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Cell Surface Carbohydrates of Preimplantation Embryos as Assessed by Lectin Binding

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Preimplantation embryos were obtained from the uteri and oviducts of 2 strains of mice, Swiss CD-1 and B_6CBA. After removal of the zona pellucida by treatment with pronase, FITC-lectins were bound to the embryonic cell surfaces at either 4°C or 37°C. Both morula and blastocyst stage embryos bound the following lectins, FITC-ConA, FITC-WGA, FITC-RCA_{II} and FITC-RCA_I. No difference in binding was observed between the morula stage and the blastocyst stage within each mouse strain for each specific lectin. However B_6CBA embryos bound less FITC-ConA and FITC-WGA than the corresponding Swiss CD-1 embryos. The topographical arrangement of the lectin receptors was observed to differ between 4°C and 37°C for FITC-Con A, FITC-RCA_{II}, and FITC-RCA_I. While lectins bound at 4°C showed a pattern of continuous labeling, the same lectin at 37°C showed aggregation of lectin receptors into patches indicating lateral mobility of these receptors within the embryonic cell membranes. In contrast FITC-WGA bound at 4°C and 37°C demonstrated continuous labeling of embryos at both temperatures. FITC-fucose binding protein did not bind to Swiss CD-1 embryos.

The invasiveness of trophoblastic cells of mouse blastocysts was studied by culturing isolated embryos without prior enzyme treatment on reconstituted collagen gels. After 4 days in BME containing only glutamine and bovine serum albumin as supplements, the embryos shed their zona pellucida and implanted into the collagen gel as indicated by zones of lysis in proximity to the embryonic cells when analyzed by scanning electron microscopy.

Key words: cell surfaces, carbohydrates, implantation, lectin binding

Implantation of the mammalian embryo into the maternal uterus occurs at the blastocyst stage of development. Prior to implantation, development of the embryo is characterized by cleavage of the fertilized ovum into successively smaller cells without concomitant cellular growth. Cleavage of the embryo occurs within the zona pellucida, a mucoprotein covering of the embryo which is shed just prior to implantation. At the blastocyst stage, the first morphological evidence of cell differentiation can be recognized

Abbreviations: BME – Basal Medium of Eagle; FITC – fluorescein isothiocyanate; ConA – concanavalin A; WGA – wheat germ agglutinin; RCA_I – Ricinus communis agglutinin of mol. wt. 120,000; RCA_{II} – Ricinus communis agglutinin of mol. wt. 60,000.

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by the segregation of 2 cell populations, the inner cell mass which gives rise to the embryo proper and the trophoblast cells which eventually form the embryonic part of the placenta (1). Although the trophoblastic cells are primarily involved in the early stages of implantation and invasion of the uterine wall, this process is necessary for the initiation of further differentiation of the inner cell mass into the various germinal layers, ultimately giving rise to tissues and organ systems typical of the mammalian embryo.

Although implantation has been studied morphologically in a number of animal species (2–6), biochemical events which mediate the process are virtually unknown. The discovery of implantation-associated proteases in uterine secretions of both the mouse (7, 8) and the rabbit (9) only at the time of implantation may be the first biochemical insight into this process. Present available evidence (7, 9) indicates these proteases are synthesized and secreted by the maternal tissues and may be involved in mediating attachment of the blastocyst to the uterus (7). Moreover, implantation probably involves changes in both the embryonic blastocyst as well as the maternal uterus. The first event in implantation, i.e., the attachment of embryonic cells to uterine cells, must involve a cell-cell interaction between these 2 cell types. The nature of this interaction must be specified by the external surface components of the 2 heterotypic cellular membranes. Later events in implantation such as those associated with trophoblastic invasion of the endometrium may be regulated by proteolytic activities either on the surface of, or secreted by the trophoblast cells.

