INFLUENCE OF CONCANAVALIN A, WHEAT GERM AGGLUTININ, AND SOYBEAN AGGLUTININ ON THE FUSION OF MYOBLASTS IN VITRO

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ABSTRACT

Although muscle cell fusion was shown to be an energy-requiring process, release of myoblasts from an EGTA fusion block could be accomplished with Earle's balanced salt solution (containing 1.8 mM Ca^{++}) free of glucose or any other energy-producing metabolite.

The effect of concanavalin A, abrin, and the lectins from wheat germ, soybean, and *Lens culinaris* on myoblast fusion was examined with synchronized myoblast cultures upon release from fusion block. At a concentration of 15 μg/ml, these lectins were found to inhibit the fusion process to the extent of 62%, 41%, 32%, 8%, and 19%, respectively. Concanavalin A inhibition could be prevented by α-methyl-D-mannoside. The inhibitory effect of all the lectins except abrin could be reversed by changing to the normal, serum-containing medium. The number of binding sites determined for ^{125}I-labeled concanavalin A, wheat germ, and soybean agglutinins was 3.4×10^7, 6.1×10^7, and 1.7×10^6, respectively. Although myoblasts were found to have about twice as many binding sites for wheat germ agglutinin as for concanavalin A, concanavalin A was determined to be twice as effective as wheat germ agglutinin as an inhibitor of myoblast fusion. These findings raise the possibility that specific cell surface glycoproteins may be an important factor in this process.

Embryonic muscle cells can be grown in culture (11) and, therefore, in vivo myogenesis, which closely corresponds to in vitro myogenesis (9), has become accessible to experimentation by cell culture techniques. Shainberg et al. (33) established an absolute requirement for Ca^{++} in the process of myogenesis and they (34), as well as Paterson and Strohman (28), succeeded in synchronizing the process by manipulation of the Ca^{++} levels in the culture medium. As Bischoff and Holtzer (3) pointed out, the fusion process, which begins the normal chain of events of myogenesis, must involve at least two separate reactions: (*a*) cell recognition (or specific adhesion), and (*b*) subsequent membrane-membrane interaction culminating in cell fusion.

Cell recognition and interactions during the process of morphogenesis are believed to be mediated by specific macromolecular components located on the cell membrane and between cells. It

has been suggested that these components function as the tissue-specific cell ligands and phenotypic determinants (recognition sites) of the cell surface (17, 18, 26). Although very few of these cell aggregation-promoting macromolecules have been identified to date (24, 13, 12), in each case they have proven to be large molecular weight glycoproteins. In recent years, the use of lectins (plant agglutinins) (20) has been introduced as a probe for these surface glycoproteins (for a recent review see reference 27).

The aim of the present work was to use lectins as an interacting probe for glycoproteins which may be involved in critical steps of myogenesis. It will be shown that concanavalin A (Con A) and, to a much lesser extent, wheat germ agglutinin (WGA) inhibit the myogenesis process. The possible reasons for this inhibition and their significance will be discussed.

METHODS AND MATERIALS

Culture Conditions

Cultures of thigh muscle cells from 12-day chicken embryos were prepared essentially as described by Konigsberg et al. (15). The medium consisted of Eagle's MEM, horse serum (Grand Island Biological Co., Grand Island, N. Y.), and chick "embryo extract" (high-speed supernate of a 50% extract of 11-day embryos in Earle's balanced salt solution) in the ratio 16:3:1, containing 1% antibiotic solution (equal parts of penicillin and streptomycin). The tissue was dissociated by treatment with 0.05% of crude collagenase (Worthington Biochemical Corp., Freehold, N. J.) for 10 min. The cells were preplated for 25 min at 37°C to remove fibroblasts which quickly adhere to the dish (39). The cells were then diluted in the culture medium to a concentration of 1×10^6 cells per ml and 0.3 ml of this suspension was added to each 35 mm gelatin-coated dish containing 2 ml of medium. The cultures were then incubated at 37°C in a humidified atmosphere of 5% CO_2-95% air. The medium was replaced once after 24 h.

