DEVELOPMENTALLY REGULATED LECTIN IN EMBRYONIC CHICK MUSCLE AND A MYOGENIC CELL LINE

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Summary: Soluble extracts of embryonic chick pectoral muscle and myoblast clone L6 agglutinated trypsin treated glutaraldehyde fixed rabbit erythrocytes. Agglutination activity was blocked by thiodigalactoside, lactose and related saccharides but not by many other saccharides. Agglutination activity of chick pectoral muscle extracts increased at least one order of magnitude between 8 and 16 days of chick embryo development, as the pectoral muscle differentiated. With L6 myoblasts there was a three-fold increase in activity of the extracts as the myoblasts fused to form multinucleated myotubes.

Developmentally regulated lectins have been identified as the cellular slime mold Dictyostelium discoideum differentiates from a vegetative form to a cohesive form (1-3). These lectins, assayed as agglutinins of erythrocytes are similar to the well known plant lectins since agglutination activity can be blocked by specific simple saccharides (1-3). Another species of cellular slime mold, Polysphondylium pallidum, also contains at least one developmentally regulated lectin that is discriminable from those from D. discoideum (4,5). Evidence has been presented that these lectins can be detected on the surface of cohesive cells and that they mediate the species-specific cohesion of cellular slime molds by interaction with species-specific cell surface oligosaccharides (1,4,6-8).

Recently, Teichberg et al. (9) have reported that a number of tissues including the electric organ of Electrophorus electricus and muscle contain lectin activity as assayed with trypsinized rabbit erythrocytes. This finding raised the possibility that these lectins have some functional similarity to those in cellular slime molds. As a first step in evaluating this possibility, we determined if the lectin activity in extracts of embryonic chick

muscle and of the differentiating myoblast clone L6 (10) were developmentally regulated. We found that in embryonic chick pectoral muscle extractable lectin activity was present at low levels early in development and rose strikingly with differentiation. A similar but less striking increase in lectin activity was observed with differentiation of L6 myoblasts in culture.

Materials and Methods: Fertilized White Leghorn chicken eggs were obtained from Hen-X and incubated in our laboratory under standard conditions (11). At any given day of development embryo morphology agreed fairly closely with that described by Hamburger and Hamilton (11). Retarded or defective embryos were discarded. A starting culture of L6 myoblasts was obtained from the laboratory of Dr. David Schubert and was cultured in 20% fetal calf serum as described by Schubert et al. (12). Approximately 5 X 10^5 cells were suspended in 120 ml of medium and 15 ml aliquots were innoculated in 100 mm diameter tissue culture dishes. Under these culture conditions cell multiplication continued for at least 2 days; and by 5 days of culture prominent cell fusion was observed. In the ensuing few days large myotubes formed.

Agglutinins from embryonic pectoral muscle were extracted with 9 vol of 0.15 M NaCl, 2 mM EDTA, 2 mM dithiothreitol (this mixture is hereafter called DES) containing 0.3 M lactose. The yield of agglutinin was lower if extracts were prepared in DES without lactose. The extracts were sedimented at 100,000 X g for 1 hr and the supernatant was dialyzed exhaustively against DES for assay of agglutination activity. Similar extraction and dialysis conditions were used with the myoblasts except that the ratio of the volume of DES with 0.3 M lactose to that of the volume of cells to be extracted was about 30:1. Cells were removed from the plate with a rubber policeman after washing 3 times with 0.15 M NaCl and addition of the extraction medium.