Of the cell surface components most often thought to be involved with cell recognition phenomena, the carbohydrate-protein complexes have been the most extensively studied (10), albeit primarily in nonembryonic cells. One notable exception is the extensive work on embryonic avian cells done in the laboratory of Moscona (11). Studies on the cell surface carbohydrates of mammalian preimplantation embryos are even more limited. Pinsker and Mintz (12) showed that radioactive glucosamine could be incorporated into preimplantation mouse embryo cells in vitro, and that at least one of these components changed with preimplantation development between cleavage and blastocyst stages.

The use of lectin-binding properties of membranes of embryonic cells (11) as well as nonembryonic cells has yielded considerable amounts of information concerning the molecular architecture of animal cell surfaces (see review by Nicolson, Ref. 13). The biological significance of changes in lectin-binding capacities at different functional states such as those associated with transformation are still controversial (14). Nevertheless, recent studies on mammalian ova (15–18) and preimplantation embryos (18) have focused on the use of lectins to compare the carbohydrate components of the zona pellucida with those of the embryo proper as well as how these change during preimplantation development. These studies have demonstrated that most of the monosaccharides common to glycoproteins, i.e., α-D-mannose, N-acetyl-D-glucosamine, β-D-galactose, and N-acetyl-D-galactosamine, are also found on both the zona pellucida and the unfertilized, as well as the fertilized, hamster ovum. These monosaccharides are presumed to exist as part of the oligosaccharide structures of glycoproteins and/or glycolipids. Changes in the amounts of these sugar components, as measured by a difference in quantity of lectin bound and assessed microscopically are difficult to quantitate but seemed to decrease as the embryo reached the blastocyst stage (18).

The present study was undertaken to extend the previous findings (18) and especially to focus on the embryo just prior to implantation. This was done by comparing the fluorescent lectin binding properties of the blastocyst and the morula, the embryonic

stage just prior to blastocyst formation. In addition, implantation in vitro was assessed using reconstituted collagen gel substrata and analyzed by scanning electron microscopy.

MATERIALS AND METHODS

Swiss CD-1 mice and the F_1 hybrid of C57BL/6 and CBA strains (hereafter called B_6CBA for brevity) were housed in vivarium facilities and kept on a 12 h dark-12 h light cycle. The females were superovulated by injection with 5 international units (IU) of follicle stimulating hormone (Gestyl^R, Organon, Oss, Holland) followed 48 h later by 4 IU of lutinizing hormone (Antuitrin "S"^{R}, Parke Davis). After the final injection, the females were mated with fertile males. The presence of a vaginal plug the following morning (day 0) was indicative of successful mating. In some experiments animals were mated without prior superovulation. No difference in experimental results was obtained between the 2 classes of embryos. On day 3.5 the animals were sacrificed and the embryos were flushed from the uterus and Fallopian tubes by insertion of a 30 gauge needle into the ovarian end of the tube and flushing isolation medium through the tube and uterus (19). Isolation medium consisted of basal medium of Eagle (BME, Gibco, Grand Island, New York) containing 1% Ficoll (Sigma, St. Louis, Missouri), and 0.3% bovine serum albumin and was also used for holding the embryos until collection was completed. Embryos from a number of females were combined and only morula and blastocyst stages were retained. The zona pellucida was dissolved by incubating the embryos in 0.2% RNAse-free Pronase (Calbiochem, LaJolla, California) in BME containing 0.5% polyvinylpyrollidone (K & K Laboratories, Inc., Plainview, New York) for 10–20 min at 37°C. The embryos were then washed with isolation medium.

While no dye exclusion tests for viability of the embryos were done, all embryos were assumed to be viable at the time of lectin binding. Two morphological observations supported this assumption: first, nonviable embryos were readily discerned and discarded at the time of isolation by arrested developmental stage and/or disintergrating blastomeres; second, patching phenomena, observed at 37°C with some lectins (see Results), requires a living cell.