EGTA Fusion-Blocked Cultures

Fusion-blocked cells were grown under identical conditions except that EGTA (1.9 mM) was added to the medium. When larger quantities of fusion-blocked cells were desired for ^{125}I-lectin-binding studies, the cells were diluted after preplating to a concentration of 1×10^7 cells per ml, and 0.3 ml of this suspension was added to each gelatin-coated 250 ml (75 cm^2) tissue culture flask containing 15 ml EGTA media. The cells were incubated as described above and harvested after reaching confluency (usually about 50 h after plating) as follows: each bottle was rinsed with 5 ml of chelate solution containing (g/liter): EDTA, 0.2; NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.15; KH_2PO_4, 0.2; and glucose, 0.2. Subsequently, 10 ml of the chelate solution were added to each culture flask. After incubation for 30 min at 37°C, the cells, which could then be easily removed by gentle shaking, were washed three times in cold phosphate-buffered saline (PBS), pH 7.0, and were adjusted to the desired concentration (usually 10^8 cells/ml). Since the size of the cells was not uniform, the concentration of cells was usually checked by protein determination; 10^8 cells correspond to 8.5 mg protein. The harvested cells were used immediately for ^{125}I-lectin-binding studies.

Staining and Counting Procedure

The dishes were washed with PBS, dehydrated with ethanol in three steps, 30%, 70%, 100% (10 min each), and stained with 10% aqueous Giemsa for 30 min. The dye was removed thoroughly with H_2O and the surface of the dish was covered with glycerol. Nuclei were counted in randomly selected fields at $\times$ 200 (phase) using an ocular counting grid. Three or more nuclei within the same membrane constituted a positive fusion locus. The degree (percent) of fusion was determined by counting the nuclei within myotubes of three or more nuclei and dividing by the total number of nuclei.

Lectins

Con A and soybean agglutinin (SBA) were very much appreciated gifts from Drs. J. S. Tkacz and H. Lis, respectively. Abrin and *Lens culinaris* lectin were generously supplied by Dr. A. McPherson. WGA was prepared by a combination of the procedures of Le Vine et al. (16) and Marchesi (23). Golden Harvest raw wheat germ obtained locally was washed twice with 2 liters of cold acetone. The resulting powder was dried in air overnight at 4°C and extracted three times, each time with 1 liter of H_2O by stirring for 1 h at 4°C. The material was filtered through cheesecloth after each extraction, and the pooled extract was centrifuged at 13,000 *g* for 15 min. The supernatant liquid was heated to 60°C, kept at this temperature for 10 min, and then cooled. The solid that precipitated was removed by centrifugation at 13,000 *g* for 15 min, and ammonium sulfate was added to the supernatant fluid to a final concentration of 40% saturation. The solution was stirred for several hours at 4°C and the precipitate collected by centrifugation at 13,000 *g* for 20 min was dissolved in H_2O. The resulting aqueous solution was dialyzed against H_2O, and the precipitate that formed during dialysis was removed by centrifugation. The supernatant fluid was dialyzed overnight against 0.25 M NaCl-0.05 M $NaPO_4$ buffer, pH 7.0, and was then applied to a Sepharose 4B-Ovalbumin column previously equilibrated with the same buffer. The column was operated at room temperature and 5-ml fractions were collected. When the A_{280}

reached 0.025, the WGA was eluted with 0.1 N acetic acid. The eluted protein was dialyzed overnight against 0.01 N acetic acid and lyophilized. The resulting product was not homogeneous when subjected to SDS polyacrylamide gel electrophoresis, but the contaminating bands were removed when the chromatography step on the Sepharose 4B-ovalbumin column was repeated.

Iodination of Lectins

The lectins (usually 5–10 mg) were iodinated with ^{125}I by the method of McConahey et al. (25). The excess of ^{125}iodide was removed from Con A by exhaustive dialysis against 1 M NaCl until the ^{125}I count of an aliquot of the dialyzed protein solution corresponded to the count obtained by the method of Mans and Novelli (22), which determines TCA-precipitable protein only. On SDS-polyacrylamide gel electrophoresis, the final product showed the presence of the three customary bands, one major and two minor, corresponding to the intact subunit (mol wt 27,000) and the two smaller chains (mol wt 12,500 and 14,500, respectively) derived by chemical or enzymatic hydrolysis.

