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CHACTERIZATION OF A MONOCLONAL ANTIBODY REACTIVE WITH THE PLASMA MEMBRANE OF SOYBEAN PROTOPLASTS

By

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ABSTRACT

CHARACTERIZATION OF A MONOCLONAL ANTIBODY REACTIVE WITH THE PLASMA MEMBRANE OF SOYBEAN PROTOPLASTS

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Mouse splenocytes, immunized with protoplasts from suspension cultures of root cells of Glycine max (SB-1 cell line), were fused with murine myeloma cells. Hybridoma cultures were screened for antibodies directed against soybean protoplasts and then were cloned. One monoclonal antibody, designated MVS-1, bound to the outer surface of the plasma membrane of SB-1 protoplasts as determined by: (a) agglutination of the protoplasts; (b) binding and enrichment of the immunoglobulin by the protoplasts; and (c) saturable binding to the protoplasts by ^{125}I-labeled MVS-1.

The antigenic target of Antibody MVS-1 was a polypeptide (M_r =40,000) by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol. When the antigen was analyzed by gel filtration in phosphate buffer, the position of elution corresponded to a high molecular weight molecule (M_r=400,000). These results provided the background information for the analysis of the mobility of MVS-1 bound on the plasma membrane of SB-1 cells. Measurements using fluorescence redistribution after photobleaching yielded a diffusion constant of 3×10^{-10} cm^2 /sec for the mobility of the antigen in the soybean plasma membrane. This value is comparable to the diffusion constant of a class of receptors with relatively high mobility, as exemplified by wheat germ agglutinin, that can be modulated by the binding of other lectins such as soybean agglutinin. Therefore, Antibody MVS-1 may serve as a homogeneous probe of the mobility of cell surface antigens of soybean protoplasts and of the modulation of lateral mobility by endogenous agents.

DEDICATION

A mi esposa Tere, por su dulzura, amor y comprension, que me apoyo siempre y sin importar si los momentos fueron buenos o malos.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphatase
BSA	bovine serum albumin
Con A	concanavalin A
cpm	counts per minute
cv.	cultivar
D	diffusion coefficient
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
F-actin	filamentous or polymerized form of actin
FRAP	fluorescence redistribution after photobleaching
G-actin	globular or monomeric form of actin
IDPase	inosine diphosphatase
Ig	immunoglobulin
IgG	immunoglobulin G
L.	Linneus
min.	minutes
M_r	molecular weight
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
PA	phosphatidic acid
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
RNA	ribonucleic acid
SBA	soybean agglutinin
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TX-100	triton x-100

Tris	tris (hydroxymethyl) aminomethane
UDP	uridine-5'-diphosphate
WGA	wheat germ agglutinin

INTRODUCTION

The field of cell surface biochemistry has to deal with the problem of how interactions of the cell surface receptors with various ligands are linked to the interior metabolic machinery of the cell. It is essential to describe this signal transduction mechanism so that the fundamental control features can be understood for various biological systems such as drug and hormone-induced effects, antigen-induced immune response, as well as a number of specific cell-cell interactions including symbiotic associations.

One major difficulty in establishing a paradigm system for studying such cell surface interactions in the plant is that the necessary and sufficient components required for binding and the need for accessory factors are still undefined. Moreover, the existence of a natural barrier, the cell wall, provides additional constraints. For example, it is unknown whether receptor molecules anchored on the plasma membrane span or can selectively penetrate the cell wall to bind ligands outside. Similarly, the mechanisms by which ligands might penetrate the cell wall to bind the plasma

membrane receptors are not known.

One approach to this problem is to generate a "cell surface map" which defines as many molecules of the plasma membrane as possible, allowing thus the characterization of their physico-chemical properties. Recent advances in the technology of monoclonal antibodies suggest that this method is ideal for generating such a catalog. It is particularly advantageous that extensive prior purification of the antigenic molecules is not required to obtain the antibodies.

The soybean (Glycine max) cell line, SB-1, is particularly attractive for the generation of such catalog. First, the parent plant has the ability to fix nitrogen by a symbiotic association with the bacterium Rhizobium and thus, may present interesting features for the study of the recognition mechanisms. Furthermore, the parameters of growth for this cell line are well defined, as are the procedures for the generation of protoplasts (cells devoid of the cell wall) by enzymatic digestion. Thus, one can prepare individual plant cells with their plasma membrane intact and devoid of the hindering cell wall. The present work describes the generation of a number of monoclonal antibodies directed against the components of the SB-1

protoplast. One such monoclonal antibody, designated MVS-1, has been characterized in terms of its cell surface binding activity and antigenic target. This antibody has also been used to study the lateral mobility of the antigenic target in the plasma membrane of the soybean protoplast.

LITERATURE REVIEW

Plant cell cultures

The study of plants and plant cells has been greatly simplified by the development of tissue culture techniques. These techniques allow one to examine biochemical phenomena in a small group of cells instead of in a whole plant (1). It is normal practice to establish cultures of plant cells as callus tissue, which is a mass of cells growing on nutritive agar. Callus cultures can be established from virtually any part of the plant. These cultures are usually maintained for long periods of time by continuos subculture (2). Upon transfer to liquid medium and in the presence of rotary agitation, most callus cultures will grow in suspension. These liquid suspension cultures can be subcultured, or grown continuosly, in a manner similar to microbial continuous cultures (3).

There are a number of advantages for studying plant cells in culture as compared to whole plants:

a) Plant cell cultures can be regarded as more homogeneous than a whole plant with respect to cell types.

b) Large scale preparations can be obtained relatively fast and, in some cases, biochemical uniformity of the cells such as partial synchrony of cell division can be induced (4).

c) Cultured cells are particularly well suited for carrying out metabolic studies. Radiolabeled precursors and auxotrophic media can be easily manipulated, and the excretion of soluble or volatile substances, as well as the uptake and degradation of biochemically important substances can be readily monitored .

d) The use of incubators for the growth of cultures permits precise control of the environment, and the size of the sample becomes greatly reduced.

Nevertheless, cultured plant cells should not be expected to display all the features of whole plants. Cultured plant cells are in a unique undifferentiated state. It has been noted that different degrees of polyploidy, as well as chromosome mutations, occur in cells and plants originated from suspension cultures (5,6). Furthermore, biochemical properties of cultured cells may vary within a single batch culture. It is known that cultured carrot cells express different isozymes of aspartate kinase during different phases of growth (1). Therefore, caution must be observed when dealing with plant cells in culture since experimental results may arise from, or be influenced by, artifacts of the culture.

Plant cells of diverse origins in terms of tissue and species, can be grown in liquid suspension cultures at present. Table 1 summarizes some of those which have been

Table 1
Some Plant Cell Cultures at the
Prairie Regional Laboratory, Saskatoon (8)

Systematic Name	Common Name	Organ
Bromus inermis, cv. Manchar	brome grass	mesocotyl
Daucus carota L., cv. Royal Chantaney	carrot	root
Glycine max L., cv. Mandarin	soybean	root
Lycopersicon esculentum, cv. Starfire	tomato	hypocotyl
Manihot esculentum	cassava	petiole
Medicago sativa L.	alfalfa	root
Melilotus alba, cv. Arctic	sweet clover	root
Oryza sativa L.	rice	root
Phaseolus vulgaris L., cv. Pinto	bean	stem
Pisum sativum L., cv. Afghanistan	pea	root
Pisum sativum L., cv. Century	pea	root
Pisum sativum L., cv. Trapper	pea	root
Rosa, cv. Sceptre	rose	stem
T. aestivum L., cv. Kharkov	wheat	mesocotyl
T. aestivum L., cv. Manitou	wheat	root

Table 1 (Continued)

Systematic Name	Common Name	Organ
T. aestivum L., cv. Ramsey Durham	wheat	root
Vicia faba L.	faba bean	root

established in the last few years.

The chemical composition of different media for a number of cultures has been described. Murashige (7) summarized the major constituents as shown in Table 2. Murasshige and Skoog's (9) salt formulation has turned out to be one of the most extensively used for both callus and suspension cultures. This medium contains sucrose as the carbon source and a combination of nitrate, potassium, and ammonium salts. A different medium, called B5, has also been widely used for a variety of plant cultures including soybean (8,10). This B5 medium has essentially the same salt composition as Murashige and Skoog's medium, although at lower concentrations. Most cells will grow solely on these media and do not require other substances such as the supplements listed in Table 2.

The soybean cell line SB-1 was first established in 1964 from soybean root tissue (Table 1). The parameters of growth as well as the nutritional requirements have been well defined (10,11), and thus, the SB-1 cell line is turning out as a paradigm system for the study of the plant biochemistry. For example, the medium developed for growing this cell line is now used for a number of other plant cultures such as carrot and tomato (Table 1).

Protoplasts

Many studies on plants have been greatly simplified

Table 2
Major Constituents of Nutrient Media
for Plant Cell Culture (7)

Inorganic salts[1]	
White (11)	
Hildebrandt, Riker & Duggar (12)	
Heller (13)	
Murashige & Skoog (9)	
Organic substances	
Carbohydrate: sucrose	
Vitamins: thiamin, inositol, nicotinic acid, pyridoxin	
Amino acids and amides: arginine, aspartic acid, asparagine, glutamic acid, glutamine, and tyrosine	
Nitrogen base: adenine	
Growth regulators:	
Auxin: indoleacetic acid, indolebutyric acid, naphthaleneacetic acid, chloroethyl phosphonic acid, 2,4-dichlorophenoxy acetic acid	
Cytokinin: Benzyladenine, kinetin, 2-isopentenyladenine	
Gibberellin: Gibberellin A_3	
Natural Complexes	
Hydrolyzed protein preparations: casein or lactalbumin hydrolysates, soy peptone	
Brewer's byproducts: malt and yeast extracts	
Endosperm fluids: coconut, corn	
Fruit pulp and juice: banana, orange juice, tomato juice	
Animal byproducts: fish emulsion	

[1] Listed references for major salt formulations

by the fact that the plant cell wall can be removed leaving a cell with its plasma membrane intact. The term protoplast is used to describe these cells which are devoid of a cell wall. For example, in the presence of the enzyme lysozyme bacteria do not form a cell wall and remain viable when cultured in an isotonic sucrose solution. In fact, any cell contained within a cell wall can be considered as a protoplast (14). Both the isolation and application of protoplasts have been reviewed in detail (15-18). Initially, plant protoplasts were isolated mechanically by cutting through plasmolyzed plant tissue . This method of isolation usually resulted in a low yield of protoplasts (17). More recently, methods using enzymes to specifically digest the cell wall were developed. The major components of the cell walls of higher plants are α -cellulose, hemicellulose, and pectin (17); therefore, mixtures of cellulase, hemicellulase, and pectinase have been used to digest these components and leave spherical cells completely devoid of their cell wall.

Enzymatic isolation of protoplasts offers several advantages:

a) High yield of protoplasts.

b) Osmotic pressure is not required to shrink the cytoplasm.

c) Damage to the cells is minimal.

However, it should be noted that most cellulase and pectinase preparations usually have contaminants such as proteases and lipases, which may be harmful to the cells. This may be the only disadvantage of the enzymatic method, as compared to mechanical isolation (17).

There are several advantages to using liquid suspension cultures as source material for the isolation of protoplasts. First, the protoplasts are homogeneous with respect to cell type, as compared with protoplasts obtained from plant tissue. Second, sterile protoplast preparations are easily obtained from cultured cells. In contrast, in protoplasts isolated from leaf, variations have been found to occur from variety to variety of a same plant species depending on the efficiency of surface sterilization (19). The procedures for isolation of plant protoplasts from different suspension cultures have been well established (19,20). A number of factors influence the succesful generation of protoplasts by enzymatic digestion:

a) The condition of starting material, actively growing cells are preferable

b) An appropriate combination of cell wall-degrading enzymes.

c) An osmotically appropriate medium at the correct PH to avoid lysis of the protoplasts.

The composition of several enzyme solutions used to generate protoplasts from liquid suspension cultures of plants is listed in Table 3.

The advent of the techniques for protoplast isolation has greatly simplified the research in areas like plant somatic hybridization, and the study of plant viruses. Somatic hybridization is the fusion of isolated somatic cells from different plant types to introduce new genetic information and obtain a fusion product with new advantageous features (1,17). Only protoplasts can be used in this instance because the close contact of cell-cell plasma membranes for the fusion would be impossible with the cell wall present.

Plant protoplasts have also been used to study the viral infection process. Enzymatically digested protoplasts have been shown to rapidly take up tobacco mosaic virus. After 10 min. of incubation with the virus, 80-90% of the protoplasts are infected. This is a high infection efficiency which is advantageous for biochemical investigations (1).

Subcellular Fractionation of Plant Cells

In general, a number of organelles can be isolated from both plant cells and protoplasts (1,16,21). The use of protoplasts as compared to whole cells is desirable, since the harsh treatments required to break the cell wall

Table 3
Composition of Enzyme Solutions for Cell Wall Degradation of Cells Cultured in Liquid Media (mg/ml) (20)

	Soybean	Carrot	Vicia
Pectinase	—	—	10
Cellulase	20	20	20
Hemicellulase	10	10	—
Sorbitol	100	100	63.5
Mannitol	—	—	63.5
$CaCl_2.2H_2O$	—	—	0.9
$CaH_4(PO_4)_2.H_2O$	—	—	0.1
pH	5.5	5.5	5.5

are avoided. This results in preservation, and increased yield, of the isolated organelles.

An important aspect to consider in subcellular fractionation is the way to track the fractions of interest throughout the purification procedure. This monitoring of purification is achieved through the use of markers, either biochemical or cytochemical. Table 4 describes a number of markers, of both types, which are used in the isolation of different organelles from plant cells.