Labeling of embryos with FITC-lectins (fluorescein isothiocyanate conjugated lectins) was done according to the method of Roberson et al. (20). All FITC-lectins were obtained from Miles-Yeda, Elkhart, Indiana. Control embryos were treated with specific monosaccharides to compete off the FITC-lectin in order to demonstrate specificity of the binding. α-Methylglucoside and D-(+)-galactose were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. N-Acetylglucosamine was from Nutritional Biochemicals, Cleveland, Ohio. After these incubations the embryos were washed 3 times with isolation medium, fixed in phosphate buffered formalin (4%) containing 1% Ficoll, and mounted on microscope slides. They were examined and photographed using a Zeiss Fluorescence Photomicroscope (light source HB0200; excitation filter KP500 and UG-2, barrier filter 47). Experiments were done at both 37°C and 4°C. Embryos were labeled for various time periods from 10 to 30 min. Within each experimental group all handling of embryos, i.e., lectin binding, washing, and fixing, was done at the indicated temperature.

Embryos were also incubated in a humid atmosphere of 5% CO_2–95% air for 4 days on reconstituted rabbit skin collagen fibrils (21) in BME to which glutamine (20 mM) and 0.3% bovine serum albumen had been added. These embryos were fixed and dehydrated

through a graded alcohol series, critical point dried, coated with Au/Pd alloy (60:40) and examined in a Cambridge S4-10 scanning electron microscope.

RESULTS

Swiss CD-1 Embryos Labeled With FITC-Lectins

Figure 1 shows a blastocyst stage Swiss CD-1 embryo which was labeled with FITC-concanavalin A (ConA) at 37°C. Two points are illustrated by this photomicrograph. First the zona pellucida, the acellular mucoprotein covering of the embryo, was heavily labeled throughout. In contrast the embryo proper displayed discrete patches of fluorescent label suggesting that the ConA receptors within the embryonic cell membranes had aggregated at this temperature. To test this hypothesis, 2 sets of embryos were labeled with FITC-ConA, one at 4°C and the other at 37°C. Figure 2a shows that embryos labeled and processed at 4°C displayed an overall pattern of FITC-ConA labeling with no evidence of clusters or patches. When labeling was done at 37°C (Fig. 2b) patches of fluorescent lectin were again seen. Experiments in which lectin was bound at ambient room temperature gave similar results to those done at 37°C. Figure 2c is a photomicrograph of a control embryo which had been labeled with FITC-ConA at 37°C; the labeled lectin was then competed off of the embryo by incubation in 0.4 M α-methyl-D-glucoside, a monosaccharide known to bind tightly to the lectin. While not all of the lectin was displaced by this procedure, the fluorescence decreased drastically indicating that a great proportion of the lectin binding was specific. For all FITC-lectins studied competition experiments, conducted with monosaccharides known to specifically bind the lectin, gave similar results. In all instances the fluorescence due to FITC-lectin bound to the embryos decreased markedly but was never abolished.

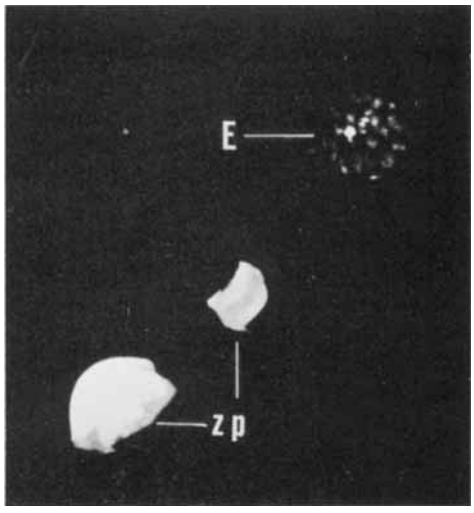

Fig. 1. Photomicrograph of Swiss CD-1 embryo and zona pellucida fragments labeled at 37°C with FITC-ConA. Note that the embryo (E) shows patching of lectin receptors while the zona pellucida (zp) does not. Magnification: 120 ×.

