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Monoclonal Antibodies Which Alter the Morphology of Cultured Chick Myogenic Cells

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Two monoclonal antibodies that cause changes in the morphology of cultured myogenic cells are described. Antibody JG9 causes myoblasts to round up and causes myotubes to become thin, cable-like structures with multiple round swellings. Antibody JG22 causes both myoblasts and myotubes to become round refractile cells poorly attached to the substratum. The effects of both antibodies are reversible. Fab fragments of JG22 can cause the morphological change. A tentative identification of the antigen recognized by JG22 is made.

Key words: monoclonal antibodies, myogenesis, cell surface

Because of their high specificity and availablility in large quantities, monoclonal antibodies are important tools for investigating the function of cell surface components. One crucial role of the cell surface is the maintenance of cell morphology in culture and in vivo; polyspecific antisera to the cell surface have profound effects on cell morphology in culture [16]. Embryonic muscle in tissue culture provide a favorable system for studying the role of the cell surface in the development and maintenance of cell shape. It is apparent that both collagen [9] and fibronectin [2,3] must be present on the substratum for normal development to occur in vitro. However, components of the cell surface that interact with these molecules remain to be defined.

In this paper, we describe two monoclonal antibodies that drastically alter the morphology of cultured chick muscle cells. The morphological changes are described and the requirements for antibody valency are investigated. Finally, a preliminary characterization of the antigen recognized by one of these antibodies is made. A preliminary communication of these results has appeared [8].

MATERIALS AND METHODS

Preparations of Monoclonal Antibodies JG9 and JG22

Monoclonal antibodies JG9 and JG22 were obtained using the procedure of Galfre et al [6]; the myeloma parent for JG9 was the NS-1 line [6], that for JG22

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was the SP2/0 line [15]. In the case of JG9, the mouse was immunized with 5-day-old cultured muscle. Cells containing 10^7 nuclei were used for both primary immunization and boosting. In the case of JG22, the mouse was immunized with freshly dissected muscle tissue from day-14 chick embryos. Each monoclonal antibody was originally detected by its ability to bind to muscle cell cultures using an indirect radioimmune assay. Hybridoma lines JG9 and JG22 were both cloned in soft agar and used to produce ascites fluid.

Muscle Cell Culture

Chick embryonic muscle cells were prepared from day-11 chick embryo thighs by mechanical disruption [4] and cultured in gelatin coated Costar 24 (Costar 3524) multiwell culture dishes at an initial cell density of 5×10^2 cells/mm^2 in CM_4 medium which consists of Dulbecco's modified Eagle's medium wih 15% horse serum, 5% chick embryo extract, nonessential amino acids (GIBCO), L-glutamine, 20 mg/ml, and 100 units/ml penicillin and streptomycin. Cytosine arabinoside was added to a final concentration of 10^{-5}M on day 3 of culture to suppress fibroblast proliferation.

Preparation of Fab Fragments of Monoclonal Antibodies

Monoclonal antibodies were partially purified from ascites fluid by precipitation with 50% saturated ammonium sulfate. Further purification by DEAE chromatography was according to Francus et al [5].

Fab fragments of monoclonal IgG were prepared by dialyzing IgG against 0.1 M ammonium acetate (pH 4.5) and then adding pepsin to 5% wt/wt IgG and cysteine to a final concentration of 0.1 M. The reaction proceeded for 12 h at 37°C and was stopped by neutralizing with NaOh. Iodoacetamide was added to a final concentration of 0.2 M and the mixture loaded onto a P-200 column (50 cm + 2 cm), equilibrated, and run in phosphate buffered saline.

SDS Gel Electrophoresis

SDS slab gel electrophoresis was run according to [12].

Binding Assays

Monoclonal antibody or Fab fragments were labeled with ^{125}I by the method of Jensenius and Williams [10] to a specific activity of about 10 μCi/μg. Assays were performed in Costar 24 multiwell dishes as follows. Antibody was added to cell monolayers in 0.5 ml of a solution of Hanks salts with 5% chicken serum (HCS) and incubated for 1 h at 4°C. The monolayers were then washed three times with chilled HCS, dissolved in 0.1 M NaOH, and counted in a gamma counter.