WGA was freed from the contaminating ^{125}iodide by reisolating the iodinated protein on the Sepharose 4B-ovalbumin column. The final product showed one band on SDS-polyacrylamide gel electrophoresis.

Iodinated SBA was first exhaustively dialyzed in 0.01 M phosphate buffer, pH 6.8 (starting buffer), then applied to a column of DEAE-cellulose and eluted with a phosphate-NaCl gradient as described by Lis and Sharon (19). SDS-polyacrylamide gel electrophoresis showed one main band and a trace of one of the other three isolectins (19).

By cutting the gels and estimating the ^{125}I content of the fragments, it was determined that the protein bands contained a preponderance of the ^{125}I present in the gels.

Protein Determination

The protein content of the lectins was determined by the method of Warburg and Christian (36) and that of the membranous proteins by Hartree's modification of the method of Lowry et al. (10).

Binding of ^{125}I-Lectins to Cells

The binding reactions were carried out in plastic tubes which had been presoaked overnight in 0.9% NaCl containing 5 mg of bovine serum albumin/ml. The reaction mixtures contained in a total volume of 0.2 ml: 250 μg of bovine serum albumin (BSA) in PBS, 5×10^6 cells (erythrocytes or EGTA fusion-blocked muscle cells), appropriate amounts of ^{125}I-lectin in PBS, and additional PBS to volume. After 30 min of incubation at room temperature the cells were washed twice with 6 ml ice-cold PBS-BSA solution and the amount of bound ^{125}I-labeled lectin was determined in a Packard Auto-gamma counter (Packard Instrument Corp., Downers Grove, Ill.). Appropriate corrections were made for nonspecific binding to the plastic tubes which accounted for less than 5% of the total counts bound.

RESULTS

The use of lectins as probes for the involvement of cell-surface glycoproteins in myogenesis was made possible by our finding that fully supplemented growth medium containing glycoprotein-rich horse serum and chick embryo extract is not necessary for the release of the EGTA fusion arrest. As shown in Table I and Fig. 1 C, as long as the concentration of Ca^{++} was 1.8 mM, the same percentage of nuclei were incorporated into syncytia whether the EGTA medium was replaced by the fully supplemented medium or by Earle's balanced salt solution (BSS). The divalent metals Mg^{++}, Mn^{++}, Ba^{++}, and Sr^{++} could not replace Ca^{++}.