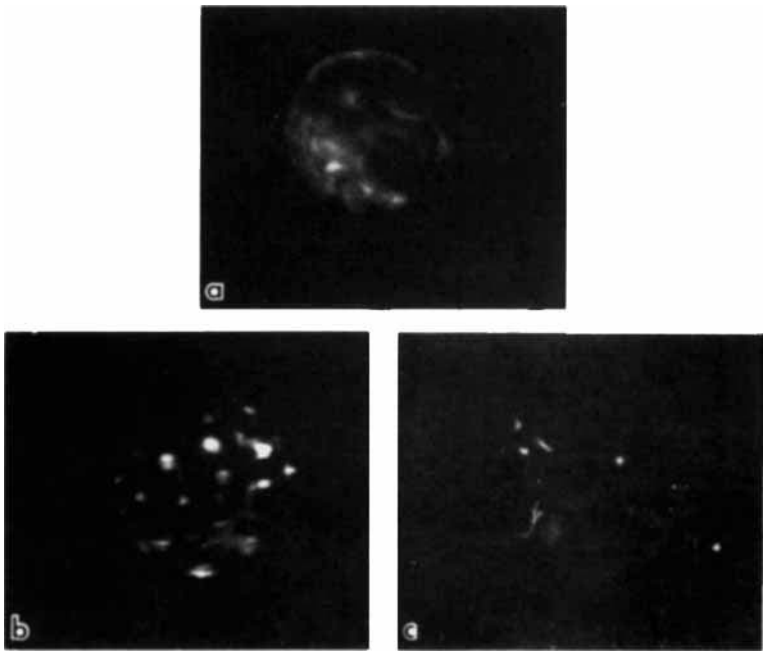

Fig. 2. Composite photomicrographs of Swiss CD-1 embryos treated at 4°C and 37°C with FITC-ConA and control embryo. a) Embryo labeled at 4°C. Note continuous distribution of label. The dark area is due to depth of focus problems in dealing with whole embryos. b) Embryo labeled at 37°C. Note patching of label on surface of embryo. c) Control embryo labeled with FITC-ConA, then incubated in 0.4 M α-methylglucoside to compete off the lectin. Note that only a small amount of fluorescence remains. Magnification: a, b) 270 ×; c) 300 ×.

Experiments in which FITC-wheat germ agglutinin (WGA) was used to label early mouse embryos are represented by the photomicrographs in Fig. 3. Embryos labeled at 4°C such as that shown in Fig. 3a displayed fluorescence on the entire surface of the embryo. In contrast to the results obtained with FITC-ConA labeling, embryos labeled with FITC-WGA at 37°C(Fig. 3b)|did not show "patching" or "clustering" of fluorescent lectin-bound receptors. The reason for this inability of WGA to induce capping, patching, or clustering of receptors is unknown but has been noted also in the case of mouse fibroblasts (22).

Other lectins whose sugar specificities are well documented were used in experiments analogous to those just described. With each lectin examined, the results were unambiguous. If the lectin bound to the embryos, all embryos were labeled. In no case was it found that fluorescence was exhibited by only some of the embryos. In addition when patching was observed to occur, all embryos displayed this phenomena. Results of this series of experiments are summarized in Table I. Embryos at both the morula stage and the blastocyst stage were examined but no qualitative differences in the lectin binding patterns were ascertained and the results for both stages are grouped together. The 2 lectins from Ricinus communis beans have been shown to be differentially inhibited (23). The lower molecular weight lectin, RCA_{II} (formerly RCA_{60}) can be inhibited by β-D-galactose- and N-acetyl-D-galactosamine-like residues while the 120,000 molecular weight species, RCA_I is specifically inhibited by β-D-galactose. Both of these

TABLE I. FITC-Lectin Binding to Preimplantation Embryos

Lectin	Concentration (μg/ml)	Number of experiments	Number of embryos	Inhibitory monosaccharides*	Monosaccharide used and concentration	Binding to embryos	Patching
FITC-Concanavalin A	780	4	32	α-D-glucose α-D-mannose	α-methyl-glucoside (0.4 M)	+	+
FITC-Wheat Germ Agglutinin	800	2	23	(N-acetyl-D-glucosamine)$_2$	N-acetylglucosamine (0.4 M)	+	–
FITC-Fucose-binding protein from Lotus tetragonolobus	100 800	2	3	L-fucose		–	
FITC-Ricinus communis Agglutinin-I	575	3	26	β-D-galactose	D(+)galactose (0.05 M)	+	+
FITC-Ricinus communis Agglutinin-II	570	1	5	D-galactose N-acetyl-D-galactosamine	D(+)galactose (0.05 M)	+	+

*See excellent review on this subject (13).

