

A MULTIVALENT LACTO-N-FUCOPENTAOSE III-
LYSYLLYSINE CONJUGATE DECOMPACTS
PREIMPLANTATION MOUSE EMBRYOS, WHILE THE FREE
OLIGOSACCHARIDE IS INEFFECTIVE

BY BRUCE A. FENDERSON, URI ZEHAVI, AND SEN-ITIROH HAKOMORI

*From the Program of Biochemical Oncology, Fred Hutchinson Cancer Research Center,
Department of Pathobiology, University of Washington, Seattle, Washington 98104*

The carbohydrate chains of glycolipids and glycoproteins at the cell surface are known to undergo many rapid changes during embryogenesis. These developmental changes include (a) alteration of branching structure in lactoseries carbohydrate chains, identified as I/i interconversion (1), (b) modification of terminal chains, responsible for the transition from Ii to ABH or SSEA-1 (reviewed in 2), (c) transition of globoseries antigen expression through P^k, P, and Forssman (3), and (d) switching of core structure synthesis from one series to another, such as SSEA-3⁺ globoseries to SSEA-1⁺ lactoseries (4). Although these structural changes have been defined clearly using various monoclonal antibodies, their physiological significance is unclear.

The appearance of SSEA-1 on the embryo surface after the third cleavage division correlates approximately in time with the onset of compaction (5). During compaction, blastomeres maximize their intercellular contacts and generate a polar distribution of microvilli (6). Identification of SSEA-1 as the X hapten (Galβ1 → 4[Fuca1 → 3]GlcNAcβ1 → R) (7-9) suggested the possibility of a 3-fucosyl-N-acetyllactosamine recognition system in the early mouse embryo. To test this hypothesis, we have studied the effect of lacto-N-fucopentaose III, its analogues, and their multivalent lysyllysine conjugates on compaction.

Materials and Methods

Purification of Oligosaccharides. A mixture of lacto-N-fucopentaose (LNFP) I, II, and III was obtained from human milk by the method of Kobata (10). LNFP I, II, and III (unreduced) were separated after acetylation by preparative thin-layer chromatography on HPTLC plates (J. T. Baker Chemical Co., Phillipsburg, NJ) using a solvent system of butylacetate-acetone-water (25:8:1). After deacetylation, purified pentasaccharides were lyophilized and stored at -20°C. Chitotriose (GlcNAcβ1 → 3GlcNAcβ1 → 3GlcNAc) was a gift from Professor Toshiaki Osawa, University of Tokyo.

Covalent Attachment of Oligosaccharides to Lysyllysine. Purified oligosaccharides were

This work was supported by grants GM23100 and AM HL31232 from the National Institutes of Health. Current address of U. Zehavi: Faculty of Agriculture, Hebrew University, P.O. Box 12, Rehovot 76100 Israel. During this investigation, U. Zehavi was under tenure of an exchange program between Hebrew University and the University of Washington, financially supported by Genetic Systems, Inc.

coupled to lysyllysine by reductive amination as described previously by Schwartz and Gray (11). This method is based on the ability of cyanoborohydride to reduce a Schiff base selectively at pH > 5. Briefly, unreduced oligosaccharide, L-lysyllysine 2HCl (Bachem Fine Chemicals, Torrance, CA), and sodium cyanoborohydride (Sigma Chemical Co., St. Louis, MO) were mixed in a molar ratio of 0.35:0.033:1.00 and dissolved in 0.2 M potassium phosphate (pH 8.0). After 72 h at 37°C, the oligosaccharide-lysyllysine conjugate was separated from free oligosaccharide, cyanoborohydride, and buffer salts by P2 molecular sieve chromatography. Oligosaccharide-lysyllysine conjugates eluted immediately after the void volume and were identified routinely in a spot test using 0.5% orcinol in a 10% H₂SO₄ spray.