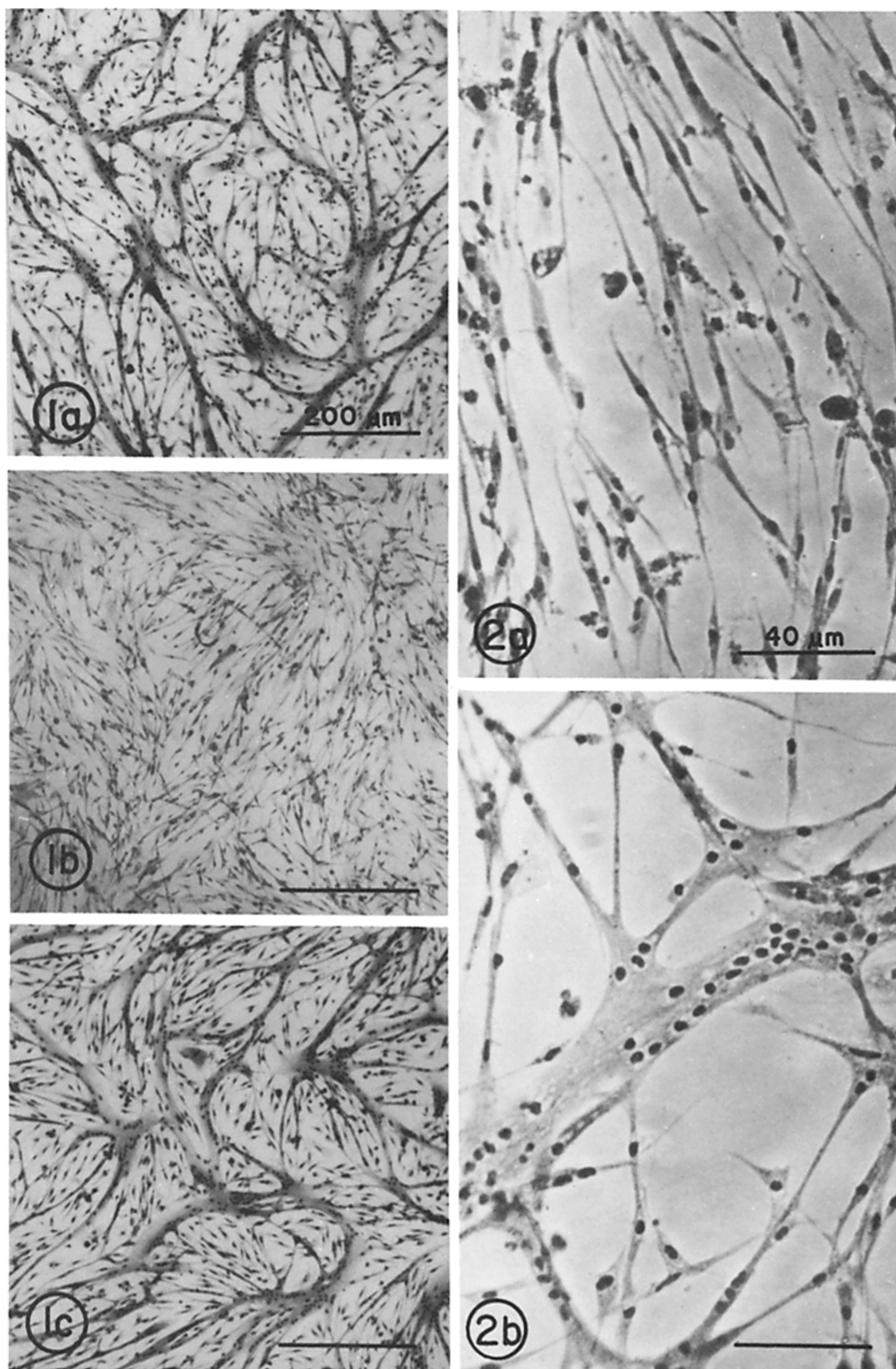
TABLE I
Release of the EGTA-Induced Fusion Arrest

EGTA-replacing Medium	Fusion
	%
Fully supplemented medium	65
Earle's balanced salt solution with 5 mM glucose	68
Earle's balanced salt solution without glucose	68

After 52 h in culture, the EGTA medium was replaced by 2 ml of the specified medium, and the cultures were incubated for 4 h more. The degree of fusion was determined as described in the text.

FIGURE 1 Phase-contrast photomicrographs (× 140) of embryonic chick muscle cells after 56 h in culture. (*a*) Cells grown continuously in culture medium containing 1.8 mM Ca^{++}; (*b*) fusion-blocked culture containing 1.9 mM EGTA· (*c*) 2 ml of Earle's BSS replaced the EGTA medium at 52 h and the culture was incubated for 4 h more.

FIGURE 2 Phase-contrast photomicrographs (× 700) demonstrating Con A effect. After 52 h in EGTA-containing cultures the EGTA medium was replaced by (*a*) 2 ml of Earle's BSS containing 32 μg of Con A/ml and (*b*) 2 ml of Earle's BSS containing 32 μg of Con A/ml and 100 mM methyl-α-D-mannoside.


1a
200 µm
1b
1c
2a
40 µm
2b

Although addition of glucose was not necessary for the process of myogenesis to occur, the presence of metabolic inhibitors in the medium interfered with cell fusion (Table II). The simultaneous addition of 5 mM glucose seemed to lessen the inhibitory effect of the metabolic poisons on myogenesis, except in the case of NaF, which is known to inhibit enolase, an enzyme required for glycolysis. It seems, therefore, that the fusion step in myogenesis is an energy-requiring process. This is also supported by the observation that the myoblasts do not fuse at 18°C, while at 0°C and in an N_2 atmosphere the cells detach from the dishes.