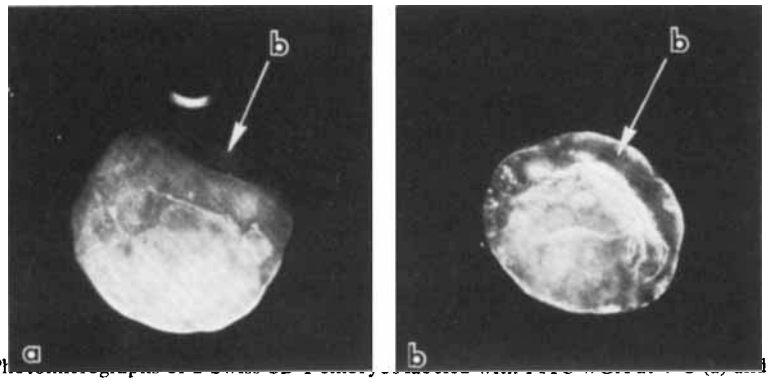

Fig. 3. Ph 37°C (b). Note that with this lectin the embryo labeled at 37°C does not show patching on cell surfaces in contrast to results obtained with FITC-ConA (see Fig. 1 and 2). The blastocoel, labeled b, a fluid-filled cavity characteristic of the blastocyst stage embryo is partially collapsed causing invagination of an area of the embryo. Magnification: 270 ×.

lectins bound to cell surfaces of the embryos and both lectins showed patching behavior when the labeling experiments were conducted at 37°C.

B_6CBA Embryos Labeled With FITC-Lectins

Additional experiments were done with B_6CBA embryos. The lectins used for binding to these embryos were FITC-ConA and FITC-WGA. Both of these lectins bound to B_6CBA embryos and embryos labeled at 37°C with FITC-ConA displayed patching phenomena. Shown in Fig. 4 is an embryo labeled with FITC-ConA. When compared to Swiss CD-1 embryos, fluorescence of B_6CBA embryos due to the FITC-lectin bound was diminished on the surfaces of these embryos (compare Fig. 4 with Fig. 1). Although these results are qualitative because of the method of assessment of lectin binding, i.e., microscopic examination, the implication of this finding is that strain specific differences in lectin binding capacity may exist. Quantitation of the amount of lectin bound to these embryos would aid in exploring this possibility.

Fig. 4. Photomicrograph of a B_6CBA embryo labeled at 37°C with FITC-ConA. Note the decreased fluorescence of this embryo compared to Swiss CD-1 embryos labeled with the same lectin. (Compare with Fig.1 or Fig. 2a, b). Magnification: 120 ×.

Culture of Swiss CD-1 Embryos on Reconstituted Collagen Gels

Embryos were flushed from the uterus and placed onto collagen gels without prior enzyme treatment. The zona pellucida was still intact after 14 h of incubation. After 4 days of incubation all of the embryos had shed their zona pellucida and implanted into the collagen gel. Figure 5 shows a scanning electron micrograph of 2 embryos and the zone of lysis around the cells which attached to the collagen gel. Zones of lysis such as this were not observed in the control collagen gel. i.e., gel incubated under exactly the same conditions except without embryos. In addition no lysis of the collagen gel was observed in the same dish as the embryos in any area not containing an embryo. Lysis was only observed in areas confluent with embryos. This indicates that the activity which lead to gel lysis was closely associated with the embryo and not disseminated throughout the culture medium.

