

Redistribution and Shedding of Flagellar Membrane Glycoproteins Visualized Using an Anti-carbohydrate Monoclonal Antibody and Concanavalin A

Robert A. Bloodgood, Michael P. Woodward, and Nancy L. Salomonsky

Department of Anatomy and Cell Biology, University of Virginia School of Medicine, Charlottesville, Virginia 22908.
Dr. Woodward's present address is Unogen, Inc., 1500 Avon Street Extended, Charlottesville, Virginia 22901.
Address reprint requests to Dr. Bloodgood.

Abstract. Two carbohydrate-binding probes, the lectin concanavalin A and an anti-carbohydrate monoclonal antibody designated FMG-1, have been used to study the distribution of their respective epitopes on the surface of *Chlamydomonas reinhardtii*, strain pf-18. Both of these ligands bind uniformly to the external surface of the flagellar membrane and the general cell body plasma membrane, although the labeling is more intense on the flagellar membrane. In addition, both ligands cross-react with cell wall glycoproteins. With respect to the flagellar membrane, both concanavalin A and the FMG-1 monoclonal antibody bind preferentially to the principal high molecular weight glycoproteins migrating with an apparent molecular weight of 350,000 although there is, in addition, cross-reactivity with a number of minor glycoproteins. Western blots of V-8 protease digests of the high molecular weight flagellar glycoproteins indicate that the epitopes recognized by the lectin and the antibody are both repeated multiple times within the glycoproteins and occur together, although the lectin and the anti-

body do not compete for the same binding sites. Incubation of live cells with the monoclonal antibody or lectin at 4°C results in a uniform labeling of the flagellar surface; upon warming of the cells, these ligands are redistributed along the flagellar surface in a characteristic manner. All of the flagellar surface-bound antibody or lectin collects into a single aggregate at the tip of each flagellum; this aggregate subsequently migrates to the base of the flagellum, where it is shed into the medium. The rate of redistribution is temperature dependent and the glycoproteins recognized by these ligands co-redistribute with the lectin or monoclonal antibody. This dynamic flagellar surface phenomenon bears a striking resemblance to the capping phenomenon that has been described in numerous mammalian cell types. However, it occurs on a structure (the flagellum) that lacks most of the cytoskeletal components generally associated with capping in other systems. The FMG-1 monoclonal antibody inhibits flagellar surface motility visualized as the rapid, bidirectional translocation of polystyrene microspheres.

PLASMA membranes are dynamic and they play many critical roles in the life history of cells. One form of plasma membrane dynamics is expressed as an energy-dependent redistribution of cell surface components induced by cross-linking with a multivalent ligand such as a lectin or antibody (14, 54). This "capping" phenomenon occurs in many eukaryotic cell types although it has been characterized most extensively in lymphocytes. Although there is evidence for multiple mechanisms for capping of different classes of cell surface receptors, a number of components of the cytoskeleton, including actin, myosin, α -actinin, and spectrin, have been implicated in at least some cases of capping (14).

Flagellar membranes are extremely dynamic; they represent a structurally and functionally unique subset of the overall plasma membrane of flagellated cells. They are especially amenable to study because flagella lack internal membranes and the act of deflagellation allows one to obtain relatively

pure preparations of this small subset of the cell's plasma membrane. Most of the work on flagellar membrane properties has used *Chlamydomonas* (8, 24, 53, 56) and *Euglena* (13); in addition, much important work has been done using ciliary membranes from *Paramecium*, *Tetrahymena*, and mollusks (4, 19). *Chlamydomonas* flagella exhibit a number of dynamic properties. The most striking of these has been termed "flagellar surface motility" and is manifested by whole cell gliding locomotion (7, 29) and by saltatory movement of polystyrene microspheres along the flagellar surface (6, 12, 25). There is also some evidence that flagellar surface motility may be involved in the early events associated with mating of *Chlamydomonas* gametes (8, 25, 53). *Chlamydomonas* flagella shed membrane vesicles into the medium (5, 32, 50) and as a consequence many flagellar membrane proteins turn over rapidly in the intact flagellum (9, 42). Goodenough and Jurivich (23) reported that antibodies generated to whole

flagella bind to the flagellar surface and redistribute to the flagellar tip, but only in gametic cells; they termed this phenomenon "tipping" in analogy to the "capping" phenomenon originally described in lymphocytes.

The principal protein component of the *Chlamydomonas reinhardtii* flagellar membrane is a pair of high molecular weight (HMW)¹ glycoproteins that migrate with an apparent molecular weight around 350,000. These glycoproteins are present in both vegetative and gametic flagella (5, 50, 58), are the major proteins exposed at the flagellar surface (10, 11, 33), represent the principal flagella membrane components that interact with the substrate during expression of flagellar surface motility (11), turn over rapidly in the intact flagellum (9, 42), and bind the lectin concanavalin A (33).

Studies using the protein glycosylation inhibitor, tunicamycin, have suggested that flagellar protein-linked carbohydrate is involved in the sexual agglutination that occurs between the flagellar surfaces of (+) and (-) gametes (30, 41, 51) and is necessary for expression of flagellar surface motility (8).

This paper reports observations using two different carbohydrate-binding probes, concanavalin A and an anti-carbohydrate monoclonal antibody, that recognize the HMW flagellar membrane glycoproteins of *Chlamydomonas reinhardtii*. Both of these multivalent ligands are redistributed along and shed from the flagellar membrane in association with flagellar membrane glycoproteins. In addition, the anti-carbohydrate monoclonal antibody inhibits flagellar surface motility, as visualized by the movement of polystyrene microspheres.

Materials and Methods

Cell Strains

Flagella were isolated from cells of wild-type *Chlamydomonas reinhardtii*, strain 21gr. This was the source of material for the mouse injections, the enzyme-linked immunosorbent assays (ELISAs), and the Western blots. Most of the immunofluorescence localization and redistribution studies were performed using the paralyzed flagella strain of *Chlamydomonas*, pf-18 (3, 58). This strain was used for two reasons: to eliminate motility of live cells so that the effect of the antibody on flagellar surface motility (as assayed by microsphere movement) could be determined, and because the FMG-1 antibody did not label live motile cells to any significant extent, although fixed 21gr cells labeled well with the antibody. For production of gametes, high efficiency mating wild-type strains (NO+ and NO-) were used. All other *Chlamydomonas reinhardtii* strains referred to in the paper were obtained from the *Chlamydomonas* Genetics Center at Duke University, with the exception of strain E2 (ts222), obtained from Dr. Jon Jarvik at Carnegie-Mellon University. *Chlamydomonas* were grown synchronously at 22°C in Medium I of Sager and Granick (44), referred to here as M (minimal) Medium. Cells were grown using an alternating cycle of 14 h light and 10 h dark. For induction of gametogenesis, vegetatively grown cells were washed into nitrogen-free growth medium (45) at a cell concentration of 10⁶/ml or lower and exposed to continuous light for a minimum of 15 h. Mating was induced by mixing equal numbers of mating type plus (NO+) and mating type minus (NO-) gametic cells together in nitrogen-free growth medium.

Preparation of Monoclonal Antibodies

Flagella were isolated from *C. reinhardtii* strain 21gr cells using the method of Witman et al. (58); the flagella were extracted with 0.05% Nonidet P-40 in 10 mM Tris, pH 7.4. The Nonidet-extractable material (Fig. 1) was boiled in 0.1% SDS, diluted in phosphate-buffered saline (PBS), and homogenized with an equal volume of Freund's adjuvant (complete adjuvant for the first injection

1. *Abbreviations used in this paper:* DIC, differential interference contrast; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HMW, high molecular weight.

of each mouse and incomplete adjuvant for subsequent injections). BALB/c mice were injected with the SDS-denatured antigens at multiple subcutaneous and intramuscular sites; mice were given a total of 100–150 µg/injection; three to four injections were spaced over a period of 6 mo. Serum from tail bleeds was assayed in an ELISA using Immulon I microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) coated with Nonidet P-40 extracts of flagella (native antigens) and a horseradish peroxidase-conjugated, affinity-purified goat anti-mouse IgG Fc antibody (Jackson Immunodiagnosics, Avondale, PA) that recognized all of the IgG subclasses but did not recognize any other immunoglobulin classes. 3 d after the last injection, the spleens from two mice that were strongly positive for anti-flagellar IgG antibodies were removed and the splenic lymphocytes fused with SP2/0 cultured mouse myeloma cells at a ratio of 4 splenocytes per myeloma cell using gradual addition of 50% polyethylene glycol followed by dilution with hybridoma medium (RPMI 1640, glutamine, pyruvate, mercaptoethanol, non-essential amino acids, NCTC 109, and heat-inactivated fetal calf serum) containing hypoxanthine and thymidine. The fusion products were distributed into ten 96-well Immulon I plates; after one day's growth at 37°C, media containing hypoxanthine, aminopterin and thymidine was added. After 14 d of HAT hypoxanthine/aminopterin/thymidine selection, the cells were returned to hypoxanthine/thymidine media.

Supernatants from wells that were positive for hybrid cell growth were assayed by the ELISA for the presence of mouse IgG. Supernatants from wells positive for mouse IgG secretion were then assayed by the ELISA using Immulon I (Dynatech Laboratories, Inc.) microtiter plates coated with native flagellar components from a 0.05% Nonidet P-40 extract of purified flagella. Wells were declared positive only when they gave a reaction that was 4× background in two subsequent ELISAs using supernatants collected at least a week apart. Supernatants that were negative on the ELISA using flagellar proteins were pooled in groups of 10 and assayed by Western blot analysis using SDS polyacrylamide slab gels of whole flagellar proteins. For those pooled supernatants that were positive, each of the individual supernatants were then screened separately by Western blots. This resulted in identification of additional positive clones. All supernatants that were positive on an ELISA for Nonidet-extracted flagellar proteins were screened in a dual ELISA procedure using the same antigen preparation; the antigen on one set of microtiter plates was treated with 10 mM sodium periodate at pH 4.5 for 1 h at room temperature under conditions where only vicinal hydroxyl groups in sugars are oxidized (59). The ratio of the signals on the control versus treated plates provided an indication of which monoclonal antibodies were recognizing carbohydrate epitopes. All supernatants that were positive on ELISA plates using detergent extracts of isolated flagella were rescreened on ELISA plates coated with freshly isolated whole flagella, either unfixed or fixed for 15 min in 1.5% glutaraldehyde. This step helped determine which of the monoclonal antibodies were recognizing flagella surface-exposed epitopes.

The FMG-1 hybridoma cell line was cloned three times by limiting dilution. Production of specific antibody by this cell line has been stable for 2 yr in continuous culture. Frozen aliquots of the line survive storage at -85°C in a low temperature freezer (So-Low Environmental Equipment Co., Inc., Cincinnati, OH) for over 12 mo; the line has survived multiple thawings without losing production of specific antibody. Typing of the immunoglobulin produced by this line was performed using a mouse immunoglobulin subtype identification kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) and found to be an IgG₁ with kappa light chains.

Ascites fluid containing the monoclonal antibody was produced by injection of FMG-1 cells into BALB/c mice that had been primed with Pristane (2,6,10,14-Tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, WI). Ascites fluid was collected, clarified, and stored frozen at -85°C.

Growth supernatant and ascites fluid derived from FMG-1 cells were purified and concentrated using ammonium sulfate precipitation and affinity chromatography on Protein A-Sepharose (21) or Affi-Gel Blue (15).

Western Blot (Immunoblot) Procedure

Proteins from whole flagella, isolated from *C. reinhardtii* strain 21gr cells, were separated by SDS PAGE using the Jarvik and Rosenbaum (26) modification of the Laemmli (28) procedure; the gels contained a 4–16% acrylamide gradient and a 2–8 M urea gradient. Acrylamide gels were stained for protein either with Coomassie Blue or the silver stain procedure of Oakley et al. (36). Electroblood transfer to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH; B.A. 83) was performed according to the method of Towbin et al. (55). After blotting, the nitrocellulose was stained with 0.1% Amido Black (Naphthol Blue Black) in 50% methanol–10% acetic acid, and then blocked in 0.1% bovine serum albumin, Fraction V (BSA) or 0.05% Tween 20 for 30–60 min. The nitrocellulose was cut into strips that were incubated in the appropriate dilution of growth supernatant or ascites fluid in PBS with 0.1% BSA (or 0.05% Tween

20) overnight at 4°C in glass tubes or sealed plastic bags (Sears Seal-N-Save) on a shaker. The strips were washed three times in PBS-BSA (or PBS-Tween 20), put in a 1:2,000 dilution of affinity-purified goat anti-mouse IgG (H&L) antibody (HyClone Laboratories, Logan, UT) or goat anti-mouse IgG Fc antibody (Jackson Immunodiagnosics) diluted in PBS-BSA or PBS-Tween 20 for 1–2 h at room temperature with shaking. The strips were then washed three times in PBS-BSA or PBS-Tween 20 and incubated in 10 mM Tris-HCl, pH 7.4, containing 1 mM diaminobenzidine (Aldrich Chemical Co.) and 0.03% H₂O₂ until color development had occurred (usually 2–5 min). The strips were then washed in distilled water and photographed immediately using Kodak Pan-X film with a blue filter. The nitrocellulose strips were then dried and rephotographed.