Table III illustrates the effect of lectins on myogenesis. Whereas SBA and *Lens culinaris* lectins had very little effect, Con A at a level of 15 μg/ml significantly reduced the degree of myoblast fusion (see also Fig. 2), while WGA at the same concentration showed substantially less inhibition than Con A. Simultaneous addition of 100 mM α-methyl-D-mannoside completely abolished the inhibitory effect of Con A. As illustrated in Table III, a normal level of cell fusion could be obtained in all lectin-treated cultures except those treated with abrin, by replacing the lectin-containing media with 2 ml of fully supplemented medium. Abrin inhibited myogenesis but also exhibited a lasting cytotoxic effect.

In view of the marked difference in the effect of the individual lectins on myogenesis, it was necessary to establish whether the degree of inhibition of fusion could be correlated with the number of binding sites of a particular lectin per muscle cell. Con A, WGA, and SBA were chosen for this study. After iodination, the number of binding sites for each lectin was determined. We first established the number of binding sites for Con A and WGA on human erythrocytes for which data are given in the literature. Thus, we obtained 1×10^6 Con A binding sites per human erythrocyte,

TABLE II

Effect of Metabolic Inhibitors On Myogenesis

Inhibitor	Concentration	Fusion*	Inhibition	Reversibility of‡ inhibition
	mM	%	%	%
No inhibitor	—	68	0	
NaN_3				
+ glucose	1.0	44	35	85
− glucose	1.0	7	89	
+ glucose	5.0	37	45	66
− glucose	5.0	8	88	
NaCN				
+ glucose	2.0	59	13	94
− glucose	2.0	42	38	
+ glucose	4.0	65	4	95
− glucose	4.0	7	89	
NaF				
+ glucose	2.0	18	73	
− glucose	2.0	26	62	
+ glucose	4.0	10	85	95
− glucose	4.0	16	76	
Dinitrophenol				
+ glucose	0.1	27	60	88
− glucose	0.1	12	82	
+ glucose	0.2	28	59	94
− glucose	0.2	12	82	

After 52 h in culture the EGTA medium was replaced by 2 ml of Earle's balanced salt solution containing the specified amount of a metabolic inhibitor with or without 5 mM glucose, and the cultures were incubated for 4 h more.

* The percent of fusion was determined by counting the nuclei within myotubes of three or more nuclei and dividing by the total number of nuclei.

‡ After 4 h of incubation in the presence of inhibitors, the cultures were rinsed and incubated with 2 ml of fully supplemented media for 6 h more.

TABLE III
Effect of Lectins on Myogenesis

Lectin	Concentration	Fusion	Inhibition	Number of counts averaged to obtain % inhibition	Reversibility* of lectin block
	$\mu g/ml$	%	%		%
No lectin	—	68	0	25	
Concanavalin A	15	26	62	3	94
	30	25	64	10	94
	60	27	60	9	94
	120	24	65	6	94
Concanavalin A plus 100 mM α-methyl-D-mannoside	30	65	4	7	
	60	62	8	2	
WGA	15	46	32	8	93
	30	49	28	8	97
	60	54	21	4	93
	120	43	36	2	
SBA	30	62	8	4	93
	60	62	8	4	90
Lens culinaris lectin	15	55	19	4	94
	30	57	16	4	98
Abrin	10	40	41	2	6
	30	33	51	2	3

After 52 h in culture, the EGTA medium was replaced with 2 ml of Earle's balanced salt solution containing the specified amount of a lectin, and the cultures were incubated for 4 h more. The degree of fusion was determined as described in the text.

