A Monoclonal Antibody that Blocks Adhesion of Chlamydomonas \(mt^+\) Gametes

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**Abstract.** During the mating reaction (fertilization) in the biflagellated alga, *Chlamydomonas reinhardtii*, \(mt^+\) and \(mt^-\) gametes adhere to each other via their flagella and subsequently fuse to form quadriflagellated zygotes. In the studies reported here, we describe a monoclonal antibody directed against an \(mt^+\) flagellar surface molecule. The antibody blocks the adhesiveness of \(mt^+\) gametes, isolated \(mt^+\) flagella, and detergent extracts thereof. It has no effect on \(mt^-\) gametes. Cyanogen bromide-activated Sepharose beads derivatized with the antibody bind only \(mt^+\) gametes; \(mt^-\) gametes and \(mt^+\) and \(mt^-\) vegetative cells are unreactive with the derivatized beads. The interaction of \(mt^+\) gametes with the beads is dynamic and cells continuously bind, detach, and rebind to the beads. Surprisingly, antibody-derivatized beads that have been incubated with \(mt^+\) gametes acquire the ability to bind \(mt^-\) gametes. Moreover, extraction of the preincubated beads with detergents releases active \(mt^+\) adhesion molecules. The evidence suggests that binding of the antibody to the flagellar surface adhesion molecules causes their release from the flagellar surface, possibly mimicking the normal mechanism of flagellar de-adhesion.

**Materials and Methods**

**Cells and Cell Cultures**

\(mt^+\) cells (the 2ig fr strain) and \(mt^-\) cells (the 645c strain) were cultured at 25°C in the minimal medium I of Sager and Granick (35) on a 12-h light-dark photoperiod as previously described (38). Gametic cells were

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1. Abbreviations used in this paper: \(mt^+\) and \(mt^-\), mating type plus and mating type minus; N-free, nitrogen free; OG, \(n\)-octylglucoside.
 obtained as previously described (38) by transferring vegetative cells (4.7 × 10⁷ cells/ml) 6 h after the beginning of the light period into the nitrogen-free (N-free) medium of Sager and Granick modified to contain 0.15 g/liter of KH₂PO₄ and 0.3 g/liter of K₂HPO₄, pH 7.2.

Preparation of Detergent Extracts of Flagella

Flagella isolated by use of the sucrose–pH shock method of Wittman et al. (30) as previously described (37), were resuspended in the n-octyl glucoside (OG) extraction buffer described by Adair et al. (4) that contained 40 mM OG, 0.1 mM diithiothreitol, 0.1 mM EDTA and 10 mM Tris, pH 7.3. Routinely, flagella isolated from 16 liters of cells were resuspended in about 2 ml of OG extraction buffer. After resuspension the sample was centrifuged for 1 h at 45,000 rpm in a rotor (50 Ti; Beckman Instruments, Inc., Fullerton, CA) in an ultracentrifuge (L-350; Beckman Instruments, Inc.) at 4°C, and the supernatant was decanted and saved. The protein concentration, determined by the Lowry method (28) using crystalline bovine serum albumin as a standard, was usually about 4 mg/ml.

Fractionation of OG Extracts on Biogel A-15M Columns

OG extracts were diluted 2:3 with 6 M urea and incubated on ice for 1 h before loading onto a column (Biogel A-15M; Bio-Rad Laboratories, Richmond, CA) (1 × 20 cm) equilibrated with elution buffer. The elution buffer contained 40 mM OG, 2 M urea, 0.1 M KCl, 10 mM EDTA, and 10 mM Tris, pH 7.2. Similar results were obtained if 0.7% n-lauryl sarkosine was used in place of OG in the elution buffer. The fractions (1 ml) were dialyzed overnight against DH₂O at 4°C (to remove the urea, which prohibited detection of adhesive activity) and assayed for adhesive activity.

