new antigenic sites (Demets et al., 1988) together with their redistribution to the flagellar tips. When gametes were treated with mAb 66.3 in the presence of colchicine, briefly fixed in glutaraldehyde and subjected to the immunofluorescence test, only weak fluorescence on some flagella was detected. However, when live cells were just treated with mAb 66.3 and fixed at subsequent time intervals, the labeling of all flagella increased dramatically and after 15 min, the label had become concentrated at the flagellar tips. The intensity of the fluorescence was then so strong that it could be photographed under incident light (Fig. 7). We do not know to what extent the apparent increase in antigenicity is simply due to the concentration of the fluorescent label at the flagellar tips. The phenomenon, called tipping, has been described by Homan et al. (1987*b*) for *C. eugametos*, using the same mAbs, and by Goodenough and Jurivich (1978) and Bloodgood et al. (1986) for *C. reinhardtii* using a polyclonal serum and mAbs, respectively, both raised against vegetative cell components. It is here sufficient

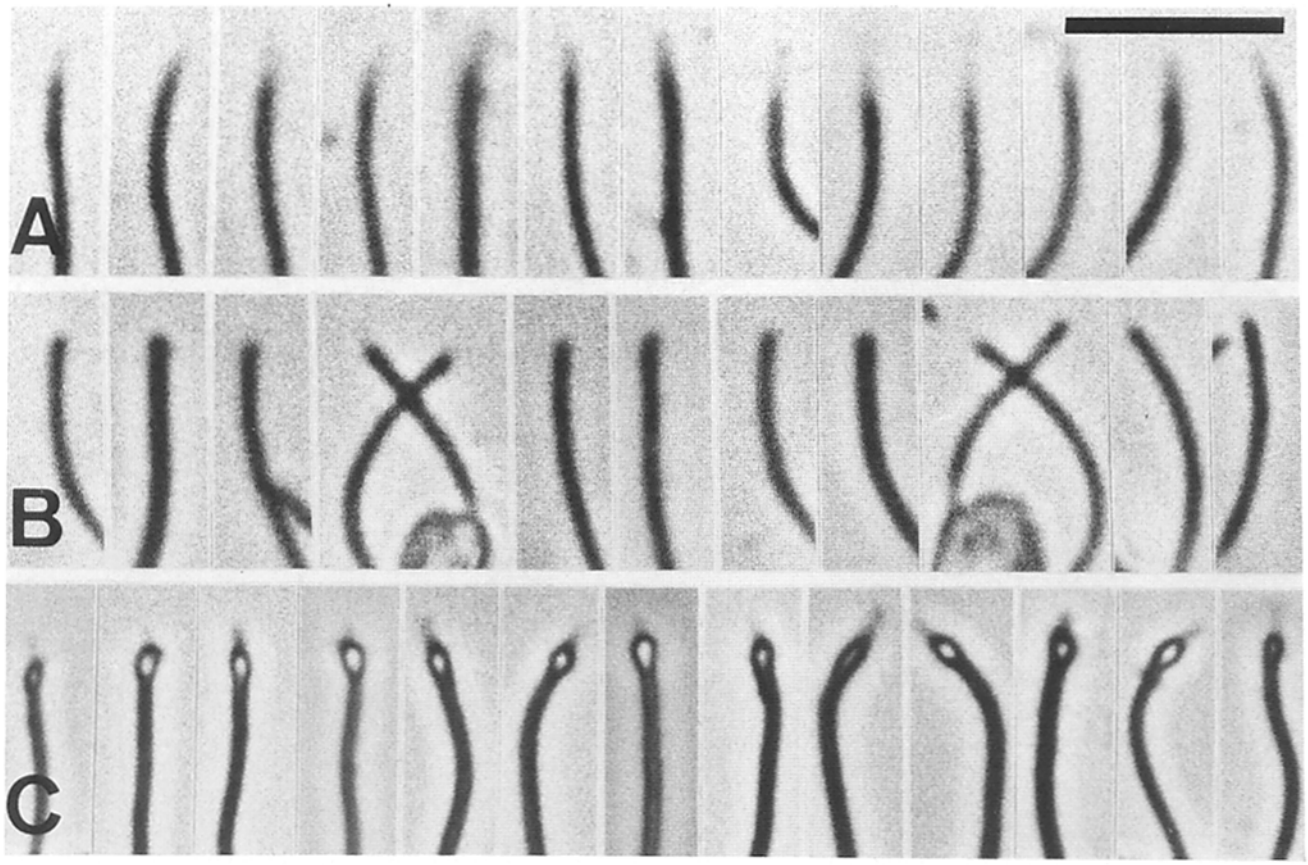

Figure 6. Flagellar tip morphology before agglutination (*A*), during sexual agglutination (*B*), and during treatment with mAb 66.3 (*C*). Cells were fixed in glutaraldehyde, washed in an alcohol series, and dry-mounted on a coverslip. After inverting the coverslip, the flagella were photographed through phase contrast optics. Bar, 5 μm.

