Lack of Evidence for the Involvement of a β-D-Galactosyl-Specific Lectin in the Fusion of Chick Myoblasts

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SUMMARY: The role of a β-D-galactosyl-specific lectin, first reported by Teichberg *et al.*, in the fusion of myoblasts *in vitro* was investigated. The concentration of this lectin in embryonic chick skeletal muscle was found to reach maximal levels at the time of myoblast fusion *in vivo*. β-D-Galactosyl-β-thiogalactopyranoside and lactose are potent inhibitors of agglutination of trypsinized rabbit erythrocytes caused by the lectin. However, at concentrations of 50 mM these compounds had no effect on either nonsynchronous fusion of myoblasts or on the release of synchronized myoblast cultures from EGTA fusion block. The presence of the agglutinin in the external membranes of chick myoblasts and myotubes could not be demonstrated. It is, therefore, concluded that the involvement of the lectin in the fusion of chick myoblasts remains questionable.

The mechanism of myoblast fusion is as yet unknown but it is generally believed (1) that this process involves at least two separate reactions: cell recognition (or specific adhesion), and subsequent membrane-membrane interaction culminating in cell fusion. Results of recent investigations suggest that surface glycoproteins (or glycolipids) play a role in cell recognition and interactions. In particular, studies on the carbohydrate-binding proteins which are capable of agglutinating erythrocytes and are present on the surface of aggregation-competent cells of several slime mold species (2) suggest a possible involvement of such agglutinins in cell adhesion. For this reason the recent report on the presence of high levels of a β-D-galactoside-specific lectin in embryonic chick skeletal muscle which agglutinates trypsinized rabbit erythrocytes (3) has attracted wide attention. Furthermore, while our work was in progress, Gartner and Podleski (4) published a series of experiments

supporting the concept that a membrane-bound lectin with specificity for β-D-galactosyl groups mediates fusion of L_6 myoblasts.

In this communication we wish to report the results of our experiments with cultured chick muscle cells in which we were unable to support the involvement of a β-D-galactoside-specific lectin in myoblast fusion.

MATERIALS AND METHODS

Culture Conditions

The culture conditions for both, cells grown in high Ca^{++} medium to permit nonsynchronous fusion and cells made to fuse synchronously by growth in EGTA-containing medium which was then changed to Ca^{++} containing BSS have been described previously (5).

Harvesting

Cells were removed from dishes by one of the following treatments: (a) Incubation at 37° for 30 min with a 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. (b) Incubation at 37° for 8 min with PBS[1], containing 5 μg of crystalline trypsin per ml.

Erythrocyte Agglutination Assay[2]

Trypsinized rabbit erythrocytes were prepared as described by Lis and Sharon (6). Material to be assayed was placed in the round-bottomed shaped wells of a microtiter plate (Linbro Chemical Co., New Haven, CT) and the volume was adjusted to 100 μl with PBS, containing 2 mM DTT. A 100 μl aliquot of a 0.5% suspension of trypsinized rabbit erythrocytes was then added to each well and the plate was rotated at 140 rpm on top of a gyratory water bath for 10 min at room temperature. Next, 100 μl aliquots of the mixed (by agitation of the plate) reaction mixtures were diluted with PBS to a volume of 10 ml and the remaining unagglutinated erythrocytes were counted in a Coulter counter (Model ZBl) using a 140 μm aperture, at the following settings: 1/amplification 1; 1/aperture current, 1/8; lower threshold at 7 and the upper threshold at 15. One hemagglutinating unit was defined as that amount of protein which is required to cause a decrease of 50% in the particle count in 10 min under the conditions described.

Protein Determination

Protein was determined by Hartree's modification of the method of Lowry *et al.* (7).

RESULTS

As illustrated in Fig. 1, the concentration of the lectin in chick embryo thigh muscle increases during the early part of embryonic development and, after reaching a maximum on the 13th day, decreases to a lower level. This time

[1] Abbreviations: PBS, isotonic saline containing 0.01 M P buffer, pH 7.0; TDG, β-D-galactopyranosyl-β-thiogalactopyranoside; BSS, balanced salt solution; HU, hemagglutinating unit; DTT, dithiothreitol.