Assaying for Adhesive Activity

The dried spot assay of Adair et al. (4) was used to assay the adhesive activity of OG extracts and column fractions. Briefly, samples were serially diluted in DH₂O and 2-μl samples were dried under vacuum onto a clean glass microscope slide. The slide was briefly rinsed with DH₂O or N-free medium and 2–3 ml of mt+ gametes in N-free medium were applied to the slide. After 2–3 min the adhesive activity of the sample was determined visually by noting the most dilute sample to which the motile flagellar surface fractions (not shown). Typically, this purification method yielded 60–100% of the starting activity with a 20–30-fold increase in specific activity. Column fractions with peak amounts of activity were dialyzed and lyophilized and used as immunogen in the preparation of monoclonal antibodies.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed on Laemmli (23) 15 × 13 × 0.1-cm slab gels made as linear 4–16% acrylamide gradient gels and also containing a gradient of 3–8 M urea (19). The stacking gel was 3% acrylamide. Gels were fixed and incubated with periodate (12) before being silver stained (31).

Production of Hybridomas

Hybridoma antibodies to *Chlamydomonas* flagellar surface molecules were generated according to the outline of Kohler and Milstein (21) as adapted by McKearn (30). Biogel A-15M column fractions that contained the peak amounts of adhesive activity were dialyzed, lyophilized, resuspended in DH₂O, and used as immunogen. The titer of the resuspended fractions was between 64,000 and 128,000. Approximately 20 μg of protein was used for each injection. For the first injection, 0.1 ml of the sample was mixed with 0.1 ml of Freund's complete adjuvant and injected subcutaneously into a 7-wk-old BALB/c mouse. Intraperitoneal injections without adjuvant were done at 2 and 4 wk, and at 7 wk the mouse was given a final intravenous injection. 3 d later it was killed by cervical dislocation during ether anesthesia, and the fusion was performed using NS-1 mouse myeloma cells.

Hybridoma Screening Procedures

Hybridoma supernatants were screened by an adhesion-blocking, functional assay. To do this, 100-μl portions of the supernatants were dialyzed against N-free medium in 28-place microdialyzers (Bethesda Research Laboratories, Gaithersburg, Md.) to reduce the ionic strength to a level compatible with this fresh water organism. 20-μl portions of the dialyzed supernatants were mixed with 20 μl of mt− gametes (~5 × 10⁶ cells/ml in N-free medium) in a 96-well plate and after 5 min at room temperature each well was examined on an inverted microscope. Samples showing (antibody-induced) isoagglutination were noted, and then 20 μl of mt+ gametes (~5 × 10⁶ cells/ml in N-free medium) was added to each well. In control wells with dia lyzed, fresh hybridoma medium there was a rapid, mating type–specific agglutination reaction yielding large clumps of aggregating cells that were readily detectable by examination with the inverted microscope. Similar aggregation was observed in most of the wells containing hybridoma supernatants. Of the 1,741 supernatants produced in several fusions and screened with this functional assay, 230 caused isoagglutination or loss of motility, and 45 interfered with adhesion of mt+ gametes. Several of the 45 that appeared to block adhesion also caused isoagglutination or loss of motility of gametes of both mating types and were not considered further. One clone, 2b40, was subcloned in soft agar and has been further characterized. Ouchterlony double immunodiffusion analysis showed that 2b40 is an IgG isotype. Ascites fluids were prepared from pristane-primed mice injected intraperitoneally with 2b40 hybridoma cells.

For some experiments, the 2b40 antibody was purified from ascites fluids either by affinity chromatography on a goat anti-mouse IgG affinity column or by use of the Affi-gel Protein A MAPS method (Bio-Rad Laboratories). Affinity-purified 2b40 was covalently linked to cyanogen bromide–activated Sepharose beads as described by Pharmacia Fine Chemicals, Piscataway, New Jersey.

Results

Partial Purification of Adhesion Molecules from mt+ Gametes

OG extracts of mt+ gametic flagella, prepared essentially as described by Adair et al. (4), were used as starting material to obtain preparations enriched in adhesion molecules. The OG extracts were fractionated by molecular sieve chromatography using a Biogel A-15M column as described in Materials and Methods. Adhesive activity was separated from mastigoneme protein and the major membrane protein with an M₉ of ~350,000, which has been shown by us and others (2, 6, 16, 19, 38, 50) to be the major protein component of flagellar surface fractions (not shown). Typically, this purification method yielded 60–100% of the starting activity with a 20–30-fold increase in specific activity. Column fractions with peak amounts of activity were dialyzed and lyophilized and used as immunogen in the preparation of monoclonal antibodies.

