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Rapid isolation of monoclonal antibodies specific for cell surface differentiation antigens

(*Dictyostelium*/plasma membrane)

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ABSTRACT **Two immunization procedures were compared for their ability to yield monoclonal antibodies that react with plasma membrane-bound differentiation antigens of *Dictyostelium*. In the first method, hybridomas prepared from BALB/c mice immunized with aggregating amoebae produced monoclonal antibodies that recognized antigens present on both growing and aggregating *Dictyostelium* amoebae. None of the monoclonal antibodies reacted with only the injected aggregation-stage cell type. In contrast, monoclonal antibodies that reacted with differentiation antigens were easily obtained by primary immunization of BALB/c mice with living aggregation-stage cells, followed by secondary immunization with a preparation of plasma membrane from aggregating cells or intact aggregating cells mixed with polyclonal BALB/c antiserum raised against undifferentiated cells. By this method, approximately 20% of all anti-*Dictyostelium* monoclonal antibodies obtained in a fusion are specific for differentiation antigens. The properties and developmental regulation of several of these antigens are described. The possible uses of this immunological method to detect unique determinants on other kinds of cells and the likely immune mechanisms that make it successful are discussed.**

Surface antigens that uniquely identify a differentiating cell type or a tumor cell in adult tissues are typically difficult to identify by immunological methods (1). Several strategies for preparing monoclonal antibodies that are specific for differentiation or tumor-associated antigens are in use. One is simply to immunize with the cell type of interest and to screen for monoclonal antibodies that are specific for the differentiated cell type. Another is a "cascade" procedure in which antibodies are prepared and used to deplete a membrane fraction of all antigens except the one desired. Such depleted extracts are then used as immunogens to prepare monoclonal antibodies that are specific for a differentiated cell type (2). Both strategies are successful, but laborious. Suppression of immune response by cyclophosphamide or induction of tolerance also has been used (3–5).

We are using monoclonal antibodies and *Dictyostelium* to study the roles of cell surface molecules in the general embryological problem of pattern formation: the subdivision of a group of apparently identical cells into different cell types. Differentiation of *Dictyostelium* amoebae begins at starvation and results in the formation of two cell types, spores and stalk cells, and possibly a third cell type, basal disk cells. Multicellular development begins when aggregation of differentiating amoebae occurs by chemotaxis. Amoebae emit and respond to pulses of cyclic AMP and move by chemotaxis to form multicellular mounds, each containing $\approx 10^5$ cells. At the end of aggregation, the apex of each mound becomes constricted to form a tip. Differentiation of the spore cell type begins near the time of tip formation (6). Differentiation of two cell types is clearly evident in the polarized, multicellular slug stage and ends in the late culmination stage when mature spores and dead stalk cells form.

A previous study showed that differentiation to form both cell types and pattern regeneration in the absence of a tip are blocked by a thermosensitive mutation that alters the cyclic AMP chemosensory system (7). Because the cyclic AMP chemosensory system is expressed only by differentiating cells, its surface components should be differentiation antigens. To identify surface components of this sensory system and to assess their role in patterning more fully, we are using the hybridoma method to prepare monoclonal antibodies that recognize surface antigens of aggregating amoebae.

We chose to immunize mice with intact differentiating (aggregating) cells to guard against the possible loss of an important antigen after cell disruption. However, we were surprised to find that, of a large number of monoclonal antibodies prepared against differentiating whole cells, all recognized only antigens present on both undifferentiated and differentiating cells. None was specific for a differentiation antigen—i.e., an antigen present on the surface of aggregating cells but absent from the surface of growing cells.

In this study we report a convenient method that allows us to overcome this difficulty and permits us to prepare monoclonal antibodies that are specific for differentiation antigens. Further, we use these antibodies to study the time of appearance of their cognate antigens in the plasma membrane.

MATERIALS AND METHODS

***Dictyostelium* Cultures.** DdC, a cycloheximide-resistant derivative of NC-4, was grown in suspension with *Escherichia coli* B/r or was starved to permit differentiation (7). Other *D. discoideum* isolates and *Polysphondylium violaceum* were provided by K. B. Raper (University of Wisconsin, Madison, WI).

