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Monoclonal Antibody Recognizing gp80, a Membrane Glycoprotein Implicated in Intercellular Adhesion of *Dictyostelium discoideum*†

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We have raised a monoclonal antibody, designated E28D8, which reacts with an 80,000-dalton membrane glycoprotein (gp80) of *Dictyostelium discoideum*. gp80 has been implicated in the formation of the EDTA-resistant adhesions ("contact sites A") which appear during development. The monoclonal antibody reacted with other developmentally regulated proteins of *D. discoideum*, confirming previous results indicating the presence of common antigenic determinants recognized by polyclonal rabbit antibodies directed to gp80. Periodate sensitivity of the determinants suggests that carbohydrate may be necessary for reactivity. Thus, the determinant recognized by E28D8 may result from a posttranslational modification common to a number of proteins. Some of the proteins that carry the determinant were preferentially localized to posterior cells in slugs. Monoclonal antibody E28D8 did not inhibit contact-sites-A-mediated intercellular adhesion. However, gp80 affinity purified on immobilized monoclonal antibody was able to neutralize the adhesion-blocking effect of rabbit antiserum to gp80. Although gp80 itself may not be essential for cell-cell adhesion, it appears to carry the determinants associated with adhesion.

The cellular slime mold *Dictyostelium discoideum* acquires the ability to form EDTA-resistant intercellular adhesions during the aggregation phase of its life cycle (10). A series of investigations by Gerisch and his colleagues (1–4) has demonstrated that the formation of these adhesions, which define "contact sites A," can be prevented by Fab antibody fragments reactive with cell surface components. A cell surface glycoprotein with an apparent molecular weight of 80,000 (gp80) has been identified as a major species which reacts with these Fab fragments (21, 22).

We have previously reported the time course of synthesis and accumulation of gp80, using polyclonal rabbit antibodies raised against purified gp80 (23). These antibodies were capable of blocking contact-sites-A-mediated adhesion. Surprisingly, the antibodies reacted with other developmentally regulated protein species as well as with gp80. We have now raised a monoclonal mouse antibody which recognizes an antigenic determinant common to some of those protein species, including gp80. This antibody binds to the surface of developed cells but fails to inhibit contact-sites-A-mediated cell adhesion. Ochiai et al. (27) have independently raised monoclonal antibodies reactive with gp80 and found that they recognize the same spectrum of proteins that we find.

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MATERIALS AND METHODS

Organisms. *D. discoideum* strains AX3 (14), HL100 (a spontaneous derivative of strain NC4), and HL80 were grown in association with *Klebsiella aerogenes* at 22°C and were developed on Millipore filters as described previously (19). Strain HL80 is a spontaneous derivative of strain HL100 with an enhanced ability to grow at 27°C. Fresh stock cultures were started every few months from desiccated spores stored on silica gel.

Generation of monoclonal antibody. (i) Immunogen. Membranes were prepared from cells of strain AX3 allowed to develop for 12 h in buffer to which pulses of cyclic AMP were added every 5 min (22). Proteins for immunization were liberated into the aqueous phase by butanol treatment of the membranes and dialyzed against 10 mM phosphate–137 mM NaCl–2.5 mM KCl (pH 7.2). About 40% of silver-staining material in this preparation migrated upon electrophoresis as material of 80,000 daltons.

(ii) Immunization. A BALB/c mouse was immunized intraperitoneally with butanol extract (containing 25 μg of protein) in complete Freund adjuvant and was boosted twice with the same material in alum (10 mg/ml) at 2 and 6 weeks after the initial immunization.

The mouse received a final 33 μg of butanol extract protein intravenously at 4 months after the initial injection. The spleen was taken for fusion 7 days later.