Selection and Characterization of 2b40, A Monoclonal Antibody that Blocks Adhesion of Only mt+ Gametes

Hybridoma supernatants were screened by use of a functional assay to identify supernatants that blocked aggregation between mt− and mt+ gametes. Several hybridomas producing adhesion-blocking antibodies were detected in our initial screens and one, designated 2b40, has been further characterized. This antibody has several properties that indicate that it is directed against an flagellar surface molecule that is intimately involved in the adhesive interaction. First, 2b40 had little visible effect on individual suspensions of mt+ or mt− vegetative or gametic cells, although some agglutination of mt+ gametes was observed at higher dilutions of the antibody. On the other hand, the antibody had a dramatic inhibitory effect on the adhesive interaction between mt− and mt+ gametes. Fig. 1 A shows the normal aggregation reaction between untreated mt− and mt+ gametes in which large clumps of agglutinating gametes form. Fig. 1 B shows that in the presence of 2b40, aggregation was completely blocked. To obtain a more quantitative assessment of the antibody inhibition of adhesion, a counter assay (Coulter

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Figure 1. Inhibition of aggregation by 2b40. mt+ and mt- gametes were mixed together with a 1/200 dilution of an irrelevant IgG1 (A) or 2b40 (B) and examined by phase-contrast microscopy. Although there was some variation from experiment to experiment, ascites fluid diluted 1/200 to 1/500 in N-free medium usually blocked adhesion completely.

Electronics, Hialeah, FL) for aggregation was used (44). The results shown in Fig. 2 indicated that half maximal inhibition of adhesion occurred at a concentration of ~10 μg/ml of antibody protein. In other experiments using an impotent mt+ mutant (imp-1; 17) which can agglutinate but not fuse, we found that the antibody would immediately disaggregate adhering mt- and impotent mt+ gametes (not shown).

In experiments in which cells of different mating types were pretreated with 2b40 and then washed into fresh, non-antibody-containing medium, we found that only pretreated mt+ gametes were affected by the antibody (not shown). There was no detectable effect on mt- gametes. Similar results were obtained when isolated mt+ gametic flagella were pretreated with the antibody. Only mt+ gametic flagella were affected. In each case an irrelevant IgG1 monoclonal antibody had no effect on adhesion.

Figure 2. Quantification of the effects of 2b40 on aggregation. mt+ gametes (8.8 × 10⁶ cells/ml in N-free medium) were pretreated for 5 min with the indicated concentrations of 2b40 purified from ascites fluid. The cells were then mixed with untreated mt- gametes. Aggregation was determined 5-10 min after the cells were mixed together by use of a counter assay (Coulter Electronics, Inc.) as described previously (44) and is presented as a percentage of the aggregation of control, untreated cells. In this experiment 63% of the control cells aggregated.

| MT+ OG Extract (ng/spot) | 7.5 | 15 | 30 | 60 | 120 |
|--------------------------|-----|----|----|----|-----|
| Treatment               | control | 2b40 | irrelevant |

Figure 3. 2b40 blocks adhesion molecules adsorbed to nitrocellulose. An OG extract of mt+ gametic flagella was diluted in 10 mM Tris, 150 mM NaCl, pH 7.4 and the indicated amounts were adsorbed to nitrocellulose strips in a dot blot apparatus (Bio-Rad Laboratories). The strips were blocked by incubation for 30 min in 1% polyvinyl alcohol (5) at 4°C and then incubated with 1% bovine serum albumin in 10 mM NaPO₄, 150 mM NaCl, pH 7.2 (control), 1 mg/ml 2b40 (2b40), or a 1/10 dilution of an irrelevant IgG1 ascites fluid (irrel.). After 1 h at 23°C the strips were removed from the incubation solutions and washed at 4°C twice with 10 mM Tris, 150 mM NaCl, pH 7.4 and twice with N-free medium. mt- gametes were incubated with the strips for 5-10 min and the strips were removed from the cell suspension, drained of excess cells, and photographed. (Staining of the cells was obviated by their natural green color.) Only 2b40 blocked adhesion of the mt- gametes to the adsorbed adhesion molecules.
Only mt\textsuperscript{+} gametes bind to 2b40-derivatized Sepharose beads. 2b40 was covalently linked to Sepharose beads and incubated with mt\textsuperscript{-} (a) and mt\textsuperscript{+} (b) gametic and mt\textsuperscript{-} (c) and mt\textsuperscript{+} (d) vegetative cells. Only mt\textsuperscript{+} gametes bound to the beads. The inset in b is a higher magnification image of mt\textsuperscript{+} gametes binding to the beads. In control experiments there was no binding of any of the four cell types to two preparations of beads derivatized with two different irrelevant antibodies.