to note that it occurred in both mating types in a manner that resembled the tipping of agglutination sites during sexual agglutination. Neither vegetative cells, light-sensitive mt^- gametes in the dark nor vis-a-vis pairs exhibited tipping when incubated with mAb 66.3.

Formation of Papillae

The climax of gamete activation is the formation of a plasma papilla by which the cell will eventually fuse with its partner. When the plasma membrane of the cell body protrudes

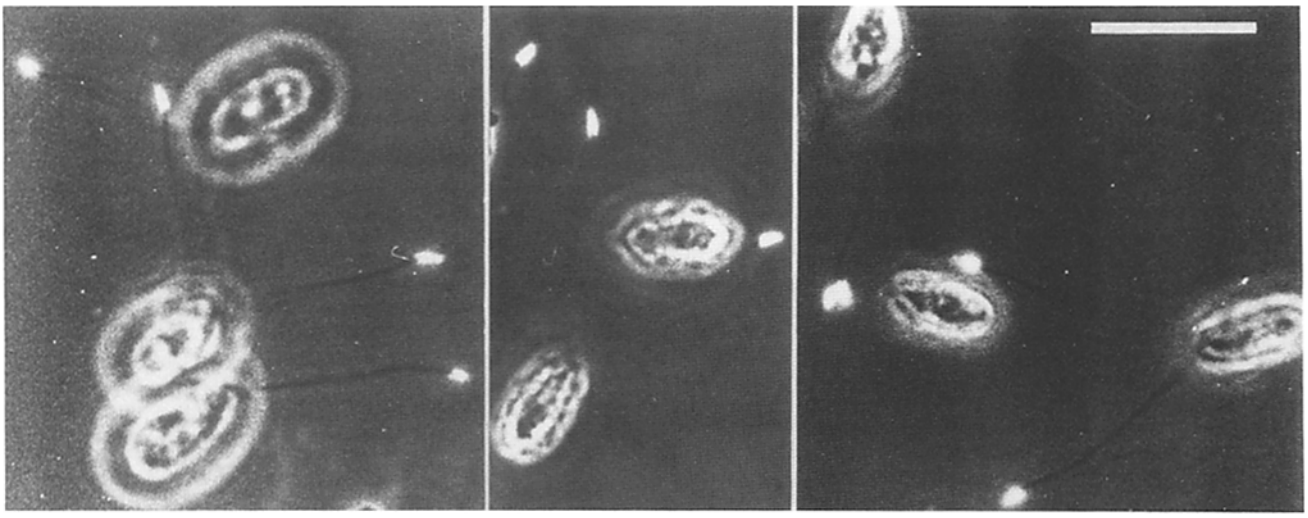

Figure 7. Redistribution of the flagellar agglutinin after treatment of mt^- gametes with mAb 66.3–FITC. mt^- gametes were treated for 1 h with antibody, fixed in glutaraldehyde and again treated with mAb 66.3–FITC to saturate the antigenic sites. The cells were then photographed under a fluorescence microscope fitted with phase contrast optics using weak incident light. In this way the fluorescent flagellar tips can be seen in relation to the rest of the cell in a single photograph. Bar, 20 μm.