The protoplast system is a particularly good starting material for the isolation of plant cell plasma membranes. If enough shearing force is applied to a whole plant cell to break the cell wall, a mixture of both external and internal membranes will be obtained since internal organelles will be disrupted. In contrast, a gentle lysis will preserve internal structures and contamination by these components in plasma membrane preparations will therefore be minimized. The methods for isolation of plasma membrane from plants and plant cells have been reviewed (21,22).

Galbraith and Northcote (23) have reported the isolation of plasma membrane from soybean protoplasts. Prior to lysis, the protoplasts were labeled with [^{35}S]sulphanilic acid (diazonium salt). This reagent has been

Table 4
Markers of Plant Subcellular Components (21)

Subcellular fraction	Markers	
	Morphological or cytochemical	Biochemical
Nuclei	Large size, Double membrane and pore complexes.	DNA
Mitochondria	Phase light microscope, Electron microscope.	Cytochrome c oxidase, Fumarase, Succinate dehydrogenase, Succinate: cytochrome c reductase.
Chloroplasts	Phase light microscope, Chlorophyll, Fluorescence, Electron microscope.	Chlorophyll, Ribulose bisphosphate carboxylase, NADP-triose phosphate dehydrogenase, Pyruvate phosphate dikinase.
Glyoxysomes	Diaminobenzidine (DAB)stain.	Catalase, Isocitrate lyase, Malate synthetase.
Peroxisomes	DAB stain	Catalase, Hydroxypyruvate reductase.
Golgi	Inosinediphosphatase (IDPase), Dictyosome stack.	Latent IDPase, Glucan synthetase I.

Table 4 (Continued)

Subcellular fraction	Markers: Morphological or cytochemical	Markers: Biochemical
Plasma membrane	Periodic acid phospho-tungstate chromate stain	K^+-ATPase, Glucan synthetase II.
Protein bodies	Dense matrix $\pm$ globoid and crystalloid inclusions, Single membrane.	Lectins, Storage proteins.

shown to derivatize cell surface lipids and proteins of erythrocytes (24). It was also shown that the radioactive reagent did not penetrate the soybean plasma membrane. After lysis, 97% of the incorporated radioactivity was found in the particulate fraction. After fractionation by continuous isopycnic gradient centrifugation, the peak of [^{35}S]radioactivity was found to be associated with the plasma membrane fractions. Analysis of enzymatic makers showed that peak Mg^{2+}-ATPase activity, an enzyme associated with the plasma membrane (21), was associated with the radioactivity. Fumarase, NADH-cytochrome c reductase and NADPH-cytochrome c reductase activities were not found in these fractions. This was expected because these enzymes are usually associated with mitochondria, nuclear envelope, and endoplasmic reticulum (21).

Although the diazosulphanilate method specifically labels the plasma membrane of soybean, it appears that certain other cell types cannot be labeled this way, as reported by Schibeci et al. (25). These researches used the iodogen method (26) to incorporate, ^{125}I into cell surface proteins of protoplasts from ryegrass endosperm cells. They reported that although Na ^{125}I penetrates the ryegrass protoplasts, this method gives almost no labeling of intracellular membranes and it was found to be one of the best for the identification of the plasma membrane

fraction from cell homogenates.

A second approach, used in parallel by Schibeci et al. (25), was the use of an adventitious monoclonal antibody which binds to the plasma membrane. Incubation of the immunoglobulin with the protoplasts led to the formation of large aggregates of plasma membrane which could be easily separated from other cellular membranes. The antibody was also radiolabeled with $Na^{125}I$ and used as a marker to track the plasmalemma throughout the isolation procedure. The importance of a cell devoid of cell wall material was stressed in this type of work since the plasma membrane was directly accessible to the coating antibody. Furthermore, a minimal disruption of organelles could be achieved by using osmotic shock lysis. Indeed, it was found that a large number of the vacuoles were released intact after lysis of the cells in a hypo-osmotic medium.

In addition to plasma membrane, the isolation of a number of other organelles from plant protoplasts has been achieved. Ohyama et al. (27) have reported the isolation of nuclei from protoplasts derived from soybean cell suspension cultures. A nuclear fraction was obtained using low speed centrifugation. It was shown that the isolated material was able to synthesize RNA precursors. This type of isolation, however, had the disadvantage that the use

of detergent to solubilize contaminant membranes also damaged the nuclear outer membrane. In contrast, nuclei with 81% intact nuclear membranes were obtained by Philipp et al. (28). They used octanol and gum arabic in the preparative media which has been reported to result in better structural preservation of the nucleus (21). These studies showed, that in Allium cepa, there was a close biochemical similarity of the endoplasmic reticulum and the nuclear envelope. Furthermore, many properties of nuclear and endoplasmic reticulum membranes from the onion tissues were similar to those of their animal counterparts.

Metaphase chromosomes have been isolated from protoplasts of tomato and tobacco suspension cultures (16). A gentle lysis of the protoplasts, as well as an enriched fraction of protoplasts in the appropiate cell cycle phase were important factors for a successful isolation of this type. Although the isolated fraction was shown to contain mainly chromosomes, their ability to direct DNA synthesis and RNA transcription has not been verified; and therefore, are useless for genetic manipulations.

Photochemically active chloroplasts from protoplasts of a number of mature plant tissues have been obtained. Wagner and Siegelman (29) have obtained chloroplasts by

suspending the cells in a hypo-osmotic buffer in which there was minimal disruption of the organelles. A mixed population of vacuoles and chloroplasts was obtained and further separated by centrifugation in a Ficoll-sorbitol buffer. Vacuoles were released after lysis of the protoplasts and remained intact for more than 20 hours in a 0.55 M sorbitol buffer. This procedure proved to be excellent for large scale preparations of vacuoles and isolation of intact, photochemically active chloroplasts.

Mitochondrial preparations can also be obtained from plant cultures and tissues (16,21). The methods for chloroplast isolation can be used to isolate both mitochondria and chloroplasts from the same starting material (16). Mitochondrial preparations have been reported to yield over 95% intact mitochondria with low endoplasmic reticulum contamination (21). Isolated mitochondria from plant cell cultures have been used for characterization and studyy of the respiratory enzymes (1).

Although relatively few studies on cell organelles from plant cells in culture have been done, their presence in several cell types has been clearly demonstrated.

Chemical Composition of the Plant Cell Plasma Membrane

One of the major problems encountered with the study

of the plasma membrane of plant cells is the fact that highly purified plasma membrane preparations are not readily obtainable. In vitro plant cell culture and protoplast geeneration techniques are valuable tools which have permitted some progress in this area.

Morphological similarities of the plasma membrane of plant cells with that of animal cells have been observed. This plant cell organelle appears as a characteristic dark-light-dark tripartite structure under the electron microscope (30). Although it is known that, in the plant, this organelle contains primarily proteins and lipids, there is little knowledge about the nature or function of these components. There are limited reports on the protein, lipid , and carbohydrate composition of highly purified membrane preparations from plant cells (30). Furthermore, these reports have not been rigorously verified. Therefore, only a very general description of these components is available. The plasma membrane of plant cells contains (on a weight percent basis) about 40% protein, 50% lipid, and an unknown amount of carbohydrate as the third major component (30). There is evidence available which has demonstrated the existence of protein molecules at the plasma membrane of plants. Radiolabeled reagents have been used to label the surface of intact protoplasts, showing that a number of proteins on the

plasma membrane are accessible to the chemical reaction (23,25). In their plasma membrane purification from soybean cells, Galbraith and Northcote (23) showed that the outer surface of the protoplast was able to incorporate diazotized [^{35}S]sulphanilic acid into proteins as determined by a saturable incorporation of radioactivity in trichloroacetic acid cell precipitates during the time course of the labeling. Schibecci et al. (25) have also succeeded in labeling the surface of ryegrass endosperm protoplasts with ^{125}I (26). They found up to 70% of the radioactivity specifically incorporated into trichloroacetic acid precipitates from the cell. These experiments suggest that there are cell surface proteins exposed to the environment which are accessible to the chemical reactions. In a different experiment, [^{14}C]-leucine incorporation was tracked in the onion stem (31) and the radioactivity was shown to appear first in the endoplasmic reticulum and later in the plasma membrane fraction.

A few proteins have been identified at the plasma membrane of plant cells. Extensive studies have been carried out on the binding of auxin to its receptor, and the protein nature of high affinity auxin-binding sites has been demonstrated (32). Although auxin binding sites are present on different cellular membranes, it has been

postulated that auxin receptors located in the plasma membrane play a primary role in tissues involved in polar auxin transport. Receptors of the auxin transport inhibitor, N-1 naphtylphtalamic acid, have also been localized on the plasma membrane of plant cells (1). Recent work by Jacobs and Gilbert (33) has demonstrated the presence of an auxin transport carrier system in the plasma membrane of pea (Pisum sativum) stem cells. A monoclonal antibody was used to fluorescently stain thin sections of tissue revealing a polar distribution of the transport system.

Phytochrome, a chromoprotein which regulates responses induced by light in plants (34), has also been associated with the plasma membrane. Evidence from studies with the green algae Mougeotia have demonstrated that phytochrome molecules are arranged either parallel or perpendicular to the plasma membrane depending on the type of light absorption to which the chromophores are sensitive (34). Similar conclusions were drawn from studies using Dryopteris filix and corn coleoptiles (34). Furthermore, correlations between naphtylphtalamic acid binding sites and phytochrome have been found in corn coleoptiles and zucchini hypocotyl hooks (34).

A number of enzymes have been also localized at the plasma membrane of plant cells. Correlations between

glucan synthetase II activity and phosphotungstate-chromate stained vesicles (presumably from plasma membrane) from onion stem preparations have been reported (21). Glucan synthetase activity has been found in plasma membrane fractions of ryegrass endosperm cells (35), although this only represented 6% of the total activity detected. Therefore, it is possible that these results arose from contamination by intracellular membranes. Van Der Woude et al. (36) have found β1-4 glucan synthetase activity to be associated with the plasma membrane and Golgi fractions of onion stem homogenates.

Substantial evidence has shown that cellulose synthesis occurs at the cell surface (21). However, the glucan synthetases presently detected in plasma membrane fractions have not been found responsible for *in vivo* cellulose synthesis.

Several other enzymes have been associated with the plasma membrane including UDP-glucose polysaccharide synthetase, and ion-stimulated ATPase (23,30,37). The function of the two former enzymes in cell wall biosynthesis or glycolipid metabolism has not been demonstrated (30). There is sufficient evidence that supports the localization of ATPase activity in the plasma membrane of all plant cells (21,30). In their studies with

soybean cells, Galbraith and Northcote (23) have found a major peak of Mg^{2+} ATPase activity associated with the plasma membrane fraction. Leonard and Van Der Woude (37) have found a similar correlation in plasma membrane fractions from corn roots. Although the exact function of the ATPase in the plant cell is unknown, a role of energy transduction to couple metabolic energy to ion transport through the membrane has been suggested (30).

Lipids are another important component of biological membranes. They provide support and fluidity to the membrane matrix. Phospholipids are the main lipids present in the membranes of plants. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) together account for approximately 70% of the total phospholipid weight of membranes from various organelles of potato tuber (38). A number of phospholipids and fatty acids have been located at the plasma membrane of this system as shown in Table 5.

A number of glycolipids are usually found in membranous structures of plant cells, including the plasma membrane. Galactolipids are commonly found in photosynthetic membranes (39). However, Sabinski et al. (39) found [^{3}H]-galactose incorporation into the plasma membrane, as well as the plastids and the cell wall of soybean suspension cultures as determined by electron microscopic autoradiography. They proposed that

Table 5
Composition of Phospholipids and Fatty Acids in Potato Tuber Plasmalemma Fractions (38)

Phospholipids	Percentage
Phosphatidylcholine	38
Phosphatidylethanolamine	47[1]
Phosphatidylinositol	15

Fatty acids[2]	Percentage
16:0	27.8
18:0	8.4
18:1	4.3
18:2	40
18:3	8.5

[1] Includes phosphatidyl serine and phosphatidyl glycerol

[2] The number before the colon represents the number of carbon atoms in the fatty acid; the number after the colon indicates the number of double bonds present

galactolipids in non-photosynthetic cells of soybean suspension cultures may be located at the plasma membrane.

Cerebrosides and similar complex lipids called phytoglycolipids have been isolated from plants (40). In animal cells these biomolecules are usually present in the plasma membrane. In the plant cell, although the details of synthesis and function of these molecules are unknown, it has been suggested that they may have a similar cellular location as seen in animal cells (40).

Soybean root and hypocotyl preparations have been reported to contain sterol compounds associated with their plasma membrane fractions (41). Sitosterol (60-70%) was the major sterol component found in both membrane preparations, stigmasterol (20% in hypocotyl and 30% in root), campesterol (10% in both), and cholesterol (0.3% in root and 2% in hypocotyl), were also present. Sterols are important components of membranes because, along with the phospholipids, their relative abundance profoundly influences membrane fluidity (42)

Cooperative thermotropic phase transitions, between a fluid phase and a gel phase, are known to occur in membrane lipids at a certain temperature which is called the transition temperature. Shorter acyl chain length and/or inclusion of unsaturation in the chains adds fluidity to membranes. Sterols, such as cholesterol tend

to restrict the motion of acyl chains of polar lipids above the transition temperature increasing their order in the membrane. Below the transition temperature, sterols tend to increase the fluidity by disrupting the ordered array of hydrocarbon chains in gel structures. This effect is caused by reduction of cohesive forces between the neighboring chains (42). The fluidity of membranes, is thus regulated by: a) the ratio of free sterols to phospholipids; b) the length of acyl chain and/or the abundance of unsaturated chains; and c) the substitution of PE or phosphatidic acid head groups for PC and vice versa since PC transition temperatures are lower than those of PE (43) or PA (44). However, generalizationns about which changes will increase or decrease membrane fluidity in biological membranes are not predictable. This is due to the fact that transition temperatures have a broad range of values which are caused by the relatively high heterogeneity of lipids and varying amounts of protein and sterols (42). In wheat (Triticum aestivum L.) protoplasts (45), the inclusion of PC molecules was found to increase fluidity of the plasma membrane but when this fluidity reached a critical level, a progressive rigidifying of the hydrophobic core was detected. These results were interpreted as a compensatory mechanism by which the cell can maintain the average fluidity of its

plasma membrane within a particular range. This mechanism is thought to be initiated when the inclusion of PC molecules exceeds a critical level (>40%). In summary, the nature and abundance of lipids on the plant cell plasma membrane are important factors which determine fluidity or rigidness, and are specifically involved in functions of the plant such as resistance to freezing (42). When plants are grown at low temperatures, it has been found that an increased lipid synthesis occurs, in particular phospholipid (42). The fatty acid pattern also changes towards more unsatured chains.