Since 2b40 was directed against an antigen prepared from column fractions enriched with mt\textsuperscript{+} adhesion molecules prepared from OG extracts, it was important to determine if the antibody could block the adhesiveness of these extracts. To do this, various dilutions of an OG extract of mt\textsuperscript{+} gametic flagella were adsorbed to nitrocellulose strips and either left untreated or treated with 2b40 or an irrelevant IgG1. The strips were then incubated with mt\textsuperscript{-} gametes to test for mt\textsuperscript{+} adhesion molecule activity. The results, presented in Fig. 3, showed that the cells could detect very small amounts of the adsorbed adhesion molecules. Binding was observed in the control sample at 15 ng of protein per spot. Fig. 3 also shows that 2b40 completely blocked adhesion of cells to the adsorbed adhesion molecules, whereas the irrelevant IgG1 had little, if any, effect.

**Only mt\textsuperscript{+} Gametes Bound to 2b40-derivatized Sepharose Beads**

To further characterize the antibody, we covalently linked it to cyanogen bromide-activated Sepharose beads and the beads were mixed with mt\textsuperscript{+} and mt\textsuperscript{-} vegetative and gametic cells. The results shown in Fig. 4 indicated that only mt\textsuperscript{+} gametes bound to the beads. In control experiments, we found that cells did not bind to beads derivatized with an irrelevant IgG1 antibody. These results showed that the antibody is directed against a flagellar surface determinant present only on mt\textsuperscript{+} gametes. In addition, close examination of the mt\textsuperscript{+} gametes adhering to the 2b40-coated beads showed that cell walls were released by these adhering cells. That is, the mt\textsuperscript{+} gametes behaved as if the antibody-coated beads were mt\textsuperscript{-} gametes and appeared to undergo normal sexual signaling.

Since these results directly indicated that the beads contained active antibody, we attempted to use the beads to affinity purify the antigen. To do this, preparations containing adhesion molecules (OG extracts and Biogel A-15M column fractions) were passed over a column containing the 2b40-derivatized beads. The run-through was collected, and the column was eluted with a buffer containing 0.1 M glycine, and 1% dioxane, pH 2.5. The eluted fractions were immediately neutralized with K\textsubscript{2}PO\textsubscript{4}. The run-through and eluted samples were then assayed for their adhesive activity using the dried spot assay. The result was that adhesive activity was recovered in the run-through fraction and no adhesive activity was found in the eluted fraction (not shown). Control experiments showed that the elution buffer alone did not destroy the activity of adhesion molecules. These results suggested either that the antibody has a low affinity for the antigen in solution, or that the soluble molecule is not in a conformation that enables it to bind to the antibody. In separate immunoblot experiments (not shown) in which 2b40 was used to probe OG extracts of mt\textsuperscript{+} gametic flagella, none of

![Figure 4. Only mt\textsuperscript{+} gametes bind to 2b40-derivatized Sepharose beads. 2b40 was covalently linked to Sepharose beads and incubated with mt\textsuperscript{-} (a) and mt\textsuperscript{+} (b) gametic and mt\textsuperscript{-} (c) and mt\textsuperscript{+} (d) vegetative cells. Only mt\textsuperscript{+} gametes bound to the beads. The inset in b is a higher magnification image of mt\textsuperscript{+} gametes binding to the beads. In control experiments there was no binding of any of the four cell types to two preparations of beads derivatized with two different irrelevant antibodies.](image_url)
Figure 5. *mt* gametes transfer adhesion molecules to 2b40-derivatized beads. The beads were incubated overnight with *mt* gametes or *mt* vegetative cells and then washed free of the cells by several centrifugations. To determine if transfer of adhesion molecules had occurred, the preincubated beads were mixed with *mt* gametes. Only beads that had been preincubated with *mt* gametes (A) acquired the ability to bind *mt* gametes. The control beads (B) were unreactive with the *mt* gametes.