(iii) Fusion and screening. Cell fusion with SP2/0 myeloma cells, selection and growth of hybrids, and screening of culture supernatants for monoclonal antibodies were as previously described (24–26), except the affinity-purified goat anti-rabbit antibodies used in the screening were conjugated with glucose oxidase rather than with ^{125}I (25). Supernatants were tested on microtest plates onto which butanol extract had been dried and methanol fixed as described previously (25). Positive hybrids were subcloned twice and retested for binding ability. Isotypes were determined as previously described (24).

(iv) Antibody preparation and purification. Ascites fluid was prepared in pristane (500 μl)-primed BALB/c mice by intraperitoneal injection of 10^7 hybridoma cells. Ascites fluid was collected in the presence of 100 U of heparin. The fluid was clarified by centrifugation at 2,000 rpm. Immunoglobulin G (IgG) was prepared by two precipitations with half-saturated $(NH_4)_2SO_4$ followed by dialysis against 10 mM phosphate–137 mM NaCl–2.5 mM KCl (pH 7.2). This material was 90 to 95% pure IgG by polyacrylamide gel electrophoresis.

Electrophoretic analysis. One- and two-dimensional electrophoretic analysis in polyacrylamide gels containing sodium dodecyl sulfate has been described previously (9, 13). For two-dimensional gels, proteins were separated by non-equilibrium-pH gel electrophoresis towards the acidic end (5). Gels were stained with silver by the method of Morrissey (17). Proteins were transferred to nitrocellulose and stained overnight with antibodies (10 μg in 15 ml) and ^{125}I-labeled staphylococcal protein A as previously described (23, 32). For staining with monoclonal antibodies, an additional 2-h incubation with rabbit antiserum directed against mouse kappa light chains (Miles Laboratories, Elkhart, Ind.) was used (at a dilution of 1:300) between the incubations with monoclonal antibody and with ^{125}I-labeled protein A.

Affinity purification. Monoclonal antibody E28D8 was covalently attached to protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) with dimethyl suberimidate at pH 8.6 (8, 11). gp80 was purified from butanol extracts of the membranes of 12-h-developed HL80 cells by affinity chromatography on the insolubilized E28D8 as described by Ochiai et al. (27), except the gp80 was released from the column by using 50 mM diethylamine (pH 11.3) (28).

Chemical and enzymatic analysis. (i) Periodate oxidation. A 10-μl portion of affinity-purified gp80 (5 μg) or of cell lysate (10^6 cell equivalents) was mixed with 10 μl of 80 mM $NaIO_4$ on ice in the dark. After 12 h, 2 μl of 10% (vol/vol) ethylene glycol was added to each sample to neutralize any remaining periodate. Control samples received ethylene glycol at time zero.

(ii) Endoglycosidase digestion. For digestion with endoglycosidase H (Miles Laboratories), 167 ng of affinity-purified gp80 in 2 μl of 10 mM Tris (pH 7) containing 0.5 mg of cholate per ml was boiled for 2 min with sodium dodecyl sulfate (2.5 to 7.5 mg/ml). An additional sample contained no sodium dodecyl sulfate and was not boiled. The samples were cooled and made up to 19 μl with 50 mM sodium citrate (pH 5.0). Endoglycosidase H (1 mU) in 1 μl of 1 mM sodium phosphate (pH 7.1) was added to each sample. The samples were covered with silicon oil and incubated at 37°C for 20 h. The silicon oil was removed, and 20 μl of double-strength sample buffer was added. After boiling for 3 min, samples were analyzed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining or antibody staining with polyclonal rabbit antibodies against gp80. Cleavage of oligosaccharides would be detected as a change in the migration position of the protein band in the gel. Such a change was observed in parallel silver-stained samples containing 2 μg of ovalbumin rather than gp80. Negative controls included samples without added endoglycosidase H and samples analyzed without incubation at 37°C.

Digestion with endoglycosidase F (a generous gift of Steve Alexander) in the presence of 2-mercaptoethanol was performed as described by Elder and Alexander (6) and analyzed by gel electrophoresis as described above. Samples of butanol extract which had been radioiodinated with chloramine T (7) were analyzed by autoradiography after electrophoresis. In both cases, some (but not all) minor bands disappeared after digestion, indicating that the enzyme was active.