Rabbit erythrocytes for agglutination assays were prepared from 50 ml of fresh rabbit blood (collected in Alsever's medium) that had been washed 4 times with 5 vol of 0.15 M NaCl. A 4% erythrocyte suspension (by vol) in 0.1 M Na phosphate, pH 7.4, 0.05 M NaCl containing 1 mg/ml of crystallized trypsin (Calbiochem, Grade A) was incubated at 37° for 1 hr. The trypsin treated cells were then washed 4 times with 5 vol of 0.15 M NaCl and fixed in 5 vol 0.075 M NaK phosphate, pH 7.2, 0.075 M NaCl (hereafter called PBS) containing 1% glutaraldehyde for 1 hr at room temperature. Glutaraldehyde fixation was terminated by addition of 5 vol of 0.1 M glycine in PBS, 7.2, at 4°. The fixed erythrocytes were centrifuged then washed twice in 5 vol of 0.1 M glycine in PBS, 7.2, and then twice in PBS, 7.2. The cells were then stored as a 10% suspension in PBS, 7.2. Fresh rabbit erythrocytes were not agglutinated by the extracts. After trypsinization the cells were agglutinable but could not be kept for long periods of time. With glutaraldehyde fixation the cells could be maintained as stable reagents which could be used for periods of up to one month without significant loss in activity. However, there was considerable variation in the agglutinability of different batches of glutaraldehyde-treated trypsinized rabbit erythrocytes. Therefore, in each of the developmental studies shown, all the extracts were assayed against an identical preparation of fixed erythrocytes. With the embryonic chick muscle studies the extracts from all the time points studied in each experiment were prepared simultaneously and also assayed together. In the studies with L6 myoblasts at least two samples from an early time point and a late time point were prepared simultaneously for concurrent assay; but multiple experiments with the same erythrocyte preparation were combined to give the final result.

Agglutination assays were done in microtiter V plates (Cooke Engineering) using serial two-fold dilutions of the extract in DES. Each well contained

.025 ml of a 4% suspension of glutaraldehyde-fixed trypsin treated rabbit erythrocytes in PBS, 7.2, .025 ml of a dilution of the dialyzed extract, .025 ml of 0.15 M NaCl and .025 ml of 1% bovine serum albumin in 0.15 M NaCl. The fixed erythrocytes were added last after which the plates were shaken vigorously. Agglutination was determined after 90 min. Unagglutinated erythrocytes formed a clear dot on the bottom on the well whereas agglutinated erythrocytes formed a diffuse mat on the bottom of the well. The transition from a dot to an agglutinate was distinct from the end point dilution of extract that still agglutinated the cells to the next dilution of extract. For sugar inhibition studies the sugar in question was dissolved at appropriate concentration in 0.15 M NaCl and added in place of the 0.15 M

Table 1. Effect of saccharides on agglutination activity of extracts from 16 day chick embryo pectoral muscle and 5 day L_6 cultures.

Saccharide	Concentration that inhibits 50% (mM)	
	Chick muscle extract	L_6 extract
thiodigalactoside	.19	.05
α-lactose	.19	.38
α-methyl-D-galactoside	9.4	18.8
β-methyl-D-galactoside	37.5	75
D-galactose	37.5	37.5
α-D-melibiose	37.5	37.5
N-acetyl-D-galactosamine	75	75
α-L-fucose	75	75
α-D-fucose	>75	>75
D-trehalose	>75	75
α-L-rhamnose	>75	>75
D-glucose	>75	>75
α-methyl-D-glucoside	>75	>75
β-methyl-D-glucoside	>75	>75
N-acetyl-D-glucosamine	>75	>75
3-O-methyl-D-glucoside	>75	>75
D-mannose	>75	>75
α-methyl-D-mannoside	>75	>75

NaCl. The sugars used were obtained from commercial sources at the highest available level of purity. Protein was estimated by the method of Lowry et al. (13) after dialysis of the extracts against 0.15 M NaCl.

Results: Extracts of both embryonic chick muscle and L6 myoblasts contained an agglutinin of glutaraldehyde-fixed trypsin treated rabbit erythrocytes. The agglutinins were not dialyzable and agglutination activity was completely destroyed by boiling for 10 min. Dialysis overnight against DES from which dithiothreitol was omitted led to a marked loss of activity.