Immunoaffinity Chromatography of JG22 Antigen

Monoclonal antibodies were coupled to CNBr-activated Sepharose 4B as described in [14]. Coupled beads were further incubated with 0.5 M glycine for several hours to block remaining activated groups and prewashed with elution buffer before use. The concentration of IgG per ml of packed beads was 5.7 mg/ml for MOPC-21 Sepharose and 7.4 mg/ml for JG22 Sepharose. Thigh muscles from 22 day-14 chick embryos were homogenized in cold 0.25 M sucrose in 10 mM Tris pH

7.4. The homogenate was then centrifuged at 1700g for 10 min and the pellet discarded. The supernatant was centrifuged at 200,000g for 60 min. The resultant pellet (crude membrane fraction) was then dissolved in 0.5% NP40/PBS by stirring overnight. Sixty milligrams protein of this extract in 12.5 ml was incubated with 1 ml of either MOPC 21- or JG22-Sepharose for 1 h at 4°C in slowly rotating tubes. The beads were washed by centrifugation and then packed into columns and washed with 50 ml of 0.5% NP40 in phosphate buffered saline. The columns were eluted with 2.4 column volumes of 0.05 M diethylamine/0.5% NP40; the eluants were neutralized with Tris, precipitated with TCA, and analyzed by SDS gel electrophoresis.

RESULTS

Effects of Monoclonal Antibodies JG9 and JG22 on Morphology of Myogenic Cells in Culture

Monoclonal antibodies JG9 and JG22 had profound effects on the morphology of myoblasts and myotubes. They both cause cells of myoblastic appearance (defined here as spindle-shaped cells; see Lipton [13] and Konigsberg [11]) to round up and become highly refractile within several hours after addition to one-day-old or two-day-old cultures. Some, but not all, of the rounded-up cells completely detached from the dish and could be observed floating. The morphology of fibroblasts was not affected. Both antibodies also changed the morphology of myotubes: JG9 caused myotubes to become thin and cable-like with knob-like protrusions along their length, while myotubes exposed to JG22 were large, round, refractile masses. In both cases, the altered myotubes were judged to be viable by trypan blue exclusion and were seen to twitch spontaneously. The cumulative effects of both antibodies on muscle cell morphology are indicated in Figure 1.

The morphological changes caused by JG9 and JG22 were quantified by defining abnormal structures found only on antibody-treated cultures. For JG9, these were taken to be the knob-like refractile structures found along the myotubes, while for JG22, refractile masses were scored. Figure 2 shows the magnitude of the

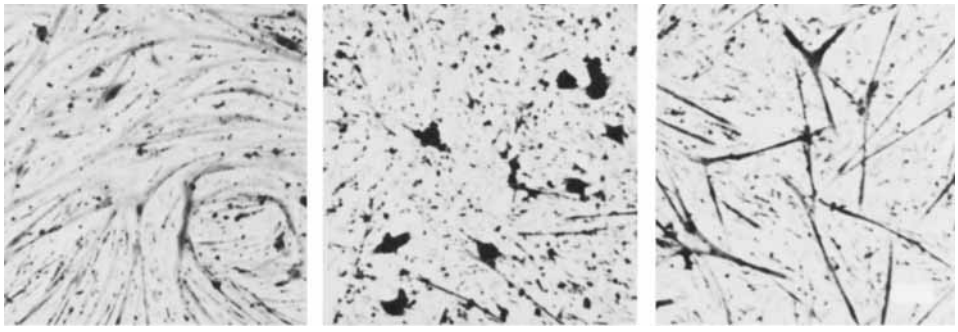

Fig. 1. Micrographs of normal muscle cultures and cultures grown in the presence of monoclonal antibodies for four days. Indicated antibody (100 μg/ml) was added to standard muscle cultures after 24 h of incubation and incubated for an additional 4 days. Cultures were fixed with 1% glutaraldehyde and stained with Sudan black. Calibration bar equals 200 μ. Left panel, no added antibody; middle panel, JG22; right panel, JG9.

morphological change as a function of added antibody concentrations. The effect varies with concentration until a plateau is reached.

It is noteworthy that the effects of JG9 and JG22 are specific. In addition to these antibodies 12 other monoclonal antibodies that have been demonstrated to bind to the surface of myogenic cells have been tested in the same manner [7]. None of these antibodies cause any detectable morphological change in either myoblasts or myotubes, indicating that the mere binding of monoclonal antibodies is not enough to induce morphological changes.