Separation of differentiated cell types. Anterior and posterior cells were isolated from slugs after 30 h of migration by manual dissection.

Immunofluorescence. The following procedures were carried out at 0 to 4°C in 20 mM phosphate buffer (pH 6.4) containing bovine serum albumin (1 mg/ml). Washed cells at 10^7/ml were incubated with monoclonal antibody (20 μg/ml) for 30 min. After one wash, the cells were incubated for 15 min with a 1/200 dilution of rabbit antiserum against mouse kappa light chains (Miles Laboratories), washed again, and incubated for 15 min in a 1/50 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibodies. After two more washes, cells were suspended to 2.5×10^6 cells per ml in 20 mM Tris-hydrochloride–100 mM NaCl (pH 8.3) and analyzed in a FACS-IV fluorescence-activated cell sorter (Becton, Dickinson & Co., Rutherford, N.J.) adjusted to detect fluorescein.

Adhesion blocking by antibodies. The effect of monoclonal and polyclonal IgGs on contact-sites-A-mediated adhesion was assayed by the method of Springer and Barondes (30). IgGs were dialyzed before use against 20 mM phosphate buffer (pH 6.4) containing 10 mM EDTA. For monoclonal antibodies, Fab fragments of goat antibodies against mouse IgG were used in place of the Fab fragments directed against rabbit IgG.

Cells which were developed for 10 h were dissociated in buffer containing 10^{-2} M EDTA (pH 6.4). Various concentrations of IgG were added along with 0.25 mg of Fab fragments per ml. The Fab fragments block agglutination of the cells by the IgG, allowing measurement of cell-cell adhesion. After 30 min of shaking at 100 rpm, the number of single cells was counted on a Coulter Counter. The decrease in single cells is due to cell-cell adhesion (30).

Fab fragments of monoclonal antibody E28D8 and polyclonal rabbit IgG were prepared as described by Maze (16). Completion of digestion was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Contact-sites-A-mediated adhesion in the presence or absence of Fab fragments was assayed as described previously (23), except the disappearance of

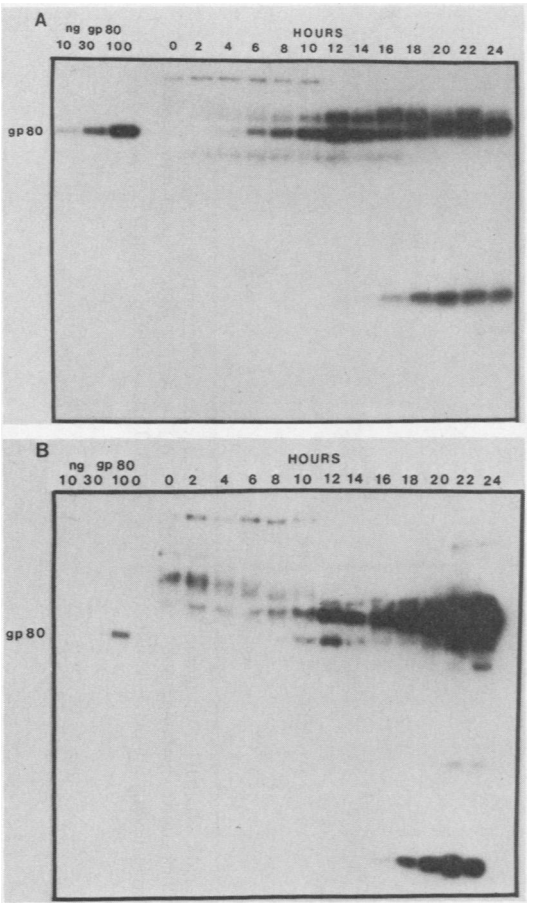

FIG. 1. Immune staining of *D. discoideum* proteins. Extracts equivalent to 5×10^5 HL80 cells from the indicated times of development were electrophoresed in 10% (A) or 8% (B) polyacrylamide gels in the presence of sodium dodecyl sulfate. After transfer to nitrocellulose, the separated proteins were stained with polyclonal rabbit IgG raised against purified gp80 and absorbed five times with vegetative AX3 cells (23) (A) or with monoclonal antibody E28D8 followed by rabbit anti-mouse kappa-chain serum (B). Bound antibody was visualized by autoradiography after incubation with ^{125}I-labeled staphylococcal protein A. Positions of purified gp80 are indicated.