Agglutination activity of myoblast extracts with titers of 1:32 and embryonic chick muscle extracts with titers as high as 1:2048 were completely inhibited by addition of 0.15 M lactose to the agglutination assays. A number of other saccharides also inhibited the agglutination reaction (Table I). The concentration of saccharides required to inhibit agglutination activity by 50% was quite similar with the agglutinins from embryonic chick muscles and L6 myoblasts, suggesting a great similarity in the binding site of these agglutinins. The spectrum of saccharides that were relatively potent inhibitors of agglutination activity of extracts from these two tissues are quite similar to those observed by Teichberg et al. (9) with extracts from electric organ of Electrophorus.

Agglutination activity of extracts from embryonic chick pectoral muscle showed a striking increase with maturation of the muscle up to about 16 days of development in ovo (Fig. 1A, 1B). Agglutination activity declined thereafter (Fig. 1A). Experiments with two different sets of extracts using two different preparations of glutaraldehyde-fixed trypsin treated rabbit erythrocytes are shown in Fig. 1A and 1B. In both experiments there was a marked increase in the specific activity of the agglutinin between 8 and 16 days of embryonic life. The major change occurred between days 10 and 12 (Fig. 1B). The results are expressed as the reciprocal of the titer divided by the milligrams protein per ml of the dialyzed extract; but similar curves were obtained when activity was expressed per gram (wet weight) of the muscle tissue used for extraction. With the erythrocyte preparation used for the

experiments in Fig. 1A the specific activity of the extracts from 16 day old embryos was about 3500, almost 2 orders of magnitude higher than the specific activity of extracts from the 8 day old embyros concurrently prepared and concurrently assayed. With another preparation of erythrocytes and with another set of extracts, concurrently prepared, specific activity at 16 days of embryonic life was only about 300, about 1 order of magnitude higher than the specific activity of extracts from 8 day old embryos concurrently prepared and assayed (Fig. 1B). The differences between Fig. 1A and Fig. 1B are due to the erythrocytes since we have assayed an extract from 16 day old chick embyros using the erythrocytes used in Fig. 1A and 1B and found specific activities of around 3500 with the former and around 300 with the latter. The difference in agglutinability of the erythrocytes may be due to special characteristics of the erythrocytes from the specific rabbit used in each case, or to slight variations in the trypsinization or the glutaraldehyde fixation conditions

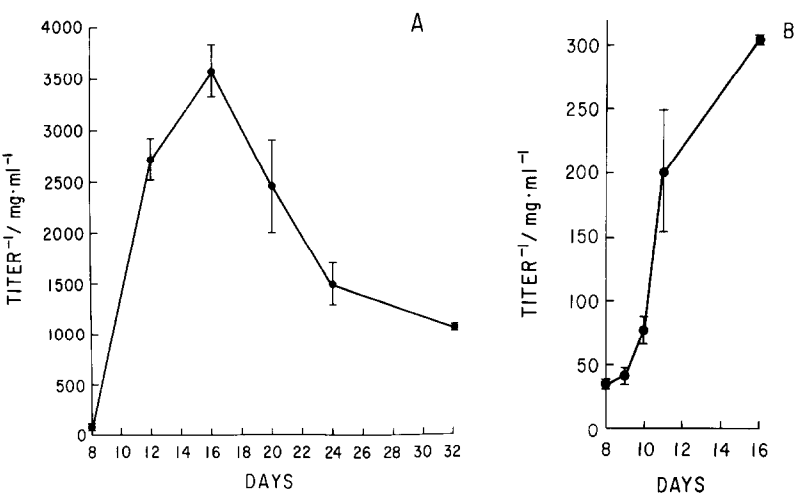

Figure 1. Specific activity of agglutinin in extracts of pectoral muscle of chick embryos or chicks whose incubation had been begun at day 0. Each point is the mean (± S.E.M.) of separate determinations made with 3 different extracts. About 16 embryos were used for each 8 day extract and smaller numbers giving approximately the same total weight of pectoral muscle were used for later time points. All extracts in (A) were prepared and assayed concurrently. A different batch of glutaraldehyde fixed trypsinized rabbit erythrocytes and a different set of extracts was used for the experiment in (B). These extracts were also prepared and assayed concurrently.

that are presently under investigation. Any of these variables could change the nature and the state of aggregation or aggregability of the saccharides on the erythrocyte cell surface with which the agglutinins presumably interact. In all these experiments agglutination could be completely blocked by lactose.