Preimplantation Embryo Culture and Compaction Assays. 4–8-cell embryos were flushed from the oviducts of Swiss Webster mice (Tyler Labs, Seattle, WA) on day 3 postcoitum. These precompaction embryos were cultured overnight to obtain a homogeneous population of fully compacted 8–16-cell morulae. Embryos were cultured in Whitten's medium under mineral oil on 50-mm petri dishes (Falcon Labware, Oxnard, CA) at 37°C in a 5% CO₂-in-air humidified incubator. On the morning of day 4, compacted 8–16-cell embryos were transferred briefly into Tyrode's salt solution containing 0.4% polyvinylpyrrolidone (pH 2.5) to remove zonae pellucidae. For compaction assays, zona-free embryos were transferred into 60- μ l droplets of Whitten's medium containing various oligosaccharides or their conjugates with lysyllysine and were cultured under oil at 37°C. Stock solutions of the oligosaccharide-lysyllysine conjugates (2 mM each) and the free oligosaccharides (20 mM each) were prepared in Whitten's medium, passed through a 0.22 μ m filter, and stored at 4°C. Effects on compaction were photographed using a Polaroid camera attached to a Nikon inverted phase microscope. Embryos were scored as compaction reversed (decompacted) if three or more blastomeres were not in close apposition.

Results

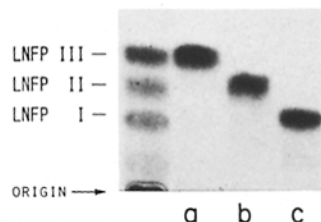
Lacto-*N*-fucopentaose I (H hapten), II (Le^a hapten), and III (X hapten) were isolated from human milk. The purity and chemical structure of these pentasaccharides are shown in Fig. 1, A and B. Multivalent haptens were obtained by coupling LNFP II, LNFP III, and chitotriose to the primary amino groups of lysyllysine. Purified oligosaccharide-lysyllysine conjugates were not reactive with fluorescamine or ninhydrin, indicating the absence of any primary amino group and suggesting, therefore, the presence of 3 moles of oligosaccharide per mole of lysyllysine. The predicted chemical structure of the LNFP III–lysyllysine conjugate is shown in Fig. 1 C.

LNFP II and III and their multivalent lysyllysine conjugates were tested for possible inhibitory effects on compaction of the early mouse embryo. As shown in Fig. 2 D, the multivalent LNFP III–lysyllysine conjugate had a dramatic effect on cultures of fully compacted 8–16-cell embryos: individual blastomeres were observed to round up and lose their close apposition of membranes. Similar results were obtained in five separate experiments. This decompaction phenomenon was not observed in cultures of embryos treated with the multivalent LNFP II–lysyllysine or chitotriose-lysyllysine conjugates (Fig. 2, C and E, respectively). Furthermore, decompaction was not observed in cultures of embryos treated with a mixture of LNFP III (unconjugated) plus lysyllysine (Fig. 2 F), suggesting the critical importance of X hapten valency.

The effect of LNFP III–lysyllysine concentration and the time of incubation on compaction are shown in Fig. 3. LNFP III–lysyllysine caused embryos to decompact at concentrations between 0.125 and 1 mM, while LNFP III (unconjugated) had no effect at concentrations as high as 5 mM (Fig. 3 A). The effect

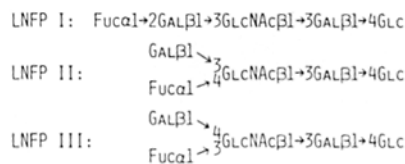
A.

SEPARATION OF ACETYLATED LACTO-N-FUCOPENTAOSE I, II, III BY THIN-LAYER CHROMATOGRAPHY.



B.

STRUCTURE OF LACTO-N-FUCOPENTAOSE I, II, III.



C.

STRUCTURE OF LNFP III—LYSYL-LYSINE CONJUGATE.

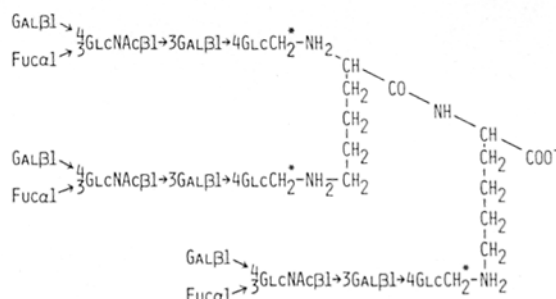


FIGURE 1. Thin-layer chromatography of unreduced, acetylated pentasaccharides in butylacetate-acetone-water (25:8:1), revealed by 0.5% orcinol in 10% H_2SO_4 (A). LNFP III (lane a), II (b), and I (c) were purified from the original mixture as described in Materials and Methods. The chemical structure of these purified pentasaccharides is shown in B. The chemical structure of the LNFP III-lysyllysine conjugate is shown in C (Glc- CH_2^* represents a 1-deoxy-D-glucosyl residue without pyranose ring).