Mammalian Cell Culture Conditions. The nonsecreting, 8-azaguanine-resistant mouse plasmacytoma line SP2/0-Ag14 was grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% gamma globulin-free horse serum (KC Biological, Lenexa, KS), penicillin, and streptomycin. Cultures were maintained between 10^5 and 7×10^5 cells per ml at 37°C in a humid chamber containing 5% CO_2.

Immunization and Preparation of Hybridomas. In protocol I, BALB/c mice (12–16 weeks old) were immunized by intraperitoneal (i.p.) injection of 0.5–1.0 ml of a phosphate-buffered saline (PBS: 0.15 M NaCl/10 mM sodium phosphate, pH 7.2) suspension of $1–5 \times 10^6$ *Dictyostelium* aggregation-stage amoebae per ml. The animals were "boostered" 14 days later either by injection of 0.5 ml of cells (1–5

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$\times 10^6$ per ml in PBS) into the tail vein or by i.p. injection of 0.5–1.0 ml of cells as above. Sixty to seventy-two hours later, dissociated spleen cells were prepared from the immunized animals and were fused with SP2/0 cells by using 50% (wt/vol) polyethylene glycol (PEG 1500, Fisher) (8).

Monoclonal antibodies that are specific for aggregating cells were obtained by protocol II, a modification of the above procedure. BALB/c mice were immunized by i.p. injection of 10^6 vegetative amoebae in 0.5 ml of PBS and boostered 14 days later. Mice were bled from the eye to obtain 0.1–0.3 ml of polyclonal serum from each mouse. The limiting dilutions of polyclonal antisera that gave positive ELISA reactions (see below) with whole vegetative amoebae were typically 1:500. A second set of BALB/c mice, immunized 14 days earlier with intact aggregating amoebae (100 μg of protein per ml in PBS) were immunized (i.p.) with either a mixture of 0.5 ml of intact aggregating cells (10^6 per ml in PBS) and 0.5 ml of polyclonal serum raised against vegetative amoebae (fusion 3, Table 2) or a mixture of 0.5 ml of plasma membrane from aggregating cells (100 μg of protein per ml) and 0.5 ml of polyclonal serum raised against vegetative amoebae (fusions 4 and 5, Table 2). The animals were boostered 14 days later with a similar mixture of aggregating cells or plasma membrane and polyclonal serum raised against vegetative cells. Sixty to seventy-two hours later, spleen cells were obtained from these immunized mice and were used to prepare hybridomas as above.

Screening of Hybridomas and Preparation of Monoclonal Antibodies. Vegetative amoebae or aggregating amoebae were washed first in KPM (7), then in PBS, and were resuspended in PBS at 3×10^6 cells per ml. Cells were distributed in 100-μl aliquots into PBS-rinsed polyvinyl chloride (PVC) microwell plates and were attached to the plate by centrifugation ($200 \times g$, 5 min). Cells were fixed [1% (vol/vol) glutaraldehyde final concentration, 20 min, 20°C], washed with PBS, and treated with 100 mM glycine and 1% bovine serum albumin (Sigma A-4503) in PBS for 30 min to eliminate nonspecific binding of monoclonal antibodies. As an alternative to assaying whole cells, plasma membrane-enriched subcellular fractions were prepared (9), resuspended in PBS at 15 μg of protein per ml, and distributed in 50-μl aliquots into PVC microwells. After 2 hr at 20°C, each well was emptied, washed four times with PBS, filled with 100 μl of 0.1% bovine serum albumin/0.05% sodium azide/PBS, sealed with tape, and stored at 4°C for up to 3 days before use. Hybridomas producing antibodies specific for *Dictyostelium* membrane antigens were detected by an ELISA technique. PVC microwells coated with amoebae were washed in PBS, washed once for 30 min in 3.0% bovine serum albumin in PBS, filled with 50 μl of hybridoma culture supernatant, and incubated for 2 hr at 20°C. Wells were then washed four times with 0.05% sodium azide in 50 mM sodium phosphate (pH 7.0) (solution B) and filled with 75 μl of a 1:200 dilution of β-galactosidase-conjugated $F(ab')_2$ sheep or rabbit anti-mouse IgG heavy and light chains (Bethesda Research Laboratories) in solution B. After 2 hr at 20°C, each well was washed four times in solution B and filled with 75 μl of solution B containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside at 100 μg/ml. After 30–60 min at 20°C, a blue color develops in wells containing antibody specific for *Dictyostelium* surface antigens. For ELISA assays using PVC plates coated with plasma membrane preparations rather than whole cells, the procedure above was followed, except that solution B was replaced by solution A (0.05% Tween 20/0.05% sodium azide/PBS, pH 7.2). Hybridomas in all wells that were positive against aggregating amoebae or plasma membrane of aggregating amoebae were subcloned at least twice by limiting dilution and passaged until a stable line was established. Most positive clones (≈95%) were stabilized. Supernatants of only stabilized cell lines were tested for reactivity against vegetative and aggregating cells, and the preparations of screening reagent and substrate were identical among the fusions reported. Purified antibodies were prepared from culture supernatants or from ascites fluids by ammonium sulfate fractionation.

