EVIDENCE THAT A MEMBRANE BOUND LECTIN MEDIATES FUSION OF L_6 MYOBLASTS

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Summary: The external membranes of L_6 myoblasts are shown to possess lectin activity which resides in protein molecules. Thiodigalactoside blocks agglutination of formalinized, trypsin treated rabbit red blood cells caused by the lectin. Thiodigalactoside at a concentration which neither inhibits cell division nor decreases cell yield prevents fusion of myoblasts. It is suggested that this protein which has lectin activity also is an essential participant in the membrane events which cause fusion of myoblasts to form myotubes.

L_6 is a permanent cell line derived from rat skeletal muscle (1). L_6 is particularly interesting because it differentiates in-vitro. Myoblasts proliferate and are the undifferentiated form of L_6. Fusion of the myoblasts results in the formation of multinucleate electrically active myotubes (1)(2). Myotubes do not proliferate; they are the differentiated form of L_6. We are interested in characterizing the membrane components which participate in fusion.

Glycoproteins are thought to be important components of cell-cell recognition and interaction (3). It has been proposed that interactions of glycoproteins may be the basis of cell fusion and other inter-cellular interactions. According to this model, the specificity of the process is determined by the carbohydrate binding specificity of the participating glycoprotein(s). In agreement with this model, Rosen, et al. have presented evidence supporting the possibility that an erythrocyte agglutinating glycoprotein synthesized by the slime mold Dictyostelium discoideum may mediate intercellular adhesion which results in the formation of a multicellular structure (4). The results of other investigations are consistent with the involvement of membrane glycoproteins in the process of intercellular interactions (5)(6).

Recently, Teichberg, et al demonstrated that extracts of electric organ tissue of Electrophorus electricus contain a protein capable of agglutinating trypsin treated rabbit red blood cells (7), such proteins are designated lectins. Lectins with similar specificity were demonstrated in both embryonic and adult chick pectoral muscle and in adult rat soleus and diaphram muscles (7). Our interest in the process of fusion of myoblasts lead us to determine if a protein(s) with lectin activity is involved in fusion of L_6 myoblasts.

Materials and Methods:

Buffer: The standard buffer used was 2mM in $NaPO_4$, 2mM in dithiothreitol and contained 0.9% NaCl and was at pH 7.5.
Growth Conditions: L_6 cells were grown at 37° in Dulbeccos Modified Eagles medium (8) supplemented with 10% fetal calf serum in an atmosphere containing 5% CO_2 and 95% air and 100% humidity on Corning tissue culture dishes. Cells were removed from the dishes and dissociated for transfer or counting by incubation at 37° in Eagles medium containing 0.25%(W/V) Viokase (GIBCO).
Preparation of the Lectins: Each 100mm dish containing L_6 cells was washed 3 times with buffer after the medium was removed. One ml of buffer was added to each plate and the cells removed by scraping with a rubber policeman. The suspended cells were transferred to a glass homogenizer and the plate washed with 0.5ml of buffer and the buffer was added to the contents of the homogenizer. The cells were homogenized in the cold. The cell extracts were spun at 36,000 rpm for 70 minutes in a 42.1 rotor in a Beckman L2-75B ultracentrifuge. The supernatant fluid was removed from each tube and used as a source of soluble lectins. Each pellet was washed once with buffer and then resuspended and homogenized in 4 ml of buffer. The particulate material was sedimented by the procedure described above. Each pellet was suspended in 1 ml of buffer and homogenized. The soluble material and the particulate material were tested for lectin activity as described below.
Red Blood Cells: Rabbit red blood cells were treated with trypsin using the method of Lis and Sharon (9). The trypsin treated red blood cells were formalinized using the method of Butler (10).
Erythrocyte Agglutination Assay (4)(11): Agglutination of erythrocytes was assayed in Microtiter "V" plates (Cooke Engineering). Each "V" plate contains 8 rows of 12 small wells with conically shaped bottoms. A calibrated dropping pipette (Cooke Engineering) was used to add approximately 25 µl of buffer to each well. Then 25 µl of the test material was added to the first well of a row of wells. Two-fold dilutions of the test materials were prepared using a Takatsy microtitrator. The microtitrator was filled by dipping it into the well containing the material to be tested. The microtitrator containing 25 µl of a 1:2 dilution of the presumed lectin was transferred to the next well and rotated to cause mixing. This process was repeated 4 times producing a two-fold dilution series in 5 wells. Next, 25 µl of a 2.5% suspension of formalinized, trypsin treated rabbit red cells were added to the wells of each row. The contents of each well in a row were stirred using the same toothpick. The contents of the wells were evaluated for agglutination after they remained at room temperature for 1.5 hours. The symbol "4+" indicates the densest uniform mat of agglutinated cells covering the bottom of the well. The symbol "-" indicates the control pattern which is a central button of cells with no mat of agglutinated cells covering the bottom of the well. The symbols: "3+", "2+" and "1+" indicate less extensive agglutination than "4+", but more than "-".