of LNFP III-lysyllysine on compaction was not immediate, requiring 6–8 h of culture (Fig. 3B). Decompacted embryos were fully viable, as determined by trypan blue exclusion. After 12 h of culture, however, the dramatic effect of LNFP III-lysyllysine on early embryo development was irreversible and blastocyst formation was prevented. LNFP III-lysyllysine had no effect on the morphology of blastocyst-stage embryos.

Discussion

Compaction is the first of many specific cell-cell interactions occurring rapidly during mammalian embryogenesis. Previous studies have shown that compaction requires Ca^{++} (12) and N-linked glycoconjugate biosynthesis (13). A Ca^{++} -dependent cell adhesion molecule has been identified that apparently mediates interblastomere adhesion during compaction (14, 15). Recently (16), a monoclonal antibody directed to this 124,000 mol wt glycoprotein has been found to decompact early mouse embryos.

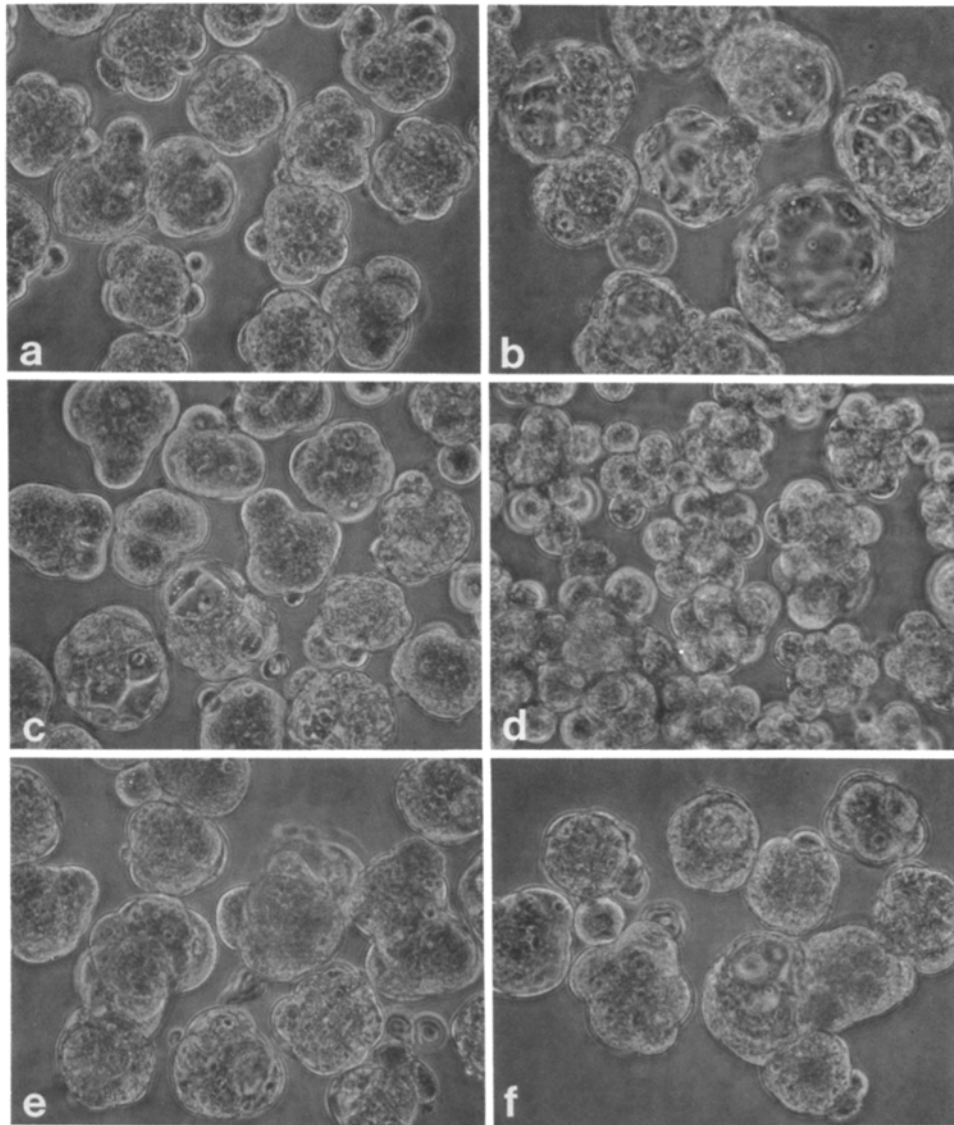


FIGURE 2. 8-16-cell compacted embryos (a) were cultured in Whitten's medium alone (b) or in Whitten's medium containing either 1 mM LNFP II-lysyllysine (c), 1 mM LNFP III-lysyllysine (d), 1 mM chitotriose-lysyllysine (e), or 5 mM LNFP III (unconjugated) plus 1 mM lysyllysine (f). Embryos are shown after 8-12 h (e-f) or 24 h (b) of culture. $\times 150$.

The results presented here suggest that a multivalent X hapten recognition system may contribute also to the process of compaction. Such a recognition system might involve a cell surface lectin, glycosyl transferase, or glycosyl hydrolase in a lock-and-key mechanism. Alternatively, the X hapten structure might regulate certain membrane proteins, including the 124,000 mol wt Ca^{++} -dependent cell adhesion molecule. Models of specific carbohydrate-carbohydrate interaction through hydrogen bonding can also be envisioned.

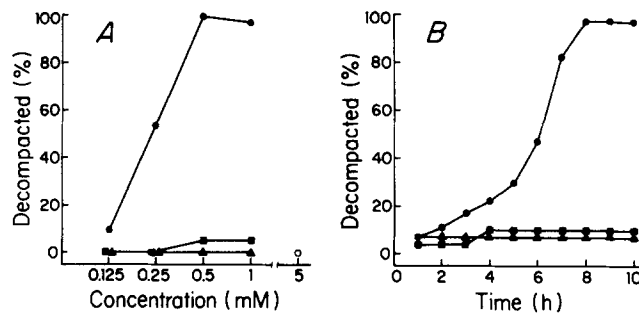