Radioimmunoassay of Antigen Expression. Amoebae, either growing in suspension with *E. coli* B/r or aggregating on non-nutrient agar plates were washed in KPM and then in PBS and distributed in 100-μl aliquots into PVC microwell plates to give 2×10^5 cells per well. The procedure above for fixing, washing, and incubating whole cells with hybridoma supernatants was followed exactly. Finally, cells were incubated with ^{125}I-labeled sheep anti-mouse whole antibody (Amersham IM 131, 8.2 μCi/μg of protein; 1 Ci = 37 GBq) for 30 min at 4°C. Wells were washed twice in cold PBS to remove unbound secondary antibody, separated by cutting with a razor blade, and assayed for radioactivity individually in a liquid scintillation counter (Tracor Analytic 6892) with Scintiverse (Fisher) as the counting medium.

Biochemical Methods. Plasma membrane-enriched fractions from growing and aggregating amoebae were prepared by Nuclepore filter lysis (9). Antigens were detected on immunoblots (10) following $NaDodSO_4$/polyacrylamide gel electrophoresis of plasma membrane fractions. Two-dimensional gel electrophoresis of plasma membrane proteins was by the method of Horst *et al.* (11).

RESULTS

Preparation of Monoclonal Antibodies. We expected that monoclonal antibodies that react with *Dictyostelium* differentiation antigens could be detected without exceptional effort. Hybridomas were prepared from mice immunized with intact aggregation-stage amoebae or with particulate plasma membrane preparations of aggregation cells. Supernatants were then screened to detect clones that produced antibody that bound to determinants present on the surface of differentiating (aggregating) amoebae but absent from the surface of undifferentiated (growing) amoebae. However, in several separate attempts, we failed to find antibodies that bound specifically to aggregating cells. All antibodies obtained bound to the surface of both vegetative and aggregation-stage cell types.

Our failure to readily detect differentiation antigens might not be surprising, considering the observation that the plasma membranes of aggregating and growing cells have quite similar protein compositions. Two-dimensional isoelectric focusing/$NaDodSO_4$/polyacrylamide gel electrophoresis of solubilized plasma membranes shows that at least 80 proteins identified in aggregation-stage membranes by silver staining are also found in plasma membrane preparations of vegetative cells (Fig. 1). Proteins enriched in the plasma membrane fraction of aggregating cells (Fig. 1 *Lower*) but not in the plasma membrane of growing cells (*Upper*) are infrequent. We note only four proteins that are of this kind. Others may exist. For example, the cyclic AMP receptor of aggregating amoebae is not seen on equilibrium gels but is observed on two-dimensional nonequilibrium isoelectric focusing gels (12). Thus, the surface of aggregating cells is not highly differentiated, and antibodies specific for this cell type might be rare.

We successfully obtained monoclonal antibodies that recognized only aggregating cells by immunizing mice with aggregating amoebae, followed by secondary immunization with particulate plasma membranes or differentiating cells mixed with polyclonal antiserum raised against intact vegetative cells (protocol II). The fraction of hybridomas that produced antibodies reactive with intact aggregating cells was reduced by a factor of ≈5, but 20% of these antibodies were specific for aggregating cells, the immunogen (Table 1).