More thorough and documented investigations on the nature and function of lipid molecules in the plasma membrane of plant cells are necessary but the lack of highly purified preparations of this organelle has been an obstacle for these purposes.

Another major component in plant plasma membrane is carbohydrate. Since proteins and lipids constitute only about 80% of the plasma membrane weight, the remaining 20% is believed to be, at least in part, carbohydrate, most likely in the form of glycolipids, glycoproteins and polysaccharides for cell wall assembly (30). This evidence comes from studies showing that lectins can bind and agglutinate plant protoplasts (30,46-49) and plasma membrane preparations (41). Williamson et al. (46) have

demonstrated concanavalin A (Con A) bindiing activity to soybean protoplasts. Travis and Berkowitz (41) also showed that vesicles from plasma membrane fractions of soybean bound ferritin-labeled Con A. More recently, Williamson (48) has been able to label leek (allium porrum L.) stem protoplasts with ferritin labeled Con A. Metcalf et al. (49) have shown a saturable binding of Con A, soybean agglutinin (SBA) and wheat germ agglutinin (WGA) on soybean protoplasts. Furthermore, fluorescence microscopy showed a characteristic ring staining of the protoplasts by the lectins. The binding of Con A, SBA, and WGA was inhibited by methyl α-D-mannoside, N-acetyl galactosamine, and N-acetylglucosamine, respectively. All these results support the view that glucose, galactose, and/or mannose moieties are present on the outer surface of the plasma membrane of the protoplasts. Galactose moieties have also been associated with the plasma membrane of protoplasts from ryegrass endosperm cells (25) due to the fact that galactose inhibits both the binding and agglutination by the galactose binding proteins, anixellin, a lectin from Anixella polypoides, and myeloma protein J539, a monoclonal antibody.

A number of sugar moieties such as glucose and galactose are also known as components of lipids found in various membranous structures from plants, including the

plasmalemma (40). Glucosyl moieties have been found as part of phytoglycolipids and cerebrosides. Because of their structural similarity with animal glycosphingolipids and cerebrosides, it is hypothesized that at least some of these molecules may be in the plasma membrane (40). In addition, glycosyl transferase activity has been localized at the cell surface where transfer of carbohydrate moieties to components of the plasma membrane occurs in at least certain plant tissues (30). Again, the lack of highly purified plasma membrane preparations has been a major hindrance in determining the carbohydrate composition of the plant plasmalemma.

Cytoskeletal structures in the plant cell

As mentioned earlier, animal and plant plasma membranes are similar in appearance by electron microscopy, and by the presence of similar amounts of protein and lipid in this structure (30). In animal cells, the plasma membrane has been further characterized in terms of mobile proteins, which can diffuse throughout the lipid bilayer. Some of these proteins are also believed to interact with cytoskeletal structures inside the cell (50-52). Receptor-cytoskeletal complexes have been postulated to be involved in regulation of cell shape, movement, and differentiation (53). These complexes are thought to consist of a metastable, tripartite structure:

a) certain surface receptors which bind to external ligands; b) microfilaments and associated proteins which are responsible for systematic movement of receptors; and c) microtubules which are responsible for reversible anchorage of receptors. This assembly is known as the cytoskeleton of the cell.

Microfilaments are mainly composed of actin. There are several forms of this protein (α, β, and γ) which are identified by their isoelectric points (54). α actin is found exclusively in muscle cells, while β and γ actins are present in all non-muscle cells (54). Finally, monomeric, or G-actin, can undergo polymerization to filamentous or F-actin. The product of this conversion can be readily observed by electron microscopy, particularly in the presence of heavy meromyosin which yields arrowhead decorated microfilaments (55). For reviews on microfilaments see references (56-59).

Microtubules are long, hollow structures of 25 nm in diameter (for reviews see references 60,61). The main protein component is tubulin which is composed of two related subunits, α and β tubulin. These subunits exist in a one to one ratio and it is thought that tubulin is an $\alpha\beta$-heterodimer. Free tubulin and microtubules are at equilibrium in the cell and this equilibrium is sensitive to various drugs such as colchicine (53), which leads to

disassembly of microtubules.

A number of cytoskeletaal proteins and structures have been identified in plant cells. Actin has been found in many plant cells and tissues. Metcalf et al. (62) have identified a protein with some of the biochemical features of actin from extracts of soybean seedlings. Pesacreta et al. (63) have demonstrated the presence of F-actin in conifer roots using fluorescently labeled phallicidin, which binds specifically to F-actin (64). Evidence for the presence of actin in plant cells has also been provided by the decoration of microfilaments with heavy meromyosin, which can be observed under the electron microscope (55). These complexes have been observed in soybean (65) and the green algae Mougeotia (66),among many plant tissues. More recently, Shah et al. (67) have reported the complete nucleotide sequence of a soybean gene that encodes actin. The deduced amino acid sequence revealed similarities with muscle and cytoplasmic actin at the N-terminus of the protein molecule.

Microfilaments have been implicated in cell movement (53,68) and their abundance has been determined to be higher in elongating cells. Parthasarathy and Muhlethaler (69) examined elongating cells of twelve species of plants and found that microfilament structures were present at the periphery of these particular cells and they were

oriented parallel to their longitudinal axis. Metcalf et al. (65) found an increased concentration of actin in radicles and root tips of soybean seedlings and plants as compared with the petioles and cotyledons.

Light microscopy observations of plant cells have demonstrated cytoplasmic movements of chloroplasts and other granules. Evidence indicates that this cytoplasmic streaming is also associated with the microfilament network (68). In the algae Nitella, bundles of actin filaments, each filament about 5 nm in diameter were located parallel to the direction in which the streaming occurs (70). When cytochalasin B, a drug which disrupts microfilaments (53), was added to the system, the cytoplasmic streaming was stopped (71). In the algae Vaucheria, localized illumination of the cell resulted in the migration of the chloroplasts towards the illuminated area along with the formation of a network of actin filaments (72).

Microtubules, the other major component of the cell cytoskeleton, have been found in the meristematic cells of Allium cepa L. by immunofluorescence staining with antibodies against porcine brain tubulin (73). Isolation of microtubules from Vigna seedlings and fern sperm flagella has also been reported (61). More recently, the isolation of the protein subunit tubulin from suspension

cultures of tobacco (Nicotiana tabacum) cells was reported (74).

In the plant cell, most microtubules are located in the cortex of the cell with the same orientation as the cellulose microfibrils that are being deposited in the cell wall. Since, in most plants, cellulose synthesis occurs at the cell surface, it is thought that the microtubules may play a role in guiding the assembly of such microfibrils (75). Other important activities which are guided by cortical microtubules include the movement of chromosomes to the poles of the dividing cell (76), and the formation of the pragmoplast fibers which control the deposition of the cell plate after cell division (76).

In addition, soybean cells are known to have mobile receptors facing the outside of the plasma membrane (49). Lectins have been demonstrated to bind to soybean protoplasts and the mobilities of the lectin-receptor complexes have been measured using the technique of fluorescence recovery after photobleaching. Soybean agglutinin, concanavalin A, and wheat germ agglutinin yielded different values of lateral mobility: SBA and Con A receptors had overall diffusion (D) values of $5x10^{-11}$and $7x10^{-11}cm^2$ /sec, whereas WGA receptors were faster with a D value of $3x10^{-10}cm^2$ /sec. An interesting fact was that pretreatment of the protoplasts with SBA or Con A reduced

the mobility of WGA 6-fold. This result suggested that binding of a lectin to a population of receptors could alter the mobility of other cell surface receptors. Furtther experiments (77) implicated a role for the cytoskeleton in this effect. The addition of colchicine, a drug which impairs cytoskeletal functions by depolymerizing microtubules, partially reversed the modulation by SBA. When lumicolchicine, a derivative of colchicine that does not bind microtubules (78) was added, there was no reversal of the modulation. These results suggested that binding of ligands to receptors on the plasma membrane of plant cells can affect the distribution of cytoskeletal structures.

In summary, the components of the cytoskeletal network have been identified in the plant cell. Interactions of the cytoskeleton with mobile plasma membrane receptors may lead to the transformation of stimuli into biochemical responses inside the cell. Microtubules and microfilaments have also been shown to play a role in the movement and guidance of a number of cell organelles and structures in plant cells.

MATERIALS AND METHODS

Immunization and Hybridoma Cell Cultures - The SB-1 line of soybean (Glycine max) cells was kindly provided by Dr. G. Lark (Department of Biology, University of Utah, Salt Lake City, UT) and were grown in suspension cultures as previously described (49). Cellulase and pectinase were used to prepare protoplasts (49); fluorescence microscopy after Calcofluor staining and scanning electron microscopy showed neither the characteristic fluorescence indicative of cell wall material (79) nor cellulose microfibrils (80), respectively.

Protoplasts (5×10^5) were suspended in phosphate-buffered saline (PBS) and were mixed and emulsified with an equal volume of Complete Freund's adjuvant (Difco, Detroit, MI). The suspension was injected intraperitoneally into male Balb/c mice. Three booster injections were given at one week intervals with the same number of cells in Incomplete Freund's adjuvant. Three days after the final booster injection, a mouse was sacrificed. The serum was tested for antibodies directed against the SB-1 cells by one of the primary screening assays (see below) and the splenocytes were used for fusion with the myeloma cell line NS-1-Ag 4/1 (Cell

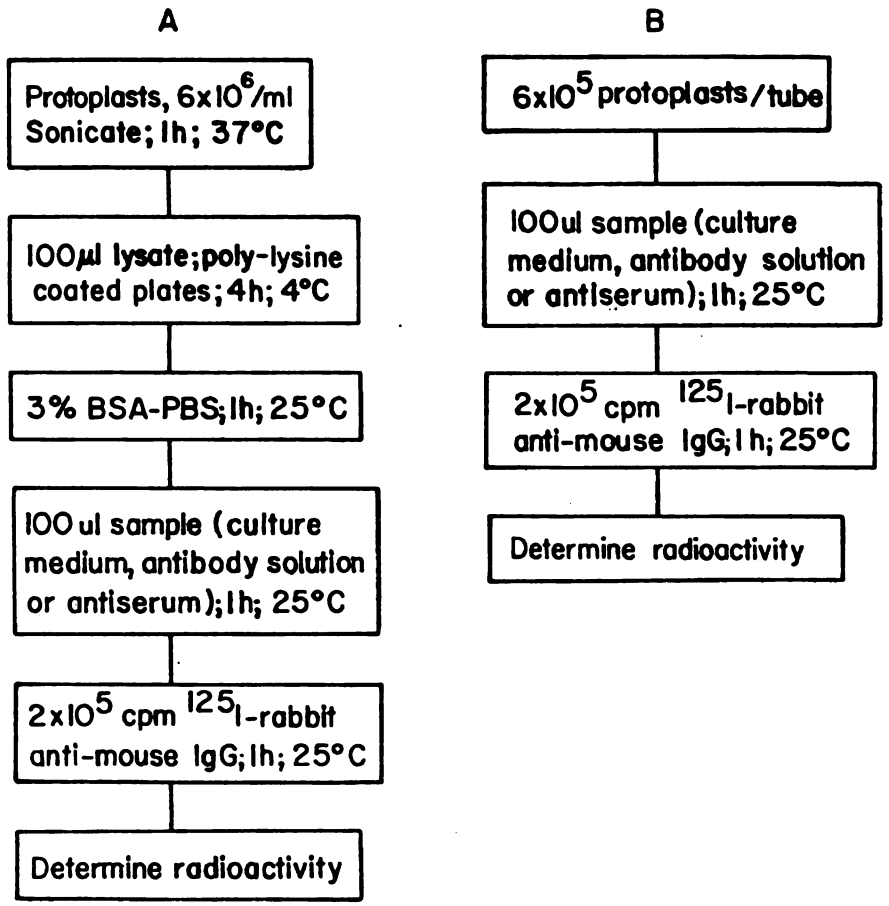
Distribution Center, Salk Institute, La Jolla, CA).

The fusion was carried out with polyethylene glycol 1500 (81), at a 10:1 ratio of immune spleen cells to myeloma cells. Hybridoma cultures were selected and cultured as described (81). Hybridoma cultures showing positive reaction in one of the screening assays (see below) were cloned by limiting dilution (82).

Screening Assays for Hybridoma Cultures Producing Antibodies Against SB-1 Protoplats - Two different assays, designated (a) "solid phase" binding assay and (b) "whole cell" binding assay, were developed to screen for hybridoma cultures producing antibodies directed against SB-1 protoplasts. These two assays are schematically diagrammed in Figure 1 and will be described briefly below.