single cells was followed by using an electronic particle counter (Coulter Electronics, Hialeah, Fla.).

RESULTS

Isolation and analysis of monoclonal antibody E28D8. From a fusion involving a BALB/c mouse immunized as described above, we recovered a hybrid cell line, designated E28D8, which secretes a monoclonal antibody that reacts with gp80, as detected by immune precipitation of ^{125}I-labeled butanol extracts or by immune staining of purified gp80 after gel electrophoresis, followed by transfer to nitrocellulose (Fig. 1). The antibody was typed as an IgG1 antibody containing kappa light chains.

Proteins reactive with E28D8. We reported previously that polyclonal antibodies raised against purified gp80 also react with several developmentally regulated protein species of *Dictyostelium* (23). These proteins were also detected by staining with monoclonal antibody E28D8 (Fig. 1), thus providing direct evidence for at least one antigenic determinant shared by all of these species. Although they are qualitatively similar, the staining patterns of E28D8 and

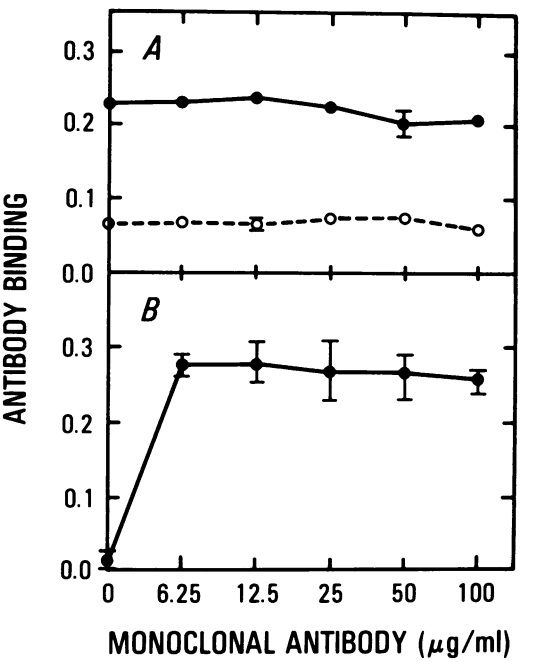

FIG. 2. Competition of monoclonal with polyclonal antibody binding. Microtest plate wells were coated with butanol extract (240 μg/ml, 25 μl per well) by drying and methanol fixation. After blocking with phosphate-buffered saline containing 10 mg of bovine serum albumin per ml, wells were incubated overnight at room temperature with 25 μl of the same buffer, containing the indicated concentrations of monoclonal antibody E28D8, per well. After washing, the wells were incubated for 4 h at 37°C with 25 μl of the above-described buffer containing rabbit polyclonal IgG raised against gp80 (50 μg/ml) and absorbed five times with vegetative AX3 cells (A, ●), preimmune rabbit IgG (50 μg/ml) (A, ○), or rabbit anti-mouse kappa-chain serum diluted 1:100 (to detect bound monoclonal antibody) (B). Binding of the rabbit antibodies was assayed by measuring bound enzymatic activity (arbitrary units) after a further 2-h incubation with a 1:250 dilution of glucose oxidase-conjugated goat anti-rabbit IgG antibodies (25). The error bars indicate the range of duplicate (A) or quadruplicate (B) determinations. Where no error bar is given, the range was less than the size of the symbol.