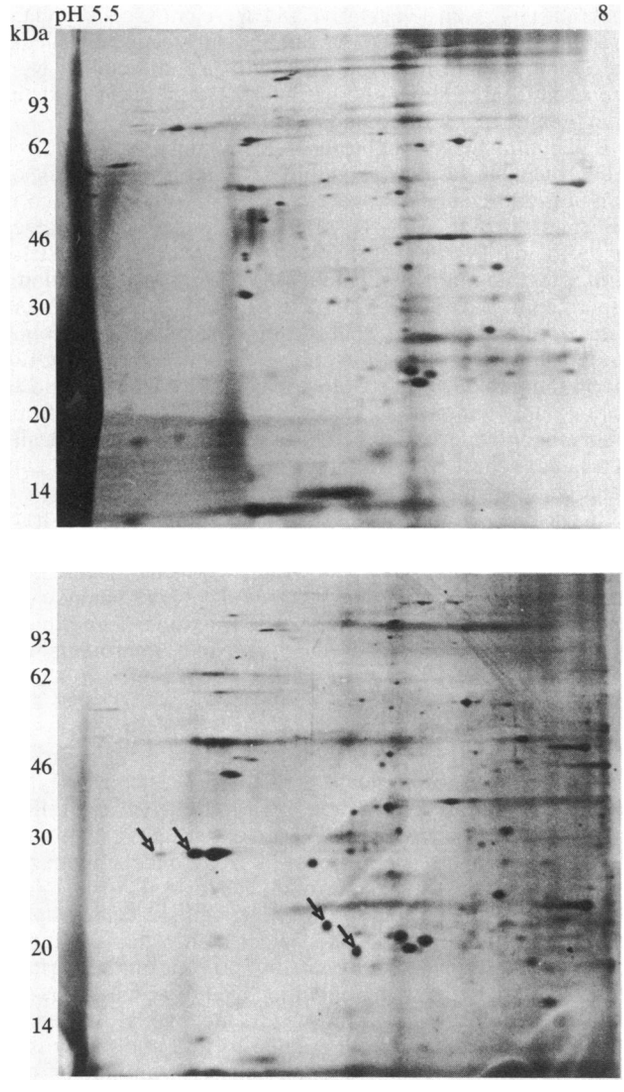

FIG. 1. Two-dimensional polyacrylamide gel electrophoresis of plasma membrane proteins. Arrows point to proteins that appear in the aggregation-stage plasma membrane preparation (*Lower*) but not in the plasma membrane preparation of vegetative cells (*Upper*). One hundred micrograms of protein from amoebae of strain DdC was loaded on first-dimension (isoelectric focusing) gels.

The two protocols differed in the IgG subclasses that they elicited (Table 2). Protocol I yielded only antibodies that react with constitutive antigens (i.e., antigens present on both undifferentiated and differentiated cells), and each antibody was either IgG1 or IgG3. In contrast, protocol II yielded antibodies reactive with both constitutive and regulated antigens, and these were either IgG2a, IgG2b, or IgG3. No IgG1 molecules were detected when protocol II was used. We do not know whether the IgG1 class was excluded because only a relatively small number of antibodies were generated by protocol II.

Table 1. Production of monoclonal antibodies specific for differentiation antigens

Fusion	Protocol	Number of hybridomas assayed	Number of positive clones	Number of specific antibodies
1	I	480	67	0
2	I	368	35	0
3	II	420	13	3
4	II	430	14	3
5	II	384	12	2

Positive clones were identified as producers of antibodies that bind to aggregation-stage amoebae. Most antibodies that bind to aggregation-stage amoebae also bind to vegetative amoebae, except as noted for fusions 3–5, from which antibodies specific for aggregation-stage amoebae were obtained.

