Normal and Dystrophic Rat Retinal Pigment Epithelia Display Different Sensitivities to Plant Lectins

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Previous studies showed that cultured retinal pigment epithelial (RPE) cells from the Royal College of Surgeons (RCS) rat incorporate 50% less fucose into a number of cell-surface glycoproteins compared with controls. The cause of reduced fucose incorporation may be a generalized defect in glycoprotein processing in the RCS rat RPE. This hypothesis has been further explored by comparing the relative sensitivities of normal and dystrophic rat RPE to the toxicity of plant lectins of various specificities. Freshly isolated RPE cells from normal and dystrophic rats were cultured in the presence of increasing concentrations of lectin. For each lectin, the concentration at which less than 10% of the cells survived was determined. These tests showed that the dystrophic RPE cells were more sensitive to lectins that bound to core sugar moieties such as mannose and N-acetylglucosamine; normal RPE cells were more sensitive to lectins which bound to the more terminally located sugars, galactose and N-acetylgalactosamine. Overall, the results suggest that decreased incorporation of fucose into the RCS RPE may be due to failure of the dystrophic RPE to add either N-acetylgalactosamine or galactose (to which fucose is subsequently added) to oligosaccharide structures.


In the Royal College of Surgeons (RCS rd^- p^-) rat, the retinal pigment epithelial (RPE) cells do not phagocytose shed retinal debris, and retinal degeneration ensues. One possible cause of this defect is that some component in the plasma membrane of the RCS rat RPE is missing or defective. The purpose of this ongoing research was to analyze the protein components of the RCS RPE plasma membrane to determine if any biochemical differences could be detected. In previous reports, evidence was presented that cultured RPE from the RCS rat display reduced incorporation of $^3$H-fucose into a number of cell-surface glycoproteins. This suggested that the RCS RPE does not elaborate the carbohydrate portion of glycoproteins (and possibly glycolipids) in the same manner as normal cells.

One technique that has been widely used to characterize glycosylation mutants, or cells that possess an aberration in glycoprotein–glycolipid processing machinery, is the lectin toxicity assay. Plant lectins (some of which are toxic to mammalian cells) act primarily by recognizing a particular carbohydrate receptor on the cell surface. An increased or decreased sensitivity of a cell to the toxic effects of a particular lectin can reflect accurately even a subtle alteration of cell-surface carbohydrate. The specificity of the lectin tested provides information about the type of sugar residues that are most accessible at the cell surface. Use of this technique has provided further evidence that the RPE cell of the RCS rat displays a subtle difference in cell-surface carbohydrate compared with controls. Additionally some information about the types of sugar residues present in reduced quantity on the RCS RPE cell surface was deduced, based on the specificities of the lectins used.

Materials and Methods

Cell Culture

Ten-day-old RCS and Long Evans (LE) rats were killed by a method that complied with the ARVO Resolution on the Use of Animals in Research. The RPE cells were isolated and established in tissue culture by a previously published method. They were plated in 96-well culture plates at 10,000 cells per well in 100 µl of growth medium. For lectin toxicity tests, fetal bovine serum concentration was reduced to 10%.

Lectin Toxicity Assays

Lectins were added to cultured cells by one of two methods. In some experiments, lectin was added to
the wells of 96-well tissue culture plates at the desired concentrations in 50 μl of growth medium, followed by RPE cells (10,000/50 μl). Alternatively, cells were plated without lectin in 100 μl of growth medium. After 1–4 days, lectin was added at the desired concentration. Initial screening of each new lectin was done by testing a wide range of concentrations (10–500 μg/ml). If the lectin was toxic, then it was further tested at smaller increments, in a narrower concentration range.

The results were quantitated by counting the number of live cells per 10 × 10 grid at 100× magnification. The dead cells were easily distinguished from live cells by morphology; they were rounded into dense balls and not attached to the dish. Live cells were attached and flattened. The percent survival was determined by comparing the number of live cells in each lectin-containing well with that in the control (lectin-free) well. The concentration of lectin at which less than 10% of cells survive was defined as the D10.21

Lectins

The lectins were purchased from Sigma (St. Louis, MO) or Vector (Burlingame, CA). Stock solutions (0.5–1 mg/ml) were made in serum-free medium or water and then filter sterilized. These stock solutions were stable for at least 3 months, with the exceptions of Pisum sativum (PSA) and Ricinus communis 120 (RCA 120). The PSA aggregated during storage in either water or medium and had to be refiltered prior to each use. The RCA 120 became more toxic with storage but lost its differential toxicity with respect to RCS and LE RPE. Therefore, fresh dilutions of RCA 120 were prepared for each experiment.

