Use of a monoclonal antibody recognizing a cell surface determinant to distinguish prestalk and prespore cells of *Dictyostelium discoideum* slugs

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**SUMMARY**

Double labelling experiments on *Dictyostelium discoideum* cells at different developmental stages were carried out using monoclonal antibodies MUD1 (prespore specific), MUD9 (strong label on prestalk and anterior-like cells) and a fluorescence-activated cell sorter. The monoclonal antibody MUD9, which recognizes the surface of prestalk and anterior-like cells strongly and prespore cells weakly, is also present on the surface of vegetative amoebae and on mature stalk cells but not on the spore surface. Sharing of an antigenic determinant between vegetative, prestalk and anterior-like cells is consistent with these cells being 'less differentiated' than prespore cells.

**INTRODUCTION**

It is commonly supposed that the cell surface plays an important role in morphogenesis, but details are still sparse. One of the best studied systems is that of different blood cell types and this has advanced through the use of monoclonal antibodies and flow cytometry (Loken & Stall, 1982). Monoclonal antibodies are particularly powerful tools for detecting and probing small differences that can occur in protein structure on the cell surface during differentiation (Williams, Galfre & Milstein, 1977). We have studied one of the simplest multicellular organisms, the slug stage of the cellular slime mould *Dictyostelium discoideum*. Our aim is to understand the basis of proportion regulation and this requires identification of the different cell types, their quantification and biochemical characterization. Techniques have been developed which achieve separation to

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single cells (Voet, Krefft, Mairhofer & Williams, 1984) and cell type specific molecules have been characterized using monoclonal antibodies and flow cytometry (Krefft, Voet, Mairhofer & Williams, 1983). Using these techniques in conjunction with immunohistochemistry and protein blotting methods, a surface antigen with \( M_r 32 \times 10^3 \) has been characterized that is specific to prespore cells (Krefft et al. 1983; Gregg, Krefft, Haas-Kraus & Williams, 1982) and several other prespore-specific antigens have been found (Grant, Krefft, Voet & Williams, 1985). We have made further attempts to discover cell-type-specific surface molecules using flow cytometry and monoclonal antibodies.

Here, using a monoclonal antibody, MUD9, we have discovered an antigen that is initially present on vegetative amoebae, but partially lost from prespore cells while remaining on prestalk and anterior-like cells in the slug of \( D. \) discoideum. In mature fruiting bodies stalk surfaces are still labelled with this antibody.

This approach will be useful in mapping out the surface of differentiated cells in the \( D. \) discoideum slug.

**MATERIALS AND METHODS**

**Growth and development of \( D. \) discoideum**

The V12-derived strains NP84 and NP73 were grown on SM agar in association with \( K. \) aerogenes and slugs were prepared as described previously (Gregg et al. 1982). For the experiments involving a developmental time course, \( 5 \times 10^7 \) amoebae were deposited on black Millipore filters (HABAG 04700) as described in detail earlier (Krefft et al. 1984) so that cells from each stage of the life cycle of \( D. \) discoideum could be collected.

**Monoclonal antibodies**

The monoclonal antibody MUD9 was obtained after i.p. injection of a Balb/c mouse (aged 6 weeks) with \( \sim 1 \times 10^7 \) whole slug cells (strain NP84) in 0.9 % NaCl. After 6 months the mouse was injected a second time i.p. with \( \sim 3000 \) slug tips in 0.9 % NaCl. Slug tips were collected under a binocular microscope with a glass capillary tube in 0.9 % NaCl and immediately frozen in liquid N\(_2\). After three weeks, a final \( \sim 3000 \) isolated slug tips were injected i.v. in 0.9 % NaCl. Three days after the i.v. boost the spleen was removed and \( 1 \times 10^7 \) spleen cells were fused with \( 1 \times 10^7 \) myeloma cells (cell line Ag 8/653, a non-secreting NS1 derivative). Hybridomas were selected in HAT medium and cloned three times by limiting dilution (de St. Groth & T.Scheidegger, 1980). The IgG-producing hybridomas (detected after a plaque test) were screened in the cell sorter for cell specificity with vegetative amoebae, slug cells and spores. Tissue culture supernatant was used for the assays. Monoclonal antibody MUD1 (Gregg et al. 1982) was used to label prespore cells. MUD1 was purified from ascites fluid through a Protein A-Sepharose CL-4B (Pharmacia) column and the IgG-fraction was separated to F(ab\(_2\))-fragments according to Goding (1976). F(ab\(_2\))-fragments (5-7 mg protein) were lyophilized, resuspended in 2.5 ml of 0-05 m-Na\(_2\)CO\(_3\):0-15 m-NaCl buffer pH 9.5 and dialysed in a Visking dialysis tubing (Type 8/32, Serva) against the same buffer for one hour at 4°C. The dialysis buffer was exchanged with 30 ml of the same buffer containing 10 mg FITC Isomer 1 (Code Nr. 3-280 Nordic Immunology, London) and dialysed for 15 h against the FITC in the dark at 4°C. The F(ab\(_2\))-FITC-MUD1 was dialysed against PBS (0-01 m-Na/K-phosphate, 0-17 m-NaCl pH 7-4) until no FITC could be detected in the dialysis buffer. For the double labelling experiments, MUD1-F(ab\(_2\))-FITC was used at a protein concentration of 0.02 mg ml\(^{-1}\) (FITC label of MUD1 was 8.5 mol FITC mol\(^{-1}\) IgG; Formi, 1979).
Sample preparation for flow cytometry