Table 2. Subclasses of monoclonal antibodies elicited by protocols I and II

Fusion	Protocol	Monoclonal antibody	Expression of antigen*	Antibody subclass
1	I	mAb1.1	Constitutive	IgG1
		mAb1.2	Constitutive	IgG3
		mAb1.3	Constitutive	IgG3
		mAb1.4	Constitutive	IgG1
		mAb1.5	Constitutive	IgG1
		mAb1.6	Constitutive	IgG1
		mAb1.7	Constitutive	IgG3
		mAb1.8	Constitutive	IgG1
2	I	mAb2.1	Constitutive	IgG3
		mAb2.2	Constitutive	IgG3
		mAb2.3	Constitutive	IgG1
		mAb2.4	Constitutive	IgG3
3	II	mAb3.1	Regulated	IgG2b
		mAb3.2	Regulated	IgG2b
		mAb3.3	Constitutive	IgG2a
4	II	mAb4.1	Regulated	IgG3
		mAb4.2	Regulated	IgG2b
		mAb4.3	Regulated	IgG3
5	II	mAb5.1	Regulated	IgG2a
		mAb5.2	Regulated	IgG2a

*Constitutive antigens are present on both undifferentiated and differentiated cells. Regulated antigens are present only on differentiating cells.

Expression of Differentiation Antigens During Development. We used a radioimmunoassay to quantitate the relative level of expression of differentiation antigens revealed by protocol II. Differentiation antigens recognized by monoclonal antibodies mAb3.1 and mAb4.2 first appeared on the surface at 1–2 hr poststarvation and attained their maximal level at 4 hr (Fig. 2). Aggregation was not visually apparent by the fourth hour. However, between 2 and 4 hr after starvation, cells become responsive to artificial pulses of cyclic AMP delivered by micropipettes (13). Thus, functional differentiation of the cell surface begins early in development. The level of mAb3.1 and mAb4.2 antigens remained constant during the next 8 hr, the period when aggregation occurred. A minimum estimate for the abundance of these mAb3.1 and mAb4.2 antigens was 10^3 molecules per cell.

Constitutive antigens recognized by mAb1.3, mAb3.3, and mAb3.4 were present at an ≈50% higher level, and the antigen of mAb1.8 was 2.5- to 3-fold more abundant than the other antigen.

Plasma membrane-enriched fractions prepared from growing and aggregating amoebae were electrophoresed in $NaDodSO_4$/polyacrylamide gels, transferred onto nitrocellulose, and probed with several monoclonal antibodies and ^{125}I-labeled secondary antibody. Fig. 3 shows that the mAb1.8 antigen was present in at least 11 bands and the mAb3.4 antigen was present in 14 or more bands obtained from the plasma membrane fraction of aggregating amoebae. The same bands, with no apparent additional bands, were also found in plasma membrane preparations of growing amoebae. Thus, these antigens were common to several proteins that were expressed on the surface of growing cells and on amoebae that had differentiated to the aggregation stage. All monoclonal antibodies prepared by protocol I identified either several (>3) bands or yielded only a smear