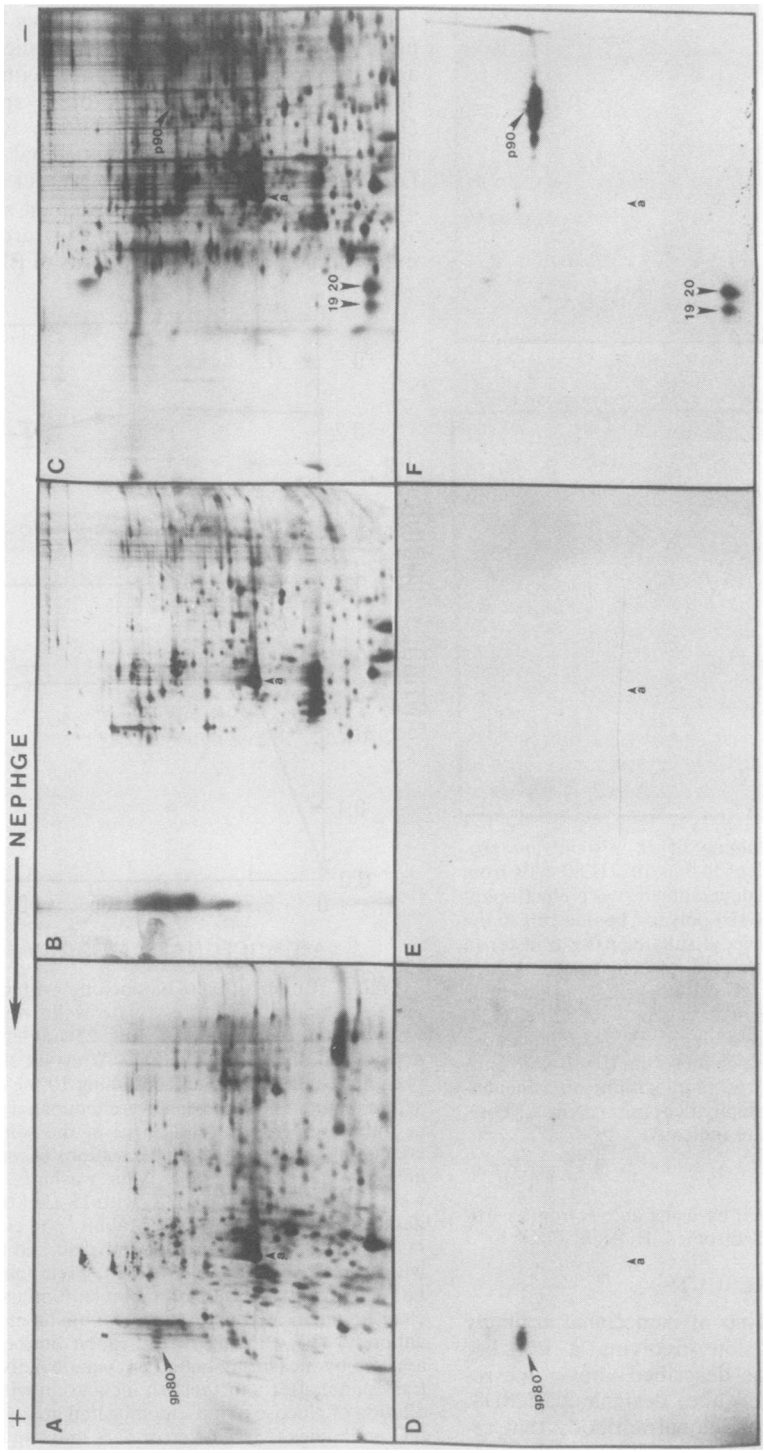

FIG. 3. Non-equilibrium-pH gradient electrophoretic analysis (acidic NEPHGE) of aggregation-stage and slug cells. Cells were taken after 12 h of development on filters (A and D) or were manually dissected from slugs after 30 h of migration (anterior cells, B and E; posterior cells, C and F). Proteins were stained with silver (A–C) or transferred to nitrocellulose and stained with monoclonal antibody E28D8 (D–F). The position of actin (a) is indicated. 19, 20, Specific protein numbers from Morrissey et al. (19).