* After 4 h of incubation in the presence of lectins, the cultures were rinsed and incubated with 2 ml of fully supplemented media for 6 more h.

well within the range of 1.2×10^6 reported by Schnebli and Bächi (32), 2.2×10^6 reported by Philips et al. (29), and 6.8×10^6 reported by Edelman and Millette for rodent erythrocytes (6). Similarly, our value of 2×10^7 WGA binding sites per human erythrocyte is comparable to the value of 4.9×10^6 reported by Adair et al. (1). Furthermore, our Scatchard plot (31) was monophasic for Con A and biphasic for WGA, in agreement with Schnebli and Bächi (32) and Adair and Kornfeld (1).

Due to the scarcity of iodinated material and the significant nonspecific binding of labeled lectins to solid surfaces, binding studies on EGTA fusion-blocked cells had to be performed after harvesting the cells with chelate solution, as described in Materials and Methods. A possibility of some changes in the cell surfaces under these conditions cannot be excluded. Harvesting was accomplished after the same elapsed time in culture as that before the determination of the inhibitory effects of unlabeled lectins. After harvesting, the PBS-washed muscle cells were adjusted with PBS to a concentration of 10^8 cells/ml, and were used immediately for binding studies.

The Scatchard plots for the three lectins tested shown in Fig. 3 reveal that there are only 1.7×10^6 binding sites for SBA per muscle cell, a figure 20–36 times less than for Con A or WGA. The degree of lectin inhibition of the fusion process of myogenesis, however, does not seem to be solely a function of the number of binding sites per muscle cell. Using a molecular weight for Con A of 110,000 (tetramer), there are twice as many binding sites per muscle cell for WGA (6.1×10^7) as for Con A (3.4×10^7). Assuming that the biphasic

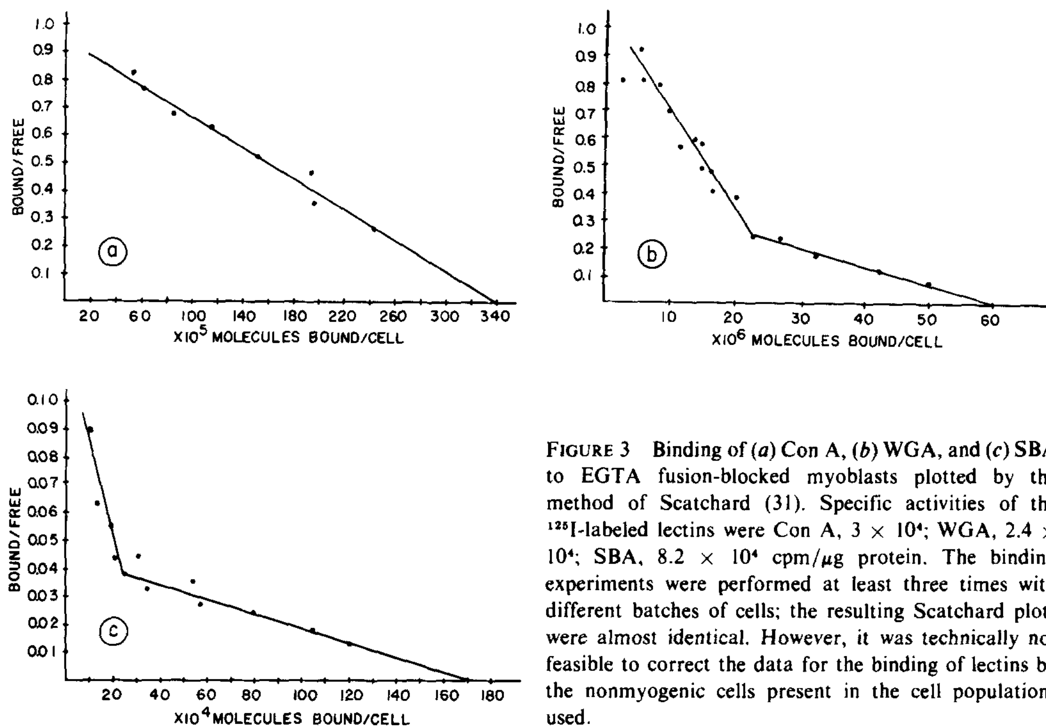


FIGURE 3 Binding of (*a*) Con A, (*b*) WGA, and (*c*) SBA to EGTA fusion-blocked myoblasts plotted by the method of Scatchard (31). Specific activities of the ^{125}I-labeled lectins were Con A, 3×10^4; WGA, 2.4×10^4; SBA, 8.2×10^4 cpm/μg protein. The binding experiments were performed at least three times with different batches of cells; the resulting Scatchard plots were almost identical. However, it was technically not feasible to correct the data for the binding of lectins by the nonmyogenic cells present in the cell populations used.