For the time course experiments, a 3x3 mm square of the Millipore filter was excized at intervals of 30 min and samples were prepared by harvesting the developing aggregates and dissociating them to single cells (Krefft et al. 1984). For the experiments with slugs of different ages (1–7 days), four individual slugs were collected per sample and single cells were prepared as described earlier (Krefft et al. 1983). For double labelling experiments the staining procedure was as follows; 0-1 ml of MUD9 supernatant was added to the washed cells together with 0-1 ml TRITC-conjugated goat anti-mouse IgG-F(ab)2 (Code Nr. 4450 Medac, Hamburg FRG) diluted to 1:20 in PBS and incubated on ice for 30 min; after the first incubation cells were centrifuged for 15 s in an Eppendorf centrifuge, the supernatant was removed by suction, followed by the addition of 0-1 ml of MUD1-F(ab)2-FITC. Subsequently the cells were incubated a second time for 30 min on ice. After the last incubation cells were analysed directly in a flow cytometer (model FACS IV Becton Dickinson Sunnyvale, CA) at ~1000 cells sec⁻¹ (Krefft et al. 1983). An argon-ion laser (Model 164-05, Spectra Physics, Mountain View CA) operating at 488 nm with an intensity of 0-5 W was used as an excitation source for FITC and an additional argon-ion laser (Model 164-09) operating at 514 nm with an intensity of 0-5 W was used for TRITC.

The data analysis techniques used have been described elsewhere (Voet, Krefft, Mairhofer & Williams, 1984).

Frozen sections

*D. discoideum* aggregates, slugs, mid-culmination stage, fruiting bodies and stalks were fixed for between 1 to 4 h at 4°C in 4 % paraformaldehyde buffered in 0.1 M-sodium phosphate at pH 7.4, washed and embedded in OCT (Tissue tek II, TK Vogel Ltd, Goettingen, FRG) and sectioned as previously described (Gregg et al. 1982). Sections were then indirectly stained with monoclonal antibody MUD1 or MUD9 and FITC-coupled goat anti-mouse IgG-F(ab)2 (Medac, Code 4350, Hamburg, FRG).

RESULTS

Identification of prestalk cells with MUD9

Fig. 1A shows that MUD9 predominantly recognized the prestalk region of *D. discoideum* slugs. Although a small amount of label could be detected on prespore cells, the surfaces of prestalk cells were brightly stained. In comparison MUD1 labels only the surfaces of prespore cells, whereas prestalk cells are devoid of any labelling (Fig. 1B).

The quantitative analysis of single cell preparations of 2-day-old slugs of *D. discoideum*, strain NP84, in the flow cytometer is shown in Fig. 2A. There was a clear separation of the two cell populations, a labelled MUD1 (FITC) prespore (psp) population and an unlabelled population of prestalk (pst) cells, which confirmed the results shown in Fig. 1B. Fig. 2B shows MUD9 labelling of separated slug cells. Both cell types were labelled with MUD9 (TRITC), but prespore cells have lower amounts of antibody MUD9 labelling than prestalk and anterior-like cells. This confirmed the results in Fig. 1A, where the prestalk region was more heavily labelled in frozen sections. Note that in Figs 2A and 2B the relative sizes of the prespore and prestalk cell populations were the same in both figures. Prespore cells were smaller than prestalk cells (psp at ~23 and pst at ~30 on the arbitrary scale of the contour plot).