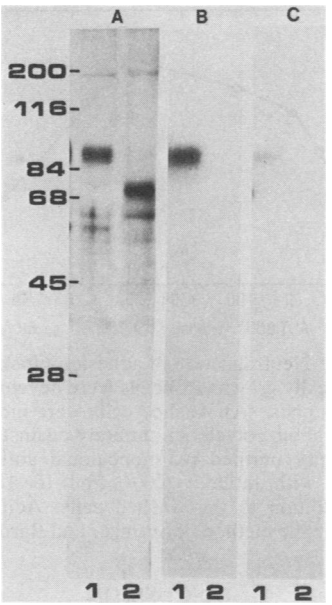

FIG. 4. Periodate oxidation of gp80. Affinity-purified gp80 was treated with 80 mM sodium periodate as described in the text and then analyzed in 10% polyacrylamide gels. Proteins were stained with silver (lane A) or transferred to nitrocellulose and stained either with rabbit polyclonal antibodies against gp80 which had been absorbed five times with vegetative cells (lane B) or with monoclonal antibody E28D8 (lane C). Well 1, control for periodate treatment of gp80; well 2, periodate-treated gp80. Molecular weight standards are indicated in kilodaltons.

the polyclonal rabbit antibodies show quantitative differences (Fig. 1). At between 6 and 20 h of development, both antibody preparations reacted with gp80 and a protein of 95,000 daltons (p95). Starting at 14 h and continuing through the remainder of development, proteins of 90,000 daltons (p90) and 25,000 daltons (p25) accumulated which reacted with the antibodies. When compared with the other reactive species in these gels, the gp80 band was much more intensely stained by the polyclonal antibodies than by the monoclonal antibody. On the other hand, the monoclonal antibody stained p90 very strongly late in development. This suggests that the polyclonal antibodies recognize specificities, other than that recognized by the monoclonal antibody, which are present predominantly or exclusively on gp80. This interpretation is strengthened by experiments which showed that saturating amounts of the monoclonal antibody do not inhibit binding of the polyclonal antibodies to partially purified preparations of gp80 (Fig. 2).

Localization of E28D8-reactive proteins. At least two different differentiated cell types can be identified in *Dictyostelium*, beginning at the tight aggregate stage. In migrating slugs, these cell types are predominantly localized in the anterior and posterior regions of the slug, respectively (15, 18). In manually dissected preparations, p90 and p25 were localized exclusively in posterior cells (Fig. 3). By comparing silver-stained with antibody-stained two-dimensional gels (Fig. 3), we identified the two spots corresponding to p25 with the posterior-specific spots 19 and 20 identified by Morrissey et al. (19). Neither gp80 nor p95 was observable in extracts of slugs which had been migrating for 30 h, indicating that after they accumulate during aggregation, they are degraded during prolonged migration. p95 is a very basic protein which did not migrate into the acidic non-equilibrium-pH gels but could be recognized on basic gels by staining with the monoclonal antibody (data not shown).

Chemistry of the determinant recognized by E28D8. gp80 is known to be highly glycosylated (22) and phosphorylated (4, 29). Digestion of the purified protein with periodate abolished its ability to react (after transfer to nitrocellulose) with either monoclonal antibody E28D8 or the polyclonal rabbit antibodies (Fig. 4). Furthermore, when whole lysates were treated with periodate, all reactivity with these antibodies was lost (data not shown). These results suggest that the presence of carbohydrate is necessary for the reactivity of the molecule with the antibodies.

Endoglycosidase H specifically cleaves high-mannose N-linked oligosaccharides from glycoproteins (31), including some, but not all, oligosaccharides from *D. discoideum* (12). Endoglycosidase F is a newly described enzyme which cleaves both complex and high-mannose N-linked oligosaccharides (6). Purified gp80 was resistant to digestion by either of these enzymes (data not shown).