Photomicrography

The cells were photographed using a Nikon (Tokyo, Japan) inverted microscope (Diaphot-TMD) with a Nikon 35-mm camera attached to the front port. The 10× objective was used for photography, giving a final magnification of 25× on the film (Kodak TMAX-400; Rochester, NY) plane.

Results

Lectin Toxicity Assays

A number of cell lines possessing mutations in glycosylation enzymes have been isolated on the basis of decreased sensitivity to the toxic effects of plant lectins.9,10 Altered sensitivity to toxicity of lectins, applied extracellularly, has been found to reflect accurately the changes in cell-surface carbohydrate resulting from mutations that cause a disruption in the synthesis of oligosaccharides which are found in both glycolipids and glycoproteins.11 The rationale for the current study was: if the cell-surface carbohydrate expressed by the RCS RPE is different from that of normal RPE, then this difference will be reflected in the relative sensitivities of the two cell types to the toxic effects of plant lectins. Furthermore, the specificity of the lectins will provide information about the types of sugar moieties that are altered in the RCS RPE.

Initially, a panel of lectins of varying specificities was tested for toxicity to RPE cells (Table 1). Many of the lectins tested (notably, most N-acetylglucosamine [GlcNAc] specific, many galactose [Gal] specific, and all fucose specific) were not toxic to the rat RPE.

Dystrophic RPE cells were more sensitive to wheat germ agglutinin (WGA) than were normal cells. This could reflect differences in either GlcNAc or sialic acid content on the surface of RCS RPE.14,22–24 Unfortunately, succinylated WGA, specific only to GlcNAc, was not toxic to the RPE cells.

Mannose binding lectins of three types (concanavalin A [Con A], PSA, and Lens culinaris hemaggulutin-45)-Dyst > normal

N-acetylglucosamine binding

Wheat germ agglutinin (WGA)

Succinylated wheat germ agglutinin

Bandiera simplicifolia II

Mannose binding

Concanavalin A (Con A)

Pisum sativum (PSA) (req’s Gal-6GlcNAc-Asn)

Lens culinaris (LCh) (req’s Fuc-6GlcNAc-Asn)

Galactose and N-acetylglactosamine binding

Ricinus communis (RCA 120)

Sophora japonica (SJA) (β-linked GalfNAc and Gal)

Phaseolus vulgaris agglutinin E (PHA-E)

Phaseolus vulgaris agglutinin L

Dolichos biflorus (a-GalNAc)

Peanut agglutinin (Gal/3l-3GalNAc)

Soybean agglutinin (α- and β-GalNAc)

Bandiera simplicifolia I (a-GalNAc and Gal)

Erythrina crystalagalli (Gal/1-4GlcNAc)

Vicia villosa (aGalNAc)

Fucose binding

Lettus tetragonolobus

Anguilla anguilla

Ulex europaeus

Sialic acid binding

Limulus polyphemus (LPA)

Elderberry bark lectin (EBL)

O-linked oligosaccharides

Jacalin

Sensitivity

Dyst > normal

Not toxic

Not toxic

Dyst > normal

Dyst > normal

Normal > dyst

Normal > dyst

Dyst > normal

Not toxic

Not toxic

Normal > dyst

Not toxic

Not toxic

Normal = dyst

Normal = dyst

Dyst = normal

* Not toxic up to 500 μg/ml.

Table 1. Lectins surveyed for toxicity to rat RPE
nin [LcH]) were all toxic to the rat RPE, and dystrophic RPE was more sensitive in every case. The Con A binds to accessible mannose residues;\textsuperscript{25,26} PSA and LcH also bind to mannose residues but display stringent requirements in oligosaccharide structures for binding. Both PSA and LcH require fucose to be present in α1–6 linkage to the GlcNAc bound to aspara-
gine in complex N-linked oligosaccharides.\textsuperscript{27} Furthermore, PSA binding is enhanced by terminal mannose residues, while LcH favors exposed GlcNAc residues, in N-linked oligosaccharide structures.\textsuperscript{27} Therefore these results suggest that core mannose and GlcNAc residues are more exposed in the RCS RPE than in normal cells.\textsuperscript{28}