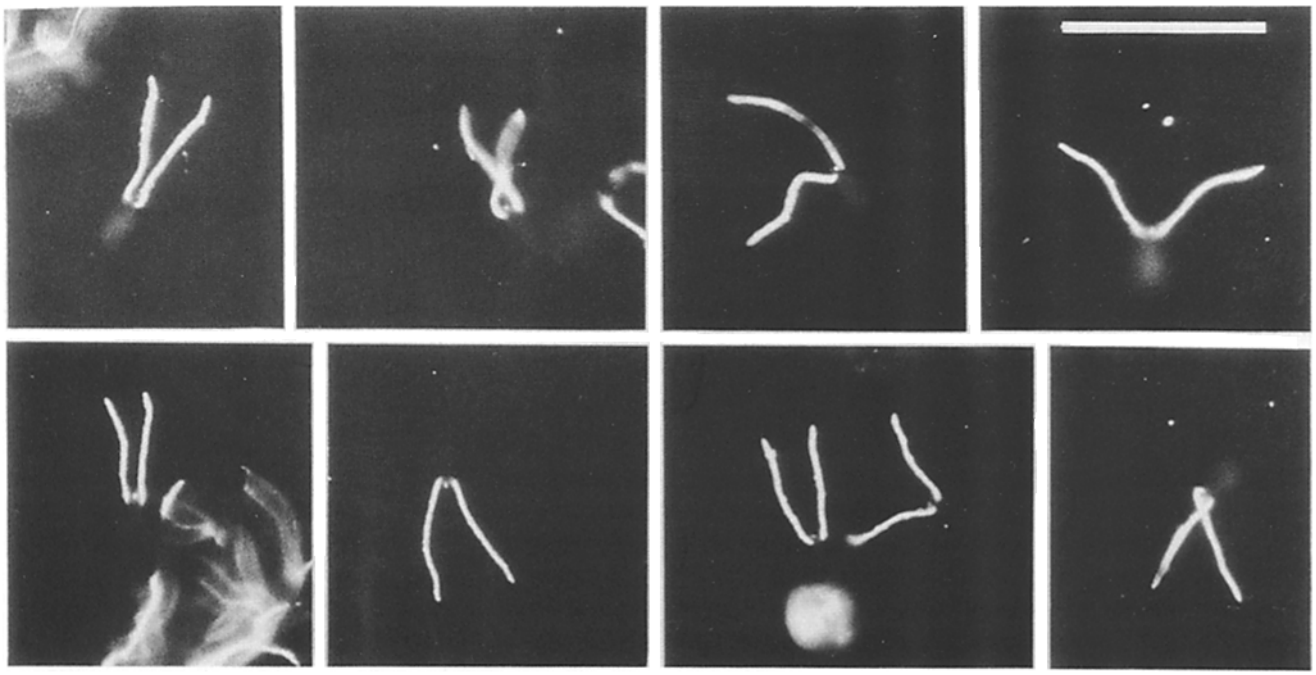

Figure 8. Induction of papillae by mAb 66.3 in mt^- gametes. Gametes were treated with 200 μg/ml mAb 66.3 for 60 min. They were then fixed in glutaraldehyde, washed, and tested for the presence of papillae in an indirect immunofluorescence test using mAb 44.2. While the whole plasma membrane surface is highly antigenic for this antibody, only surfaces exposed outside the cell wall can be labeled, viz., the flagella and papillae. Bar, 20 μm.

through the cell wall, membrane antigens are exposed that can be detected on the mt^- cell with mAb 44.2 (Musgrave et al., 1986; Homan et al., 1987*a*). In an immunofluorescence assay, the presence of papillae on a large number of cells can be easily judged (Fig. 8). When mt^- cells were treated with high concentrations of mAb 66.3, they were induced to form papillae. The mAb concentration needed was 40 times higher than that needed to induce a change in flagellar movement or tipping (Table II). It was similar to that needed to block the agglutinability of live mt^- gametes, implying that most of the flagellar agglutinins had to be occupied before the induction signal was strong enough to trigger the outgrowth of papillae.

Fusion Competence

Having looked at the individual effects of mAb 66.3 on live gametes, we wondered what the general effect on the sexual competence of the cells was. On the one hand, agglutination is inhibited, but on the other hand, the gametes are activated for cell fusion. With this in mind, the treatment of mt^- gametes with different concentrations of mAb 66.3 was tested for its effect on cell fusion. The experiment was deliberately performed with cells that exhibited a relatively low level of mating competence, to detect positive as well as negative effects. Only 25% of the control cells had fused after 40 min with an excess of mt^+ gametes. The results are presented in Fig. 9. The treatment had both negative as well as positive effects on cell fusion, dependent upon the concentration and the duration of the treatment. An optimal effect (maximum pair formation) was produced by intermediate concentrations of antibodies (10 and 20 μg/ml) added together with the mt^+ gametes, i.e., without a pretreatment of the mt^- gametes. The positive effect became less with a pretreatment, and the optimal concentration shifted to the lower end of the scale. High concentrations that had previously been shown to block agglutination, always had a strong negative effect on mating competence, and with pretreatment, even lower con-