Effect of antibodies on contact-sites-A-mediated adhesion. Although monoclonal antibody E28D8 reacted with gp80 (and other proteins), it had no effect on the EDTA-resistant cell adhesion mediated by contact sites A when tested either as Fab antibody fragments (Table 1) or as whole IgG

TABLE 1. Effects of monoclonal antibody E28D8 Fab fragments on adhesion[a]

Fab fragment concn (mg/ml)	% Single cells	
	Mean	Range (n = 3)
0.0	54.4	47.9–64.2
0.7	52.0	43.5–65.5

[a] Adhesion of 11-h-developed bacterially grown AX3 cells was assayed in the presence or absence of Fab fragments as previously described (23). Single cells were determined by electronic particle counting.

molecules by the method of Springer and Barondes (30) (Fig. 5). However, E28D8 bound specifically to the surface of developed cells as assayed by immunofluorescence analysis with a fluorescent-activated cell sorter (data not shown).

We previously reported that rabbit polyclonal antibodies which had been absorbed three times with vegetative AX3 cells were still capable of blocking EDTA-resistant adhesion (23). Recent experiments have shown that more thorough absorption (five times) can remove the adhesion-blocking ability of these antibodies (Fig. 5) while leaving unaffected their ability to stain multiple *D. discoideum* proteins, including gp80 (Fig. 1). Fab fragments prepared from these antibodies also failed to block adhesion (data not shown).

These results indicate that binding of antibodies to gp80 is not sufficient for the adhesion-blocking activity. However, gp80 affinity purified on monoclonal antibody neutralized the ability of the unabsorbed rabbit serum to block adhesion (Fig. 6). The affinity-purified material contained gp80 but no observable p95, p90, or p25 in silver-stained gels. Therefore, gp80 appears to carry the determinants involved in adhesion blocking.

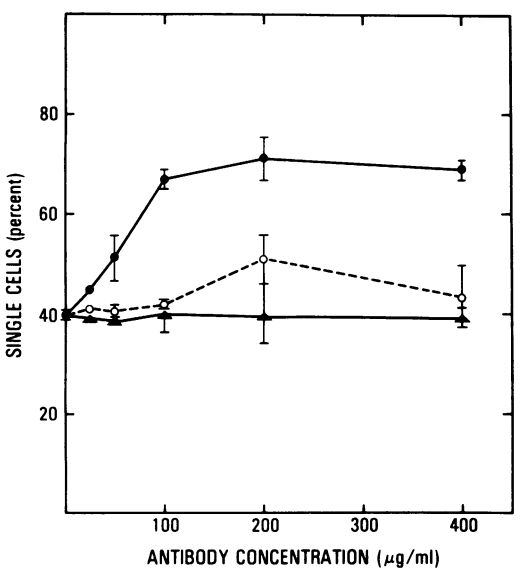

FIG. 5. Effect of antibodies on cell adhesion. Axenically grown AX3 cells were developed for 10 h on filter pads. Washed cells were incubated on ice with the indicated concentrations of monoclonal antibody E28D8 (○), rabbit polyclonal antibody against gp80 (●), or rabbit antibodies absorbed five times with vegetative AX3 cells (▲). Adhesion was assayed by the method of Springer and Barondes (30), using Fab fragments (0.25 mg/ml) of goat anti-mouse IgG (○) or of goat anti-rabbit IgG (●, ▲). The error bars indicate the range of triplicate determinations.