A variation of this Western blot procedure was performed in order to identify glycoproteins that bind concanavalin A. After the electroblotting step, the nitrocellulose was blocked for 2 h at room temperature or overnight at 4°C with 0.1% BSA in a lectin blot buffer containing 10 mM Hepes, pH 7.1, 0.1 mM CaCl₂, 0.1 mM MnCl₂, 0.15 M NaCl, 0.1% Tween 20, and 0.005% Thimerosal. The nitrocellulose was then washed twice in fresh lectin blot buffer containing 0.1% BSA, incubated with 2 µg/ml of biotinylated concanavalin A (Vector Laboratories, Inc., Burlingame, CA) in lectin blot buffer, and incubated in 0.5 µg/ml horseradish peroxidase-conjugated avidin (Vector Laboratories, Inc.) in lectin blot buffer for 1 h at room temperature. The nitrocellulose was washed twice in the lectin blot buffer and incubated in 1.0 mM diaminobenzidine and 0.03% hydrogen peroxide in 10 mM Tris, pH 7.0, in order to obtain color development.

ELISA Procedure

Intact purified flagella or Nonidet P-40 extracts of whole flagella were incubated overnight at 4°C in 96-well Immunon I microtiter plates. For the blocking step and all washing steps, the wells were filled to the top; for all other steps, 100 µl of each reagent were added to each well using a Titertek 8 place pipettor (Flow Laboratories, Inc., McLean, VA). For periodate experiments, the plates were treated as previously described (59). The plates were blocked using 0.1% BSA in PBS at pH 7.0 for 30 min at 37°C, washed four times with BSA-PBS, and incubated with the monoclonal antibody for 1 h at 37°C; various dilutions of growth supernatant, Protein A-purified antibody, or ascites fluid were used. Plates were washed five times with BSA-PBS and incubated for 1 h at 37°C in a 1:3,000 dilution of peroxidase-conjugated affinity-purified goat anti-mouse IgG (H&L) antibody (HyClone Laboratories) in BSA-PBS or a 1:5,000 dilution of peroxidase-conjugated affinity-purified goat anti-mouse IgG Fc antibody (Jackson Immunodiagnosics) in BSA-PBS. The plates were washed five times in BSA-PBS and incubated for 60 min in 1 mM 2,2'-Azinobis (3-ethylbenzothiazolonesulfonic acid) in 0.1 M citrate buffer (pH 5.0) containing 0.002% hydrogen peroxide. Plates were read on a Titertek Multiscan (Flow Laboratories, Inc.) using a 405-nm filter. Control wells lacking the flagellar antigen or the monoclonal antibody were run on all plates.

Immunofluorescence Microscopy and Redistribution Studies

A. Concanavalin A Labeling and Redistribution. To determine the unperturbed distribution of concanavalin A binding sites, cells were fixed for 15 min at room temperature or 4°C in 1.5% glutaraldehyde in M medium, washed twice in M medium containing 1% glycine, incubated in 50 µg/ml fluorescein isothiocyanate (FITC)-concanavalin A (Vector Labs) for 30 min at 4°C in the dark with shaking, washed with M medium in the cold, and rapidly warmed to 23°C. Aliquots were removed in the cold, before and after washing, and at various times after warming the sample to 23°C, fixed in 1.5% glutaraldehyde in M medium for 15 min and then washed twice in 1% glycine in M medium. FITC-wheat germ agglutinin (a lectin that does not label the surface of *Chlamydomonas*) was used as a control for the concanavalin A labeling experiments. The FITC-concanavalin A staining was eliminated by addition of excess unlabeled concanavalin A or α -methyl-D mannose.

B. FMG-1 Antibody Labeling and Redistribution. To determine the unperturbed distribution of the FMG-1 monoclonal antibody, cells were fixed for 15 min at room temperature or 4°C in 1.5% glutaraldehyde in M medium, washed twice in M medium containing 1% glycine, and incubated in undiluted FMG-1 hybridoma growth supernatant or a 1:100 dilution of ascites fluid or Protein A-Sepharose-purified antibody for 30 min at a cell concentration of 1–5 × 10⁶/ml. The cells were then washed twice in M medium and incubated in 10 µg/ml affinity-purified goat anti-mouse IgG (H&L) antibody (HyClone Laboratories) in M medium at 4°C for 20 min in the dark with shaking. For redistribution studies, live cells (1–5 × 10⁶/ml) were incubated in FMG-1 hybridoma growth supernatant (previously dialyzed for 2 h or more against M

medium) for 30 min on ice. It was critical to keep the cells as cold as possible during the incubation and subsequent washes in order to avoid any premature redistribution from occurring. After the labeling period, the cells were washed free of the monoclonal antibody using ice-cold M medium and half the sample was warmed to a particular temperature (between 10 and 30°C) to induce redistribution. Aliquots were removed from the sample in the cold before and after washing and at various times after warming the sample, and then fixed in 1.5% glutaraldehyde in M medium. The cells were washed twice with 1% glycine in M medium and labeled with the FITC-labeled second antibody as described above. A mouse monoclonal antibody, made against brain β -tubulin, that recognizes *Chlamydomonas* β -tubulin was used as a control for nonspecific labeling and for entry of the primary or secondary antibody into the fixed cells; no labeling was ever observed except when the fixed cells were permeabilized with Nonidet P-40 detergent before incubation with the anti-tubulin monoclonal antibody.

C. Photography. Cells were observed using a Leitz Ortholux II microscope equipped with differential interference contrast (DIC) and fluorescence optics. Most photographs were taken using a Zeiss 63× planapo objective lens; DIC-fluorescence pairs of photographs were recorded using a Leitz 100× fluoritar objective lens. Photographs were taken using Kodak TRI-X film and an ASA setting of 1600; the film was developed in Diafine (Acufine, Inc., Chicago, IL). In some cases, an automatic photometer was used for photography. However, when it was necessary to compare control versus experimentally treated cells, photographs were taken using a manual exposure so that control and experimental cells were exposed for the same period of time under the same optical conditions. Similarly, the negatives were printed using identical exposure and development conditions in order to facilitate comparison.

Pronase Digestion Studies

A 0.05% Nonidet P-40 extract of flagella, isolated from strain 21gr cells, was digested at 37°C for 24 h in 1 mg/ml pronase (Calbiochem-Behring Corp., La Jolla, CA) in 10 mM CaCl₂, 0.1 M Tris-HCl, pH 8.0, according to the method of Sefton (47). FMG-1 antibody binding to control and pronase-digested samples was analysed using Western blot (immunoblot) analysis and a special ELISA procedure designed specifically for glycopeptides (20).

V8 Protease Digestion Experiments

A. Basic Enzyme Digestion Procedure. A 0.05% Nonidet P-40 extract of isolated *C. reinhardtii* strain 21gr flagella was incubated at 37°C in 10 mM Tris-HCl, pH 7.4, 0.05% SDS, with or without 0.05 mg/ml *Staphylococcus aureus* V-8 Protease (Miles Scientific Div., Naperville, IL) (17). At various times (10 min–24 h) after the incubation was started, aliquots were removed from the control and protease-containing samples, sufficient 10% SDS was added to bring the sample to a concentration of 1% SDS, an equal volume of Laemmli electrophoresis sample buffer (28) was added, the sample was immediately boiled for 2 min, and all samples were then stored frozen at –85°C. SDS PAGE and Western blot analysis were performed as described above.

B. FMG-1-derivatized Bead Adsorption. V-8 protease and control incubations were performed for 60 min at 37°C as described above; the digestion was terminated by addition of SDS to a final concentration of 1% and boiling of the sample. An equal volume of buffer containing 80 mM NaCl, pH 8.3, 300 mM NaCl, 10 mM EDTA, 5% Triton X-100 and 100 U/ml Trasylol (aprotinin) was added to the sample in order to complex the SDS with the Triton X-100 so it would not interfere with antibody binding. To derivatize Protein A-Sepharose beads with the FMG-1 mouse monoclonal antibody (IgG₁ subclass), it was necessary to use a sandwich technique in which a rabbit anti-mouse IgG antibody was bound to the Protein A by its Fc region; this antibody then bound the FMG-1 mouse monoclonal antibody. Protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) were washed in a buffer containing 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 100 U/ml Trasylol (aprotinin), 10 mM Tris-HCl, pH 8.3. The beads were incubated in 1.6 mg/ml affinity-purified rabbit anti-mouse IgG (H&L) antibody overnight at 4°C on a rotator. After several rinses in the buffer described above, half of the beads were incubated overnight at 4°C in hybridoma growth supernatant containing FMG-1 monoclonal antibody; the other half of the beads was left in buffer and constituted the control beads. The V-8 protease-digested or control flagellar extract sample was then incubated at room temperature for 3 h with either control or antibody-derivatized beads. The beads were washed and then boiled in Laemmli electrophoresis sample buffer; the beads were pelleted and the supernatants loaded on an SDS polyacrylamide slab gradient gel.

C. Concanavalin A Bead Adsorption. V-8 protease and control incubations were performed for 60 min at 37°C as described above; the incubation was terminated by adding SDS to a final concentration of 1% and boiling the

sample. An equal volume of buffer containing 30 mM Tris-HCl, pH 7.4, 1 M NaCl, and 5% Triton X-100 was added to each sample in order to complex the SDS with Triton X-100 so that the SDS would not inhibit binding of the peptides to the immobilized lectin. Concanavalin A-Sepharose (Pharmacia Fine Chemicals) was washed twice in a buffer containing: 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.1% Triton X-100, 0.1 mM MnSO₂, and 0.1 mM CaCl₂. The beads were incubated with the V-8 protease-derived peptides or the undigested control preparation for 1 h at room temperature on a rotator; the beads were pelleted at 1,000 g for 2 min at 4°C and washed four to five times in the buffer described above. The beads were then boiled in Laemmli electrophoresis sample buffer, pelleted at 5,000 g, and the supernatant was loaded onto an SDS polyacrylamide gradient slab gel.

Results

Characterization of an Anti-carbohydrate Monoclonal Antibody (FMG-1) Recognizing the Major Flagellar Membrane Glycoprotein and Comparison with the Lectin Concanavalin A

The antigen used to immunize BALB/c mice was a 0.05% Nonidet P-40 extract of whole flagella purified from *Chlamydomonas reinhardtii* strain 21gr cells (Fig. 1). The dominant protein component in this extract is an HMW glycoprotein migrating with apparent molecular weight of 350,000; under optimal electrophoretic conditions, this HMW glycoprotein region can be separated into two glycosylated components (9, 10). Characterization of the resulting mouse monoclonal antibodies that recognized *Chlamydomonas reinhardtii* flagellar membrane components yielded three major categories of monoclonal antibodies:

(a) Monoclonal antibodies recognizing carbohydrate epitopes on flagellar membrane glycoproteins; most but not all of these antibodies recognized the HMW (350,000-mol-wt) glycoproteins.

(b) Monoclonal antibodies recognizing carbohydrate epitopes on a flagellar membrane acidic glycolipid.

(c) Monoclonal antibodies recognizing protein epitopes associated with flagellar membrane proteins. In most cases, the epitopes recognized by these antibodies are not accessible at the external surface of the flagellar membrane; some but not all of these antibodies recognized the HMW (350,000-mol-wt) glycoproteins.

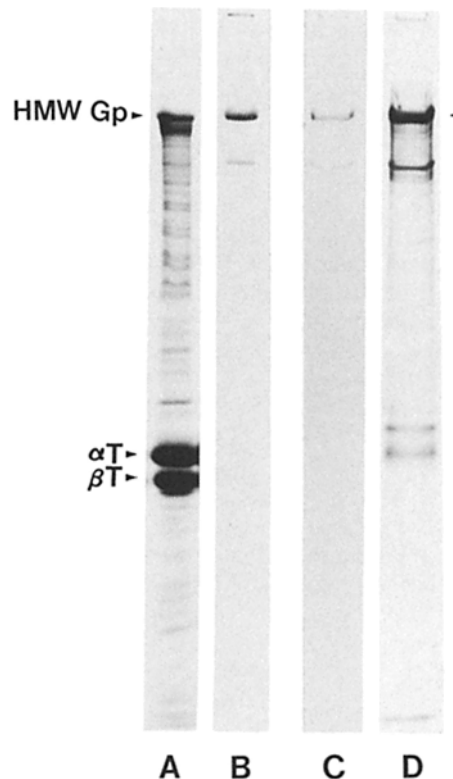


Figure 1. SDS polyacrylamide gel. Lanes A-C are stained with Coomassie Blue and lane D is stained using a silver stain procedure. Lane A contains whole flagellar proteins from *C. reinhardtii* strain 21gr. Lane B contains a 0.05% Nonidet P-40 extract of the flagella shown in lane A. Lane C shows another Nonidet P-40 extract obtained from a different preparation of strain 21gr flagella. Lane D is the exact same gel lane shown in lane C after restaining with a silver stain procedure with greatly increased sensitivity relative to the Coomassie Blue stain. The principal membrane component, consisting of HMW glycoproteins migrating with apparent molecular weight of 350,000, is labeled *HMW Gp*. The α - and β -tubulins are labeled αT and βT . The material shown in lanes B-D comprises the immunogen injected into the mice used to prepare hybridomas secreting monoclonal antibodies recognizing flagellar membrane components.