[2] A more detailed description of the assay and purification of the lectin by affinity chromatography will be published elsewhere.

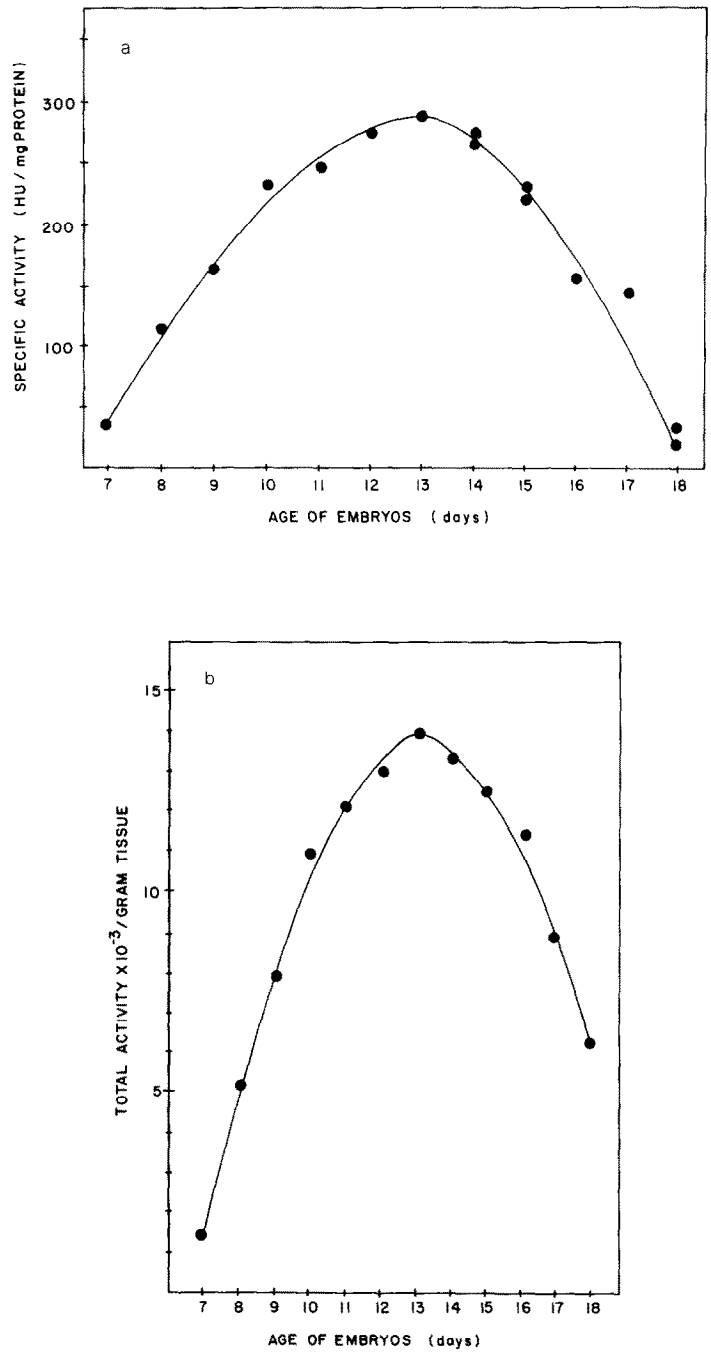

Figure 1: Lectin levels during embryonic development. (a) Specific activity (b) Total activity. The excised thigh muscle was homogenized, the homogenates were sonicated for 1 min and, after appropriate dilutions with PBS-DTT, were assayed as described in the text.