nature of the Scatchard plot for WGA reflects the existence of two classes of receptors for which the lectin has different affinities, and further assuming that only one type of receptor is involved in that component of WGA binding which inhibits fusion, extrapolation of the first part of the curve to the abscissa yields 2.9×10^7 WGA binding sites per cell, a value about equal to the number of binding sites calculated for Con A (3.4×10^7 sites per muscle cell). However, inhibition of myogenesis by Con A is almost twice as effective on a molar basis as that of WGA.

Regardless of the amount of lectins added to the cultures, inhibition of myogenic cell fusion by Con A or WGA could not be increased past maxima of 60% and 30%, respectively. At the time of addition of lectin to the cultures, there were about 10^6 cells per dish, to which 30 μg of lectins were added (2 ml of a solution containing 15 μg per ml). According to the Scatchard plots, the amount of added lectin is already equal to or in excess of the number of binding sites available on the cells.

It was impossible to obtain a uniform curve showing a functional decrease of inhibition with decreasing Con A or WGA concentration below 15 μg per ml. Lowering the concentration of lectins below 15 μg/ml produced variable degrees of fusion depending on the number of cells per dish.

DISCUSSION

Among the five lectins tested, Con A and WGA reversibly inhibited the fusion process in myogenesis to the extent of 60% and 30%, respectively. We have found that the EGTA fusion-blocked muscle cells have 20–36 times fewer binding sites for SBA, one of the lectins which showed little effect on the process of cell fusion. It is assumed that the same is probably true for the relatively ineffective lectin from *Lens culinaris*. Abrin was prohibitively cytotoxic (Table III). These results raise the question as to whether the lectin-induced inhibition is due to (*a*) the binding of the lectin(s) to specific glycoprotein(s) essential for the fusion process, or (*b*) to nonspecific steric hindrance which results in the prevention of sufficient intercellular contact required for cell fusion. Either or both of these explanations could apply.

The steric hindrance mechanism was suggested by Ludwig et al. (21) and by Rott et al. (30) who found that pseudorabies virus-induced cell fusion of rabbit kidney cells could be prevented by Con A (21) and that Con A was effective in inhibiting

paramyxovirus SV5-induced cell fusion of BHK cells (30). The differential effects of Con A and WGA on myogenesis may be due to the difference in the size of the two lectins: tetrameric Con A has a mol wt of 110,000, whereas WGA's mol wt is 23,500. According to Fambrough (8), well developed myotubes were formed in culture in the presence of α-bungarotoxin, a molecule of mol wt 9,000 which binds specifically and essentially irreversibly to the acetylcholine receptors on the surface of the muscle cells. A partial answer to this question of the role of lectin size might be found by preparing trypsin-fragmented so-called "monovalent" Con A (35, 5) and checking its effect on the cell fusion process of myogenesis.

However, Con A may be more effective as an inhibitor as a result of some property, other than bulk, which WGA lacks. It has been suggested that in Novikoff tumor cells (38) and in mouse leukemia line L1210 (14) the Con A and WGA binding sites reside on different glycopeptides and perhaps on different glycoproteins as well. Also, unlike WGA, Con A is a mitogenic lectin which can induce profound surface and intracellular alterations in lymphoid and perhaps other tissues (7).

Since Con A is known to inhibit specifically other cell fusion processes, such as the fertilization of the sea urchin *Authocidaris crassipina* (after exposure of the sperm to Con A) (2), conjugation of opposite gametes of chlamydomonas (37), and of opposite mating types (strains 5 and 21) of yeast, *Hansenula wingei* (4), the possible role of specific Con A receptor molecules in the fusion process of myogenesis merits further attention.

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