Agglutination activity in extracts from differentiating L6 myoblasts also showed a change with differentiation (Fig. 2). Activity was relatively low with two days of incubation at which time cells were largely dividing myoblasts; and reached a peak after about 5 days of culture, at which time extensive fusion and multinucleated forms were observed. The increase in spontaneous activity of extracts from the differentiating L6 cultures is much less striking than that observed with differentiation of chick pectoral muscle, particularly in view of the fact that the assay is not linear but is based on serial two-fold dilutions. However, the results were consistent in all experiments done and were statistically significant (Fig. 2).

With all erythrocyte preparations studied the specific activities with

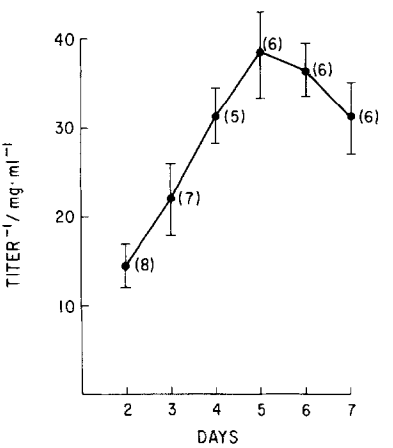

Figure 2. Specific activity of agglutinin in extracts of L6 myoblasts cultured for the indicated periods of time. The results are shown as the mean ± S.E.M. of the number of experiments indicated in parenthesis. The specific activity of the 5 day extracts differed significantly from the 2 day extracts ($P < .01$) and from the 3 day extracts ($P < .05$) but not from any of the others.

the L6 extracts were about the same. In all cases when concurrent extraction and assay were done, the extracts from 16 day old embryonic chick pectoral muscle extracts were at least an order of magnitude higher than those from the 5 day L6 cultures. This need not, however, indicate that there are more agglutinin molecules in the embryonic chick pectoral muscle. Rather, it is possible that the agglutinins in the L6 extract are just less potent with the particular test erythrocytes used in these studies.

Discussion: The results clearly indicate that extracts of embryonic chick pectoral muscle and L6 myoblasts contain agglutinins that are inhibited by a similar group of saccharides and that, by this criterion, are also quite similar to the lectin identified from electric organ of Electrophorus (9). With both chick pectoral muscle and L6 myoblast extracts, agglutinin activity increased with differentiation, although the findings were much more striking with the embryonic pectoral muscle.

The nature and function of the agglutinins described here is not known. We presume that the component of the pectoral muscle from which the agglutinins are extracted is the muscle cells since a similar agglutinin is present in the L6 cell line which gives rise to tissue that closely resembles skeletal muscle. We also presume that the agglutinin exists in a polyvalent form in vivo and might therefore function to cross-link complementary oligosaccharides. However, the possibility that the agglutinin functions as an enzyme in vivo, either a glycosyl transferase or a glycosidase, cannot be excluded. Studies with cellular slime molds have shown that developmentally regulated lectins are present on the cell surface of cohesive cells and play a role in cell cohesion (1-8). This raises the possibility that the agglutinins observed here might be present on the surface of differentiating muscle cells, possibly to play a role in muscle cell fusion or the formation of neuromuscular junctions. The period of appearance of agglutinins between 9 and 16 days of embryonic life is the same as the period over which chick pectoral

muscle cells are stopping mitosis (14) and beginning fusion; but a possible relationship between these processes requires more detailed study.

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