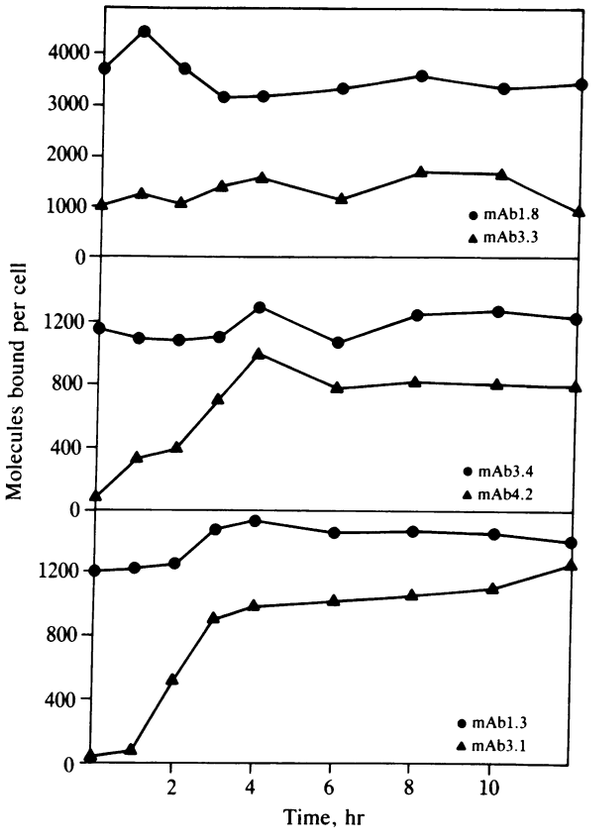

FIG. 2. Radioimmunoassay of antigen levels during early differentiation of strain DdC. Separate aliquots of one preparation of developing cells were used to test for the presence of the six antigens. The same preparation of iodinated secondary antibody was used for all determinations. Zero time is the time at which starvation was begun.

on immunoblots. This result is frequently experienced by others who prepare antibodies to *Dictyostelium* surface molecules. The reactive determinants are known as "common antigens." We presume that these common antigens are

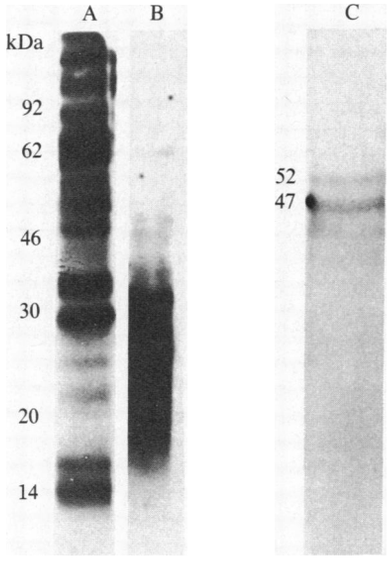

FIG. 3. Immunoblot analysis of antigens. mAb3.4 (lane A) and mAb1.8 (lane B) detect constitutive antigens and mAb3.1 (lane C) identifies a regulated antigen(s) (52 and 47 kDa). The plasma membrane preparation (100 μg per lane) was from aggregating cells.

Table 3. Distribution of antigens among slime molds

	Constitutive						Regulated	
Strain	1.1	1.3	1.7	1.8	3.3	3.4	3.1	4.2
D. discoideum								
NC-4	+	+	+	+	+	+	+	+
DdC	+	+	+	+	+	+	+	+
WS10	+	+	+	+	−	+	+	+
WS396B	+	+	+	+	−	w	w	+
WS521	+	+	+	+	−	+	+	+
WS698	+	+	+	+	−	+	w	+
D. purpureum	−	−	−	−	+	+	+	−
D. mucoroides	−	−	−	−	+	+	−	−
D. rosarium	−	−	−	−	−	−	−	−
D. minutum	−	−	−	−	−	+	w	−
Polysphondylium violaceum	−	−	−	−	−	−	−	−

w, Weak binding; +, strong binding; −, no binding.

part of the oligosaccharide chains of glycoproteins, as found for other monoclonal antibodies (14). In contrast, the differentiation antigen(s) recognized by mAb3.1 was limited to only two polypeptide bands, at 52 kDa and 47 kDa (Fig. 3, lane C). The limited distribution of this antigen, as compared to the constitutive antigens, probably accounts for the lower level of binding in Fig. 2. Because mAb3.1 antigen is heat-sensitive and is present on the surface of tunicamycin-treated cells (data not shown), the epitope is probably part of the polypeptide chain.

Distribution of Antigens Among Different Strains. We expected that protocol II might reveal polymorphisms within a species by preventing an immune response to immunodominant antigens. As a way to detect polymorphisms and species specificity, we analyzed the pattern of antigen distribution among several natural isolates of *D. discoideum* and other cellular slime mold genera and species. The organisms tested differed in the chemoattractant and receptor used for chemotaxis during aggregation. They also differed in mating type.

Table 3 shows the distribution of several antigens on independent natural isolates of *D. discoideum*, other *Dictyostelium* species, and another genus of slime mold. All amoebae were collected at the aggregation stage of each species. Constitutive surface antigens (csAg) 1.1, 1.3, 1.7, and 1.8 were reactive with monoclonal antibodies prepared by protocol I. These were species-specific and did not show polymorphism among several natural isolates of *D. discoideum*. The same result was found for two other monoclonal antibodies tested against several of these strains. Thus, immunization with whole *D. discoideum* amoebae (protocol I) elicited predominantly species-specific monoclonal antibodies.