Fig. 1. Survival of normal (■) and dystrophic (□) RPE vs increasing concentrations of lectin. (A) Survival in WGA, at 13 days; data taken from exp. #3 (Table 2). (B) Con-A, day 5, exp. #2. (C) LcH, day 5, exp. #2. (D) RCA, day 6, exp. #3. (E) Expansion of 0–0.3 μg/ml from (D) to show toxicity at low lectin concentrations. (F) SJA, day 14, exp. #1.
Three of the Gal, N-acetylgalactosamine (Gal-Nac)-binding lectins (RCA120, Sophora japonica [SJA], and Phaeocontulus vulgaris agglutinin E [PHA-E]) were toxic to the RPE. Of these, RCA120 and SJA were more toxic to normal cells, and PHA-E was more toxic to dystrophic cells. Both RCA and SJA bind to oligosaccharides containing β-linked Gal and GalNAc. PHA-E specifically recognizes biantennary complex oligosaccharides containing two outer galactoses and a bisecting N-acetylgalactosamine linked β1,4 to the β-linked mannose residue at the core. Therefore, the difference in sensitivity of normal and dystrophic RPE to RCA and SJA may reflect binding to a specific class of β-linked Gal residues different from those recognized by PHA-E. Alternatively, the difference may be due to binding to GalNAc (more often found in O-linked carbohydrate). Unfortunately, with the exception of Jacalin, other Gal and GalNAc binding lectins of greater specificity, which may have shed further light on this question, were not toxic to the RPE (Table 1).

There is strong evidence that Jacalin binds preferentially to Gal in O-linked oligosaccharides. Normal and dystrophic RPE were equally sensitive to the toxic effects of Jacalin, suggesting that there was no major disruption of addition of Gal to O-linked carbohydrate in the RCS RPE.

Results with sialic acid binding lectins Limulus polyphemus (LPA) and elderberry bark lectin (EBL) were variable and not reproducible. At times the RCS cells were slightly more sensitive; opposite results were obtained in other experiments. In conclusion, the lectin toxicity test could not be used reliably with RPE cells to probe the sialic content of the cell surface. This may have been due to the fact that the sialic acid-binding lectins were not very toxic (>200 µg/ml was required to kill the cells).

Of the lectins that were toxic, each was further tested to determine the relative toxicity to normal and dystrophic rat RPE. Dose-response curves were generated for each lectin of interest. Figures 1A–C show dose–response curves for lectins (WGA, Con A, and LcH) that were more toxic to dystrophic than to normal RPE. The difference in sensitivities of normal and dystrophic cells was small but reproducible. Figures 1D–F show the curves for RCA120 and SJA, lectins that were more toxic to normal RPE. From such graphs, the D10, or concentration at which less than 10% of the cells survived, was determined. Each lectin for which there was a slight difference in sensitivity was tested three times in separate experiments.

Table 2 shows sets of D10 values for both normal and dystrophic RPE for each of the lectins tested. In some cases, a small difference in sensitivity of dystrophic and normal cells to one lectin was corroborated by an even larger difference in D10 for a lectin of similar specificity (for example, Con A and LcH). A test was conducted to determine if slight errors in cell counting (ie, number of cells plated) could affect the D10 values observed. The RPE cells were plated at 8000, 10,000 and 12,000 cells per well in WGA at 20 µg/ml. By day 12, less than 10% of the cells survived in each well. Therefore, the error involved in using a hemocytometer to count cells would not significantly affect the outcome of these tests.

To determine if slight differences in growth rate of the normal and dystrophic RPE could be responsible for these apparent differences in lectin toxicity, another test was done. Lectin was not added until cells reached confluence (day 4). Even when lectin was added later, the differences in sensitivity between normal and dystrophic cells was observed. However, killing of the established cells occurred more slowly, possibly due to loss of access of lectin to the basal surfaces of the cells.

### Table 2. D10 values* of toxic plant lectins for normal and dystrophic RPE

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Exp. #</th>
<th>Normal D10 (µg/ml)</th>
<th>Normal D10 (µg/ml)</th>
<th>Dystrophic D10 (µg/ml)</th>
<th>Morph. change</th>
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* Each value was determined in a separate experiment. While the actual D10 value for each lectin varied slightly, the differences between normal and dystrophic D10 values were consistent and reproducible.

† Greater than 10% of cells alive at 200 µg/ml.