Also demonstrated in Fig. 5 is the fact that cells of the embryo at this stage of culture are not uniform with regard to either size or surface topography. Very small cells as well as much larger ones were seen in all embryos examined. Smooth-surfaced and rough-surfaced cells were seen on all embryos probably reflecting various stages of the cell cycle (24). The significance and origin of the translucent bridge connecting the 2 embryos

Fig. 5. Low magnification scanning electron micrograph of 2 embryos "implanted" into a gel of reconstituted collagen fibrils. Note the variety of cells found in these embryos at this stage. A zone of lysis (indicated by arrowheads) can be seen around each embryo. C) collagen fibrils; E) embryo. Magnification: 1,000 ×.

is unknown. Figure 6 is a higher magnification scanning electron micrograph which illustrates the great profusion of microvilli on blastocyst cell surfaces. Of particular interest is the area in which knob-like projections rather than slender microvilli are seen. The significance of this surface specialization remains to be determined.

DISCUSSION

Binding of lectins to surfaces of mammalian embryos is a powerful tool for elucidating the chemical nature of carbohydrate moieties. This information would be virtually impossible to obtain by other means due to scarity of biological material. The use of lectins for which specificity of binding has been documented is essential for interpretation of results. While early studies on binding sites indicated that lectins bound primarily to terminal sugars of an oligosaccharide chain, more recent studies have demonstrated that some lectins bind to sugars within the oligosaccharide moiety (13). From the lectin binding experiments reported here, it can be concluded that α-D-glycosides (such as α-D-glucose and α-D-mannose), N-acetyl-D-glucosamine (singly or as the dimer), β-D-galactose and/or N-acetyl-D-galactosamine are all present, presumably as constituents of glycoproteins and

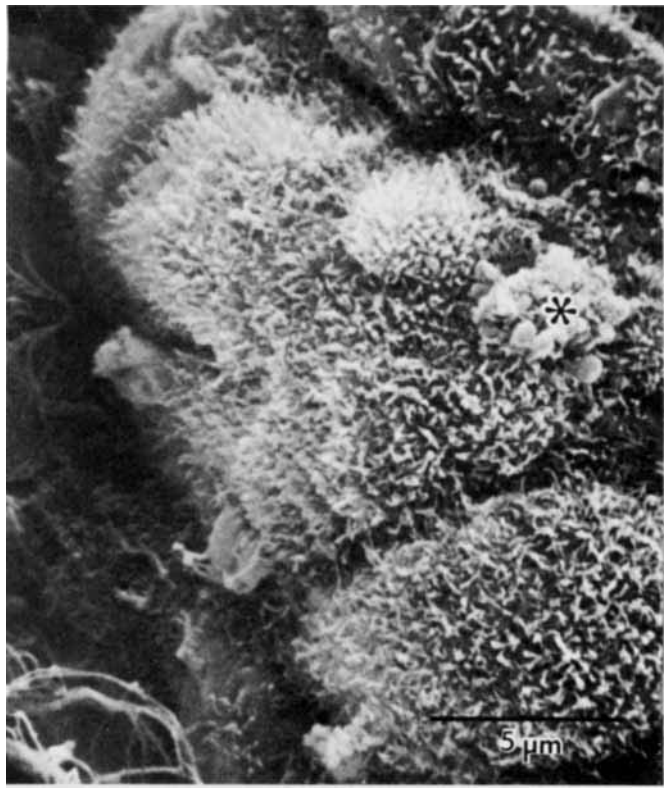

Fig. 6. Higher magnification scanning electron micrograph of a few cells of an embryo after 4 days in culture on reconstituted collagen fibrils. Note the profusion of microvilli, some of which are knob-like (*). Magnification: 5,000 ×.

glycolipids, on cellular surfaces of preimplantation embryos. These results agree with previously reported lectin studies from the laboratory of Nicolson (16–18) who along with his colleagues demonstrated that these lectins bound to both the zona pelucida (16, 17) and unfertilized egg plasma membranes (16) of hamster, mouse, and rat as well as to hamster preimplantation embryos (18). The present experiments further extend these data by focusing in depth on 2 later stages of preimplantation development, the morula stage and the blastocyst stage which contains about 64 cells (25).

In all stages of preimplantation embryos studied, no evidence for the existence of L-fucose-like residues could be obtained. Neither hamster zona pellucida, unfertilized and fertilized eggs (18), or mouse morula and blastocyst embryos bound lectins specific for this sugar. These results indicate that L-fucose may be absent or present in low amounts that cannot be detected by the present procedure. In this regard early avian embryos (blastoderm stage) showed no evidence of L-fucose-like residues (26).