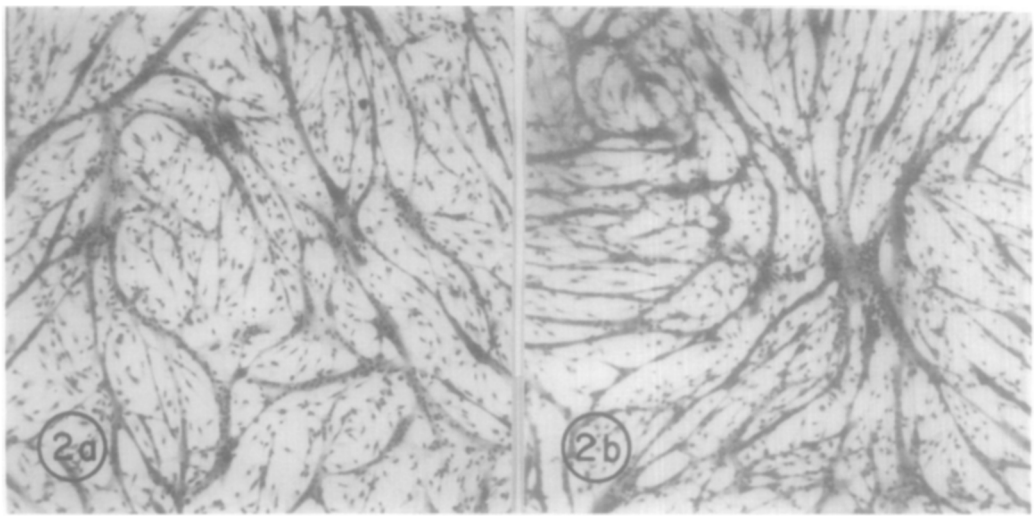

Figure 2: Phase contrast photomicrographs (X40) demonstrating that the presence of TDG at 50 mM had no effect on the fusion of chick myoblasts *in vitro*. Cultures of thigh muscle cells from 12-day chick embryos were prepared as previously described (5). After 50 h in culture the dishes were washed with PBS, dehydrated with ethanol and stained with 10% aqueous giemsa for 30 min. (a) Control cultures without TDG. (b) The myoblasts were seeded and grown in the continuous presence of 50 mM TDG.

course coincides with the period of embryonic development in which muscle fusion has been shown to occur *in vivo* (8).

As previously reported by Teichberg *et al.*, among the saccharides tested, TDG and lactose were most effective in inhibiting the agglutinin activity. In our hands TDG at 25 μM and lactose at 0.1 mM completely inhibited agglutination caused by 1 HU. However, in contrast to the experiments reported by Gartner and Podleski (4), in which the presence of 15 mM TDG in the culture medium inhibited the fusion of L_6 myoblasts, neither TDG nor lactose at concentrations up to 50 mM[3] had any effect on the growth, morphology, or fusion of chick embryo myoblasts. This is illustrated in Fig. 2 where the phase-contrast photomicrographs of 50 h cultures with and without TDG clearly show no difference in the degree of fusion. It could be argued that the horse serum in the growth medium

[3]This represents a vast excess of TDG over the small amount (1 mM) which can be bound by the lectin-containing "chick embryo extract" present in the growth media.

TABLE I

Synchronous Fusion of Chick Myoblasts in the Presence of Thiodigalactoside or Lactose

Agglutinin Inhibitor During the Release of EGTA Fusion Arrest	Fusion %	Inhibition %
None	69%	
Thiodigalactoside, 50 mM	69%	0
Lactose, 50 mM	68%	0

Fusion-blocked cells were grown in the presence of 1.85 mM EGTA. After 50 h in cultures the EGTA-containing medium was replaced by Earle's BSS, alone or with the inhibitors, and the cultures were incubated for 4 h more. Nuclei were counted in randomly selected fields at X200 (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.

contains glycosidases which hydrolyze lactose. However, this objection does not apply to the synchronous fusion of cultured chick myoblasts which was accomplish ed in BSS without serum supplement. As illustrated in Table I, the synchronous fusion was also not affected by the agglutinin inhibitors and the same percentage of nuclei was incorporated into syncytia whether or not TDG or lactose were present in the medium during the release of the EGTA fusion arrest by BSS, containing 1.8 mM Ca^{++}.