In contrast, most antibodies produced by protocol II revealed polymorphisms among both constitutive and regulated antigens. For example, constitutive antigens csAg3.3 and -3.4 were not present on all natural isolates of *D. discoideum*, although they were found on other *Dictyostelium* species. These antigens are probably borne by a surface molecule that is polymorphic among slime mold species. Regulated surface antigen rsAg3.1 also showed polymorphism, whereas another regulated surface antigen, rsAg4.2, was species-specific.

DISCUSSION

When whole cells are used as immunogen, the ease with which one isolates a differentiation antigen-specific monoclonal antibody will decline if the undifferentiated cell and its differentiating counterpart are still quite similar in surface

composition. This may partially explain our failure to observe such antigens after immunization with very early aggregation-stage cells, which differ little from vegetative cells (15). Others have used whole cells to identify differentiation antigens more easily when the immunizing cells were more advanced in differentiation (16, 17). Thus, a method that efficiently yields monoclonal antibodies specific for differentiation antigens on minimally differentiated cells can be valuable.

The method we describe for identifying *Dictyostelium* cell surface differentiation antigens is quite reliable and has the advantage of being easy and relatively rapid. We expect that its use will be expanded to study other cell types. For example, it may help to identify tumor-associated antigens with greater ease. The method has already been successfully used to prepare a monoclonal antibody that is specific for a bovine lymphocyte surface antigen that escaped detection by monoclonal antibodies prepared by conventional methods (G. A. Splitter and J. Burkeholder, personal communication). In their use of this method, they concurred with our finding that immunization with a mixture of intact cells and polyclonal antibodies reduces the total number of hybridomas reactive with the infected cells. Further, the fraction of positive hybridomas that produce an antibody specific for the immunizing cell type is increased. Thus, the method appears to be general.

The rationale for our procedure was suggested to us by the early observation of suppression of tumor rejection by injection of immune sera from tumor-bearing animals (18, 19). However, the mechanism by which this method restricts the immunological response of mice is still unknown. We doubt that polyclonal serum actually "masks" or sterically prevents an antigen from participating in the immune response, because too little antibody is added to ensure that all determinants are complexed by their cognate antibody. Some determinants undoubtedly are free and accessible to the immune system. We favor, instead, a model that is based on Jerne's network hypothesis (20), which predicts that the coinjected polyclonal serum will induce synthesis of anti-idiotype antibodies. Anti-idiotype antibodies synthesized by a second BALB/c mouse will prevent synthesis of antibodies that have identical or crossreacting idiotypes that are similar to those found on antibodies in the polyclonal serum. Thus, antibodies that react with constitutive antigens will not be synthesized if they have the same idiotype as those antibodies provided in the polyclonal serum. This prediction is supported by our observation that antibodies elicited by protocols I and II are of different IgG subclasses.

This method for preparing monoclonal antibodies that react with particular antigens of interest has several useful features. First, it is much less time consuming than cascade methods (2) that require cycles of immunoprecipitation to deplete a complex mixture of unwanted antigens. Second, it has the potential of permitting one to add different combinations of antibodies to the primary immunization mixture in order to elicit a selective response against antigens that are not recognized by antibodies in the mixture. Such a selective response is not possible by using cyclophosphamide or cyclosporine to prevent a response against unwanted antigens. Third, this method may permit *Dictyostelium* workers as well as those in other fields to overcome the "common-antigen" problem. Many cell surface and lysosomal proteins bear oligosaccharide chains that are similar (possibly identical) on otherwise dissimilar proteins. These oligosaccharides are strong immunogens and confound attempts to prepare monoclonal antibodies that uniquely identify a single species of glycoprotein molecule. Although the common-antigen problem can be overcome by inducing tolerance to the common antigen, such a procedure requires prior preparation of sources of common antigen that are not contaminated by the novel antigen against which a specific monoclonal antibody is being sought. Thus, the method we report here has the advantage of bypassing the need for antigen purification.

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