FIGURE 3. Dose response (A) and time course (B) for decompaction by the multivalent LNFP III-lysyllysine conjugate. 8-16-cell compacted embryos (15-60 per group) were cultured in the presence of LNFP III-lysyllysine (●), LNFP II-lysyllysine (■), chitotriose-lysyllysine (▲), or LNFP III (5 mM, unconjugated) plus 1 mM lysyllysine (○). The percentage of decompacted embryos was scored after 10-12 h of culture (A) or scored hourly for 10 h (B). The concentration of oligosaccharide-lysyllysine conjugates used in the time course assay (B) was 1 mM. Similar results were obtained in replicate assays.

The fact that a multivalent LNFP III hapten was able to decompact embryos, whereas free oligosaccharide was ineffective (Figs. 2 and 3), suggests the critical importance of X hapten valency. Carbohydrate recognition in other biological systems has been shown to require a high density or "clustering" of sugar residues (17). Thus, it may be significant that the X hapten is apparently carried by the early mouse embryo on a highly branched lactosaminoglycan (embryoglycan) (reviewed in 18). Embryoglycan purified from F9 embryonal carcinoma cells, however, did not decompact embryos; instead, it caused rapid agglutination (unpublished observations). Thus, the decompaction phenomenon appears to be associated with lower molecular weight compounds. The limits of molecular weight and valency required for decompaction versus agglutination remain to be studied. Multivalent oligosaccharide-lysyllysine conjugates should prove useful for future studies on the functional role of cell surface carbohydrates in cell-cell interactions.

Summary

A multivalent lacto-*N*-fucopentaose (LNFP) III-lysyllysine conjugate was observed to decompact preimplantation mouse embryos. Decompaction was not obtained with free oligosaccharides (LNFP II and III), nor with multivalent LNFP II-lysyllysine or chitotriose-lysyllysine conjugates. These results suggest a role for X hapten recognition during compaction and suggest further that X hapten valency may play a key role in modulating this developmental process.

Received for publication 10 July 1984 and in revised form 15 August 1984.