Table I. Characteristics of the FMG-1 Anti-carbohydrate Monoclonal Antibody

(a) Mouse IgG ₁ , kappa light chain	(j) The antibody binds uniformly along the flagellar surface of fixed cells or live cells kept at 4°C of paralyzed flagella strains (pf-18, pf-1, E2)
(b) Binds to <i>Chlamydomonas reinhardtii</i> flagellar glycoproteins that are native, denatured with SDS, reduced with dithiothreitol, or fixed with glutaraldehyde	(k) The antibody does not bind to motile flagella on live cells of various motile strains, although it does bind to these flagella after fixation of the cells or isolation of the flagella
(c) Exhaustive pronase treatment of detergent-solubilized flagellar membrane proteins does not abolish binding	(l) The antibody agglutinates cells by their flagellar surfaces but not by their cell walls
(d) Pronase treatment of whole cells (live or fixed) results in a loss of antibody binding	(m) Treatment of cells with the antibody inhibits flagellar surface motility, as judged by polystyrene microsphere movement
(e) Periodate oxidation of the flagellar glycoproteins abolishes binding	(n) The antibody redistributes along the flagellar surface and is subsequently shed into the medium in a temperature-dependent manner
(f) Binding of the antibody is not affected by the presence of concanavalin A or α -methyl-D-mannoside	(o) The antibody does not recognize any components in <i>Chlamydomonas moewusii</i> , in whole mouse serum, in bovine brain supernatant, or in a crude glycolipid extract of <i>Chlamydomonas reinhardtii</i> whole cells or isolated flagella
(g) Binding of concanavalin A is not affected by the presence of the antibody	
(h) V8 protease digestion yields multiple peptides, all of which bind both concanavalin A and the FMG-1 antibody	
(i) The antibody recognizes cell surface-exposed epitopes on the flagellar membrane, the cell body plasma membrane, and the cell wall	

The majority of the monoclonal antibodies obtained fell into the first category and appeared to recognize carbohydrate epitopes common to the cell wall glycoproteins, to the major flagellar membrane glycoproteins, and to certain minor flagellar membrane components; this is not surprising since the flagellar membrane has an extensive glycocalyx (43, 58) and the principal protein components of the flagellar membrane are heavily glycosylated (33). One such anti-carbohydrate monoclonal antibody (designated the FMG-1 antibody for flagellar membrane glycoprotein antibody #1) was chosen for detailed study and for comparison with concanavalin A, the principal lectin that binds to the *Chlamydomonas* flagellar surface (31, 33, 34, 57).

The characteristics of the FMG-1 monoclonal antibody are summarized in Table I. Observations that collectively suggest that this antibody is recognizing a carbohydrate epitope include (a) cross-reactivity with cell wall glycoproteins and a number of minor flagellar components; (b) presence of multiple copies of the epitope on the flagellar glycoproteins (see below); (c) insensitivity of the epitope to pronase treatment; and (d) sensitivity of the epitope to periodate oxidation under conditions specific for carbohydrate epitopes (59).

Figs. 9A and 15A illustrate the distribution of binding sites for the FMG-1 antibody and concanavalin A, respectively, on whole cells of strain pf-18; this pattern is the same whether the cells are labeled live at 4°C and then fixed, or are prefixed in 1.5% glutaraldehyde and then labeled at 4°C or room temperature. The flagella give a uniform distribution of label, both flagella always label, and 100% of the flagellated cells in a population are labeled. Although the flagella on glutaraldehyde fixed pf-18 cells often appeared subjectively to give an increased fluorescent signal relative to live cells when labeled with the FMG-1 antibody, quantitative ELISAs using plates coated with whole, freshly isolated pf-18 flagella that were either treated with glutaraldehyde or untreated exhibited identical levels of antibody binding. The intense fluorescent labeling over the cell body of intact cells represents binding to the cell wall. Two observations suggest that the monoclonal antibody also binds to the plasma membrane over the cell body. The cell wall-less mutant, CW-15 (18), exhibits labeling over the entire cell body; transmission electron microscopic analysis (18; Bloodgood, R. A. unpublished data) revealed no cell wall material in this mutant. In addition, quadriflagellate dikaryons lacking a cell wall were obtained shortly after mating NO+ and NO- gametes, and exhibited a clearly detectable layer of fluorescent labeling at the cell surface relative to controls. Although not unique to that region of the plasma membrane associated with flagella, the carbohydrate epitopes recognized by the FMG-1 antibody appear to be enriched in the flagellar membrane over the cell body plasma membrane. Comparative Western blots of purified cell walls and purified flagella suggest that the epitopes recognized by the FMG-1 monoclonal antibodies are associated with very different polypeptides in these two cellular compartments.

Live cells of a wide variety of motile strains (21gr, CW-15, NO+, 137c+, 137c-, C8, C9, UTEX 90) of *Chlamydomonas reinhardtii* do not exhibit flagellar staining with the FMG-1 antibody while these same cells, after fixation in glutaraldehyde, do exhibit staining of the flagella. In all of these strains, cell walls are well stained with the antibody before or after fixation. In the case of paralyzed flagella strains (pf-18, pf-1, ts222 [E2]), the flagella of both live and fixed cells stain with

the antibody. Gametic and vegetative cells exhibit a similar uniform pattern of staining of the flagellar surface and the cell wall after fixation. Quadriflagellate zygotes also exhibit uniform flagellar staining after fixation.

The pattern of cell wall labeling with the FMG-1 monoclonal antibody and concanavalin A is similar. The FMG-1 antibody always labels the wall uniformly except for two very intense spots of labeling at the bases of the flagella (see especially Figs. 3B, 9D, 11A). Since these structures appear to be at the same level as the cell wall, it is speculated that they represent the tunnels through which the flagella emerge. Snell (52) has characterized flagellar collars that are thought to represent the material lining these tunnels. Although these same sites at the flagellar bases are usually observed with fluorescent concanavalin A labeling, it is sometimes observed that concanavalin A will label a large region of the anterior end of the cell wall more intensely than the rest of the wall (Fig. 15B-H).

The Western blot patterns for the FMG-1 antibody (see Figs. 2A, 5E, 6) and concanavalin A (Fig. 2B) (using SDS

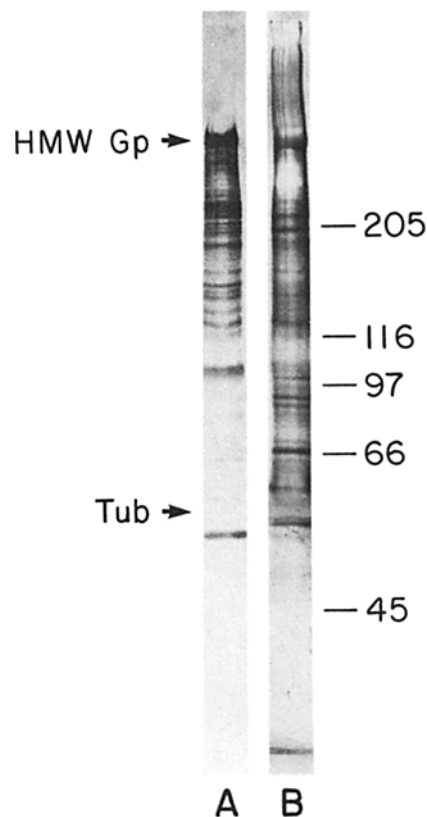


Figure 2. Nitrocellulose blots of gels containing whole 21gr flagellar proteins. Lane A shows the components recognized by the FMG-1 monoclonal antibody (visualized using a horseradish peroxidase-conjugated second antibody). Lane B shows the components recognized by the lectin concanavalin A (visualized using biotinylated concanavalin A followed by horseradish peroxidase-conjugated avidin). In each case, the principal component recognized consists of the HMW glycoproteins (labeled *HMW Gp*). When the nitrocellulose blots were stained with Amido Black, this component was the only membrane component visible, testifying to the extreme sensitivity of the enzyme-linked detection methods used. The spectrum of minor components recognized by the antibody and the lectin are very different. The position where tubulin (*Tub*) and various molecular weight standards migrate are indicated.

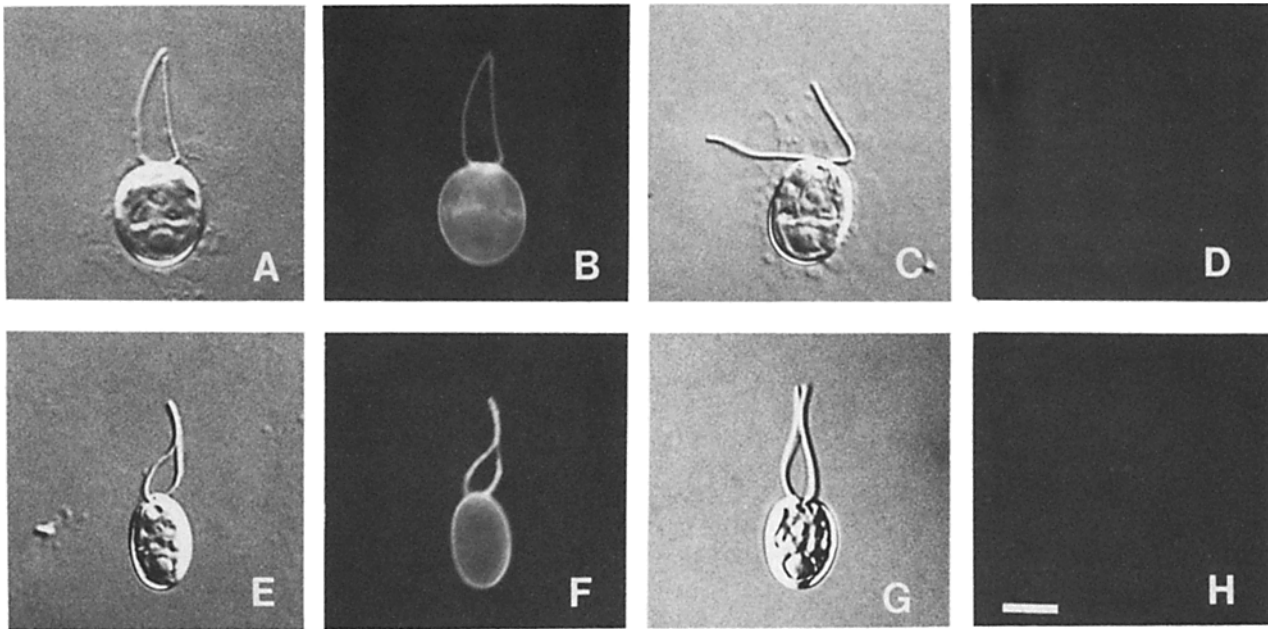


Figure 3. Effect of periodate oxidation of glutaraldehyde-fixed strain pf-18 cells on the pattern of labeling with FITC-concanavalin A (A–D) or with the FMG-1 monoclonal antibody and FITC-labeled second antibody (E–H). The cells shown in C, D, G and H were pre-treated at room temperature for 1 h with 10 mM periodic acid at pH 4.5. The cells shown in A, B, E, and F were treated in the same manner except the periodic acid was eliminated from the buffer. Figures A, C, E, and G were photographed using DIC optics while figures B, D, F, and H were photographed using epifluorescent optics. Bar, 5 μ m.

PAGE of whole flagellar proteins) are both dominated by the HMW glycoproteins although there are a number of cross-reactive minor components of lower molecular weight. The pattern of reactivity to these lower molecular weight components differs considerably between the antibody and the lectin (Fig. 2). The sensitivity of the Western blot technique being used over-represents these minor components, which are not well visualized on silver-stained gels (see Fig. 6). The complexity of the Western blot pattern for the FMG-1 antibody and concanavalin A could be due to common carbohydrate groups on distinctly different protein moieties and/or could be due to proteolysis; the latter possibility cannot be ruled out, although the inclusion of a mixture of protease inhibitors (aprotinin, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor) at all stages in the flagellar purification procedure does not alter the complexity of the pattern observed. The hybridoma cell line producing the FMG-1 monoclonal antibody has been cloned at least three times and this has not altered the Western blot pattern obtained using this antibody. Varying the antibody concentration and the incubation time of the monoclonal antibody used in the Western blot procedure demonstrates that the maximum signal is achieved by an antibody concentration of 4 μ g/ml and an incubation time of 30 min at room temperature; these conditions are met or exceeded in all of the Western blots shown.