We were not able to demonstrate agglutination of erythrocytes by intact myoblasts, although homogenates prepared from such cells had ample activity. The following cells in both, the normal and EGTA fusion-arrested series were tested: single cell suspensions prepared by dissociation of the 11- or 12-day chick embryo thigh muscle with crude collagenase, intact monolayers at different times of culture, and cells removed from the dishes by EDTA or trypsin treatment. Although such cell suspensions routinely showed positive agglutination behavior when tested in the wells of microtiter plates, a close microscopic

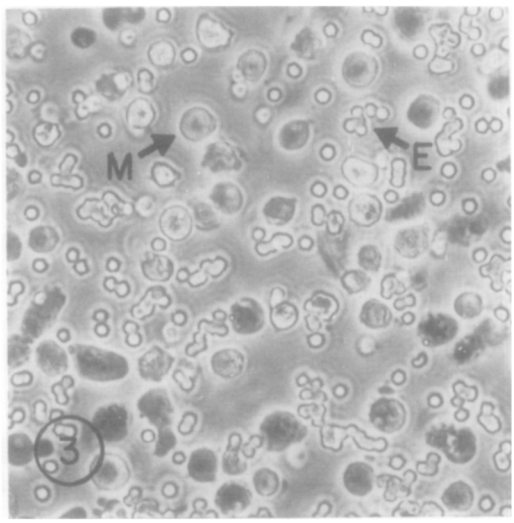

Figure 3: Phase contrast photomicrographs (X200) demonstrating that intact myoblasts do not agglutinate trypsinized rabbit erythrocytes. After 50 h in EGTA fusion-arrested cultures the cells were removed from the dishes by EDTA treatment, washed, and suspended in PBS-DTT. Aliquots of this suspension were rotated with erythrocyte suspensions on microtiter plates as described in the text. The reaction mixtures were then subjected to microscopic investigation. M, a myoblast; E, an erythrocyte agglutinate.

examination failed to detect undisrupted (Trypan Blue excluding) cells surrounded by erythrocytes (Fig. 3). Instead, the erythrocyte aggregates were located at random. It is possible that sufficient amounts of lectin were released from a small percentage of disrupted cells to cause a positive agglutination test when assayed on microtiter plates.

DISCUSSION

The β-D-galactoside-specific hemagglutinating activity reported by Teichberg *et al.* was shown to be present in particularly high amounts in chick embryonic muscle and in tissue cultures of nerve and muscle, i.e., in rapidly differentiating systems (3). In view of the recent reports on the involvement of carbohydrate-binding proteins in slime mold differentiation (2), it seems reasonable to suspect that this lectin may play a role in muscle differentiation, more specifically in the fusion step. This belief is supported by the fact

that highest concentrations of the lectin were found in the period of embryonic development characterized by intense muscle fusion *in vivo*. However, our results of experiments designed to investigate this hypothesis are in conflict with the evidence presented by other investigators (4). In our hands, the two potent inhibitors of the lectin's agglutination activity, TDG and lactose, did not prevent the muscle cell fusion. Furthermore, we were not able to demonstrate agglutination of trypsinized rabbit erythrocytes by intact myoblasts or myotubes in monolayers. It must be stressed, however, that our experiments used primary chick myoblast cultures whereas Gartner and Podleski (4) worked with a continuous line, L_6, derived from rat skeletal muscle. It is possible that the L_6 line may, in analogy to other continuous (mostly virus transformed) cells, lack a surface component(s) which covers the lectin on the surface of normal myoblasts. In this regard, the relatively mild trypsin treatment used in our experiments to remove the cells from dishes did not appear to uncover the lectin since such cells did not agglutinate trypsinized rabbit erythrocytes Nevertheless, until the differences between our results and those of Gartner and Podleski (4) are resolved, the role of the lectin in muscle fusion remains in doubt. Closer examination of the function of this interesting agglutinin will become possible when sufficient amounts of the purified material become available.

ACKNOWLEDGEMENT

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