(a) "Solid phase" binding assay: The procedures for this assay were adapted from a similar radioimmunoassay for actin detailed previously (65). Soybean protoplasts (6×10^6/ml) were frozen at -80°C, thawed, and then placed in a waterbath sonicator for 1 h; the resulting suspension was termed cell lysate. Polyvinyl microtitre plates (96-well, Costar, Rochester Scientific. Rochester, NY) were coated in sequence with 100 ul of each of the following materials (i) poly-L-lysine (100 ug/ml in 10 mM Tris, pH 7.2; Miles Laboratories, Elkhart, IN), 3 h at

Figure 1: Schematic diagram illustrating the key steps in the two screening assays used to detect hybridoma cultures producing antibodies directed against SB-1 protoplasts. (A) "solid phase" binding assay. In some assays, the target antigen which was deposited in the microtitre wells consisted of the whole cell lysate. In other assays, specific fractions derived from the whole cell lysate were used as the target antigen. (B) "whole cell" binding assay. In some assays, the incubation was carried out in 50 mM Tris, 0.5 mM $CaCl_2$, 10% (w/v) sorbitol, pH 7.5, which preserved the intactness of the protoplasts. In other assays, phosphate-buffered saline, which resulted in broken cells, was used.


A
Protoplasts, 6x10^6/ml
Sonicate; 1h; 37°C
100µl lysate; poly-lysine coated plates; 4h; 4°C
3% BSA-PBS; 1h; 25°C
100 ul sample (culture medium, antibody solution or antiserum); 1h; 25°C
2x10^5 cpm 125I-rabbit anti-mouse IgG; 1h; 25°C
Determine radioactivity
B
6x10^5 protoplasts/tube
100ul sample (culture medium, antibody solution or antiserum); 1h; 25°C
2x10^5 cpm 125I-rabbit anti-mouse IgG; 1h; 25°C
Determine radioactivity

room temperature; (ii) cell lysate, 4 h at 4°C; and (iii) bovine serum albumin (BSA; 3% (w/v) solution in PBS), 1 h at room temperature. After steps (i) and (iii), the wells were incubated at room temperature for 1 h with 100 ul of the solution to be tested for the presence of mouse antibodies against SB-1 cells (hybridoma culture supernatant solution or immune serum). The binding of the mouse antibody was quantitated by ^{125}I-labeled (26) rabbit anti-mouse immunoglobulin (Miles Laboratories) (100 ul, 2 x 10^5 cpm, 1 h at room temperature). After each incubation with antibody, the wells were washed three times with 0.3% (w/v) BSA in PBS. The polyvinyl plates were cut into individual wells and the amount of radioactivity bound to each well was determined in a gamma counter.

In some experiments, various fractions derived from cell lysates were tested for the presence of the antigenic target of the monoclonal antibody by a variation of the "solid phase" binding assay described above. The fraction to be tested was deposited in wells of Immulon-2 plates (Dynatech, Alexandria, VA) and the binding of the monoclonal antibody was quantitated following the same procedure as above, starting at step (iii), the addition of BSA in PBS.

(b) "Whole cell" binding assay: Protoplasts (100 ul

of a 6 x 10^5/ml suspension) were incubated at room temperature for 1 h in glass tubes (6 x 50 mm) containing 100 ul of the solution to be tested for the presence of mouse antibodies against SB-1 cells. The buffer used for this incubation was either (i) 50 mM Tris-HC1, 0.5 mM CaC1 , 10% (w/v) sorbitol, pH 7.5 (Buffer A), which preserved the osmolarity and intactness of the protoplasts or (ii) PBS, which resulted in some broken cells. The cells were washed three times by centrifugation (100 g, 5 minutes) and resuspension in equal volumes of 0.3% (w/v) BSA in the appropriate buffer. The amount of mouse antibody bound to the SB-1 protoplasts was quantitated by ^{125}I-labeled rabbit anti-mouse immunoglobulin (100 ul, 2 x 10^5 cpm, 1 h at room temperature). The cells were again washed four times by centrifugation and resuspension. The amount of radioactivity bound to the cell pellet was determined.

Isolation and Characterization of Monoclonal Antibody from Clone MVS-1

Monoclonal antibody produced by clone MVS-1 was isolated from the supernatant solution of culture medium by affinity chromatography on a column (2.1 x 1.1 cm) of agarose derivatized with goat anti-mouse immunoglobulin (Sigma). The column was equilibrated with 10 mM phosphate buffer (pH 8.0). The bound murine monoclonal antibody was eluted with 0.1 M citrate buffer (pH 3.0).

Alternatively, monoclonal antibody from clone MVS-1 was isolated from ascites fluid obtained from mice bearing the hybridoma tumor. For ascites tumors, Balb/c mice were primed with Pristane (Aldrich, Milwaukee, WI) by intraperitoneal injection. Three days after the last injection, 3 x 10^6 cells of clone MVS-1 were inoculated in the peritoneal cavity (82). Ascites fluid from tumor-bearing mice was clarified by centrifugation (10,000 g, 5 min). The majority of the non-immunoglobulin proteins were precipitated by adding Rivanol (Sigma; 0.65 ml of a 3% (w/v) aqueous solution per 1 ml of ascites fluid)(83). After centrifugation (12,400 g, 15 min, 4°C), the supernatant solution was made 5% (w/v) in NaCl and passed through a Whatman filter (No. 2). Solid ammonium sulfate was then added to the filtrate until 40% saturation at 25°C. After centrifugation (12,400 g, 15 min, 4°C), the pellet was dissolved in water and then was subjected to precipitation (45% ammonium sulfate) and centrifugation as above. The pellet was dissolved in water and dialyzed against buffer before use. The immunoglobulin fractions were identified by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) (Sigma) (84) and by the screening assay for antibodies directed against SB-1 protoplasts as described above.

Binding of Antibody MVS-1 to SB-1 Protoplasts

Antibody MVS-1 was labeled with ^{125}I (Amersham, Arlington Heights, IL) using the iodogen, chloroglycoluril (26). The specific radioactivity of the resulting preparation was 6.5 x 10^8 dpm/mg. Protoplasts (3 x 10^4) were incubated at room temperature for 1 h with ^{125}I -labeled Antibody MVS-1 (0.25-50 ug/ml). The cells were washed four times by centrifugation (100 g, 5 min) and resuspension in equal volumes of 0.3% (w/v) BSA in Buffer A. After transfer to a fresh tube, the amount of radioactivity bound to the cell pellet was determined in a gamma counter and the data was analyzed by the method of Scatchard (85).

For agglutination assays, soybean protoplasts (3 x 10^4 cells/ml) were incubated at room temperature with 150 ug/ml of Antibody MVS-1 in glass tubes with gentle agitation. Agglutination was scored after 30 min visually under an Olympus inverted microscope. To test the specificity of the agglutination reaction, normal mouse immunoglobulin was tested in parallel.

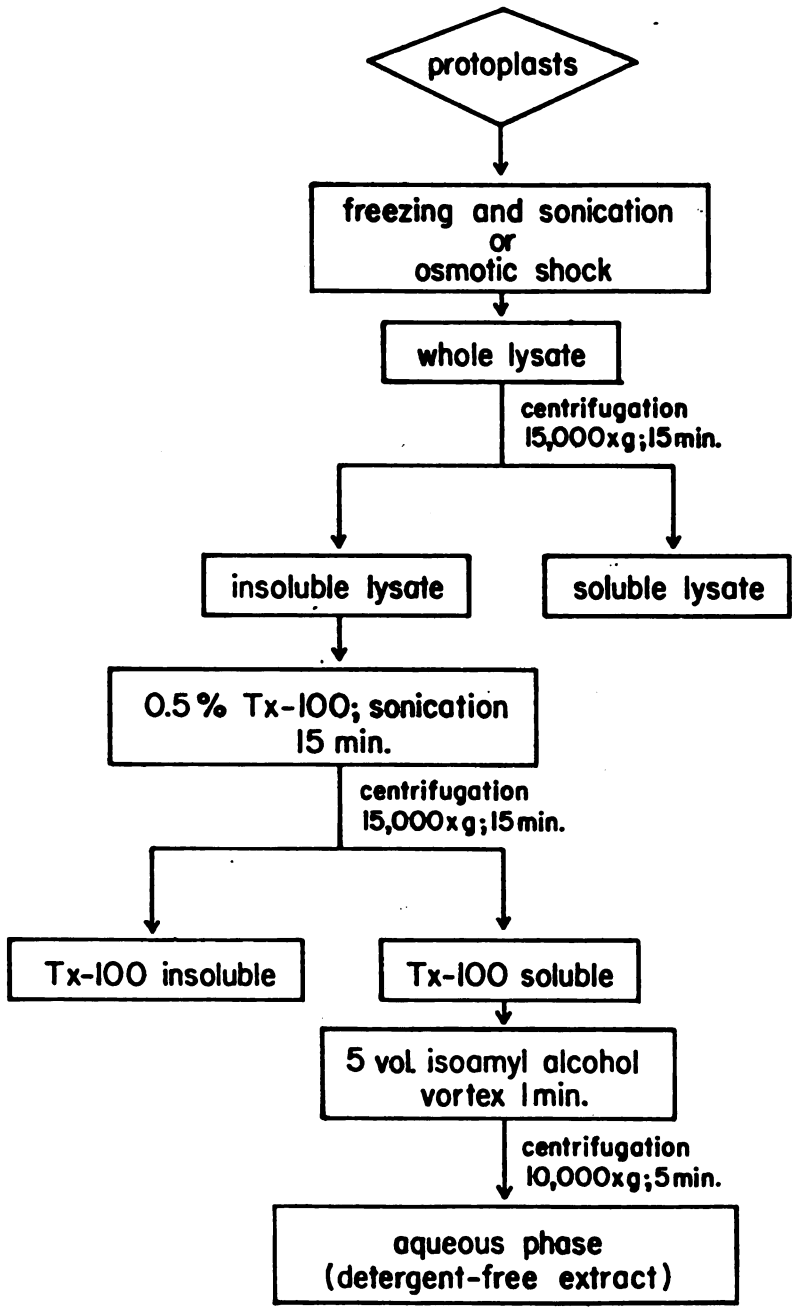
To test whether intact SB-1 protoplasts can absorb monoclonal antibody from culture supernatant of cloned hybridoma cells, clone MVS-1 and the parent NS-1 myeloma cells were labeled by culturing in medium containing [^{35}S]methionine (Amersham) (0.1 $mC_i/10^6$ cells) and no unlabeled methionine for 12 h. The supernatants from the

clone MVS-1 and NS-1 cultures were collected and yielded 10^5 cpm/ml and 2 x 10^3 cpm/ml, respectively, of radioactive proteins precipitable by 3% (v/v) trichloracetic acid. Protoplasts (10^5 cells) were incubated for 1 h at room temperature in Buffer A containing equal amounts of radioactivity (2 x 10^4 cpm) derived from the supernatant of clone MVS-1 or NS-1 cultures. The cells were washed with Buffer A by centrifugation (100 g, 5 min) and resuspension. Radioactivity bound to the protoplasts was released with 0.1 M citrate, 10% (w/v) sorbitol, pH 3.0. After 30 min at room temperature, the protoplasts, which showed no lysis when examined under the light microscope, were pelleted by centrifugation (100 g, 5 min). The radioactive proteins in the supernatants were precipitated by trichloracetic acid and subjected to polyacrylamide gel electrophoresis and fluorography. The above protocol was modified from a similar one previously used to demonstrate that the binding of a ligand to cell surface receptors can be used to enrich the relative proportion of that protein out of a mixture (86).

Fractionation of Lysates From SB-1 Protoplasts

Several fractionation steps were carried out (Figure 2): (a) separation of whole cell lysate into soluble and pellet fractions by centrifugation at 15,000 g for 15

Figure 2: Schematic diagram illustrating the fractionation steps carried out on lysates of SB-1 protoplasts.


protoplasts
freezing and sonication
or
osmotic shock
whole lysate
centrifugation
15,000xg;15min.
insoluble lysate
soluble lysate
0.5% Tx-100; sonication
15 min.
centrifugation
15,000xg;15min.
Tx-100 insoluble
Tx-100 soluble
5 vol. isoamyl alcohol
vortex 1min.
centrifugation
10,000xg;5min.
aqueous phase
(detergent-free extract)

min; (b) extraction of the pellet fraction of cell lysate with Triton X-100 (Research Products Int., Elk Grove, IL; 50 mM potassium phosphate, 0.13 M KCl, pH 7.5 (Buffer B) containing 0.5% (v/v) TX-100 with sonication at 25°C for 15 min followed by centrifugation at 15,000 g for 15 min) to yield TX-100 soluble and TX-100 insoluble fractions; and (c) extraction of the TX-100 soluble fraction with 5 volumes of isoamyl alcohol to yield the aqueous and organic phases (87). All fractions were tested for Antibody MVS-1 binding activity by the "solid phase" binding assay. The organic phase contained the detergent and was discarded. The aqueous phase ("antigen-enriched" fraction), contained the antigenic target of Antibody MVS-1.

Identification of the Antigenic Target of Antibody MVS-1

To identify the molecule bound by Antibody MVS-1, two different radiolabeling procedures were carried out on the proteins of SB-1 cells. First, SB-1 cells were cultured in $^{35}SO_4^{2-}$ (New England Nuclear, Boston, MA; 0.5 mC_i per 50 ml culture) for 72 h. Protoplasts were prepared, lysed and fractionated as described above to yield a ^{35}S-labeled preparation of the "antigen-enriched" fraction (aqueous phase after isoamyl alcohol extract). The ^{35}S-labeled "antigen-enriched" fraction was subjected to immunoaffinity purification.