Periodate oxidation of flagellar components under conditions that affect only vicinal hydroxyl groups in carbohydrates (59) results in loss of binding of the FMG-1 antibody to its epitopes when assayed by immunofluorescent labeling of whole cells fixed with glutaraldehyde (Fig. 3), by the ELISA using Nonidet P-40 extracts of flagella (Fig. 4), and by Western blot analysis using SDS PAGE of whole flagella proteins (Fig. 5). Periodate oxidation of the cell surface also eliminates the

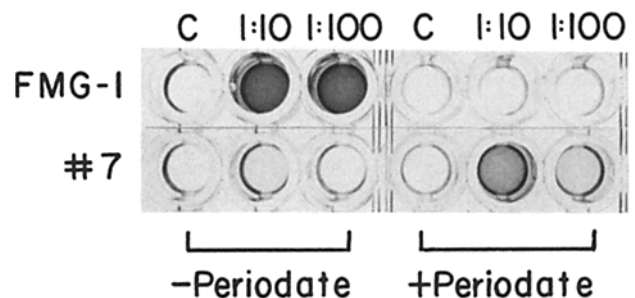


Figure 4. Effect of periodic acid oxidation of flagellar membrane proteins on the binding of two different monoclonal antibodies, one recognizing a carbohydrate epitope (FMG-1) and the other recognizing a protein epitope (#7) on the HMW flagellar membrane glycoproteins, as visualized using an ELISA. The microtiter plate was coated with a Nonidet P-40 extract of strain 21gr flagella after which the three rows of wells on the right were treated for 1 h at room temperature with 10 mM periodic acid at pH 4.5. After incubation with sodium borohydride, the plate was washed and growth supernatants containing the two monoclonal antibodies were placed in the indicated wells. C indicates control wells in which no primary antibody was placed; 1:10 and 1:100 refer to the dilutions of the hybridoma growth supernatants. Periodate pre-treatment of the flagellar membrane glycoproteins abolishes the binding of the FMG-1 monoclonal antibody but increases the amount of binding of the #7 monoclonal antibodies. The Western blot patterns for these two antibodies are shown in Fig. 5.

binding of FITC-concanavalin A (Fig. 3). The effect of extensive pronase digestion of the Nonidet P-40 flagellar extract was assayed using Western blots and an ELISA designed for use with glycopeptides (see Materials and Methods). After digestion under conditions where no cross-reactive polypeptides were visible on the Western blot of SDS polyacrylamide

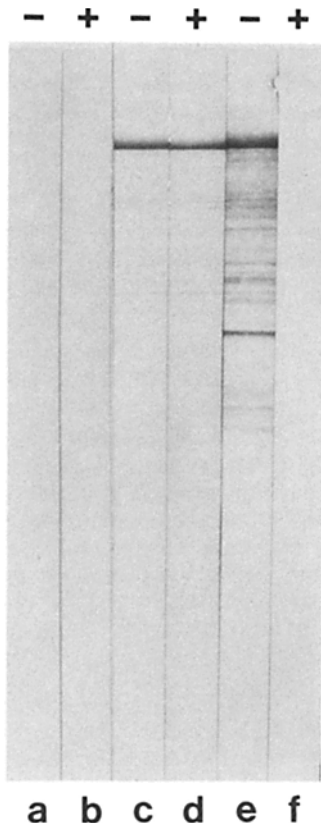


Figure 5. Effect of periodate oxidation on the binding of the FMG-1 monoclonal antibody to flagellar glycoproteins as judged by Western blot (or immunoblot) analysis. Whole flagellar proteins were separated by SDS PAGE and then transferred to nitrocellulose. The nitrocellulose containing the flagellar proteins was cut into numbered strips; half the strips were treated for 1 h at room temperature with 10 mM periodic acid at pH 4.5. All strips were treated with sodium borohydride and washed before being incubated with the monoclonal antibodies. Strips *a* and *b* were incubated with the hybridoma growth medium alone; strips *c* and *d* were incubated in a growth supernatant containing a monoclonal antibody (#7) recognizing protein epitopes on the HMW glycoproteins; strips *e* and *f* were incubated in growth supernatant containing the FMG-1 monoclonal antibody. Strips *b*, *d* and *f* were pre-treated with periodic acid; the binding of the FMG-1 monoclonal antibody was entirely abolished by this treatment while the binding of the other monoclonal antibody (recognizing protein epitopes on the same HMW glycoproteins) was unaffected.

gels, the ELISA exhibited reactivity with the FMG-1 antibody (data not shown). Coupled with the periodate observations, this observation strongly suggests that the FMG-1 antibody is recognizing a carbohydrate epitope.

Although the FMG-1 monoclonal antibody and concanavalin A both bind to the principal HMW flagellar membrane glycoproteins and both exhibit similar immunofluorescent labeling patterns on whole cells, the two carbohydrate-binding ligands do not compete for binding to the same epitopes. Concentrations of soluble concanavalin A up to 1,000 $\mu\text{g}/\text{ml}$ and α -methyl-D-mannoside up to 100 mM have no effect on the binding of the FMG-1 antibody to its epitope as assayed by the ELISA or Western blot analysis. Pre-labeling of the flagellar surface with excess FMG-1 monoclonal antibody does not affect the labeling of the flagellum with FITC-concanavalin A.

Each HMW Glycoprotein Molecule Contains Multiple Binding Sites for Concanavalin A and for the FMG-1 Monoclonal Antibody

Preparations enriched in the HMW flagellar glycoproteins can be obtained by brief (5–10 min) extraction of flagella from 21gr cells at 4°C with 0.05% Nonidet P-40 (Fig. 6, lane *S-O'* and Fig. 7*A*). Digestion of these preparations with *S. aureus* V-8 protease results in the generation of a number of distinct peptides; every major peptide that is observed by silver staining contains binding sites for the FMG-1 antibody (Fig. 6). This is in distinct contrast to a number of other anti-flagellar monoclonal antibodies (those recognizing protein epitopes) that reacted with only a single one or a small subset of the V-8 protease-derived peptides. A 30-min V-8 protease digest of the Nonidet P-40 extract enriched for the HMW flagellar glycoproteins was divided into two aliquots; one half of the sample was incubated in concanavalin A-Sepharose beads and one half was incubated in Protein A-Sepharose beads derivatized with the FMG-1 monoclonal antibody (using rabbit anti-mouse IgG as an intermediate ligand). The peptides that were bound by the beads were examined by Western blot analysis using the FMG-1 antibody (Fig. 7). All of the V-8 protease-derived fragments that bound to the antibody-derivatized beads were also bound to the concanavalin A-derivatized beads. This suggests that the antibody- and lectin-binding sites occur together and are repeated multiple times along the length of the glycoproteins as is portrayed in the diagram shown in Fig. 8. There were no significant peptides generated by the V-8 protease digestion that were not bound by the concanavalin A or the FMG-1 antibody-derivatized beads. Two rounds of adsorption of the V-8 protease digest with concanavalin A-Sepharose beads depleted the solution of virtually all antibody-reactive material as judged by Western blot analysis of the concanavalin A-Sepharose bead supernatants. Protein A beads alone or derivatized only with the rabbit anti-mouse IgG (H&L) antibody did not bind any of the V-8 protease-induced peptides. As noted above, concanavalin A and the FMG-1 antibody do not compete for binding to the same carbohydrate epitopes.

Concanavalin A and the FMG-1 Monoclonal Antibody Are Redistributed along and Shed from the Flagellar Surface in a Similar Manner

When live *Chlamydomonas reinhardtii* strain pf-18 cells are labeled with the FMG-1 antibody or FITC-concanavalin A at 0–4°C and washed in the cold to remove unbound antibody of lectin, the flagella remain uniformly labeled as long as the cells are maintained at or below 4°C. However, when the cells are warmed to 23°C, the monoclonal antibody or lectin redistributed along the flagellar surface in a very characteristic manner (Figs. 9 and 15); these figures represent a true time sequence in a population of cells and do not represent an ordering of different images seen in the same time point into an arbitrary sequence. The label begins to clear from the proximal end of the flagella (Fig. 9, *B* and *C*; and Fig. 15*B*) and the unlabeled area gradually increases until all of the label is aggregated at the tips of the flagella (Figs. 9*A* and 15*D*). These aggregates then appear to migrate as a unit from the distal ends of the flagella down to the base of the flagella, where they give the appearance of “headlights” (Figs. 9*H* and

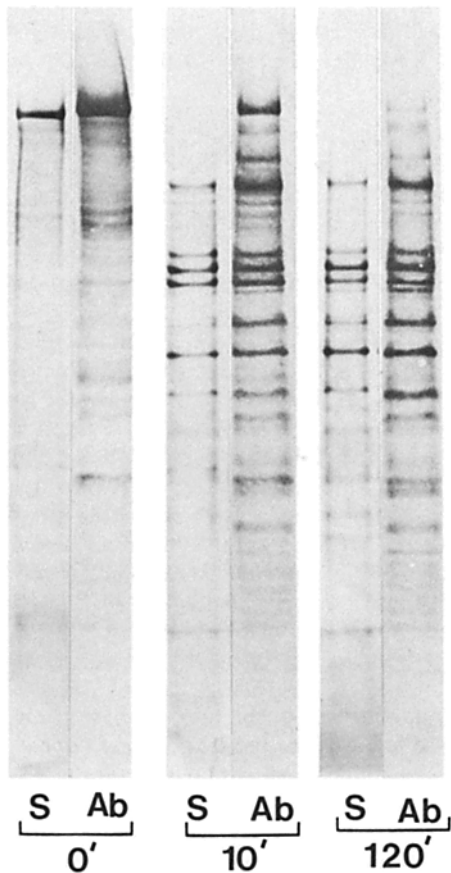


Figure 6. Binding of the FMG-1 monoclonal antibody to peptide fragments of the HMW flagellar glycoproteins generated by V-8 protease digestion. A Nonidet P-40 extract of strain 21gr flagella was digested with V-8 protease for various periods of time; digestion was terminated by boiling an aliquot in Laemmli electrophoresis sample buffer after which the samples were analyzed by SDS PAGE. Parallel samples were run on a single slab gel. Half the gel was stained for protein with a sensitive silver stain technique; the other half was blotted onto nitrocellulose paper and labeled with the FMG-1 monoclonal antibody. Samples shown were taken immediately after addition of the protease (0 time) or after 10 min and 120 min of digestion at 37°C. The silver stain pattern (S) is shown on the left side of each pair while the immunoblot pattern obtained using the FMG-1 monoclonal antibody (Ab) is shown on the right side of each pair.

15 F). The label is subsequently lost from the flagella, presumably by being shed into the medium; fluorescent aggregates can be observed free in the medium during the later stages of the redistribution process. This entire sequence of events is completed within 15 min at 23°C. No redistribution or change in intensity of the label associated with the cell wall occurs during incubation of the cells at 23°C. This process of antibody and lectin redistribution on the flagellar surface occurs quite synchronously in a healthy population of cells and the kinetics of this process can be followed by scoring the percentage of cells exhibiting various patterns of flagella fluorescence at various times after warming the cells (Figs. 10 and 16); these data argue that the majority of cells go through a temporal sequence of stages similar to that portrayed in Figs. 9 and 15. Variability is observed in how long it takes a particular batch of cells to complete the redistribution process; however, the sequence of stages in the redistribution process

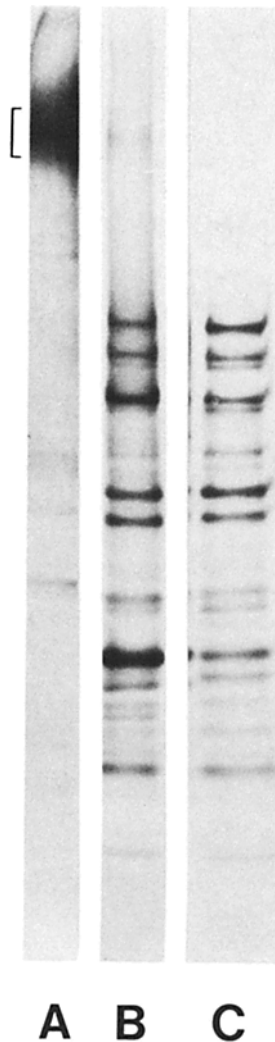


Figure 7. Comparison of the V-8 protease-derived polypeptides recognized by concanavalin A and by the FMG-1 monoclonal antibody. A Nonidet P-40 extract of strain 21gr flagella enriched in the HMW flagellar glycoproteins (bracket in lane A) was digested for 30 min with V-8 protease. The digestion was terminated by boiling the sample in 1% SDS; control samples were incubated in the absence of the V-8 protease. Control and digested samples were incubated with Sepharose beads derivatized with concanavalin A or with the FMG-1 monoclonal antibody. The beads were washed extensively and the bound peptides were released by boiling the beads in Laemmli gel sample buffer. The resulting samples were separated by SDS PAGE and then blotted onto nitrocellulose followed by immunolocalization using the FMG-1 monoclonal antibody. A very similar set of V-8 protease-derived peptides was bound by the lectin beads (lane B) and the antibody beads (lane C).