References

1. Kapadia, A., T. Feizi, and M. J. Evans. 1981. Changes in the expression and polarization of blood group I and i antigens in post-implantation embryos and teratocarcinomas of mouse associated with cell differentiation. *Exp. Cell Res.* 131:185.
2. Hakomori, S., M. Fukuda, and E. Nudelman. 1982. Role of cell surface carbohydrates in differentiation: behavior of lactosaminoglycan in glycolipids and glycoproteins. *In*

- Teratocarcinoma and Embryonic Cell Interactions. T. Muramatsu, G. Gachelin, A. A. Moscona, and Y. Ikawa, editors. Academic Press, Inc., New York. 179-200.
3. Willison, K. R., R. A. Karol, A. Suzuki, S. K. Kundu, and D. M. Marcus. 1982. Neutral glycolipid antigens as developmental markers of mouse teratocarcinoma and early embryos: an immunologic and chemical analysis. *J. Immunol.* 129:603.
 4. Kannagi, R., N. R. Cochran, F. Ishigami, S. Hakomori, P. W. Andrews, B. B. Knowles, and D. Solter. 1983. Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *EMBO (Eur. Mol. Biochem. Org.) J.* 2:2355.
 5. Solter, D., and B. B. Knowles. 1978. Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. USA.* 75:5565.
 6. Handyside, A. H. 1980. Distribution of antibody- and lectin-binding sites on dissociated blastomeres from mouse morulae: evidence for polarization at compaction. *J. Embryol. Exp. Morph.* 60:99.
 7. Gooi, H. C., T. Feizi, A. Kapadia, B. B. Knowles, D. Solter, and M. J. Evans. 1981. Stage-specific embryonic antigen involves $\alpha 1 \rightarrow 3$ fucosylated type 2 blood group chains. *Nature (Lond.)* 292:156.
 8. Hakomori, S., E. Nudelman, S. Levery, D. Solter, and B. B. Knowles. 1981. The hapten structure of a developmentally regulated glycolipid antigen (SSEA-1) isolated from human erythrocytes and adenocarcinoma: a preliminary note. *Biochem. Biophys. Res. Commun.* 100:1578.
 9. Kannagi, R., E. Nudelman, S. B. Levery, and S. Hakomori. 1981. A series of human erythrocyte glycosphingolipids reacting to the monoclonal antibody directed to a developmentally regulated antigen, SSEA-1. *J. Biol. Chem.* 257:14865.
 10. Kobata, A., K. Yamashita, and Y. Tachibana. 1978. Oligosaccharides from human milk. *Methods Enzymol.* 50:216.
 11. Schwartz, B. A., and G. Gray. 1977. Proteins containing reductively aminated disaccharides: synthesis and chemical characterization. *Arch. Biochem. Biophys.* 181:542.
 12. Ducibella, T., and E. Anderson. 1975. Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. *Dev. Biol.* 47:45.
 13. Surani, M. A. H., S. J. Kimber, and A. H. Handyside. 1981. Synthesis and role of cell surface glycoproteins in preimplantation mouse development. *Exp. Cell Res.* 133:331.
 14. Hyafil, F., D. Morello, C. Babinet, and F. Jacob. 1980. A cell surface glycoprotein involved in the compaction of embryonal carcinoma cells and cleavage stage embryos. *Cell.* 21:927.
 15. Ogou, S., T. S. Okada, and M. Takeichi. 1982. Cleavage stage mouse embryos share a common cell adhesion system with teratocarcinoma cells. *Dev. Biol.* 92:521.
 16. Shirayoshi, Y., T. S. Okada, and M. Takeichi. 1983. The calcium-dependent cell-cell adhesion system regulates inner cell mass formation and cell surface polarization in early mouse development. *Cell.* 35:631.
 17. Lee, Y. C., R. R. Townsend, M. R. Hardy, J. Lonngren, J. Arnarp, M. Haraldsson, and H. Lonn. 1983. Binding of synthetic oligosaccharides to the hepatic Gal/GalNAc lectin: dependence on fine structural features. *J. Biol. Chem.* 258:199.
 18. Muramatsu, T., H. Muramatsu, G. Gachelin, and F. Jacob. 1982. High molecular weight carbohydrates on embryonal carcinoma cells. *In Teratocarcinoma and Embryonic Cell Interactions.* T. Muramatsu, G. Gachelin, A. A. Moscona, and Y. Ikawa, editors. Academic Press, Inc., New York. 143-156.