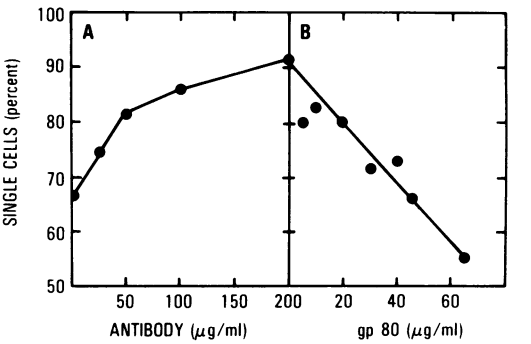

FIG. 6. Neutralization of adhesion-blocking activity. Axenically grown AX3 cells were developed for 10 h on filter pads. (A) Washed cells were incubated on ice with rabbit polyclonal antibody against gp80. (B) gp80 affinity purified on monoclonal antibody was incubated with antibody (200 μg/ml) for 18 h at 4°C before addition to the washed cells. Adhesion was assayed by the method of Springer and Barondes (30).

DISCUSSION

In a previous paper (23), we reported immunological cross-reactivity between gp80 and other *D. discoideum* proteins detected at various stages of development. Since polyclonal rabbit antibodies were used for that study, it was difficult to rule out completely the possibility that the cross-reactivity resulted from the presence of undetected low levels of the cross-reacting proteins in the gp80 preparation used for immunization. We have now provided direct evidence, using monoclonal antibody E28D8, that the various protein species in question do share at least one antigenic determinant. Post-translational glycosylation may be involved in generating the common determinant, since periodate treatment abolished the reactivity of all of the proteins with the monoclonal antibody. Ochiai et al. (27) recently have reported immune staining results which are very similar to ours, using a monoclonal antibody which was independently raised against gp80.

Müller et al. (22) have reported that about 25% of the mass of gp80 is carbohydrate. Our results are consistent with this, as periodate digestion reduced the apparent molecular weight of the molecule from 80,000–85,000 to about 70,000. Thus, we would expect to be able to detect loss of the oligosaccharide from gp80 after endoglycosidase digestion as a shift in band position on gel electrophoresis. Ivatt et al. (12) recently have reported that most mature oligosaccharide chains in *D. discoideum* synthesized early in development are resistant to endoglycosidase H. Since gp80 is synthesized during this period (23), it is not surprising that it is resistant to endoglycosidase H. It is also resistant to endoglycosidase F.

The monoclonal antibody recognizes gp80 and p95, both of which appear at the time that the cells become adhesive and are degraded in migrating slugs. Both are phosphoproteins but appear to be otherwise unrelated since the phosphorylated peptides generated from the two proteins by partial proteolysis with either *Staphylococcus aureus* V8 protease or chymotrypsin are distinctly different (29). Whereas gp80 is a highly acidic protein, p95 is highly basic. The other proteins (p90 and p25) recognized by the monoclonal antibody appear later in development and accumulate in prespore but not prestalk cells. The function of these proteins is unknown.

There are several possible explanations for the fact that monoclonal antibody E28D8 had no detectable effect on contact-sites-A-mediated adhesion. First, antibodies may have to bind to a particular antigenic determinant or determinants on the gp80 molecule to block adhesion, and the monoclonal antibody may not be directed against one of these determinants. The rabbit antiserum binds to determinants on the cells not recognized by the monoclonal antibody (Fig. 2) and stains gp80 relatively more strongly (Fig. 1); its ability to block adhesion may be related to its reactivity with these specific determinants. Second, more than one different determinant may need to be covered to block adhesion; in this case, no single monoclonal antibody will be sufficient. Third, monoclonal antibody E28D8 may be directed against a potential target site for the blocking of adhesion, but the antibody may not be of sufficient affinity to block.

Finally, gp80 may not be the physiologically relevant adhesion molecule but may still share antigenic determinants with the physiological target. These determinants would be recognized by the rabbit antiserum to gp80 but not by the monoclonal antibody. In any case, it appears that gp80 carries the determinants responsible for the ability of rabbit serum to block cell-cell adhesion, since E28D8-affinity-purified gp80 can neutralize this activity. Since these determinants may be carried on several different proteins, it remains to be shown which ones are essential for cell-cell adhesion.

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