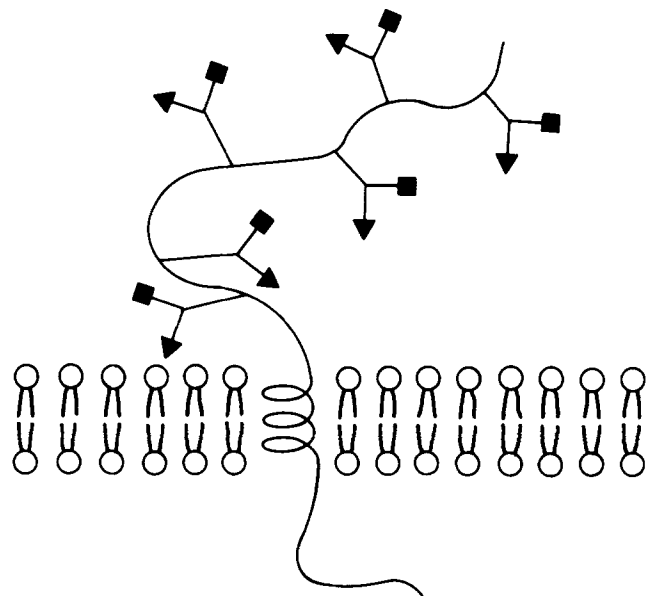


Figure 8. Diagram illustrating the proposed model for the predominant glycoprotein of the flagellar membrane. The externally exposed portion of the glycoprotein possesses multiple sites of glycosylation; each carbohydrate structure contains independent binding sites for both concanavalin A (■) and the FMG-1 antibody (▲).

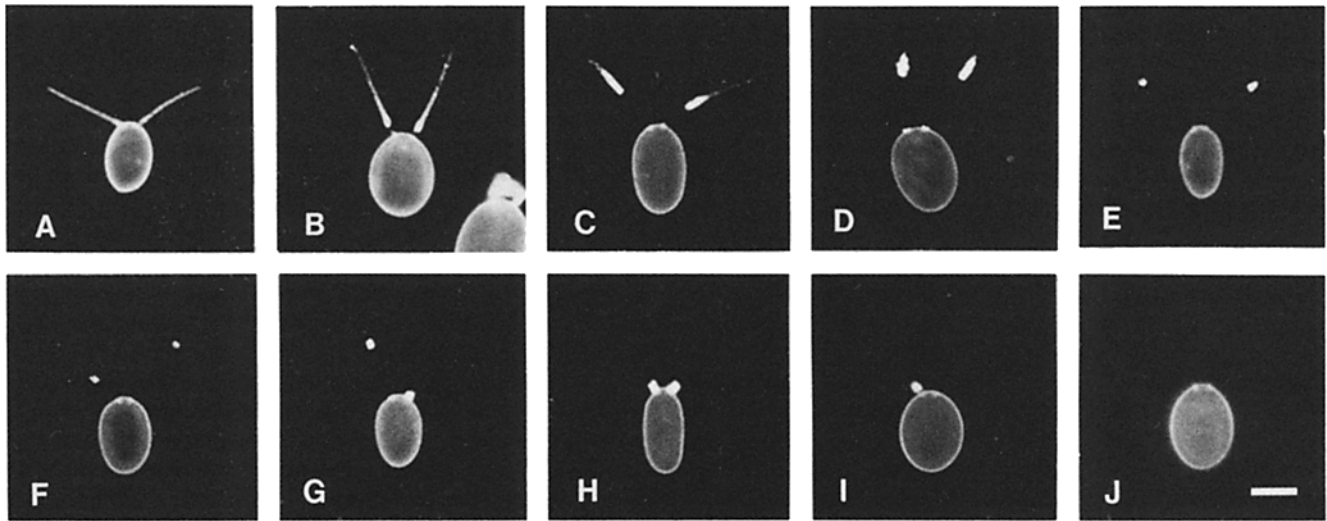


Figure 9. The pattern of redistribution of the FMG-1 monoclonal antibody along the flagellar surface of live strain pf-18 cells. Live cells were incubated with the monoclonal antibody on ice and the excess antibody was removed by washing in ice cold M medium. The cells were rapidly warmed to 23°C and aliquots of cells were fixed with glutaraldehyde at various time intervals after warming. After fixation, all of the samples were incubated with FITC-labeled second antibody, washed, and photographed using epifluorescence optics. The cell in *A* was fixed while the cells were still in the cold. The other cells were fixed at 5 min (*B-F*), 10 min (*G* and *H*), and 15 min (*I* and *J*) after warming. Bar, 5 μ m.

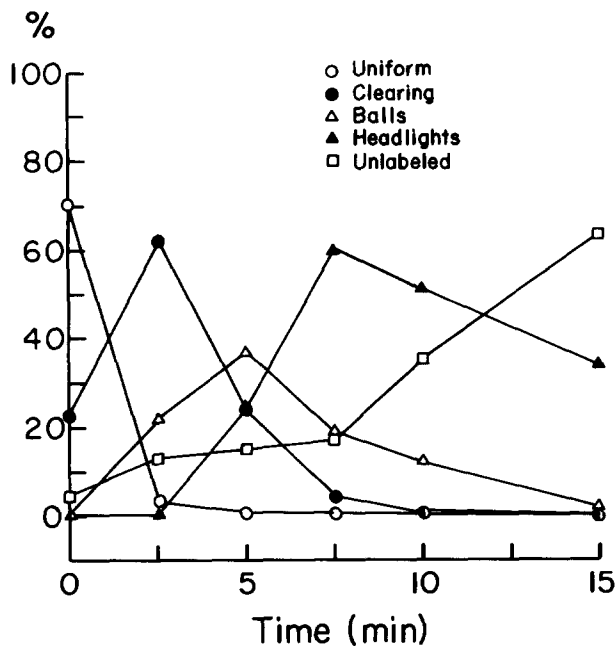


Figure 10. Quantitation of the kinetics of redistribution of the FMG-1 monoclonal antibody along the flagellar surface. At various times after warming, samples of cells were fixed and processed. 500 cells were scored at each time point and placed within one of five categories describing the redistribution image. *Uniform* corresponds to the image in Fig. 9*A*; *Clearing* corresponds to the images in Fig. 9, *B-D*; *Balls* corresponds to the images in Fig. 9, *E* and *F*; *Headlights* corresponds to the images in Fig. 9, *H* and *I*; *Unlabeled* corresponds to the image in Fig. 9*J*.

is always the same. Note that these data have been obtained from a population of cells that are fixed at various times after warming and the position of the monoclonal antibody was determined using the indirect immunofluorescence procedure. To determine whether the pattern of redistribution of the antibody along a single cell in the population is the same

as that observed by sampling the population at various time intervals, we have also followed the redistribution of fluorescein-conjugated FMG-1 monoclonal antibody along the flagellar surfaces of individual live pf-18 cells labeled at 4°C and then allowed to warm to room temperature while being observed in the fluorescence microscope. The temporal pattern of antibody redistribution observed on single live cells mirrors that shown in Fig. 9 for the entire population. These observations are difficult to make because of the rapid bleaching of the fluorescein and because of problems inherent in keeping cells under a coverslip healthy while being exposed to the excitation irradiation. Preliminary observations have also been made using FITC-concanavalin A on individual live pf-18 cells. Although early stages in the redistribution process have been difficult to visualize, it has been possible to observe directly the movement of the aggregates of FITC-concanavalin A (such as those shown in Fig. 15, *C* and *D*) along the entire length of individual flagella on live cells.

The flagella appear to be morphologically unaltered during the process of redistribution and shedding of the antibody and lectin (Fig. 11), although it is sometimes observed that there are subtle thickenings on the flagellar surface (as viewed by DIC microscopy) that correlate with the position of the fluorescent aggregates; previous studies have reported that aggregates of material form on flagella exposed to rabbit polyclonal antibodies made to whole flagella (23) or flagellar membranes (10). A striking feature of this redistribution phenomenon is that, in the vast majority of cases, each flagellum on a single cell exhibits the exact same pattern at the same time as its companion flagellum (Fig. 9, *A-E* and *H*, Fig. 11, Fig. 13, and Fig. 15, *A-D* and *F*); the activity of the two flagella seem to be highly coordinated. Fig. 9, *F*, *G*, and *I* and Fig. 15, *E* and *G*, are among the very rare exceptions where the two flagella on a single cell exhibit different localizations of the antibody or lectin. The redistribution of the FMG-1 monoclonal antibody occurred over the temperature of 10–30°C and the rate of redistribution was very temperature

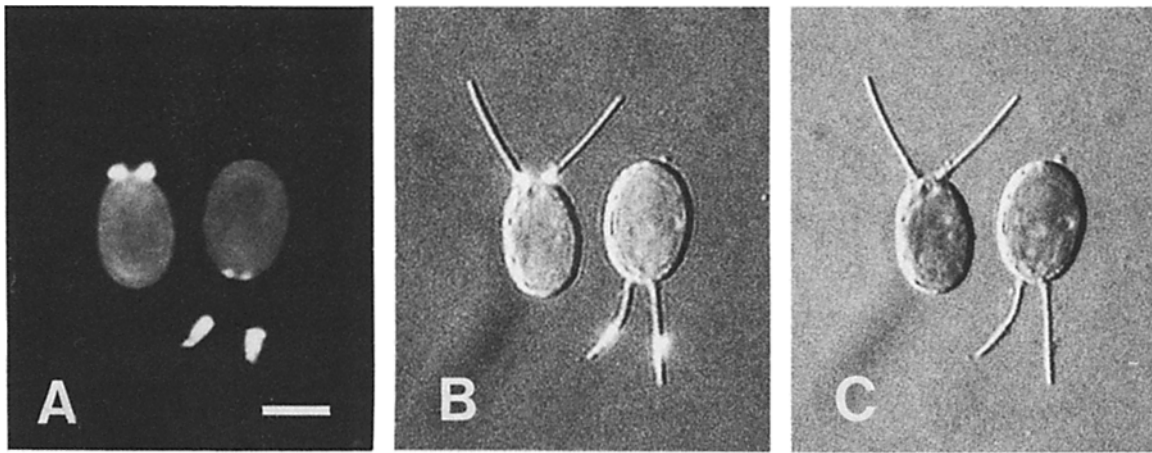


Figure 11. Comparison of the fluorescence and DIC images of two cells exhibiting different stages of redistribution of the FMG-1 antibody. *A* was photographed using epifluorescence optics. *C* was photographed using DIC optics. *B* was photographed using both fluorescence and DIC optical systems simultaneously. Bar, 5 μm .

dependent, occurring much faster at 30°C than at 15°C (Fig. 12). However, redistribution could be observed to occur even at lower temperatures; if the cells were allowed to warm to temperatures even slightly above 4°C during the post-labeling washing period, some of the early stages of clearing (such as those shown in Fig. 9*B* and Fig. 15*B*) could be observed. High molar ratios of EGTA/calcium (13:1) in the medium have no effect on the antibody redistribution. The redistribution of the FMG-1 antibody along the flagellar surface is not inhibited by concentrations of cycloheximide (10 $\mu\text{g}/\text{ml}$) that have been shown previously (12) to completely inhibit protein synthesis in the pf-18 strain of *Chlamydomonas reinhardtii*.

Strain pf-18 cells are immotile because they lack the central pair microtubule complex (3, 58). Other paralyzed flagella mutants of *C. reinhardtii* (pf-1, ts222) exhibiting different defects from that of pf-18 (26) also exhibit temperature-dependent redistribution of the FMG-1 monoclonal antibody. As pointed out above, wild-type, motile strains of *Chlamydomonas* cannot be assessed for their ability to redistribute the FMG-1 monoclonal antibody because live cells of motile strains do not appear to bind the monoclonal antibody to any significant extent.

To determine whether the HMW flagellar glycoproteins were redistributing in parallel with the redistribution of the FMG-1 monoclonal antibody, cells were incubated with the monoclonal antibody at 4°C and the cells were washed and warmed to 23°C to induce redistribution. At various time intervals after warming, aliquots of cells were fixed in glutaraldehyde; half of each sample was labeled with FITC second antibody in order to localize the monoclonal antibody and half of each sample was labeled with FITC-concanavalin A in order to localize the flagellar glycoproteins. The two samples exhibited very similar patterns of redistribution at each time point examined (Fig. 13) indicating that most of the surface-exposed, concanavalin A-binding flagellar glycoproteins were indeed redistributing along with the FMG-1 antibody.