The immunoaffinity isolation procedure was modified from that reported previously (88). Antibody MVS-1 or normal mouse immunoglobulin was added (12 ug in 100 ul) to individual wells of an Immulon-2 plate and incubated at 4° C for 24 h. The wells were then flooded with a solution of 3% (w/v) BSA in Buffer B for 2 hours at room temperature, followed by washing three times with a 0.3% (w/v) BSA-Buffer B solution. The ^{35}S-labeled or ^{125}I -labeled "antigen enriched" fraction (100 ul) was added and incubated overnight at 4° C. After washing seven times with 0.3% (w/v) BSA in Buffer B, the radioactivity bound to the individual Immulon-2 wells was extracted with 100 ul of buffer containing SDS (0.1% (w/v)) and subjected to gel electrophoresis. Alternatively, the "antigen-enriched" fraction was chromatographed on a Biogel A 1.5 column (Bio Rad Laboratories, Richmond, CA; 64 x 1 cm) equilibrated at 4° C with: (a) Buffer B, (b) 0.5% (v/v) TX-100 in Buffer B, and (c) 10 mM β -mercaptoethanol in Buffer B. Individual fractions were tested for binding of Antibody MVS-1 by the "solid phase" binding assay.

Distribution of the Antigenic Target of Antibody MVS-1 in Soybean Seedlings

Soybean [Glycine max (L.) Merr. var. Amsoy 71] seedlings were prepared as previously described (65).

Root, stem, leaves, and cotyledons were homogenized in 10 mM imidazole-HCl buffer, pH 7.5, with a mortar and pestle and filtered through cheesecloth. Samples, with equal amounts of protein, were deposited in poly-L-lysine derivatized microtitre plates, and the binding of monoclonal antibody was quantitated by the "solid phase" binding assay as described starting from step (iii).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in SDS was performed according to Laemmli (84) with running gel 0f 5-16% (w/v) and stacking gel of 4% (w/v) acrylamide. Samples were dissolved in 0.1% (w/v) SDS, 0.01% (v/v) β -mercaptoethanol and 60 mM Tris, pH 6.8, and boiled for 3 min. After electrophoresis, the gels were fixed for 30 min in 10% (v/v) trichloroacetic acid and stained with Coomassie Brilliant Blue. After destaining, the gel was dried and in certain cases, radioactive bands were detected by fluorography using Kodak X-Omat R (XAR-5 film (89) or by autoradiography using XRP-5 film (90).

Fluorescence Redistribution After Photobleaching (FRAP)

Antibody MVS-1 or monovalent Fab fragments (82), were derivatized with morpholinorhodamine isothiocyanate (Research Organics, Cleveland, OH) (2). All steps were done at 4° C. The sample was dialyzed against (a) 0.15 M NaCl for 1 day with 3 changes of buffer; (b) 0.05 M

bicarbonate-buffered saline (1.96 g Na_2CO_3, 2.66 g $NaHCO_3$, 8.8 g NaCl per liter) pH 8.5, for 5 h; (c) 0.05 M bicarbonate-buffered saline, pH 9.2, for 2 h. The sample was incubated with a 30 fold molar excess of dye (5 mg/ml in dimethylsulfoxide) with respect to protein, for 16-18 h in the dark. The reaction was terminated by addition of glycine to a final concentration of 0.1 M, followed by dialysis against PBS with 0.1 M glycine, pH 7.2, overnight. Unincorporated, free dye was removed by gel filtration on a Sephadex G-25 column (35 x 1.2 cm) equilbrated in PBS. The fluorescently derivatized material was concentrated using a Centricon microconcentrator (30,000 molecular weight cut-off; Amicon Corp., Danvers, MA) and stored at -20°C.

Protoplasts were prepared for photobleaching experiments by the following procedure: (a) protoplasts (10^6/ml in Buffer A) were incubated with the fluorescent probe (final concentrations for the intact immunoglobulin (Ig): 50,100, 278 ug/ml; for Fab: 440 ug/ml) for 1 hr at room temperature; (b) the protoplasts were washed three times by centrifugation (410 g for 4 min) and resuspension in 1 ml of Buffer A; (c) after the final wash, the cells were resuspended in 50 ul of Buffer A. Slides and samples were prepared as previously described (49).

The technique of FRAP (91) was used to measure the

lateral mobility of the fluorescently-labeled antibody-antigen complex in the plasma membrane of protoplasts. The experimental optics and electronics have been described in detail elsewhere (92). Briefly, a Leitz Ortholux II fluorescence microscope, equipped for incident illumination, is used to focus an argon laser beam (diameter 1 um) onto the sample and collect the fluorescence for detection and subsequent processing by photon-counting electronics. The angular orientation of the incident laser beam, and hence the location on the sample of the focused spot along the scan axis, is controlled by a servo-activated galvanometric optical scanning mirror. Morpholinorhodamine emission was monitored using an incident wavelength of 514.0 nm in combination with a Leitz TK580 dichroic mirror and a K570 barrier filter. All measurements were made with a X40/0.65 NA dry objective. The analysis of the redistribution of fluorescence after a localized photobleaching pulse was done by a normal-mode analysis (91).

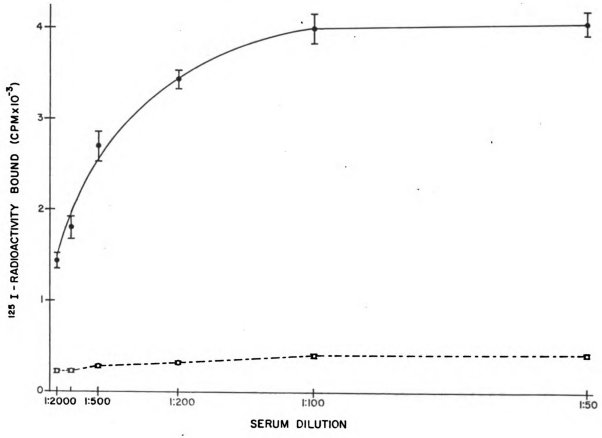
RESULTS

Hybridoma Cultures and Monoclonal Antibodies Reactive with Soybean Protoplasts

Mice were immunized *in vivo* with protoplasts derived from SB-1 cells and their sera were tested for antibodies directed against the immunnogen with the two assays outlined in Figure 1. The serum collected from the mouse, whose spleen cells were subsequently used for fusion, showed strong reactivity in the screening assay using the "whole cell" binding assay (Figure 3). Over the entire concentretation range tested, this primary screening assay can clearly distinguish normal mouse (preimmune) serum from serum containing antibodies directed against SB-1 protoplasts. Similar results were obtained when the other screening assay was used, in which antibodies were tested for their binding to cell lysates of SB-1 protoplasts immobilized on plastic plates (see Figure 1A).

The spleen cells of this particular immune responsive mouse were fused with NS-1 myeloma cells and hybridoma cultures that produce antibodies reacting with SB-1 protoplasts were detected by the same primary screening assays. Representative results from the "whole cell" binding assay, carried out on the supernatant

Figure 3: Dose-response curve of immune (●——●) and preimmune (o----o) sera in the assay of antibody activity directed against SB-1 protoplasts. The experiments were carried out using the "whole cell" binding assay in phosphate buffered saline described under Materials and Methods. Concentrations are expressed as dilutions of the sera obtained directly from the mice before and after immunization. The data represent the averages of triplicate determinations ($\pm$ SEM).


^{125}I - RADIOACTIVITY BOUND (CPM x 10^{-3})
4
3
2
1
0
1:2000
1:500
1:200
1:100
1:50
SERUM DILUTION

solution from several cloned cultures, are shown in Figure 4. Several possitive clones were found; these exhibited 1.6-2.0 fold greater binding activity as compared to that seen with the supernatant from the parental myeloma NS-1 cell line. Essentially the same results were obtained using the "solid phase" binding assay except that in this case, clone 21 showed no binding activity compared to the NS-1 controls and was therefore a negative clone by this assay.

Isolation and Characterization of Antibody MVS-1

Clone 1 was one of the earliest clones established in the monoclonal antibody screening program and several lines of evidence indicated that it secreted an antibody reactive with the outer surface of the protoplast plasma membrane (see below). This culture (named 1MV) was recloned by limiting dilution and all the subclones were tested for antibody binding activity in the "whole cell" binding assay. At least five subclones showed binding activity significantly above the NS-1 control (Figure 5). One subclone, designated MVS-1, was used for all subsequent experiments. Cells of clone MVS-1 were cultured in defined medium (82). Ammonium sulfate (40%) precipitation of the supernatant of clone MVS-1 cells in defined medium yielded antibody activity in the primary screeninng assays. This fraction was subjected to

Figure 4: Representative results obtained using the "whole cell" binding assay to screen hybridoma cultures producing antibodies directed against SB-1 protoplasts. The supernatant solution from the parent myeloma line, NS-1-Ag 4/1, was used as a control. The data represent the averages of triplicate determinations ($\pm$ SEM).

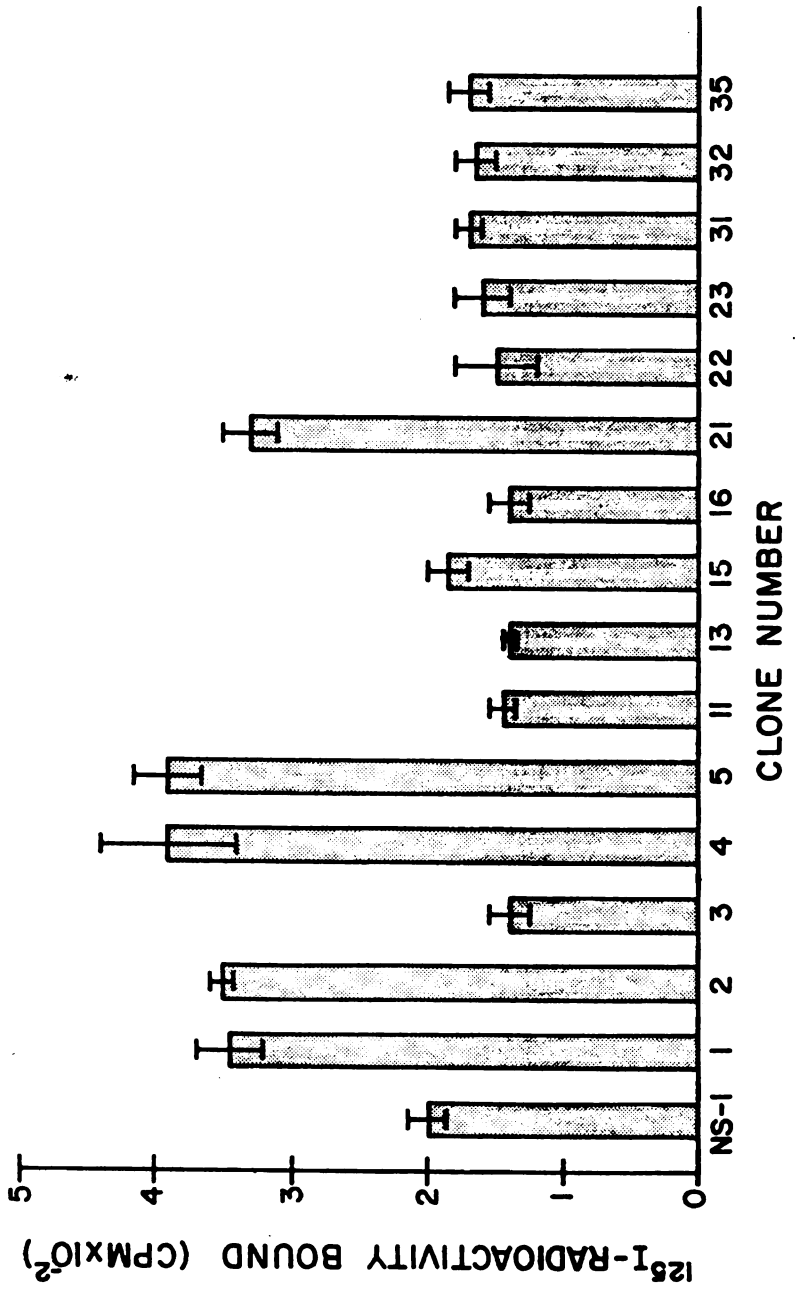
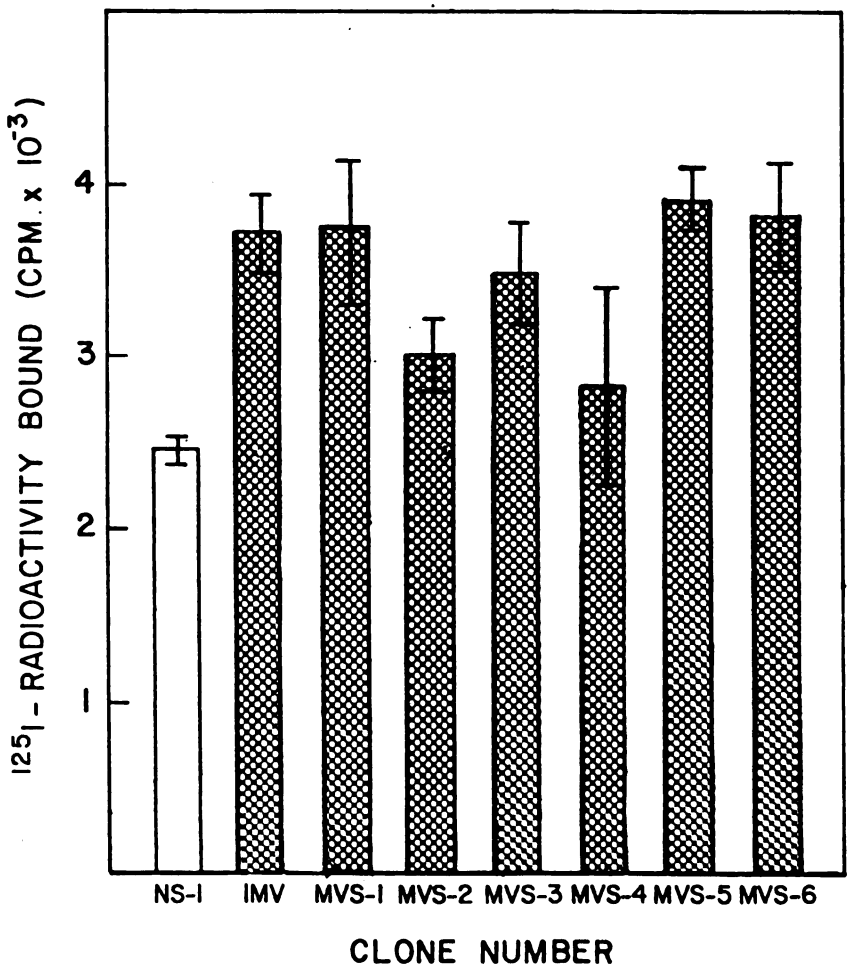

^{125}I-RADIOACTIVITY BOUND (CPMx10^{-2})
0
1
2
3
4
5
NS-1
1
2
3
4
5
11
13
15
16
21
22
23
31
32
35
CLONE NUMBER

Figure 5: Representative results obtained using the "whole cell" binding assay to screen subclones from Clone 1MV. NS-1-Ag 4/1 cell culture supernatant solution was used as control. The data represent the averages of triplicate determinations ($\pm$ SEM).