**mt* Gametes Transfer Adhesion Molecules to Antibody-derivatized Beads**

In the course of the experiments with the bead–cell mixtures, we observed by phase-contrast microscopy that the *mt* gametes were in a dynamic interaction with the 2b40 beads. The cells continuously adhered to and de-adhered from the beads. This was similar to gamete–gamete interactions that occur during the mating reaction as well as to the gamete–flagella adhesion and de-adhesion that we have described previously (40, 44). To investigate the possibility that the cells might transfer active adhesion molecules to the beads, cells that had been incubated with *mt* gametes were washed free of the cells by differential centrifugation and were mixed with *mt* gametes. Fig. 5 shows that *mt* gametes bound to beads previously incubated with *mt* gametes (Fig. 5 A) but failed to adhere to beads that had been incubated with *mt* vegetative cells (Fig. 5 B). *mt* gametes were also still able to bind to the beads previously incubated with *mt* gametes, indicating that unreacted antibodies remained accessible on the beads (not shown). These results suggested that *mt* gametes transferred adhesion molecules to the beads.
We decided, because of these observations, to do a modified affinity purification of the antigen by incubating \( mt^+ \) gametes with the 2b40-derivatized beads and then eluting the antigen from the beads. To do this, \( mt^+ \) gametes were incubated overnight with 2b40 beads. As a control, \( mt^- \) gametes and \( mt^+ \) vegetative cells were also incubated with the beads under the same experimental conditions, except that the incubation with the vegetative cells was done in N-containing vegetative medium. In some experiments, we observed that the vegetative cells bound to the beads. It is likely that this was a nonspecific binding because it did not occur routinely. Also, the vegetative cells that bound to the beads were not removed by addition of soluble 2b40, whereas \( mt^+ \) gametes were immediately removed by this treatment.

At the end of the incubation the beads were collected on a sintered glass filter, washed extensively with N-free medium, and tested for their ability to bind \( mt^+ \) and \( mt^- \) gametes. Only the beads previously incubated with \( mt^+ \) gametes supported adhesion of \( mt^+ \) gametes. The beads that had been incubated with \( mt^+ \) vegetative cells or \( mt^- \) gametes did not bind \( mt^+ \) gametes. Beads from all three incubations, however, were still able to bind \( mt^- \) gametes. The beads were eluted with detergent-containing solutions, the eluates assayed for adhesion molecule activity in the dried spot assay and their protein composition was analyzed by SDS PAGE.

The bioassay showed that the eluate from the beads incubated with \( mt^+ \) gametes had a titer of 16,000, whereas the eluates from the controls showed little if any activity above background. Equal proportions of the eluates from each bead preparation were analyzed by SDS PAGE and the results are shown in Fig. 6. The \( mt^- \) sample had primarily one protein with an \( M_r \) of \( \sim 270,000 \), whereas the \( mt^+ \) vegetative and \( mt^- \) gametic eluates both had polypeptides with \( M_r \)'s of \( \sim 350,000 \) corresponding to the \( M_r \) of the major membrane protein of the flagellum. The amounts of these polypeptides present in the eluates varied from experiment to experiment. On the other hand, the \( mt^+ \) gametic eluates showed a band in the stacking gel that was found consistently only in \( mt^+ \) gametic eluates. Since this band was in the stacking gel it was not possible to determine its molecular mass or even to establish if it was a single molecule or a complex or aggregate of several molecules. Nevertheless, this band becomes a prime candidate for the 2b40 antigen, and appears to have properties similar to \( mt^+ \) agglutinin molecules first identified by Adair et al. (4).