When cells were double labelled with MUD1 (green fluorescence) and with MUD9 (red fluorescence), there was a clear separation of both labelled
populations (Fig. 2C). Prestalk cells were labelled only with MUD9 and no label of MUD1 could be detected on the prestalk cells. The prespore cell population was labelled with both monoclonal antibodies MUD1 and MUD9.

*MUD9 and MUD1 labelling of cells from different developmental stages of D. discoideum*

Separated cells from different stages of the asexual life cycle of *D. discoideum* which were double labelled with monoclonal antibodies MUD9 and MUD1 were examined in the flow cytometer (Fig. 3).

Vegetative amoebae of *D. discoideum* (Fig. 3, 1 h) were heavily labelled with MUD9 (red fluorescence), while MUD1 (green fluorescence) showed no label at all. After 7 h of development (Fig. 3, 7 h), when aggregates had formed, the MUD9 label was reduced by about a third of the intensity as compared to the label of vegetative amoebae (veg. amoebae, 1 h ~24 and aggregates, 7h ~16 on the arbitrary scale of the contour plots). The amount of antigen recognized by MUD9 on prestalk cells remained constant during the rest of development. MUD9 labelling on prespore cells decreased progressively until the surface label disappeared when spores were formed (Fig. 3, 8–14 h). The MUD1 label on prespore cells began to appear when tips were formed on the aggregates (Fig. 3,

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**Fig. 1.** Fluorescence staining of frozen 5 µm sections of *D. discoideum* (NP84) 3-day-old slugs. (A) Indirect staining with FITC of monoclonal antibody MUD9 showing predominant labelling of prestalk and anterior-like cells and little prespore cell labelling. (B) Indirect staining with FITC of monoclonal antibody MUD1 showing labelling of prespore cells only and absence of label in the prestalk region. The small black region in the prespore area represents a hole in the section.
Fig. 2. Dual parameter flow cytometry histograms of cells separated from four individual slugs of *D. discoideum*, strain NP84. Experiments are visualized as contour plots (dots indicating regions containing 1% or more, and lines at 10% intervals from 5% to 95% with respect to the highest peak in the histograms). Prespore cells = psp; prestalk cells = pst. (A) Cells labelled with monoclonal antibody MUD1 and goat anti-mouse F(ab)2-FITC, green fluorescence. (B) Cells labelled with monoclonal antibody MUD9 and goat anti-mouse F(ab)2-TRITC, red fluorescence. (C) Double labelling of cells with monoclonal antibody MUD1 (green fluorescence) and MUD9 (red fluorescence). The small increase of the MUD9 label on prespore cells in Fig. 2C in comparison to Fig. 2B is caused by the contribution of green fluorescence to the red fluorescence detector. Percentages of prestalk/prespore proportions in A, B and C are ~33% pst/67% psp (mean values).
Fig. 3. Five contour plots from stages of a time course study of aggregation and fruiting of *D. discoideum*. Three millimetre squares of black Millipore filters were excized, the cells dissociated and double labelled with monoclonal antibody MUD9 (red fluorescence) and MUD1 (green fluorescence). The experiments are visualized as contour plots as in Fig. 2. 

- (1 h) is 1 hour after vegetative amoebae were plated on the Millipore filter: these cells were at a preaggregative stage.
- (7 h) is 7 hours, which corresponds to aggregates just prior to tip formation.
- (8 h) is 8 hours, which corresponds to aggregates with tips.
- (11 h) is 11 hours, which corresponds to slugs, although the resolution of the separated cells is not as clear as when only individual slugs are examined (Fig. 2C).
- (14 h) is 14 hours, which corresponds to fruiting bodies, with strain NP84.
At this stage the prespore cells began to lose the MUD9 label and only the small population of the prestalk and anterior-like cells remained strongly labelled with MUD9.

Mature spores did not carry the antigens recognized by MUD1 or MUD9 on their surfaces (Fig. 3, 14 h). It was not possible to separate stalk cells, which are enveloped in a cellulose sheath, so mature stalk cells were not observed in the flow cytometer profile (Fig. 3, 14 h).

A 5 μm section through a mature stalk (Fig. 4A) showed surface labelling of stalk cells with the monoclonal antibody MUD9. Although mature spores showed no MUD9 cell surface label, when a fruiting body was sectioned it was possible to detect small amounts of MUD9 label inside the spores. MUD9 labelling of sectioned mid-culmination stages (Fig. 4B) already exhibited brightly labelled stalk cells. In comparison, MUD1 labelling of sections of the same stage showed no label on the stalk, but labelling inside the spores (Fig. 4C).