^{125}I - RADIOACTIVITY BOUND (CPM x 10^{-3})
1
2
3
4
NS-1
IMV
MVS-1
MVS-2
MVS-3
MVS-4
MVS-5
MVS-6
CLONE NUMBER

polyacrylamide gel electrophoresis in SDS. In the presence of β-mercaptoethanol, this material showed two polypeptides (M_rs of 55,000 and 23,000) (Figure 6b).

We have also metabolically labeled cultures of clone MVS-1 with [^{35}S]methionine. Analysis of the radioactive polypeptides in the supernatant of these labeled cultures by gel electrophoresis also showed that the predominant polypeptides were the heavy and light chains of immunoglobulin (M_rs of 55,000, and 23,000). These results indicate that the product of clone MVS-1 was a mouse IgG molecule.

Evidence for a Cell Surface Target of Antibody MVS-1

Several lines of evidence indicated that the target antigen of Antibody MVS-1 is located on the outer surface of the SB-1 cell plasma membrane. First, SB-1 protoplasts were incubated with supernatants derived from [^{35}S]methionine-labeled cultures of clone MVS-1 and NS-1 cells. After washing, the bound radioactivity was re-extracted and analyzed by SDS gel electrophoresis in the absence of β-mercaptoethanol. One predominant band was recovered from SB-1 protoplasts incubated with the hybridoma supernatant, with a M_r=150,000 Figure 7). These results suggest that the protoplast can bind and selectively enrich the immunoglobulin molecule relative to other polypeptide(s) present in the supernatant of the ^{35}S

Figure 6: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of immunoglobulin derived from hybridoma clone MVS-1. The immunoglobulin was isolated from clone MVS-1 cultured in defined medium (8). The composition of the running gel consisted of a gradient in acrylamide (5-16% (w/v))and the gel was stained with Coomassie Blue. (a) Electrophoresis in the presence of β-mercaptoethanol. (b) Electrophoresis in the absence of β-mercaptoethanol. The arrows indicate the positions of migration of molecular weight markers: myosin (212,000); bovine serum albumin (68,000); aldolase (40,000); and myoglobin (17,800).

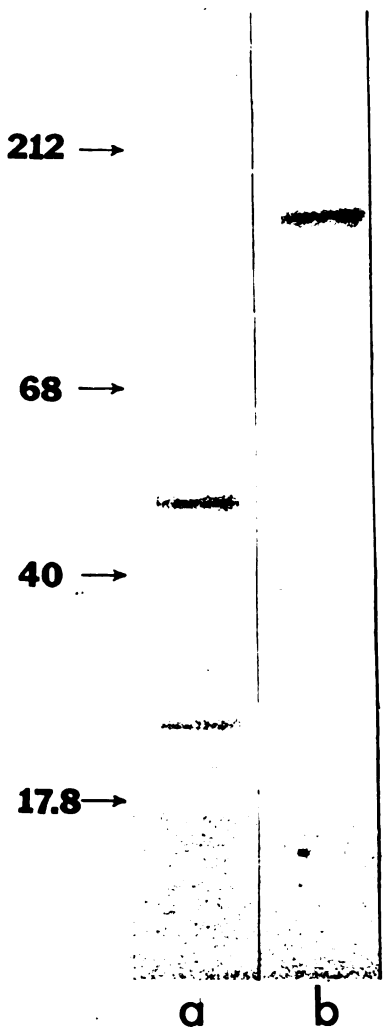
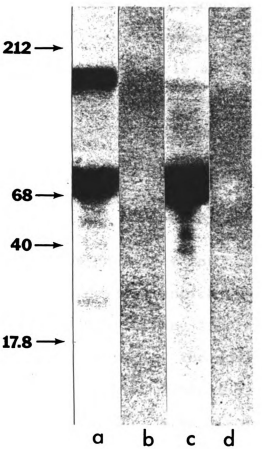

212
68
40
17.8
a
b

Figure 7: Polyacrylamide gel electrophoretic analysis of the polypeptide composition of the supernatant of clone MVS-1 before and after binding to SB-1 protoplasts. (a) Supernatant of [^{35}S]methionine labeled clone MVS-1 before incubation with SB-1 protoplasts. (b) [^{35}S]Methionine labeled polypeptide(s) from clone MVS-1 supernatant that was bound and reextracted from SB-1 protoplasts. (c) Supernatant of [^{35}S]methionine labeled NS-1 cultures before incubation with SB-1 protoplasts. (d) [^{35}S]Methionine labeled polypeptide(s) from NS-1 supernatant after incubation and extraction from SB-1 protoplasts. Protoplasts (10^5 cells) were incubated for 1 h at room temperature containing equal amounts of radioactivity (2×10^4 cpm) derived from the supernatant of MVS-1 or NS-1 cultures. The electrophoresis was carried out on samples containing 1000 cpm of radioactivity in polyacrylamide gels (5-16% (w/v) in acrylamide composition) in the absence of β-mercaptoethanol. The arrows indicate the positions of migration of molecular weight markers.


212
68
40
17.8
a
b
c
d

-labeled cultures. Second, purified Antibody MVS-1 can agglutinate the protoplasts (Figure 8). In contrast, normal mouse immunoglobulin failed to agglutinate the same cells. Third, ^{125}I-labeled Antibody MVS-1 bound to intact SB-1 protoplasts with a dose-response curve that showed saturation (Figure 9). Analysis of the binding data by the method of Scatchard (85) indicated that there were at least 1.5 x 10^6 antigenic sites per protoplast exposed at the cell surface (Figure 9, inset).

Fractionation and Enrichment of the Antigen in Lysates of SB-1 Protoplasts

A fractionation procedure for lysates of SB-1 protoplasts was developed to enrich for the antigenic target of Antibody MVS-1 (Figure 2). At each step of the fractionation, the presence of the antigenic target was tested using the "solid phase" binding assay (Figure 1A). When the cell lysate of SB-1 protoplasts was centrifuged to yield supernatant and precipitate fractions, Antibody MVS-1 bound only to the precipitate (Table 6). The precipitate fraction of the cell lysate was further partitioned by extraction with TX-100. Both the material solubilized by TX-100 and the TX-100 insoluble material contained some antigenic activity. When the TX-100 soluble fraction was further extracted with five volumes of isoamyl alcohol to remove the detergent, the aqueous

Figure 8: Agglutination of SB-1 protoplasts by monoclonal Antibody MVS-1. (a) Antibody MVS-1 (150 ug/ml). (b) Control mouse immunoglobulin (150 ug/ml). Protoplasts (3 x 10^4 cells/ml) were incubated with the antibody for 30 min at room temperature in 50 mM Tris, 0.5 mM $CaCl_2$, 10% (w/v) sorbitol, pH 7.5.

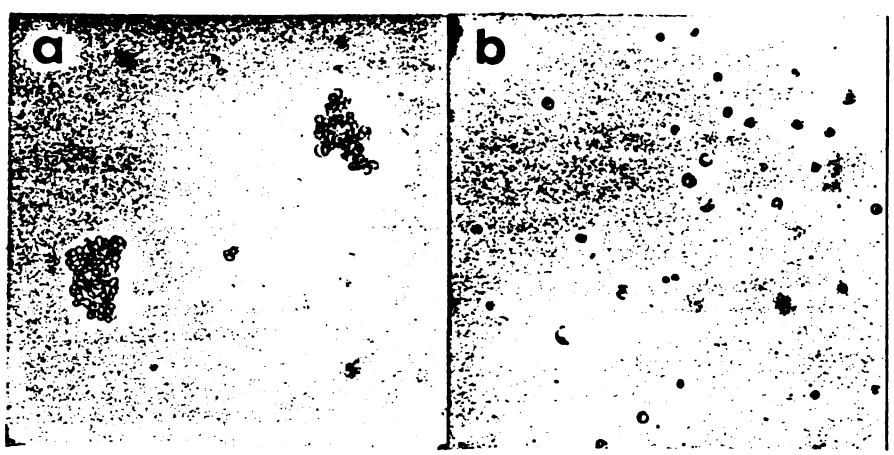

a
b

Figure 9: Concentration dependence of the binding of ^{125}I-labeled Antibody MVS-1 (6.5×10^8 dpm/mg) to SB-1 protoplasts. The experiment was carried out in 50 mM Tris, 0.5 mM $CaCl_2$, 10% (w/v) sorbitol, pH 7.5, at room temperature. The data represent the averages of triplicate determinations (± SEM). The inset represents the analysis of the binding data by the method of Scatchard (13). The straight line was fitted by the least squares method, which yielded a correlation coefficient of 0.66.

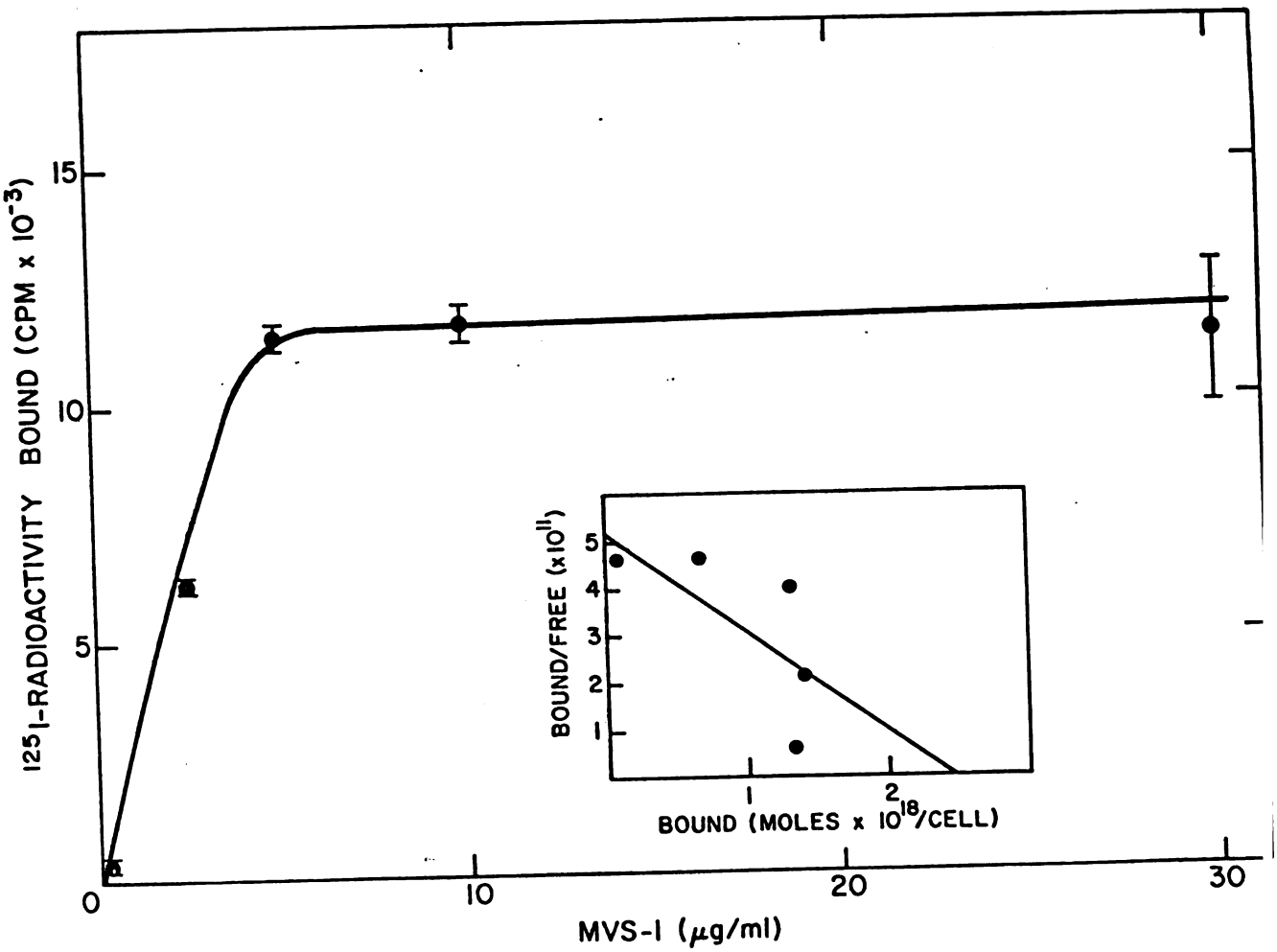

^{125}I-RADIOACTIVITY BOUND (CPM x 10^{-3})
15
10
5
0
10
20
30
MVS-I (μg/ml)
BOUND/FREE (x10^{11})
5
4
3
2
1
1
2
BOUND (MOLES x 10^{18}/CELL)

TABLE 6

Binding of Antibody MVS-1 to Various Fractions of Lysates of SB-1 Protoplasts

Sample	Specific Binding[1] (cpm/ug protein)
Cell lysate	308±46
Lysate pellet	1963±335
Lysate supernate	17±2
TX-100 insoluble fraction	285±84
TX-100 soluble fraction	559±63
Aqueous phase of isoamyl alcohol extract	1343±329

[1]The samples were deposited in wells of microtitre plates and the binding of Antibody MVS-1 was quantitated by the "solid phase" binding assay described in Materials and Methods. The data are expressed as specific binding (cpm of Antibody MVS-1 bound minus cpm of normal mouse immunoglobulin in bound) and are normalized to the amount of protein in the samples bound on the mocrotitre wells. The data represent the averages of triplicate determinations (±SEM).

phase showed and enrichment of 2.3-2.4 fold in terms of specific binding activity (Table 6). These results are consistent with the notion that the antigenic target was a polypeptide component of a membrane fraction of SB-1 protoplasts.