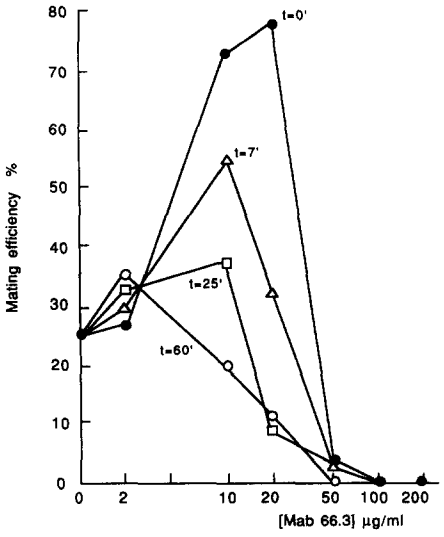

Figure 9. Effect of mAb 66.3 on mt^- mating efficiency. Batches of mt^- gametes were treated with different concentrations of mAb 66.3 for different lengths of time before adding mt^+ partners. After 40 min, during which the same concentrations of mAb 66.3 were present, the mixture was fixed in glutaraldehyde. The number of fused mt^- gametes was then calculated as a percentage of the total number of mt^- cells. This is referred to as the mating efficiency. The length of the mAb pretreatment in minutes is expressed within the figure as $t = x'$. The antibody concentrations are plotted on a log scale.

Table III. Responses Exhibited by mt⁻ Gametes of C. eugametos Treated with mAb 66.3 (Class A) or mAb 66.6 (Class B) or their Fabs, Compared with Responses Induced during Sexual Agglutination with mt⁺ Gametes

Response	66.3		66.6		mt⁺ gamete
	mAb	Fab	mAb	Fab	
Inhibition of agglutination	+	+	+	−	NR
Twitching	+	−	+	−	+
FTA	−	−	−	−	+
Agglutinin tipping	+	−	+	−	+
Formation of papillae	+	−	+	−	+

NR, not relevant; (+ and −) response and no response.

centrations inhibited fusion. Nonetheless, antibodies can have a stimulating effect on fusion. In this respect, it is significant to note that Fab fragments from mAb 66.3 always inhibited *mt⁻* agglutination and cell fusion and never stimulated them. This is significant because the Fabs were unable to activate the gametes (Table III). For example, they did not induce the exposure of more agglutinins, or tipping. Thus the only effect they seem to have is to block agglutination. When *mt⁻* gametes were treated with Fabs from mAb 66.3, and subsequently cross-linked with goat anti-mouse IgG, tipping soon became evident. However, we have not tested this combination of treatments for any other effects on sexual activation. In conclusion then, it seems that it is the cross-linking of agglutinins that activates the gametes for fusion.

Discussion

The *mt⁻* agglutinin from *C. eugametos* is a poor antigen in mice. The immune response was dominated by antibodies recognizing epitopes containing methylated sugars, which do not occur on either *mt⁻* or *mt⁺* agglutinins, but are prevalent on several of the major flagellar glycoproteins (Gerwig et al., 1984; Homan et al., 1987*a*) that contaminate the antigen preparation. Because the synthesis of these sugars is a strain-specific property (Schuring et al., 1987), by selecting different mating types with different patterns of methylation, one can use the antibodies as mating type–specific labels. However, it has hampered the production of agglutinin-specific antibodies. Only by using the hyperimmunization scheme presented here and by selecting for antibodies blocking agglutinability, were such mAbs eventually produced. The fact that these mAbs initially gave negative results when screened in an ELISA or an immunofluorescence assay, illustrates how easily they can be overlooked. Significantly, none of the mAbs raised against *C. reinhardtii* agglutinins has so far proved to be agglutinin specific (Adair et al., 1985; Snell et al., 1986), and only one, again selected in an adhesion-blocking assay, has been shown to recognize the sexual binding site (Snell et al., 1986).