If cells are labeled with the FMG-1 antibody, allowed to redistribute, fixed, and then labeled with the FMG-1 antibody a second time before incubation with the FITC-labeled second antibody, labeling appears on the cleared areas of the flagella

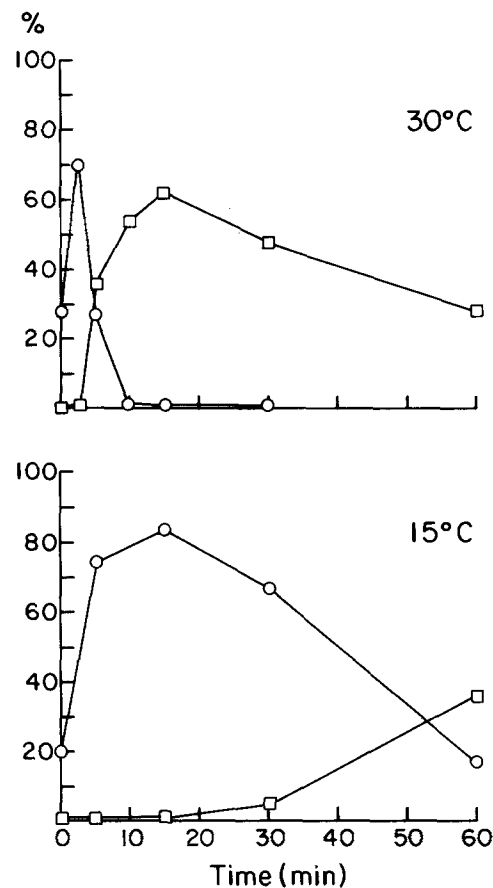


Figure 12. Effect of temperature on the kinetics of redistribution of the FMG-1 monoclonal antibody. Cells were labeled with the monoclonal antibody and then warmed to either 15 or 30°C. Images were scored as in Fig. 11. For simplicity, the kinetics of only two redistribution intermediates were graphed for each temperature. Open circles represent clearing stages (Fig. 9, *B-D*) and open squares represent the headlight stage (Fig. 9*H*).

(Fig. 14). This is interpreted to reflect turnover and insertion of new copies of the HMW flagellar membrane glycoproteins, as has been previously reported to occur (9), subsequent to clearing of the copies that had bound the first round of

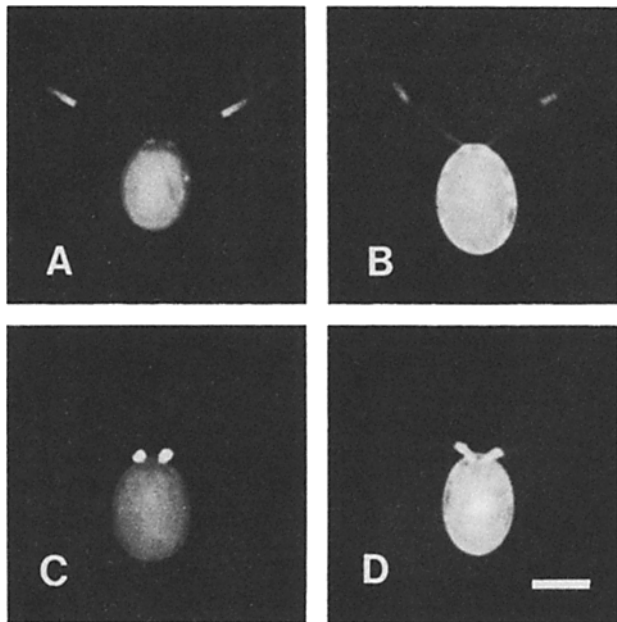


Figure 13. Co-redistribution of the FMG-1 monoclonal antibody with the concanavalin A-binding flagellar membrane glycoproteins. Cells were incubated with the FMG-1 monoclonal antibody in the cold, washed in the cold, warmed to 23°C, and allowed to redistribute. After various time intervals, two aliquots of cells were fixed in glutaraldehyde. One aliquot from each time point was labeled with FITC-labeled second antibody (goat anti-mouse IgG) while the other aliquot was labeled with FITC-concanavalin A. *A* and *C* illustrate stages of redistribution of the FMG-1 monoclonal antibody as detected using FITC-labeled second antibody. *B* and *D* illustrate stages of redistribution of concanavalin A-binding flagellar membrane glycoproteins as detected using FITC-labeled concanavalin A. *A* and *B* were taken from the 15-min sample while *C* and *D* were taken from the 30-min sample. Bar, 5 μm .

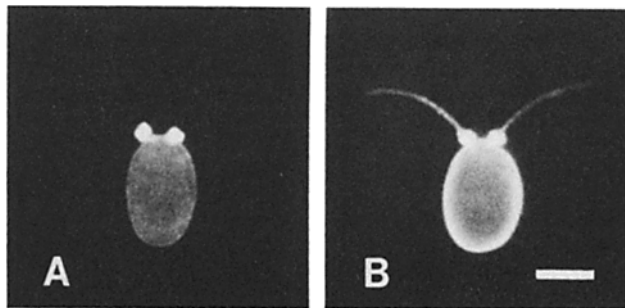


Figure 14. Relabeling of flagella with a second round of FMG-1 monoclonal antibody after redistribution. Cells were labeled with the FMG-1 monoclonal antibody in the cold, washed in the cold, and warmed to room temperature. After various time intervals, two aliquots of cells were fixed in glutaraldehyde. One aliquot from each time point was immediately labeled with FITC-labeled second antibody. *A* is such a cell from the 15-min time point. The other aliquot of fixed cells was relabeled a second time with the FMG-1 monoclonal antibody, washed, and then labeled with FITC-labeled second antibody. *B* is such a cell from the 15-min time point. Bar, 5 μm .

monoclonal antibody; it could, however, reflect the fact that some of the original copies of the membrane glycoproteins did not bind antibody during the first exposure to the monoclonal antibody and hence were not cleared.

The FMG-1 Monoclonal Antibody Inhibits Flagellar Surface Motility As Visualized by the Movement of Polystyrene Microspheres along the Flagellar Surface

Live pf-18 cells were incubated at room temperature in growth medium containing various concentrations of FMG-1 monoclonal antibody that had been purified on Protein A-Sepharose; the cells were assayed for polystyrene microsphere adhesion and movement by the methods previously described (12). The cell density was kept low in order to minimize clumping of cells by the antibody and the cells were kept in the continuous presence of the antibody during the measurements of microsphere adhesion and movement. Comparable concentrations of a similarly purified β -tubulin monoclonal antibody (clone 27BC) that did not exhibit any binding to the flagellar surface were used as controls. The anti-tubulin monoclonal had no effect on microsphere adhesion to or movement along the flagellar surface at any concentration tested. Increasing concentrations of the FMG-1 monoclonal antibody resulted in a graded increase in the number of microspheres bound to the flagellar surface. In contrast, the FMG-1 monoclonal antibody inhibited polystyrene microsphere movement dramatically at concentrations of 25 $\mu\text{g}/\text{ml}$ or higher. FAB' fragments of the FMG-1 monoclonal antibody were generated by conventional procedures (38) but their binding to the flagellar glycoproteins was found to be too low (as judged by the ELISA) to test their effect on microsphere adhesion and movement.

Discussion

The cell surface plays many important roles in the life of the cell. It is involved in cell shape changes, cell motility, cell-cell interactions, cell-substrate interactions, uptake of materials from and release of materials into the extracellular compartment, reception of stimuli (often in the form of polypeptides and small molecules that bind to specific cell surface receptors), and transfer of information from the surface to the interior of the cell. Plasma membrane glycoproteins have been shown to be intimately involved in many of these functions; in at least some cases, the carbohydrate moieties are necessary either for the proper insertion or the proper functioning of the glycoprotein (37, 48).

The flagellar surface of *Chlamydomonas* plays important roles in the lifestyle of this single-celled eukaryotic organism. In addition to the typical swimming behavior that involves dynamic events associated with the flagellar axoneme, *Chlamydomonas* can exhibit an alternative form of whole cell locomotion, which is a gliding motility resulting from the interaction of the flagellar surface with a solid or semi-solid substrate (7, 29). This dynamic cell-substrate interaction appears to be mediated by the principal HMW glycoproteins of the flagellar surface (11). Fertilization in *Chlamydomonas* is initiated by contact between gamete-specific flagellar surface-exposed glycoproteins (24, 53, 56); adhesion between the flagella of the (+) and (-) gametes is followed by other dynamic flagellar surface events essential for achieving cell-cell fusion (53). The predominant protein components in the flagellar membrane of both gametic and vegetative *Chlamydomonas reinhardtii* are a small group of HMW glycoproteins migrating with apparent molecular weights in the neighborhood of 350,000 (5, 10, 58). In addition, gametic flagellar

surfaces possess smaller amounts of gamete-specific HMW glycoproteins involved in the early events of mating (1, 56).

In the present study, we have localized carbohydrate sites on the flagellar surface and the cell wall using two carbohydrate-binding reagents: the lectin concanavalin A, which recognizes α -D-mannosyl and α -D-glucosyl residues associated with glycoproteins and glycolipids, and a monoclonal antibody that recognizes unknown but repeated carbohydrate epitopes associated with the HMW flagellar membrane glycoproteins. The characteristics of the FMG-1 monoclonal antibody are summarized in Table I: the principal evidence for the epitope recognized by this antibody being composed of carbohydrate rests on the following observations: (a) extensive pronase treatment of the antigen does not abolish antibody binding; (b) periodate oxidation under conditions that are specific for vicinal hydroxyl groups in sugars (59) abolishes antibody binding; (c) the epitopes are repeated multiple times along the polypeptide, and (d) the antibody cross-reacts with cell wall glycoproteins.

The carbohydrate epitopes being examined in this study are found greatly enriched on the flagellar plasma membrane although the same carbohydrate epitopes are also found associated with the general cell body plasma membrane and with cell wall glycoproteins. Both polyclonal (35) and monoclonal (49) antibodies raised against *Chlamydomonas* cell wall glycoproteins have been reported to cross-react with flagellar membrane components. Several of the monoclonal antibodies raised to gametic flagellar membrane glycoproteins also cross-react with cell wall components (2, 27). Where examined, these cross-reactive polyclonal and monoclonal antibodies appear to be recognizing carbohydrate groups.

Examination of wild-type dikaryons shortly after gamete fusion and vegetative cells of the cell wall-less mutant CW15 indicate that the carbohydrate epitopes recognized by concanavalin A and the FMG-1 monoclonal antibody are also found associated with the surface of the cell body plasma membrane albeit in a reduced density relative to the flagellar plasma membrane. A number of observations suggest that the flagellar plasma membrane of *Chlamydomonas reinhardtii* is structurally and functionally distinct from the rest of the cell's plasma membrane. Unique features of the flagellar surface include: (a) longitudinal arrays of intramembrane particles (5, 50); (b) a prominent fuzzy coat or glycocalyx (43); (c) mastigonemes (43); (d) flagellar surface motility (gliding motility and microsphere movement) (6, 7, 29); and (e) attachments to the underlying cytoskeleton (axoneme) (6, 43). The pattern of staining with concanavalin A and the FMG-1 monoclonal antibody best correlates with the distribution of the fuzzy coat or glycocalyx that covers the entire surface of vegetative and gametic flagella. Currently, there is no strong evidence in *Chlamydomonas* for the existence of any protein or glycoprotein that is distributed solely on that subset of the plasma membrane associated with the flagella. The best candidate for such a protein component might be the mating-type specific sexual agglutinins (1, 56); however, there is evidence from *C. eugametos* that there is a large pool of the sexual agglutinin glycoprotein associated with the cell body plasma membrane (39). Bouck (13) has evidence for a *Euglena* flagellar glycoprotein being unique to the subset of the plasma membrane associated with the flagellum and reservoir.

The *Chlamydomonas* flagellar surface displays a number of

dynamic properties; these include (a) rapid turnover of flagellar membrane proteins (9, 42); (b) flagellar surface motility as exhibited by polystyrene microsphere movements (6, 12, 25) and whole cell gliding motility (7, 29); and (c) a process similar to lymphocyte capping (termed "tipping") in which lectins and antibodies redistribute along the flagellar surface (23; this report). The phenomenon of flagellar surface motility is rapid, local, and bidirectional, and is unlikely to be related to the slower, global redistribution of glycoprotein-associated carbohydrate epitopes described in the present report. Indeed, the presence of the FMG-1 monoclonal antibody in the medium at a concentration of 25 μ g/ml or higher inhibits flagellar surface motility as visualized using polystyrene microspheres. The temperature-dependent redistribution of concanavalin A and the anti-carbohydrate monoclonal antibody reported here has a striking resemblance to the capping phenomenon described on the bulk plasma membrane of many mammalian cell types (14, 54). Many, but not all, cases of capping on lymphocytes and other mammalian cells have been associated with the underlying cytoskeleton. In particular, cytochalasin B has been reported to inhibit capping, and actin, myosin, α -actinin, and spectrin have been reported to co-cap with surface receptors (for review see reference 14). In the case of the flagellum, the cytoskeleton consists of the axoneme and, with the exception of an actin-like polypeptide localized to the outer dynein arm (40), the cytoskeletal proteins normally associated with capping in mammalian cells are absent from the *Chlamydomonas* flagellum. At the present time, the mechanism responsible for the redistribution of the flagellar carbohydrate epitopes is unknown but is thought to be different from that responsible for flagellar surface motility. A low free calcium concentration in the medium results in an inhibition of flagellar surface motility (12) but has no effect on the antibody redistribution phenomenon. The present study has demonstrated that the energy-dependent redistribution of the anti-carbohydrate monoclonal antibody is accompanied by the redistribution of the HMW glycoproteins possessing the carbohydrate epitopes recognized by this antibody. It is assumed that the redistribution occurs because there are multiple binding sites for concanavalin A and the FMG-1 monoclonal antibody on each HMW flagellar glycoprotein molecule allowing a cross-linking of this population of molecules in the plane of the flagellar membrane. However, it is conceivable that the ligands being used in this study are merely visualizing a movement or flow of materials in the plane of the flagellar membrane that is occurring in the absence of cross-linking with these multivalent ligands. The test of this hypothesis is to determine whether the monovalent FAB' fragments of the FMG-1 antibody or succinylated concanavalin A will redistribute along the flagellar surface. Thus far, FAB' fragments of the FMG-1 antibody produced by the method of Parham (38) have not exhibited enough binding to the epitope (as judged by the ELISA) to allow us to use them for immunofluorescence studies; similarly, FITC-succinyl concanavalin A has not provided enough signal to answer this question.