Identification of the Antigenic Target of Antibody MVS-1

To identify the molecule bound by Antibody MVS-1, SB-1 cells were cultured in the presence of $^{35}SO_4^{2-}$ and protoplasts were prepared, lysed, and fractionated to yield a ^{35}S-labeled preparation of "antigen-enriched" fraction (aqueous phase after isoamyl alcohol extraction). When this labeled fraction was subjected to immunoaffinity purification and SDS gel electrophoresis, one predominant polypeptide band (M_r=40,000) was observed in samples purified by Antibody MVS-1 but not in control samples (Figure 10; Table 7). These results suggest that the antigenic target recognized by Antibody MVS-1 was a protein (M_r=40,000).

The "antigen-enriched" aqueous phase derived from unlabeled SB-1 protoplasts was subjected to gel filtration on a Biogel A 1.5 column. When the presence of the antigenic target in each of the individual fractions eluting from the column was quantitated by the "solid phase" binding assay, we found a single peak of activity (Figure 11a). The position of elution of this material,

Figure 10: Polyacrylamide gel electrophoretic analysis of the antigenic target of Antibody MVS-1 after immunoaffinity isolation. SB-1 cells were cultured in $^{35}SO_4^{2-}$ to label the cellular components. The cells were lysed and fractionated; the aqueous phase of the isoamyl alcohol extract was subjected to immunoaffinity isolation with Antibody MVS-1 bound to Immulon-2 wells. The radioactive material bound on the wells was analyzed on polyacrylamide gels (5-16% (w/v) in acrylamide) in the presence of β-mercaptoethanol. The arrows indicate the positions of migration of molecular weight markers. (a) ^{35}S-labeled material bound and extracted from Immulon-2 wells coated with normal mouse immunoglobulin. (b) ^{35}S-labeled material bound and extracted from Immulon-2 wells coated with Antibody MVS-1. Approximately 900 cpms were electrophoresed in each lane.

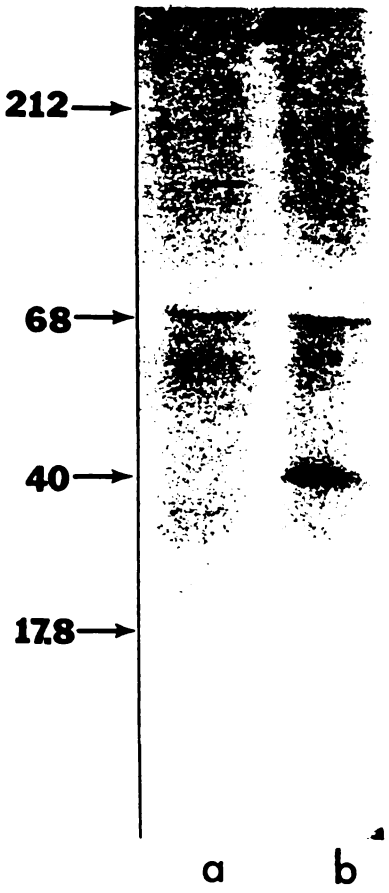

212
68
40
17.8
a
b

TABLE 7

Molecular Weight of the Antigenic target of Antibody MVS-1 under Different Conditions

Detergent	Reducing Agent	M_r
SDS	β - Mercaptoethanol	40,000
TX-100	β - Mercaptoethanol	400,000 + 30,000
TX-100	none	400,000
none	none	400,000

in the absence of any detergent or reducing agent, corresponded to a M_r of 400,000 (Figure 11a; Table 7). Detergent alone (TX-100) did not produce any change as only a 400,000-dalton peak was observed (Figure 11b; Table 7). When 10 mM β-mercaptoethanol, either with or without TX-100, was added in the buffer, smaller peaks were found in addition to the 400,000-dalton peak (Figure 11b; Table 7). These results strongly suggest that the antigenic target of Antibody MVS-1 formed high molecular weight aggregates, either with itself or with other types of molecules.

Distribution of the Antigenic Target of Antibody MVS-1 in Soybean Seedlings

Homogenates from different organs of soybean seedlings were diluted into aliquots containing equal concentrations of protein. When these aliquots were bound to microtitre plates and tested for Antibody MVS-1 binding activity, all samples showed reactivity to a similar extent (1.4-1.7 fold more binding than NS-1 controls) (Figure 12). Equal concentrations of protein from lysates of SB-1 protoplasts were also tested and the level of reactivity was comparable to that obtained with the homogenates. These results indicate that the antigenic target of Antibody MVS-1 was not restricted to cultured soybean cells but was ubiquitous in all parts of the

Figure 11: Chromatographic characterization of the antigenic target of Antibody MVS-1. The aqueous phase of the isoamyl alcohol extract derived from SB-1 protoplasts was chromatographed on a column (64 x 1 cm) of Biogel A 1.5 equilibrated at 4°C with: (a) Buffer B; (b) Buffer B containing 0.5% (v/v) Tx-100, and (c) Buffer B containing 10 mM β-mercaptoethanol. Fractions were assayed for the presence of the antigenic target by the "solid phase" binding assay. This binding is expressed on the ordinate scale as the ratio of the binding of MVS-1 immunoglobulin to the binding of normal mouse immunoglobulin as detected by [^{125}I]rabbit anti-mouse immunoglobulin. The arrows indicate the positions of elution of molecular weight markers: A-blue dextran; B-apoferritin (480,000); C-immunoglobulin G (150,000); D-bovine serum albumin (68,000); and E-myoglobin (17,500). V_o and V_t are the exclusion and total volumes of the column, respectively.

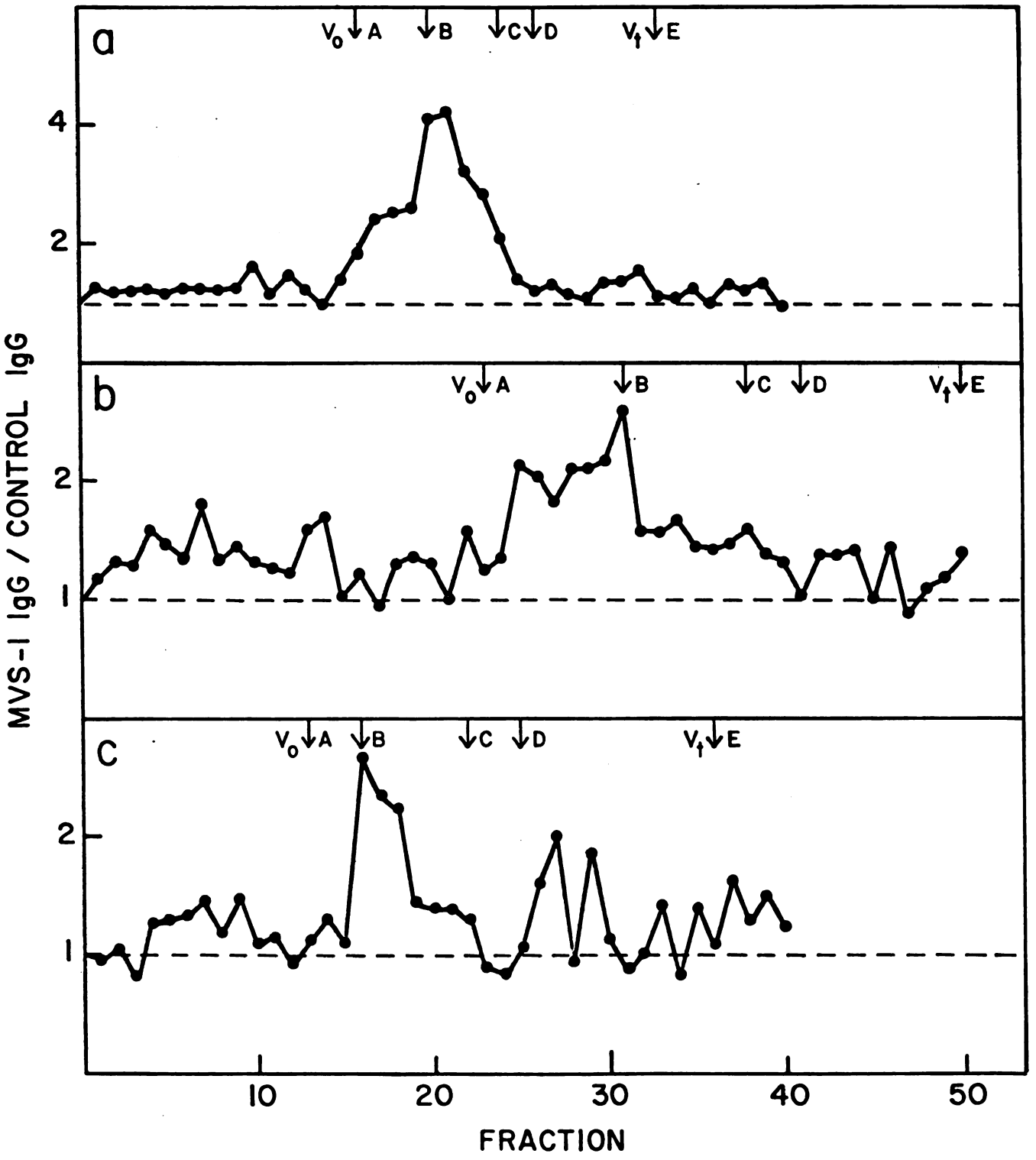
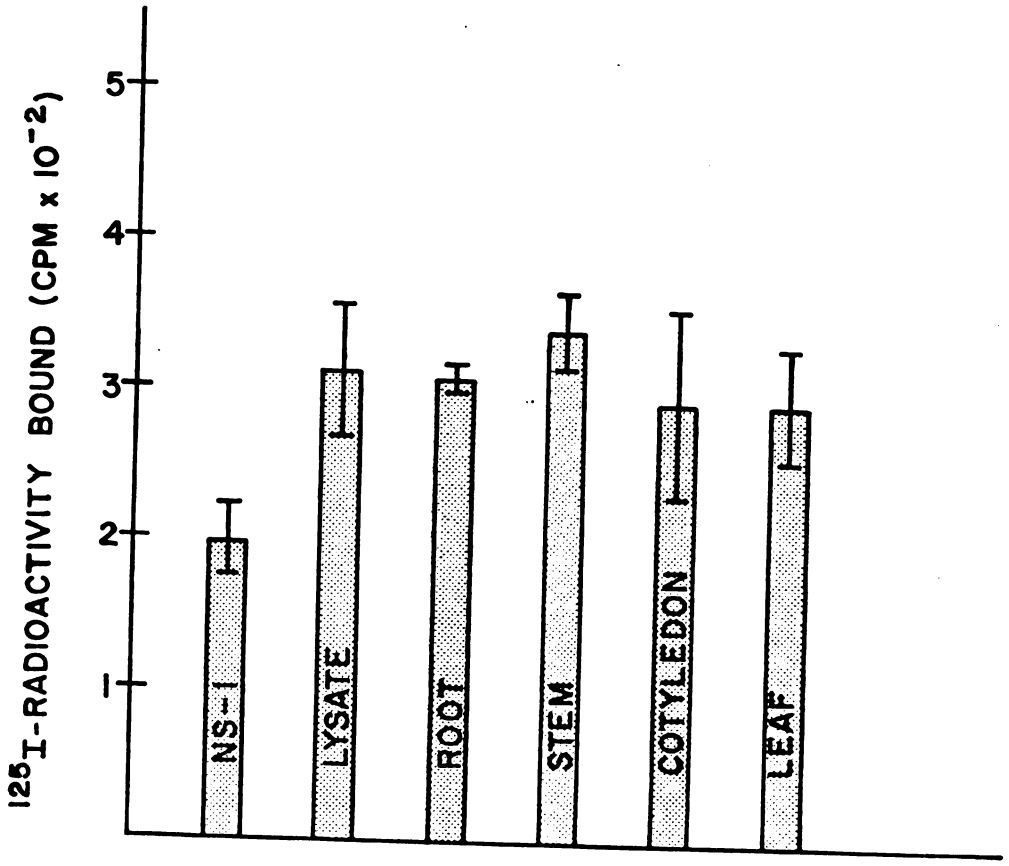

a
b
c
V_0
A
B
C
D
V_t
E
4
2
1
MVS-1 IgG / CONTROL IgG
10
20
30
40
50
FRACTION

Figure 12: Distribution of the antigenic target of Antibody MVS-1 in soybean seedlings. SB-1 lysate and homogenates from organs of the seedlings were tested by the "solid phase" binding assay. The value of NS-1 control is the average of the controls tested for each organ. The data represents averages of triplicate determinations ($\pm$SEM).