Lateral mobility of membrane receptors has been postulated to account for the patching and capping phenomena observed when cells are incubated at physiological temperatures with molecules which can bind to the cell surface and at the same time form oligomeric structures (13). Tetrameric lectins such as ConA bind to sugar residues on cell surfaces; each subunit of the lectin can bind a sugar residue. Reaction of the multivalent lectin with sugar moieties on different molecules leads to aggregation of receptors yielding patches or caps (see review, Ref. 13). In the present study patching was observed to occur with ConA, RCA_{II}, and RCA_{I}. Thus preimplantation embryonic cells are similar to mature animal cells in this regard. Of importance is the fact that an energy dependent system, i.e., a living cell, is necessary for this phenomena to occur. This was strikingly demonstrated in Fig. 1 in which the zona pellucida, a nonvital extracellular coat, while labeling intensely did not show patching. In contrast to results obtained with ConA and RCA lectins, embryos labeled with WGA did not display lateral mobility of receptors for this lectin: embryos labeled at both 4°C (Fig. 2a) and 37°C (Fig. 2b) showed a continuous pattern of label. These results are in conflict with reports which indicated that this lectin induced patching of WGA-receptors on mammalian eggs (16, 18). However, mouse fibroblasts labeled with WGA did not show clustering of receptors (22). One reason for these discrepancies could be developmental – the unfertilized egg may have WGA-receptors whose mobilities are greater than those found within blastocyst stage embryos or mature cells.

Qualitative differences in the lectins bound or the labeling patterns at 4°C and 37°C between embryos from different mouse strains were not apparent. Species-specific differences between hamster, rat, and mouse zona pellucida have been reported (17). While all zona pellucida bound all the lectins examined, the quantity of lectin necessary to induce agglutination differed between the 3 species. This result may imply a quantitative difference in the number of lectin receptors present on zona pellucida from different animals. While microscopic examination is not a quantitative method for evaluating amounts of lectin bound, preliminary results from the present experiments suggest that different strains of mouse embryos may have different lectin-binding capacities. Whether these differences reflect genetic variance remains to be demonstrated.

While earlier studies found no developmental differences in lectin-binding properties between the fertilized egg, the 2-cell stage, and the 8-cell stage a decrease in lectin binding was noted at the blastocyst stage (18). As many biochemical events occur during development of the embryo from cleavage stages to the blastocyst stage, it was of interest to explore the question of whether this decrease in lectin binding occurred precipitously at the blastocyst stage or if it was a more gradual change. The experiments reported here

demonstrated that both the blastocyst embryo and the morula stage embryo bound the same amounts of lectin as assessed microscopically. These results taken together with the previous ones indicate that decrease in lectin binding is a gradual change that occurs between the 8-cell stage and the blastocyst stage. This change in the number of binding sites for lectins could reflect either a change in total number of sites synthesized or masking of specific sites by the addition of other sugars to the oligosaccharide chain. Alternatively, degradation of binding sites by developmentally regulated glycosidases could account for the observed decrease in lectin receptors.

The biological significance of changes in the lectin-binding properties during preimplantation development of mammalian embryos remains to be determined, although it has been speculated that these changes are related to alterations in embryonic cell surfaces necessary for implantation (18). Perhaps unmasking of certain sugar residues on the embryo is necessary for recognition by cells of the endometrium. The in vitro implantation system described in this report should aid in elucidating the biochemistry of implantation and what role, if any, lectin receptors play in this important biological event.

The scanning electron micrographs demonstrate that collagen, one of the components of the endometrium, can serve as suitable substrata for culture of early mouse embryos (27) and for studying implantation in vitro. The finding of discrete areas of lysis surrounding the "implanted" embryos suggests that proteolytic activities arising from the trophoblast cells may be involved in this biological phenomena. The fact that no other areas of lysis were observed in this culture suggests that this activity is severely restricted perhaps by being bound to the cell membranes of the embryo.

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