Reversibility of MAb-Mediated Effects

An experiment was performed in which cultures were exposed to MAbs JG9 and JG22 for varying periods of time. MAb (200 μg/ml) was added to a series of day-1 muscle cultures, and one set of these cultures was incubated until day 5 in the presence of the MAb while other sets had the MAb-containing medium replaced with fresh medium on days 2, 3, or 4. All cultures were then incubated until day 5 and scored for morphological changes. The results of this experiment, shown in Figure 3, indicate that antibody must be continuously present in order to obtain the maximum morphological change. Direct and frequent observations indicate that antibody-treated cells gradually flatten and assume a more normal appearance when antibody is removed.

Role of Antibody Valency

The requirement for antibody valency was investigated by preparing Fab fragments of JG9 and JG22 as detailed in Materials and Methods. The Fab fragments chromatographed on a P200 column at nearly the same position as marker rabbit Fab fragments (data not shown). Figure 4 shows the SDS gel profiles of the Fab fragments obtained; the Fab fragments contain the appropriate light chain and

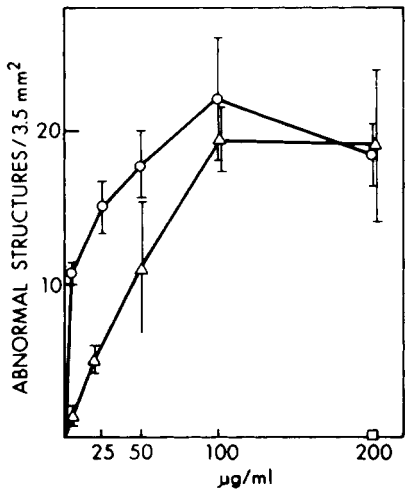

Fig. 2. Magnitude of morphological change as a function of antibody concentration. Antibody to the concentration indicated on the abscissa was added to day-1 cultures; cultures were scored for morphological change on day 5 and the number of abnormal structures per 3.5 sq mm plotted on the ordinate. Results expressed as the mean of triplicates ± SD: ○, JG22; △, JG9; □, MOPC-21.

heavy chain fragment. The relative biological activity of these fragments compared to their respective IgGs indicates that JG22 Fab fragments can induce the full morphological change, while JG9 Fab fragments are only partially able to do so (Fig. 5) It is also clear that a much higher concentration of JG22 Fab is necessary when compared to the intact IgG. Therefore in the case of JG22 at least, cross-linking of the antigen is not necessary in order to induce the morphological effect.

JG9 and JG22 Inhibit Each Other's Binding

Since monoclonal antibodies JG9 and JG22 both affect cell morphology, an experiment to determine the independence of their binding site was performed. In the experiment in Figure 6, the binding of ^{125}I-IgG was assayed in the presence and absence of competing unlabeled antibodies. The left panel shows that JG22 IgG is nearly as effective a competitor for the JG9 site as JG9 IgG itself. The JG9 Fab is a much weaker competitor. The right panel shows the corresponding data for JG22. JG22 IgG is the best competitor, but JG22 Fab and JG9 IgG are still effective competitors. Thus, JG9 and JG22 must recognize antigens that are either spatially or structurally related. Several other antibodies do not compete for JG9 or JG22 sites (data not shown).

Identification of a Polypeptide Recognized by Monoclonal Antibody JG22

The identity of the antigen recognized by monoclonal antibody JG22 was investigated by affinity chromatography of a detergent extract of embryonic muscle membranes. Details of the procedure are in Materials and Methods and the results are shown in Figure 7. A 138,000 dalton protein was specifically eluted from the JG22-Sepharose column but not from the control MOPC-21-Sepharose column. Also eluted from these columns was a protein comigrating with actin, with more of

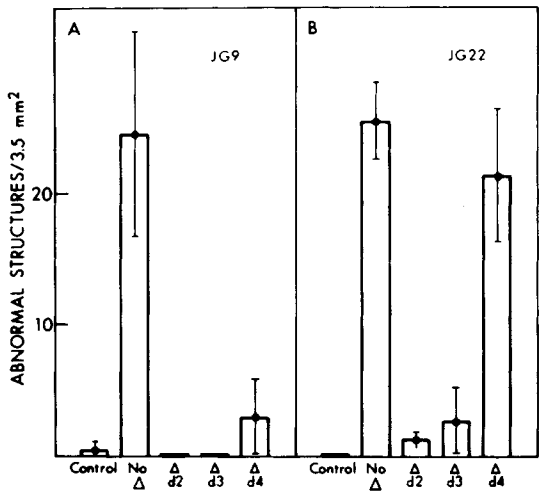

Fig. 3. Reversibility of morphological change. Antibody was added at a concentration of 200 μg/ml on day 1 of culture. Media was then removed and replaced with fresh media on days 2, 3, or 4 (Δd2, Δd3, Δd4) or not at all (no Δ) and scored for abnormal structures on day 5. Panel A, JG9; panel B, JG22.