^{125}I-RADIOACTIVITY BOUND (CPM x 10^{-2})
1
2
3
4
5
NS-1
LYSATE
ROOT
STEM
COTYLEDON
LEAF

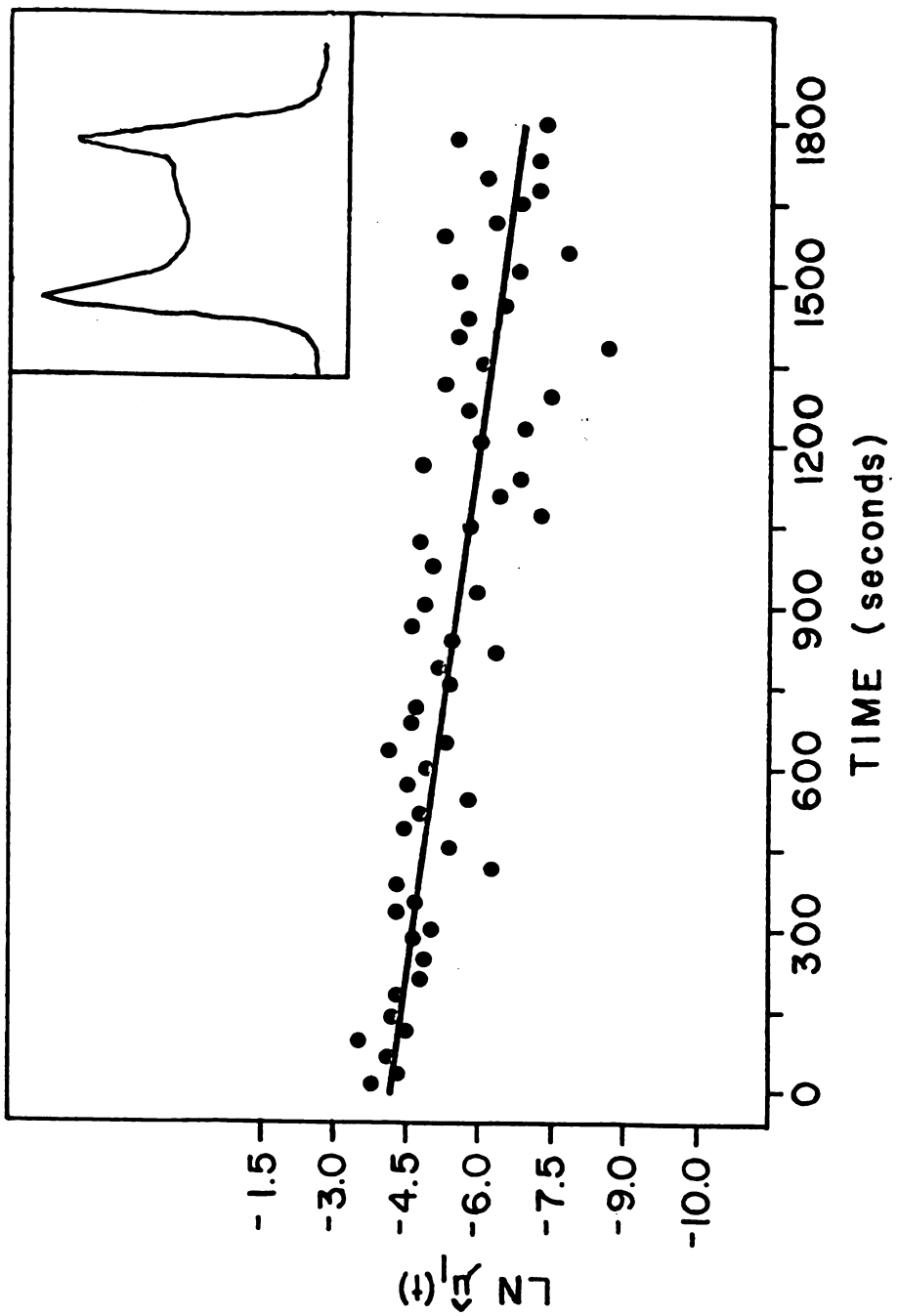
germinating seed as well.

Lateral Mobility of Antibody MVS-1 Bound on SB-1 Protoplasts

To test whether Antibody MVS-1 could be used as a probe for measuring the mobility of the antigenic target on the cell surface, it was labeled with rhodamine isothiocyanate. This derivative bound to the SB-1 protoplasts with a diffuse distribution over the membrane as observed by fluorescence microscopy. A fluorescence scan across a labeled protoplast is shown in the inset of Figure 13. The two peaks of fluorescence demonstrate that the fluorescent antibody is bound predominantly at the plasma membrane, giving more intense fluorescence at the edges of the cell. Data from a representative photobleaching experiment is plotted in Figure 13. The semilogarithmic plot describes the time course of the fluorophore concentratrion distribution after the photobleaching pulse. Each point represents a complete fluorescence scan across the cell. The data from this and other experiments did not show any deviations from the initial slopes of the semilogarithmic plot; therefore, the data was analyzed using a single exponential decay.

The diffusion coefficient calculated from the data plotted in Figure 13 (100 ug/ml immunoglobulin) is 2.2 x 10^{-10} cm^2/sec. This D value is similar to that obtained

Figure 13: Semilogarithmic plot of $\mu_1(t)$ (experimental estimate of the normalized first moment of the fluorophore concentration distribution) as a function of time after photobleaching on a SB-1 protoplast labeled with morpholinorhodamine Antibody MVS-1. D= 1.1 x 10^{-10} cm^2/sec. The inset shows a scan across the protoplast membrane prior to the photobleaching pulse.


-1.5
-3.0
-4.5
-6.0
-7.5
-9.0
-10.0
LN $I_2^1(t)$
0
300
600
900
1200
1500
1800
TIME (seconds)

for WGA receptors (3.0×10^{-10} cm^2/sec.) (49). When photobleaching experiments were done using non saturating concentrations (50 ug/ml), or saturating concentrations (100 ug/ml) of MVS-1 immunoglobulin, there were no significant variations in D values (see Table 8). These results suggest that any crosslinking by immunoglobulin molecules, at non-saturating concentrations, does not lead to the formation of large immobile patches. The diffusion coefficient of monovalent Fab fragments was also determined, and was similar to that obtained for the intact molecule above saturation (see Table 8).

TABLE 8

Diffusion Coefficients of intact immunoglobulin and Fab fragments of Antibody MVS-1 Bound to SB-1 Protoplasts at 20°C

Antibody MVS-1 (ug/ml)	condition	D^1 ($\times 10^{-10}$ cm^2/sec)	% Recovery[1]
50	IgG	3.5 ± 1.6	62 ± 23
100	IgG	2.2 ± 1.3	61 ± 35
278	IgG	3.5 ± 2.1	52 ± 24
440	Fab fragment	3.0 ± 1.1	70 ± 15

[1]Values are expressed as mean ± standard deviation

DISCUSSION

The experiments reported here document that we have successfully generated several different hybridoma clones, each of which secretes an immunoglobulin directed against protoplasts of the SB-1 cell line. A key requirement for the success of this program was the development of rapid and yet reliable assays for screening the supernatant solutions of hybridoma cultures. We have used two differents screening assays in parallel throughout our initial screening procedure: (a) the "solid phase" binding assay; and (b) the "whole cell" binding assay.

Each assay offered its distinct advantages as well as drawbacks. For example, the "solid phase" assay was quick and required small amounts of material. However, it is by no means certain that all proteins were fixed onto the microtitre plates; those antigenic molecules which failed to bind to the "solid phase" in sufficient amounts would obviously not play a role in detecting hybridomas that may secrete monoclonal antibodies against them. This may be an explanation for the observation that hybridoma clone 21, which was positive in the "whole cell" binding assay, failed to yield a positive result in the "solid phase" binding assay. On the other hand, the "whole cell" binding

assay was more tedious to carry out and would not be practical for screening a large number (>100) of hybridoma cultures.

Using a combination of these two assays, we have obtained monoclonal antibodies directed against the outer surface of protoplasts as well as certain ones specifically localized intracellularly. For example, preliminary evidence indicates that clone 5 produces an antibody directed against actin (M_r= 45,000), whose isolation from soybean cells we had reported (62). This observation is consistent with the finding that the immune serum, obtained by in vivo immunization of mouse with soybean protoplasts, showed antibody activity against calf thymus actin in the "solid phase" actin binding radioimmunoassay (65).

Clone MVS-1, derived by subcloning clone 1MV, secreted an antibody that binds to the plasma membrane of intact SB-1 protoplasts. This conclusion is based on four independent sets of experiments: (a) agglutination of SB-1 protoplasts; (b) saturable binding of ^{125}I-labeled Antibody MVS-1 to the protoplasts; (c) enrichment through protoplast binding of the immunoglobulin molecule relative to other proteins present in the supernatant of MVS-1 cultures; and (d) fluorescence labeling, by morpholinorhodamine-derivatized Antibody MVS-1, of the

cell, showing distinct edge peaks.

The antigenic target of Antibody MVS-1 was identified, by immunoaffinity isolation techniques, as a polypeptide (M_r=40,000). This polypeptide is probably membrane-derived, a conclusion which is based on the pattern of distribution in a series of preliminary fractionations. The antigen was found in the pellet fraction of SB-1 protoplast lysates and was extractable from this pellet with the detergent TX-100. In contrast, the antigen was not soluble in organic solvents such as chloroform-methanol (unpublished observations) and isoamyl alcohol.

The molecular weight of the antigenic target of Antibody MVS-1 varies, depending on the presence or absence of detergent and/or reducing agent. Our present estimates of the molecular weights under these conditions are summarized in Table 7. In the presence of SDS and β -mercaptoethanol, the molecular weight is 40,000. In contrast, in the absence of any detergent or reducing agent, the antigenic molecule was found a high molecular weight complex (M_r=400,000). Detergent alone did not produce any change in the migration of the peak (M_r =400,000). However, when 10 mM β-mercaptoethanol was included in the buffer, peaks of smaller molecular weight were found along with the 400,000-dalton aggregate,

indicating that, at least in part, dissociation of the antigen is occuring towards smaller subunits. Similar results were obtained when both β-mercaptoethanol and TX-100 were included in the buffer. Whether this antigenic target forms high molecular weight aggregates with itself or with other types of molecules (e.g. polysaccharides) remains to be determined.

The antigen of antibody MVS-1 was found present in all tissues from soybean seedlings (Figure 12). This result demonstrates that the target molecule is not an artifact of the cells in culture, but rather, it is a ubiquitous molecule present in the cells of the plant as well.

Our original motivation for generating a monoclonal antibody, that binds the outer surface of the SB-1 protoplast, was to use it as a probe to measure the mobility of its antigenic target in the plasma membrane. Previous studies, using fluorescent lectins to measure the mobility of receptors on the plasma membrane of SB-1 protoplasts (49), showed that there were two classes of mobile receptors: (a) WGA receptors with a D value of 3×10^{-10} cm^2/sec, which is catalogued as "fast"; and (b) SBA and Con A receptors with D values of 5×10^{-11} and 7×10^{-11} cm^2/sec respectively and, therefore, catalogued as "slow". Pretreatment of the protoplasts with unlabeled SBA or Con

A before measuring WGA receptor mobility resulted in a 6-fold decrease in mobility ($D=5 \times 10^{-11}$ cm^2/sec). Furthermore, when the microtubule disorganizing drug, colchicine, was added, this modulation of mobility was partially reversed (77). Lumicolchicine, which does not disorganize microtubules, did not reverse the effect. These results suggested that the binding os SBA or Con A to the SB-1 protoplast plasma membrane affects the cytoskeleton of the cell, and in turn, modulates the mobility of other receptors.

FRAP measurements showed that the mobility of the receptor of Antibody MVS-1 belongs to the "fast" class ($D=3 \times 10^{-10}$ cm^2/sec). It would be very intriguing if this D value could also be modulated. If this turns out to be the case, the interpretation of modulation would be greatly simplified since there is no heterogeneity of receptors as in the case of lectins. When using lectins as probes, it is not known if the decrease in mobility is due to an increase of slow moving receptors, a decrease of fast moving receptors, or an intrinsic change in the mobility of the receptors, themselves.

It has also become apparent that monoclonal antibodies, such as Antibody MVS-1, could find a number of other practical applications. Of particular significance is the possibility of using the monoclonal antibody to

agglutinate and stabilize the plasma membrane during swelling and hypo-osmotic lysis for the purification of plasma membranes. Schibeci et al. (25) have used the murine myeloma protein J539, a galactose-binding immunoglobulin A, to agglutinate and isolate the plasma membrane from ryegrass protoplasts. A similar approach has been used with the lectin concanavalin A (93).

Previous experiments on the fractionation of plasma membrane and subcellular organelles have used a variety of "markers" (reviewed by Quail (21)): (a) the cytochemical phosphotungstate-chromate stain (94); (b) enzyme markers such as adenosine triphosphatase (95) and glucan synthetase II (36); and (c) specific cell surface labeling (23, 96, 97). Boss and Ruesink (97) have used ^{14}C-labeled concanavalin A to tag the plasma membrane of carrot protoplasts and to follow its fractionation in isopycnic gradient centrifugation. Similarly, Schibeci et al. (25) have used ^{125}I-labeled myeloma protein J539, a galactose-binding immunoglobulin A, to follow membrane purification from protoplasts of ryegrass endosperm cells. Once the antigenic target of Antibody MVS-1 has been mapped with respect to interior and exterior localization, it may also be used to monitor membrane purification from soybean cells (23,98).

Finally, plant lectins have been shown to bind and

agglutinate protoplasts prepared from a number of systems, including the soybean (46, 49, 77). To date, however, the molecular species of the lectin receptors have not been defined. Preliminary studies on immunoblotting extracts of SB-1 protoplasts with labeled lectins indicate that the lectins receptors represent at least a heterogeneous mixture of proteins. It is possible that some of the monoclonal antibodies generated in the present study may be directed against a particular receptor for soybean agglutinin. The availability of such a monoclonal antibody should facilitate the isolation, characterization and subcellular localization of such receptors in the soybean cell.

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