Table 1

†Type of Extract Assayed	Dilution of Test Materials 1/2	1/4	1/8	1/16	1/32	Buffer Controls (No Lectins)	
2 days soluble	4+	2+	-	-	-	-	-
* + lactose	-	-	-	-	-	-	-
+ thiodigalactose	-	-	-	-	-	-	-
+ α-methyl-D-glucoside	4+	2+	-	-	-	-	-
+ α-methyl-D-mannoside	4+	2+	-	-	-	-	-
containing .018% trypsin	-	-	-	-	-	-	-
2 days particulate	4+	2+	-	-	-	-	-
+ lactose	-	-	-	-	-	-	-
+ thiodigalactoside	-	-	-	-	-	-	-
+ α-methyl-D-glucoside	4+	2+	-	-	-	-	-
+ α-methyl-D-mannoside	4+	2+	-	-	-	-	-
containing .018% trypsin	-	-	-	-	-	-	-
intact cells	4+	4+	2+	-	-	-	-
+ lactose	-	-	-	-	-	-	-
+ thiodigalactoside	-	-	-	-	-	-	-
containing .018% trypsin	-	-	-	-	-	-	-

* Each well contains a final concentration of 10mM of the carbohydrate used to try and block agglutination of erythrocytes.

† Fetal calf serum has lectin activity in our assay, however, this lectin activity cannot be blocked by either lactose or thiodigalactoside.

Results: Extracts of cells from 12 100mm dishes containing L_6 cells were tested for lectin activity. Extracts were prepared from cells grown for 2,3,4,5,7,8,9,10,14 and 16 days. Two dishes were used for both the 2 and 3 day samples (total of 1.5ml/sample). One dish was used for each of the other

samples. Myotube formation was not extensive until about the fourth day. Cell extracts from each preparation had lectin activity. Both the soluble and particulate materials from the 2 day preparation had erythrocyte-agglutinating ability that was blocked by 10mM lactose or 10mM thiodigalactoside. Neither activity was blocked by 10mM α-methyl-M-D-glucoside or 10mM α-methyl-M-D-mannoside. Thus, the lectin activities were blocked by disaccharides with β-D-galactoside moities (see Table 1). The particulate material from each preparation contained only lectin activity which could be blocked by lactose or thiodigalactoside. In contrast, the preparations of soluble material prepared from cells grown for longer than 2 days prior to harvesting had erythrocyte agglutinating ability which was not blocked by any of the sugars tested.

The lectin activities of both the soluble and particulate fractions of 2 day old cells were demonstrated to reside in proteins by their susceptibility to trypsin. The lectin solutions containing trypsin (0.018% final concentration) were incubated at 37° for 30 minutes prior to use in a standard assay. As controls, trypsin was added to the rabbit red blood cells (same dilutions) and incubated at 37° for 30 minutes prior to using the trypsin treated erythrocytes in an assay; also erythrocytes and trypsin were tested separately in the absence of lectin for the ability to agglutinate erythrocytes. It is clear from these results (see Table 1) that the lectin activity is trypsin sensitive and therefore probably resides in protein molecules.

The membrane location of at least some of the lectin activity was demonstrated by using intact L_6 myoblasts in the erythrocyte agglutinating assay. The cells were removed from the dishes by EDTA. The intact cells had lectin activity and this activity was blocked by lactose or thiodigalactoside in cells grown for two days. Furthermore, the lectin activity of intact cells was susceptible to trypsin used as described above.