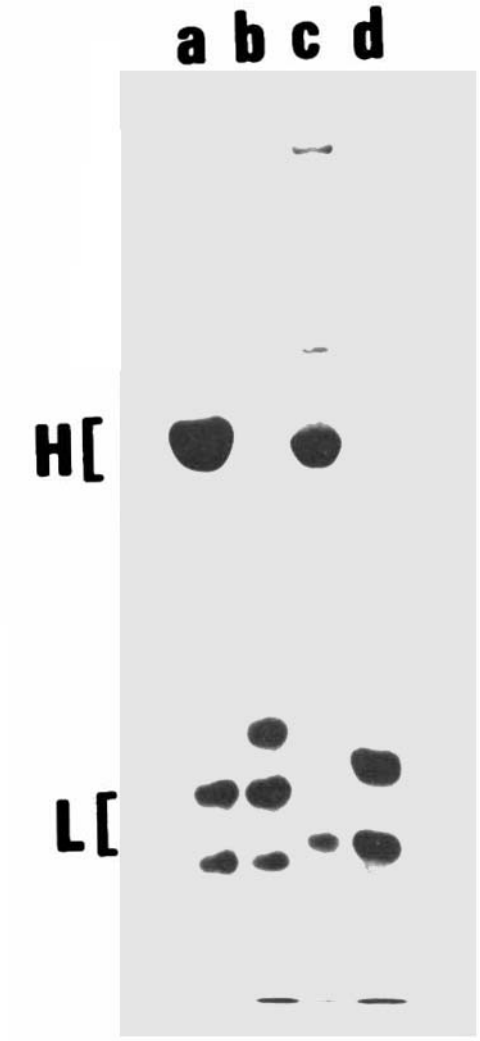

Fig. 4. SDS-polyacrylamide gel electrophoresis of hybridoma IgG and Fab fragments: lane a, JG9 IgG; lane b, JG9 Fab; lane c, JG22 IgG; and lane d, JG22 Fab. Each lane has 10 μg protein. H = heavy chain region; L = light chain region.

this protein eluting from the JG22 column than the MOPC-21-Sepharose column; small amounts of JG22 and MOPC-21 light chains eluted from the columns. In a second cycle over these columns, the 138,000 dalton band was reeluted specifically (lanes c and d). A similar band can be greatly enriched from ^{35}S-labeled extracts of cultured muscle cells (data not shown).

DISCUSSION

The central finding of this study is that two monoclonal antibodies, JG9 and JG22, are able to cause profound morphological alterations in cultured myoblasts

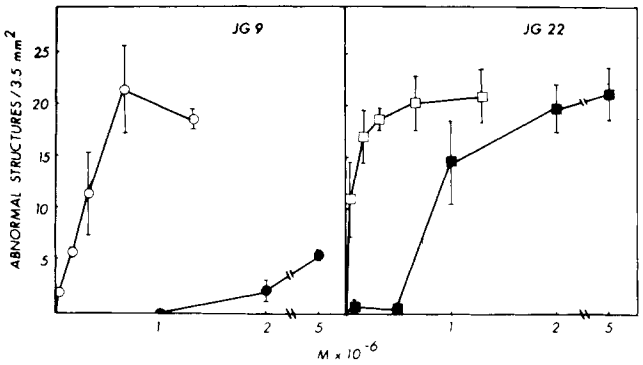

Fig. 5. Morphological change induced by IgG and Fab fragments. Antibodies were added on day 1 to indicated concentration and cultures were incubated and scored on day 5 of culture for abnormal structures. The molar concentrations of added antibody is given on the abscissa. ○, JG9 IgG; ●, JG9 Fab; □, JG22 IgG; ■, JG22 Fab.