The most surprising feature of the redistribution of carbohydrate epitopes on the flagellar surface observed in this study is that the clearing of the label into a mass at the tip of each flagellum was followed by a migration of these aggregates to the base of the flagella, from which site the labeled aggregates

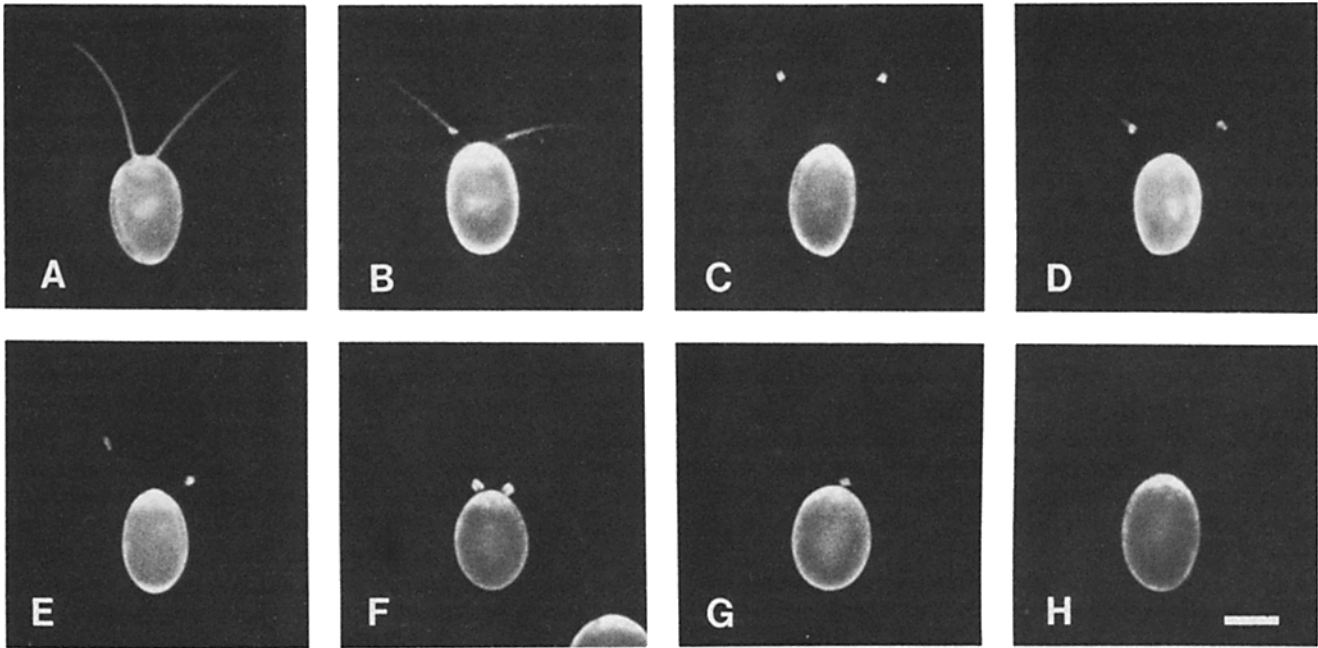


Figure 15. Pattern of redistribution of FITC-concanavalin A along the flagellar surface of live strain pf-18 cells. Live cells were incubated with the FITC-concanavalin A on ice and the excess FITC-concanavalin A removed by washing in ice cold M medium. The cells were rapidly warmed to 23°C and aliquots of cells were fixed with glutaraldehyde at various times after warming. All photographs were taken using epifluorescent optics. The cell in *A* was fixed while the cells were still in the cold. The other cells were fixed at 5 min (*B* and *C*), 10 min (*D* and *E*), and 30 min (*F*-*H*) after warming. Bar, 5 μ m.

were shed into the medium. The argument for this temporal sequence of events is best made in the FMG-1 antibody data shown in Fig. 10, where the dominant image in the population shifts from a clearing intermediate at 2.5 min to distal aggregates at 5 min to proximal aggregates (termed headlights) at 7.5 min to unlabeled cells at 10 min. More careful temporal analysis of the antibody redistribution phenomenon has shown intermediate times between those times when most of the cells are at the tip aggregate stage and when most of the cells are at the base aggregate stage (headlights) at which time the aggregates of label tend to be at intermediate positions along the flagella (as in Fig. 9*F*). The role of the headlight intermediate in the redistribution of concanavalin A is less clear (Fig. 16); although some headlight images are definitely seen (Fig. 15*F*), they often constitute a small percentage of the population. It is possible, in the case of concanavalin A, that there are two pathways; some cells may redistribute the ligand to the tips of the flagella, after which it is shed, while other cells may return the aggregated ligand to the base of the flagella, where it is subsequently shed. Using image intensification techniques, direct visualization has been made of the movement of FITC-concanavalin A aggregates along the flagellar surface of individual live cells. It is clear, however, that virtually all cells in a population redistribute both the antibody and the lectin to the distal tips of the flagella. Although the sequence of redistribution intermediates is very reproducible, the time course varies somewhat from experiment to experiment, even when performed at the same temperature. Because of that, no special significance should be assigned to the fact that the time course of redistribution for the FMG-1 antibody shown in Fig. 10 is somewhat faster than that shown for concanavalin A in Fig. 16.

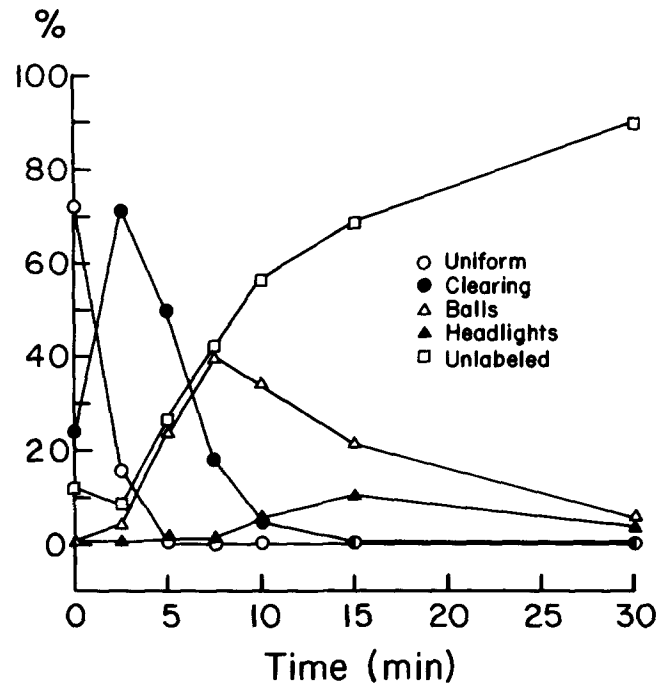


Figure 16. Quantitation of the kinetics of redistribution of FITC-concanavalin A along the flagellar surface. At various times after warming, samples of cells were fixed and 500 cells from each time point were scored as to the stage of redistribution exhibited. The categories used and the symbols on the graph are the same as those used in Fig. 10. *Uniform* corresponds to the image in Fig. 15*A*; *Clearing* corresponds to the image in Fig. 15*B*; *Balls* corresponds to the image in Fig. 15, *C-E*; *Headlights* corresponds to the images in Fig. 16*F*; *Unlabeled* corresponds to the image in Fig. 15*H*.

Goodenough and Jurivich (23) reported that an anti-flagellar polyclonal antiserum would aggregate to the tips of gametic flagella of *C. reinhardtii*; these authors reported that vegetative cells were incapable of antibody "tipping." The present report clearly demonstrates that antibody redistribution can occur on the flagella of vegetative cells of *C. reinhardtii*, at least in the case of paralyzed flagella strains.

Capping-like phenomena in many cell types result in internalization of the redistributed ligand-receptor complex into the cell by some form of endocytosis (46, 54). Endocytosis of the flagellar membrane is impossible because of the architecture of the flagellum; all areas of the *Chlamydomonas* plasma membrane either overlay the axoneme or are covered by cell wall. It is conceivable that flagellar membrane could be endocytosed at the base of the flagellum; however, initial electron microscopic observations (Levin, E. N., and R. A. Bloodgood, unpublished results) using soluble horseradish peroxidase in the culture medium as an electron dense tracer showed no signs of plasma membrane internalization into the cell. It is, therefore, not surprising that the ligand aggregates observed in the present study appear to be shed into the medium. Shedding of flagellar membrane vesicles from both vegetative and gametic cells of *Chlamydomonas reinhardtii* has been reported (5, 32, 50) and radioactive pulse-labeling studies have indicated a continuous turnover of flagellar membrane proteins (9, 42). This is consistent with the observation that new FMG-1 antibody binding sites appear on the flagellar surface subsequent to antibody-induced redistribution (Fig. 14).

The two carbohydrate-binding probes (concanavalin A and the FMG-1 antibody) used in the present study exhibit a number of similarities in that both bind to the HMW flagellar glycoproteins, both bind to virtually all of the V-8 protease-derived peptides, both label the flagellar surface uniformly, both redistribute and are shed from the flagellar surface, both bind to cell wall glycoproteins, and the binding of both is inhibited by periodate oxidation (at low pH) of the flagellar glycoproteins. The FMG-1 monoclonal antibody and concanavalin A do not compete for the same binding site. Soluble concanavalin A, even at high concentrations (1,000 $\mu\text{g}/\text{ml}$) does not inhibit antibody binding and vice versa. The soluble sugar α -methyl-D-mannoside inhibits the binding of the concanavalin A but does not affect the binding of the monoclonal antibody. The two ligands exhibit differences in the pattern of binding to minor flagellar membrane components on Western blots (Fig. 2). Taken together, these observations suggest that concanavalin A and the FMG-1 monoclonal antibody bind to different carbohydrate epitopes located on a carbohydrate structure that is repeated multiple times along the length of the HMW flagellar glycoproteins (Fig. 8).

The relatively slow redistribution of flagellar membrane proteins visualized by the use of concanavalin A and the anti-carbohydrate monoclonal antibody is a phenomenon distinct from the rapid, local, bidirectional movements of flagellar membrane domains visualized by the movement of polystyrene microspheres and referred to as flagellar surface motility (12, 25). The first of these two processes is independent of the free calcium concentration in the medium while the second has been shown previously to be dependent upon external free calcium (12). Indeed, the FMG-1 monoclonal antibody used in the present study to visualize (and perhaps also induce)

the slow clearing of the flagellar membrane glycoproteins inhibits the rapid movements of polystyrene microspheres along the flagellar surface. A similar inhibition of flagellar surface motility has been reported previously for polyclonal antibodies prepared to the high molecular weight flagellar glycoproteins (10) or to whole flagella from mating type (+) gametes (25). The antibody inhibition, taken at face value, suggests that the principal flagellar surface-exposed glycoproteins recognized by the HMG-1 glycoprotein are intimately involved in the mechanism of rapid flagellar surface motility. This is consistent with the conclusions from previous studies using polyclonal antibodies to these 350,000-mol-wt components (10), pronase digestion studies of the flagellar surface (10), and protein iodination studies using a substrate-immobilized iodination system (11). However, there always remains the possibility that the cross-linking of an abundant flagellar membrane glycoprotein by the anti-carbohydrate antibody could restrict the lateral mobility of another flagellar membrane component that is actually the one involved in the mechanism of flagellar surface motility.

Mating in *Chlamydomonas* involves a number of dynamic events on the part of the flagella. The first event in mating involves the agglutination of the flagella of the two different mating-type gametic cells; this agglutination is followed by a realignment of the contact sites between the flagellar surfaces such that the tips of the flagella become locked into register. It has been suggested that the rapid flagellar surface motility visualized by microsphere movement could be responsible for this phenomenon (8, 12, 25, 29); however, it is equally possible that a slower, directed migration of a particular class of flagellar membrane glycoproteins, such as that being visualized in the present report with the use of antibody and lectin probes, is responsible for the reorganization of the flagellar surface that accompanies mating and appears to be a prerequisite for the flagellar signaling phenomenon (24, 53). Forest et al. (22) predicted that "gametic flagella possess a vectorial concentration mechanism which, when activated by sexual or antiserum agglutination, brings dispersed signaling components in the flagellar membrane towards the flagellar tip, the signaling reaction taking place when a sufficient density of components builds up at the tip." Both anti-flagellar antibody (16, 23) and concanavalin A (16) treatment of unmated gametes have been reported to result in flagellar activation and signaling; the antibody effect was reported to require bivalent antibodies (23). Recent observations (Homan, W., and H. van den Ende, unpublished results) indicate that during mating in *C. eugametos*, certain flagellar components (including the sexual agglutinin glycoprotein) redistribute to the flagellar tip and subsequently disappear from the flagellar surface after flagella deadhere.