The antibodies fall into two classes with those in class A being the most interesting because in *mt⁻* gametes, they are specific for the *mt⁻* agglutinin. The arguments are as follows. (*a*) Both the intact antibody as well as the Fab fragment block *mt⁻* agglutinability (Table III). This implies that they bind an epitope in or very close to the sexual binding site, and consequently one may expect the epitope to be rare if not unique. (*b*) In blots of separated *mt⁻* gamete proteins, only the *mt⁻* agglutinin was labeled. Similarly, the *mt⁻* agglutinin was the only component consistently purified by affinity chromatography or immunoprecipitation using class A mAbs. (*c*) Class A mAbs did not bind to *mt⁻* cells that were not agglutinable, e.g., vegetative cells, gametes of light-sensitive strains that were kept in the dark (Kooijman et al., 1987) and gametes that had fused. In addition, these cells did not exhibit any sexual responses when treated with class A mAbs, as did *mt⁻* gametes. In the light of these data, it was a surprise to discover that the class A epitope was not *mt⁻* specific but also occurred on the *mt⁺* agglutinin (though not in the agglutination site) as well as on a second *mt⁺* glycoprotein. Perhaps both agglutinins are related, having evolved from a common gene. In support, the composition of both molecules is similar, with both being dominated by a preponderance of arabinose, galactose, hydroxyproline, glycine and serine (Samson et al., 1987). However, the class A epitope probably contains O-glycosidically bound sugars, as witnessed by its NaOH sensitivity. Therefore the cross-reactivity of these mAbs may simply reflect the common presence of an unusual oligosaccharide. They are also dissimilar in form, for while they are both large linear proteins, the *mt⁺* agglutinin is more rigid with a globular end, whereas the *mt⁻* agglutinin is stringy and without obvious asymmetry (Crabbendam et al., 1987).

Because class A mAbs are specific for the agglutinin on *mt⁻* gamete flagella, they can be used to monitor its distribution during sexual agglutination. A prerequisite is that one must be able to distinguish the mating types from each other, for the same epitope is also present on *mt⁺* gamete flagella. A solution to this problem is to use mAb 66.3 coupled to FITC, in combination with a mating type–specific mAb (e.g., mAb 44.2, see Homan et al., 1987*a*) coupled to tetramethyl rhodamine isothiocyanate. The *mt⁻* gametes can then first be selected before registering the distribution of the agglutinin via mAb 66.3.

Class B mAbs probably inhibit agglutination by sterically hindering the agglutination site, for the intact antibody inhibits agglutination but the Fab fragments do not (Table III). The antigenic epitope occurs on several flagellar glycoproteins and also the cell wall proteins. This cross-reactivity is interesting in view of Adair's experience with mAbs raised against the *mt⁻* agglutinin of *C. reinhardtii*. His group I mAbs also gave a strong immunostain with cell wall proteins of *C. reinhardtii*. Based on this result and the obvious similarity in composition, Cooper et al. (1983) speculated that the agglutinins may have evolved from cell wall progenitors. In *C. eugametos*, the wall and agglutinin glycoproteins are similar in composition (Samson et al., 1987; Schuring et al., 1987), but it is difficult to claim a significant relationship until we have more idea of the variation that exists between the glycoproteins. Nonetheless, those proteins recognized by class B mAbs are also characterized by the lack of methylated sugars in their carbohydrate content (Homan et

al., 1987*a*), so this is further justification for classifying them as a group of related compounds.

Although class A mAbs only bind the mt^- agglutinin on the mt^- flagellar membrane, they and the Fabs derived from them completely block agglutination. This indicates that the mt^- agglutinin is the receptor for the mt^+ agglutinin and thus the only component directly involved in mt^- agglutination. This is an important conclusion because until now, an alternative possibility was that the mt^+ agglutinin was recognized by an as yet unidentified receptor. In binding the mt^- agglutinin, class A mAbs seem to behave as if they were mt^+ gametes, for treated mt^- gametes reacted as if partaking in sexual agglutination (Table III). There was only one exception, normal flagellar tip activation did not occur. While it can be argued that the sub-tip region did swell, there was no apparent extension of the peripheral microtubules, as occurred during sexual agglutination. We think that the swelling is an artifact of the technique, and is due to the accumulation of antigen and antibody in this region. It is not obvious why tip activation should not occur as normal, especially because later events, such as the protrusion of the papilla, all occurred normally. This implies that the intracellular signal for papillar induction is not dependent on the normal change in tip morphology, as has been suggested for *C. reinhardtii* (Mesland et al., 1980). Because mAb 66.3 is specific for the mt^- agglutinin, it also indicates that the stimulation of one flagellar component is sufficient to induce practically all the sexual responses without the need to invoke secondary receptors at later stages of agglutination. Induction is not an all or nothing event but a graduated response, with low concentrations of antibody (or flagellar membrane material, see Mesland and van den Ende, 1978) triggering responses typical of the first stages of agglutination, such as twitching, whereas much higher concentrations are needed to trigger the formation of the papillae. This indicates that the chronological succession of events that climax in cell fusion, involves an increasing number of agglutinins to raise a particular signal level through different thresholds. This model suggests that gametes can agglutinate without being able to fuse because they do not have enough agglutinins exposed on their flagella to induce later events. It is then not necessary to invoke secondary receptors that produce a specific papillar signal, as was argued by Solter and Gibor (1977, 1978).