The primary objective of this investigation was to determine if a mem-

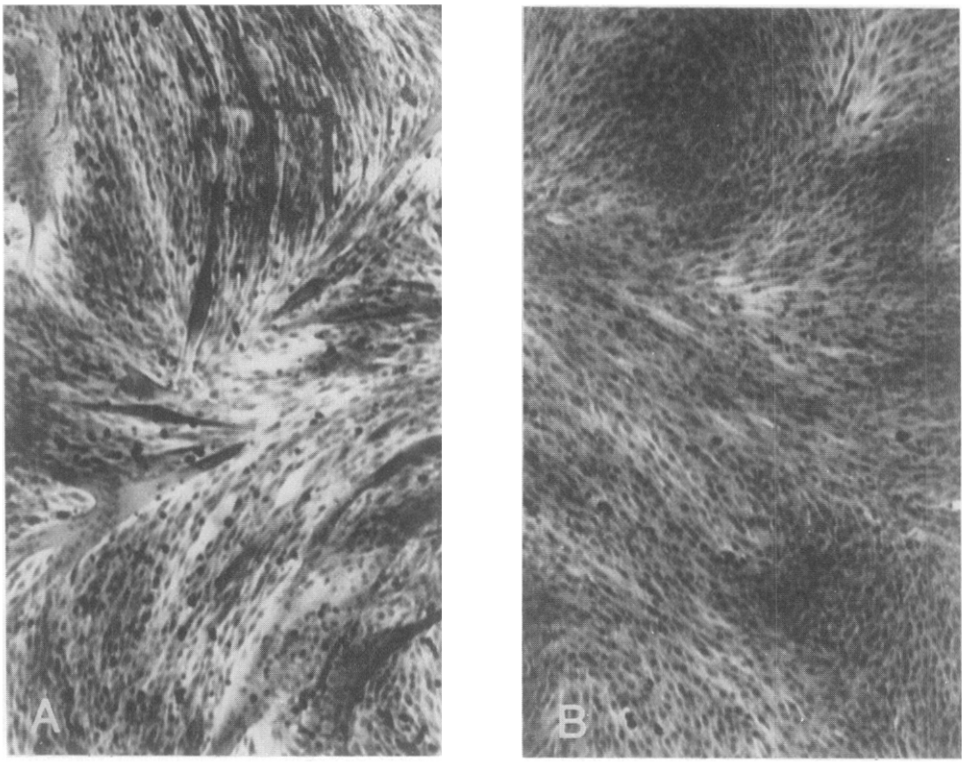

Figure 1. Thiodigalactoside inhibited fusion of myoblasts. The cells (A) on the left grew in normal medium in the absence of thiodigalactoside, note the multinucleate myotubes formed by fusion of myoblasts. The cells (B) on the right grew in normal medium containing a 15mM final concentration of thiodigalactoside. Note the absence of multinucleate myotubes. Ehrlich's hematoxylin was used to stain nuclei.

brane component with lectin activity could be implicated as participating in the process of fusion of myoblasts. The role of a lectin in fusion was evaluated by determining if thiodigalactoside, a presumed non-metabolizable analogue of lactose which blocks agglutination of erythrocytes caused by the myoblast derived lectin, could prevent the formation of myotubes. With this end in mind, eight 60mm dishes were each inoculated with approximately 40,000 cells. Twenty four hours later, cells on four control plates received fresh medium; the cells on the other four plates received culture medium which was 15mM in thiodigalactoside. The media, with and without thiodigalactoside, were replaced 4 days later and again 2 days later. It was clear that thiodigalactoside inhibited the formation of myotubes, see Figure 1. The thiodigalactoside did not significantly decrease the rate of growth of L_6 myo-

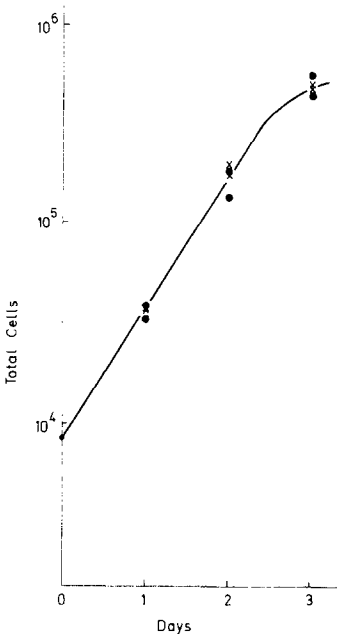

Figure 2. Myoblasts were grown at 37° in normal medium (xxx) or in otherwise identical medium containing a 15mM final concentration of thiodigalactoside. All cells grew in normal medium for 24 hours prior to the use of thiodigalactoside. The exposure of experimental cells to thiodigalactoside began at the point designated day zero on the growth curve. Cells were counted in a Neubauer hemocytometer.

blasts (see Figure 2), so the inhibition of fusion caused by thiodigalactoside is not simply an indirect effect of a decreased rate of growth or a lower cell yield. Evidence that the inhibition of myotube formation by thiodigalactoside is a specific effect is provided by the fact that neither α-methyl-D-glucoside nor α-methyl-D-mannoside used in the same way as thiodigalactoside had any effect on fusion. Thus, the inhibition of fusion caused by thiodigalactoside is specific and consequently may have been caused by the blocking of the normal function of protein molecules which have lectin activity and are present in the external membrane of myoblasts.

Discussion: The results of this investigation provide evidence that the external membranes of myoblasts contain protein molecules with lectin activity and that these protein molecules may participate in the fusion of myoblasts.

Our results also provide insights into the control of fusion. It is sur-

prising that myoblasts at all stages of development contain the membrane proteins which interact with thiodigalactoside and are presumably required for fusion. Because these proteins are always present, it is not clear how fusion is controlled. Fusion may be controlled, for example, by the late appearance of a receptor molecule for the thiodigalactoside "sensitive" membrane bound lectins. Alternatively, the thiodigalactoside binding lectins may be physiologically cryptic until a later stage of development or the density of these receptors may be a critical factor. The fact that older cultures have erythrocyte agglutinating ability which is not blocked by lactose or thiodigalactoside may reflect the appearance of a receptor molecule at a later stage of development. Hopefully, further characterization of the L_6 system will provide insight into the control of fusion.

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