The observations reported in this paper further emphasize the dynamic nature of the *Chlamydomonas* flagellar surface. It will be of future interest to compare and contrast the mechanisms underlying the two different surface phenomena common to the vegetative and gametic flagellar surfaces and to further explore the relationship of these phenomena to the flagellar changes that occur during mating in this organism.

The authors gratefully acknowledge Dr. Charles D. Little for use of the fluorescence microscope, frequent advice on fluorescence microscopy, and access to his cell culture facilities. Alice Mayer provided critical technical help in obtaining the mouse-mouse hybridomas;

additional technical assistance was provided by Fritz Reinhart and Doris Peterkin. The authors acknowledge the generosity of Dr. John Jarvik in providing the E2 (ts222) strain of *Chlamydomonas*. All other cell strains were provided by the *Chlamydomonas* Genetics Center in the Department of Botany at Duke University (funded by the National Science Foundation). Anti-tubulin monoclonal antibodies were provided by Dr. Lester I. Binder. Dr. David Benjamin provided the SP2/0 mouse myeloma cell line used in the fusions.

This work was supported by a National Institutes of Health research grant (GM28766) and a National Science Foundation research grant (DCB-8502980) to R. A. Bloodgood. M. P. Woodward was supported by a National Institutes of Health developmental biology training grant (T32 HD07192).

Received for publication 21 September 1985, and in revised form 13 January 1986.

References

- Adair, W. S., C. Hwang, and U. W. Goodenough. 1983. Identification and visualization of the sexual agglutinin from the mating-type plus flagellar membrane of *Chlamydomonas*. *Cell* 33:183-193.
- Adair, W. S., J. Long, W. B. Mehard, J. E. Heuser, and U. W. Goodenough. 1983. Monoclonal antibodies directed against the sexual agglutinin of *Chlamydomonas reinhardtii*. *J. Cell Biol.* 97(5, Pt. 2):93a. (Abstr.)
- Adams, G. M. W., B. Huang, G. Piperno, and D. J. L. Luck. 1981. Central-pair microtubular complex of *Chlamydomonas* flagella: polypeptide composition as revealed by analysis of mutants. *J. Cell Biol.* 91:69-76.
- Adoutte, A., R. Ramanathan, R. Lewis, R. Dute, K.-Y. Ling, C. Kung, and D. L. Nelson. 1980. Biochemical studies on the excitable membrane of *Paramecium tetraurelia*. III. Proteins of cilia and ciliary membranes. *J. Cell Biol.* 84:717-738.
- Bergman, K., U. W. Goodenough, D. A. Goodenough, J. Jawitz, and H. Martin. 1975. Gametic differentiation in *Chlamydomonas reinhardtii*. II. Flagellar membranes and the agglutination reaction. *J. Cell Biol.* 67:606-622.
- Bloodgood, R. A. 1977. Motility occurring in association with the surface of the *Chlamydomonas* flagellum. *J. Cell Biol.* 75:983-989.
- Bloodgood, R. A. 1981. Flagella-dependent gliding motility in *Chlamydomonas*. *Protoplasma*. 106:183-192.
- Bloodgood, R. A. 1982. Dynamic properties of the flagellar membrane. *Symp. Soc. Exp. Biol.* 35:353-380.
- Bloodgood, R. A. 1984. Preferential turnover of membrane proteins in the intact *Chlamydomonas* flagellum. *Exp. Cell Res.* 150:488-493.
- Bloodgood, R. A., and G. S. May. 1982. Functional modification of the *Chlamydomonas* flagellar surface. *J. Cell Biol.* 93:88-96.
- Bloodgood, R. A., and L. J. Workman. 1984. A flagellar surface glycoprotein mediating cell-substrate interaction in *Chlamydomonas*. *Cell Motility*. 4:77-87.
- Bloodgood, R. A., E. M. Leffler, and A. T. Bojczuk. 1979. Reversible inhibition of *Chlamydomonas* flagellar surface motility. *J. Cell Biol.* 82:664-674.
- Boucek, G. B., and S.-J. Chen. 1984. Synthesis and assembly of the flagellar surface. *J. Protozool.* 31:21-24.
- Bourguignon, L. Y. W., and G. L. Bourguignon. 1984. Capping and the cytoskeleton. *Int. Rev. Cytol.* 87:195-224.
- Bruck, C., D. Protetelle, C. Glineur, and A. Bollen. 1982. One-step purification of mouse monoclonal antibodies from ascites fluid by DEAE Affi-Gel Blue chromatography. *J. Immunol. Methods*. 53:313-319.
- Claes, H. 1977. Non-specific stimulation of the antolytic system in gametes from *Chlamydomonas reinhardtii*. *Exp. Cell Res.* 108:221-229.
- Cleveland, D. W., S. G. Fisher, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102-1106.
- Davies, D. R., and A. Plaskitt. 1971. Genetical and structural analyses of cell-wall formation in *Chlamydomonas reinhardtii*. *Genet. Res.* 17:33-43.
- Dentler, W. L. 1981. Microtubule-membrane interactions in cilia and flagella. *Int. Rev. Cytol.* 72:1-47.
- Dion, A. S., C. C. Williams, and A. A. Pomenti. 1983. Glycopeptide detection by covalent attachment to microplate wells and lectin binding. *BioTechniques*. Sept/Oct:130-134.
- Ey, P. L., S. J. Prowse, and C. R. Jenkins. 1978. Isolation of pure IgG₁, IgG_{2a}, and IgG_{2b} immunoglobulins from mouse serum using Protein A-Sepharose. *Immunochemistry*. 15:429-436.
- Forest, C. L., D. A. Goodenough, and U. W. Goodenough. 1978. Flagellar membrane agglutination and sexual signaling in the conditional GAM-1 mutant of *Chlamydomonas*. *J. Cell Biol.* 79:74-84.
- Goodenough, U. W., and D. Jurivich. 1978. Tipping and mating-structure activation induced in *Chlamydomonas* by flagellar membrane antisera. *J. Cell Biol.* 79:680-693.
- Goodenough, U. W., W. S. Adair, E. Caligor, C. L. Forest, J. L. Hoffman, D. A. Mesland, and S. Speth. 1980. Membrane-membrane and membrane-ligand interactions in *Chlamydomonas* mating. In *Membrane-Membrane Interactions*. N. B. Gilula, editor. Raven Press, New York. 131-152.
- Hoffman, J. L., and U. W. Goodenough. 1980. Experimental dissection of flagellar surface motility in *Chlamydomonas*. *J. Cell Biol.* 86:656-665.
- Jarvik, J. W., and J. L. Rosenbaum. 1980. Oversized flagellar membrane protein in paralyzed mutants of *Chlamydomonas reinhardtii*. *J. Cell Biol.* 85:258-272.
- Kosfisz, M. A., A. Clausell, S. Iman, B. Lavery, Jr., B. Border, P. Witte, and W. J. Snell. 1984. A monoclonal antibody that blocks adhesion of mt+ gametes of *Chlamydomonas*. *J. Cell Biol.* 99(4, Pt. 2):71a. (Abstr.)
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lewin, R. A. 1952. Studies on the flagella of algae. I. General observations of *Chlamydomonas moewusii* Gerloff. *Biol. Bull.* 103:74-79.
- Matsuda, Y., K. Sakamoto, N. Kiuchi, T. Mizuochi, Y. Tsubo, and A. Kobata. 1982. Two tunicamycin-sensitive components involved in agglutination and fusion of *Chlamydomonas reinhardtii* gametes. *Arch. Microbiol.* 131:87-90.
- McLean, R. J., and R. M. Brown. 1974. Cell surface differentiation of *Chlamydomonas* during gametogenesis. I. Mating and concanavalin A agglutinability. *Dev. Biol.* 36:279-285.
- McLean, R. J., C. J. Laurendi, and R. M. Brown, Jr. 1974. The relationship of gamone to the mating reaction in *Chlamydomonas moewusii*. *Proc. Natl. Acad. Sci. USA* 71:2610-2613.
- Monk, B. C., W. S. Adair, R. A. Cohen, and U. W. Goodenough. 1983. Topography of *Chlamydomonas*: fine structure and polypeptide components of the gametic flagellar membrane surface and the cell wall. *Planta (Berl.)* 147:51-56.
- Musgrave, A., P. van der Steuyt, and L. Ero. 1979. Concanavalin A binding to *Chlamydomonas eugametes* flagellar proteins and its effect on sexual reproduction. *Planta (Berl.)* 147:51-56.
- Musgrave, A., P. de Wildt, R. Broekman, and H. van den Ende. 1983. The cell wall of *Chlamydomonas eugametes*. Immunological aspects. *Planta (Berl.)* 158:82-89.
- Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105:361-363.
- Olden, K., J. B. Parent, and S. L. White. 1982. Carbohydrate moieties of glycoproteins. A re-evaluation of their function. *Biochim. Biophys. Acta.* 650:209-232.
- Parham, P. 1983. On the fragmentation of monoclonal IgG₁, IgG_{2a}, and IgG_{2b} from BALB/c mice. *J. Immunol.* 131:2895-2902.
- Pijst, H. L. A., R. J. Zilver, A. Musgrave, and H. van den Ende. 1983. Agglutination factor in the cell body of *Chlamydomonas eugametes*. *Planta (Berl.)* 158:403-409.
- Piperno, G., and D. J. L. Luck. 1979. An actin-like protein is a component of axonemes from *Chlamydomonas* flagella. *J. Biol. Chem.* 254:2187-2190.
- Ray, D. A., and A. Gibor. 1982. Tunicamycin-sensitive glycoproteins involved in the mating of *Chlamydomonas reinhardtii*. *Exp. Cell Res.* 141:245-252.
- Remillard, S. P., and G. B. Witman. 1982. Synthesis, transport, and utilization of specific flagellar proteins during flagellar regeneration in *Chlamydomonas*. *J. Cell Biol.* 93:615-631.
- Ringo, D. L. 1967. Flagellar motion and fine structure of the flagellar apparatus in *Chlamydomonas*. *J. Cell Biol.* 33:543-571.
- Sager, R., and S. Granick. 1953. Nutritional studies with *Chlamydomonas reinhardtii*. *Ann. NY Acad. Sci.* 56:831-838.
- Sager, R., and S. Granick. 1954. Nutritional control of sexuality in *Chlamydomonas reinhardtii*. *J. Gen. Physiol.* 37:729-742.
- Salisbury, J. L., J. S. Condeelis, and P. Satir. 1980. Role of coated vesicles, microfilaments, and calmodulin in receptor-mediated endocytosis by cultured B Lymphoblastoid cells. *J. Cell Biol.* 87:132-141.
- Sefton, B. 1976. Virus-dependent glycosylation. *J. Virol.* 17:85-93.
- Sharon, N., and H. Lis. 1982. Glycoproteins: research booming on long-ignored ubiquitous compounds. *Mol. Cell. Biochem.* 42:167-187.
- Smith, E., K. Roberts, A. Hutchings, and G. Galfre. 1984. Monoclonal antibodies to the major structural glycoprotein of the *Chlamydomonas* cell wall. *Planta (Berl.)* 161:330-338.
- Snell, W. J. 1979. Mating in *Chlamydomonas*: a system for the study of specific cell adhesion. I. Ultrastructural and electrophoretic analysis of flagellar surface components involved in adhesion. *J. Cell Biol.* 68:48-69.
- Snell, W. J. 1981. Flagellar adhesion and deadhesion in *Chlamydomonas* gametes: effects of tunicamycin and observations of flagellar tip morphology. *J. Supramol. Struct. Cell. Biochem.* 16:371-376.
- Snell, W. J. 1983. Characterization of the *Chlamydomonas* flagellar collar. *Cell Motility*. 3:273-280.
- Snell, W. J. 1985. Cell-cell interactions in *Chlamydomonas*. *Annu. Rev. Plant Physiol.* 36:287-315.
- Taylor, R. B., W. Duffus, M. Raff, and S. DePetris. 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by

anti-immunoglobulin antibody. *Nature New Biology*. 233:225-229.

55. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*. 76:4350-4354.

56. van den ende, H. 1985. Sexual agglutination in *Chlamydomonas*. *Adv. Microb. Physiol.* 26:89-123.

57. Wiese, L., and D. W. Shoemaker. 1979. On sexual agglutination and mating-type substances (gamones) in isogamous heterothallic *Chlamydomon-*

ads. II. The effect of concanavalin A upon the mating-type reaction. *Biol. Bull.* 138:88-95.

58. Witman, G. B., K. Carlson, J. Berliner, and J. L. Rosenbaum. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. *J. Cell Biol.* 54:507-539.

59. Woodward, M. P., W. W. Young, Jr., and R. A. Bloodgood. 1985. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *J. Immunol. Methods*. 78:143-153.