**Discussion**

Antibodies that block adhesion have been instrumental in identifying cell surface molecules involved in specific cell-cell recognition in many systems (11, 13, 14, 24, 27, 34, 46). In the present report we describe the results of our first steps in using this method to identify adhesion molecules in *C. reinhardtii*. We have used detergent extracts of \( mt^+ \) gametic flagella enriched in adhesion molecules as immunogens to prepare monoclonal antibodies that block adhesion of \( mt^+ \) gametes. The data presented here indicate that monoclonal antibody 2b40 is directed against a flagellar surface determinant intimately involved in the adhesive interaction and present only on \( mt^+ \) gametes. The antibody did not block the adhesiveness of \( mt^- \) gametes nor did \( mt^- \) gametes bind to beads coated with the antibody. In addition, \( mt^+ \) and \( mt^- \) vegetative cells were not specifically reactive with 2b40-derivatized beads. The simplest explanation for the antibody's effects are that it binds to a flagellar surface adhesion molecule at or near the active site of the molecule and thereby renders the molecule unable to interact with adhesion molecules on the surface of \( mt^- \) gametic flagella.

To our knowledge, this is the first report of a monoclonal antibody that specifically blocks adhesion of *C. reinhardtii* gametes. The Van den Ende laboratory has reported on an adhesion-blocking rabbit polyclonal antibody directed against a *C. eugametos* cell surface determinant (24, 25, 26, 43). This antiseraum reacted with several flagellar surface glycoproteins, and its action could be blocked by disaccharide moieties (25). Adair, Goodenough, Heuser, and co-workers have obtained monoclonal antibodies directed against flagellar surface molecules found only on gametic flagella (1, 3, 15). These antibodies, designated class II, react with both \( mt^+ \) and \( mt^- \) gametes, as well as with nonagglutinating mutant gametes that contain molecules closely related to the agglutinin molecules used as immunogens for the preparations of the monoclonal antibodies. The antibodies do not react with vegetative cells, and are directed against the agglutinin molecules.

**Transfer of Adhesion Molecules**

Our observation that \( mt^+ \) gametes could be separated from 2b40-derivatized beads by gentle washing procedures indicated that the interaction between the beads and the cells was a dynamic one. This is quite similar to the interaction of live gametes with each other or with flagella isolated from gametes of the opposite mating type. The results presented here, that \( mt^+ \) gametes transferred active adhesion molecules to the 2b40 beads, suggest that binding of 2b40 antibodies to adhesion molecules might induce their release from the flagellar surface. Apparently the released adhesion molecules were in a multivalent form, because adhesion sites not interacting with the antibody were available to be bound by \( mt^- \) gametes. Possibly a similar release mechanism is responsible both for loss of flagellar adhesiveness after cell fusion, and for the flagellar de-adhesion process described previously. We have shown that as a consequence of adhesion, flagellar adhesion molecule activity is lost from the flagellar surface (42, 44). On live cells, the inactivated adhesion molecules are rapidly replaced by protein synthesis. With isolated flagella or with cells incubated in cycloheximide, the inactivated adhesion molecules are not replaced, and the cells de-adhere (42).

Several mechanisms can be envisioned that could lead to inactivation of flagellar adhesion molecules during adhesion. For example, the molecules themselves or nearby molecules might modify the adhesion molecules by adding or removing a critical component. Possibly a protease is activated that partially degrades adhesion molecules and renders them inactive. Alternatively, carbohydrate moieties could be added or disulfides formed or reduced. Another explanation, which is supported by our antibody data, would be that the adhesion molecules are released from the surface of flagella of live cells during adhesion. These released molecules might still be active, but since they were no longer tethered to the flagellum they could not support cell-cell or cell-flagellum adhesion.

The similarity in the consequences of adhesion of \( mt^+ \) ga-
metes to 2b40-derivatized beads and to mt+ flagella suggests that mt+ gametes might transfer active adhesion molecules to mt− flagella. Although we showed that the adhesiveness of mt− flagella is lost as a consequence of their adhering to mt+ gametes, we never determined if the mt− flagella had acquired mt+ adhesion molecules (44). It may be that loss of flagellar adhesiveness in this situation occurs because adhesion molecules on the live cells form a complex with the adhesion molecules on the surface of the isolated flagella. This event could cause release of the adhesion molecules from the flagellum of the live cell, possibly by an active release mechanism. If this mechanism were not active in isolated flagella, the complex would remain on the surface of the isolated flagella and could be detected, because molecules transferred from the live cell had excess adhesion sites available. When two live cells interact, these putative complexes would form; but because both cells would have an intact release mechanism, the complex would be released from both flagella. Experiments to test these hypotheses are currently in progress in our laboratory.

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