No specific antigenic determinant(s) of MUD9 could be detected in cell extracts of D. discoideum using immunoblotting techniques (Towbin, Staehlin & Gordon, 1979) following SDS-PAGE, under both reducing and non-reducing conditions. We investigated plasma membranes and whole cells of vegetative amoebae, slug cells, and gradient separated prestalk and prespore cells from slugs, (Ratner & Borth, 1983), spores, stalks, and slime sheath. This may result from a sensitivity of MUD9 antigen(s) to SDS. Electroblotting techniques have failed to bind respective antigens in several other systems (O'Connor & Ashman, 1982; Brown, Molloy & Johnson, 1983; Hawkes, Niday & Matus, 1982). Blots were therefore

Fig. 4. Frozen sections (5 μm) of stalks (strain NP84) and mid-culmination stage (strain NP73) of D. discoideum cells. A and B were labelled with MUD9, and C with MUD1.
obtained from gels under non-denaturing conditions. Under non-denaturing conditions the extraction of proteins from cell extracts and cell membranes is not efficient and the separation of the proteins on gels is not as sharp as with SDS. There is a hint that the antigen recognized by MUD9 comigrates in the region of urease (Jack Bean), giving both a band and a smear, while under the same conditions the control antibody does not stain at all. This result shows that the MUD9 antigen is SDS sensitive.

**DISCUSSION**

Studies on slug cell proteins (Alton & Brenner, 1979; Coloma & Lodish, 1981; Borth & Ratner, 1983; Watts, 1984), plasma membrane proteins (West & McMahon, 1979; Parish, 1983; Das & Henderson, 1983) or use of cDNA probes (Barklis & Lodish, 1983; Mehdy, Ratner & Firtel, 1983) have shown that considerable changes occur at the time of prestalk/prespore differentiation, but that the changes are largely restricted to prespore cells. Recently, the discovery of prestalk-specific mRNAs (Chisholm, Barklis & Lodish, 1984), a prestalk enzymic marker (Oohata, 1983; Loomis & Kuspa, 1984) and monoclonal antibodies which identify autophagic vacuoles inside prestalk cells (Tasaka, Noce & Takeuchi, 1983) have made it clear that there are prestalk-specific events. Here we report on the first surface antigen that is largely prestalk specific (Fig. 1A). Our use of two monoclonal antibodies and flow cytometry has shown that the prestalk monoclonal antibody MUD9 stains strongly all cells not recognized by the prespore-specific monoclonal antibody MUD1 (Fig. 2). Therefore, like other biochemical studies, (Morrisey, Devine & Loomis, 1984) this work emphasizes the similarity between prestalk cells, located at the front of the slug and anterior-like cells which are intermingled with prespore cells. That the cells which were more strongly labelled with MUD9 were really prestalk and anterior-like cells can be inferred from their larger size (Fig. 2A and B) and the double labelling data (Fig. 2C). The prestalk and anterior-like cells are larger than prespore cells (Voet, Krefft, Mairhofer & Williams, 1984; Voet, Krefft, Bruederlein & Williams, 1985). This is not an artifact of cell clumping, since doublets would be twice the size of prespore cells in the sorter.

Unlike most prespore-specific molecules, which first appear at the time of prespore differentiation (Williams et al. 1984), the prestalk antigen discovered here is also found on vegetative amoebae. This reinforces earlier observations that have shown that prestalk cells remain similar in their morphology (George, Hohl & Raper, 1972; Schaap, 1983) and even possibly their function (Meinhardt, 1983; Fisher, Dohrmann & Williams, 1984) to vegetative or aggregating amoebae. Interestingly, the MUD9 label is not uniformly distributed on vegetative amoebae, but varies greatly from cell to cell (see Fig. 3, 1 h), presumably due to differences in the cell cycle (Weijer, Duschl & David, 1984). This was confirmed by inspecting MUD9-labelled vegetative amoebae under a fluorescence microscope.
Prestalk cell surface in D. discoideum slugs

The antigenic recognition by MUD9 in aggregates is more uniform (Fig. 3, 7h) presumably because cells in aggregates become synchronized.

That no exclusive prestalk marker was found could be explained by the prestalk proteins of the slug being either undetectable or present only in modest amounts (Borth & Ratner, 1983). This question may be resolved when the prestalk mRNAs found by Chisholm, Barklis & Lodish (1984) are sequenced and the presumptive proteins they code for are identified. Differentiation of prestalk cells is complex, and a complete picture of how these cells differentiate will require many markers. The new MUD9 marker described here contributes to our understanding of this process.

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