Is it essential that an antibody bind to the sexual agglutination site to induce the sexual responses? Apparently not, for the responses induced by mAb 66.3 can also be induced by mAb 66.6 that binds outside the sexual site (Table III). Working with *C. reinhardtii*, Claes (1977) and Goodenough and Jurivich (1978) found that polyclonal antisera raised against the flagella of vegetative cells, were also able to induce sexual responses in gametes. Because we may assume that the agglutinins were absent from the original antigen, it is unlikely that the sexual binding site of the gametes was bound and activated. One may even doubt whether the agglutinin was bound at all. Indeed, R. Kooijman (personal communication) has recently found that the lectin, wheat germ agglutinin, neither binds to the mt^- nor to the mt^+ agglutinin of *C. eugametos*, yet is an effective surrogate agglutinin. We think the lectin binds a component that is naturally complexed with the agglutinin, and therefore via cross-linking is able to induce the various responses, but it is not the binding site per se that instigates the reaction chain. Thus agglutination need not change the conformation of the agglutinins, but aggregate them into patches to produce an intracellular signal. At the same time, patching of receptors could promote their attachment to the submembrane cytoskeleton and consequently their transport to the flagellar tips. Cross-linking seems to be essential because Fab fragments did not induce tipping or any other biological response (Table III). Similarly, Goodenough and Jurivich (1978) found for *C. reinhardtii* that a polyclonal serum raised against isolated flagella induced a sexual response in gametes, whereas the Fab fragments from the same serum did not.

A significant difference seems to exist between the transport of flagellar components in *C. eugametos* and *C. reinhardtii*. In the latter, the major membrane glycoprotein can be transported over the flagellar surface (for a review, see Bloodgood, 1987). In *C. eugametos*, that does not seem to be the case, for during sexual agglutination, the major glycoproteins do not become redistributed (Homan et al., 1987*b*), and when live gametes are treated with monoclonal antibodies directed against these proteins, there is no redistribution equivalent to that described here for mAb 66.3. It is possible that the major glycoproteins in *C. eugametos* flagellar membranes are bound to a rigid submembrane skeleton. This would suggest that the transport of minor components such as the agglutinins, is restricted to tracks that lie between the rigid domains.

It is interesting that colchicine, that prevents the polymerization of tubulin, effectively inhibits tipping. Although a high concentration of colchicine was used (7.5 mg/ml) the cells were not adversely affected. Colchicine not only inhibits tipping but can also prevent the formation of agglutinating clumps, apparently by reducing agglutinability, even though the cells do remain weakly agglutinable (Demets, R., personal communication). Not surprisingly therefore, pair formation can be completely prevented by colchicine, as was first reported by Hoffman and Goodenough (1980), who used a similarly high concentration of colchicine (10 mg/ml) to treat *C. reinhardtii*. What is colchicine affecting, a tubulin-dependent transport process or some other phenomenon? Flagellar surface motility, that is usually visualized as the saltatory movement of polystyrene beads, is not inhibited by colchicine (Bloodgood, 1977; Hoffman and Goodenough, 1980). However, Hoffman and Goodenough found that the binding of the beads to the flagellar surface was clearly inhibited. Perhaps colchicine can nonspecifically affect flagellar adhesions, for example by preventing the aggregation of adhesion receptors into effective contact sites. This process may involve the polymerization of tubulin. An alternative is that tipping itself is affected, for while saltatory motility may be insensitive to colchicine, it is not the only surface transport phenomenon. Bloodgood et al. (1986) have also described the bulk redistribution of flagellar glycoproteins as a result of treating live cells with mAbs, and their data indicate that the mechanism involved is different to that which accounts for saltatory movement.

Intact mAbs have been shown to enhance sexual cell fusion even though they can block agglutination. What is the explanation of this paradox? mAb 66.3–agglutinin interactions seem to activate gametes as effectively as agglutinin–agglutinin interactions. Thus mAb-treatments that do not seriously hinder agglutinability could promote cell fusion. In particular, the ability of the antibodies to induce the exposure of more antigenic sites is important. mAb 66.3 is specific for

the agglutination site, thus more antigenic sites is synonymous with more agglutination sites. Thus a critical concentration of mAb 66.3 could block agglutination sites and yet be compensated by the exposure of new sites, which allow the gamete–gamete adhesions necessary for cell fusion.

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