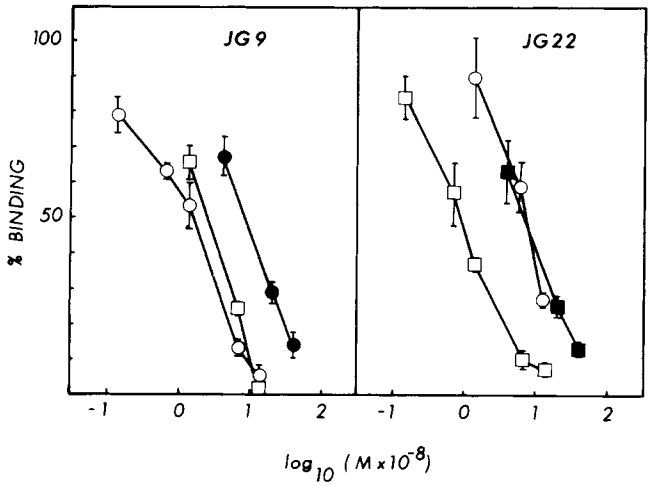

Fig. 6. Competition of ^{125}I IgG binding to day-5 chick muscle cells with unlabeled IgG and Fab fragments. Labeled antibody (10^5 cpm or about 1 ng/ml) was added to each well; maximum binding was about 1% of input cpm. Added unlabeled antibodies: ○, JG9 IgG; ●, JG9 Fab; □, JG22 IgG; ■, JG22 Fab. Abscissa: concentration of unlabeled antibody. Ordinate: Percent binding as fraction of uninhibited control.

and myotubes. The muscle cells are able to live in the presence of the antibodies for up to four days, the longest period we have tested. Thus, the change in morphology is not toxic. Furthermore, the changes are at least partially reversible, a more normal morphology being restored when the antibody is withdrawn. In the case of JG22, at least, the effect is elicited by monovalent antibody fragments.

The primary questions raised by these studies concern the mechanism of ac-

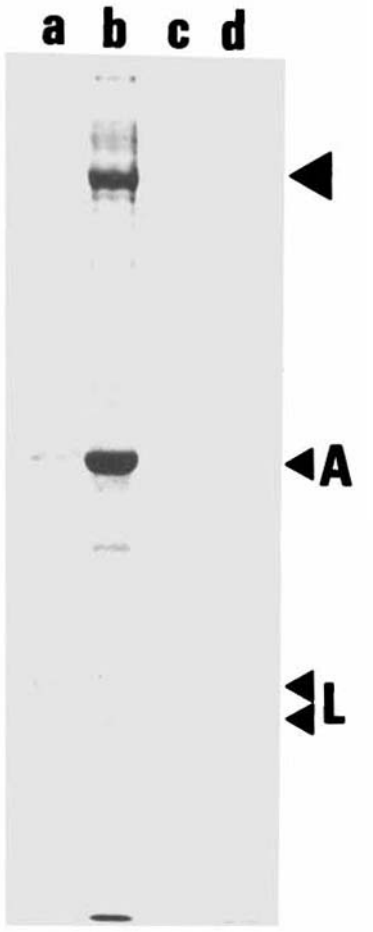

Fig. 7. Analysis of fractions from affinity column purification. Samples were prepared as described in text and analyzed on 10% SDS polyacrylamide gels stained with Coomassie blue. Lane a, eluant from MOPC-21 Sepharose; lane b, eluant from JG22 Sepharose; lane c, eluant from MOPC-21 Sepharose recycled over same; lane d, eluant from JG22 Sepharose recycled over same. Large arrow indicates 138,000 dalton band; actin (A) and IgG light chains (L) are indicated.

tion of these antibodies. The adhesion of cultured muscle cells to dishes is not just a simple interaction between the cell surface and plastic substratum. The presence of collagen on the culture dish is an essential requirement for the maintenance of normal morphology [9]. Recent evidence indicates that fibronectin is also essential for the maintenance of cell substrate adhesion by cultured myoblasts [2,3]. However, the exact roles played by collagen and fibronectin in maintaining cell shape have yet to be resolved. One plausible explanation of the effects of JG9 and JG22 is that they bind to a cell surface component(s) that interacts directly with either collagen and/or fibronectin. The antibodies would then interfere with this interaction resulting in cells with altered morphology.

A second possible mechanism would involve the cytoskeleton. Agents that disrupt the cytoskeleton drastically alter cell morphology. Furthermore, cell surface

components that form transmembrane links to the cytoskeleton have been postulated [1]. Perhaps the surface antigen(s) recognized by JG9 and JG22 influence the cytoskeleton and hence cell morphology with the effects of antibody binding being exerted through this mechanism. Further work will be necessary to decide among these and other possible mechanisms.

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