### Morphology Changes

Figure 2A shows confluent, healthy RPE cells with no lectin added. Cells are hexagonally shaped and well pigmented with visible nuclei. In certain areas,
Fig. 2. Normal and lectin-altered morphologies of cultured rat RPE. (A) Confluent culture of normal rat RPE. The arrow denotes a “dome” of cells. (B–E) Cells that were cultured for 10 days in: (B) WGA, 10 μg/ml; (C) RCA120, 0.05 μg/ml (arrows denote examples of “pointed cells”); (D) PHA-E, 50 μg/ml; (E) Jacalin, 25 μg/ml. In (E), the large arrow indicates a mass of filamentous material that was the end result of Jacalin treatment. Note the multiple dome-like formations (small arrows). (F) Dead RPE cells, following treatment with WGA at 25 μg/ml for 10 days. Magnification in these photographs is X46.

“domes” (reflecting tight junction formation and normal fluid transport) are observed. At sublethal concentrations of the lectins, the RPE often underwent peculiar morphologic changes. Interestingly, several types of morphologies were observed. Figures 2B–E show the morphologies produced by WGA, RCA120, PHA-E, and Jacalin. Each had distinctive features. With WGA (also seen with Con A) cells retracted from the culture dish into groups of four to eight (Fig. 2B). With RCA120 (also SJA and EBL) individual cells ceased to have contact and acquired sharp points at some edges, before dying (Fig. 2C). With PHA-E, RPE cells relinquished the hexagonal shape in favor of an elongated shape. Similar, but not
identical morphologies were also observed for LcH and PSA. Groups of cells were aligned in this elongation process (Fig. 2D). Jacalin (Fig. 2E) initially stimulated production of dome-like groups of cells, resembling those found in normal cultures (Fig. 2A). After several days, cells in these dome-like areas appeared to extrude filamentous material. The result was a filamentous mass in which no cells or nuclei were visible. Dead RPE cells appeared as dense, dark spheres, which were no longer attached to the culture dish (Fig. 2F). Thus it was easy to distinguish live from dead cells.

In the case of WGA, the reversibility of the morphology change was examined. Normal and dystrophic cells, which had assumed the morphology found in Figure 2B, were washed with fresh medium containing no lectin, and cultured for several days in lectin-free medium. By day 4, the cells resumed a normal shape and were again confluent. When lectin was added back to the cells, they underwent the same morphologic change. Furthermore, the differences in dose response of the normal and dystrophic cells was once again manifest in the second treatment. When lectin was removed from cells assuming the dead morphology (Fig. 2F), no reversibility was found.

These morphologic changes served as useful adjuncts to D10 values for discerning differences in the sensitivity of normal and dystrophic RPE to various lectins (Table 2). For example, in the case of Con A, morphologic changes in dystrophic cells began at 20 \( \mu g/ml \); similar changes were not seen in normal cells until 30 \( \mu g/ml \) was reached.

In summary, the dystrophic RPE were more sensitive to lectins binding to core sugar moieties, mannose (Con A, PSA, and LcH) and GlcNAc (WGA). Normal RPE cells were more sensitive to lectins that bind to the terminal sugars, Gal and GaINAc (RCA120 and SJA). However PHA-E, specific for the Gal in a particular type of biantennary complex N-linked oligosaccharide, was more toxic to dystrophic cells. Lectins binding to the most terminally located sugars, fucose and sialic acid, were not toxic enough to the RPE to be of value in lectin toxicity assays.

### Discussion

In previous reports\(^6\)–\(^8\) a group of high-molecular-weight cell-surface glycoproteins was identified in cultured rat RPE. When RCS and normal rat RPE were labeled in cell culture with \( ^3H \)-fucose, the dystrophic RPE incorporated about 50% less fucose into a number of these proteins compared with controls.\(^8\) Three possible reasons for reduced fucose incorporation were proposed. First there may have been a mutation at or near a glycosylation site in an acceptor glycoprotein. In this case the affected glycoproteins detected by two-dimensional gel electrophoresis would have to be related, or derived from the same precursor protein. Second an alteration in oligosaccharide structures at sites to which fucose is normally attached may have occurred. Third there was a lack or reduction of a particular fucosyltransferase activity or change in fucose metabolism. In another report, the first possibility was excluded by demonstrating that the high-molecular-weight fucose-labeled cell-surface glycoproteins were not highly related in structure.\(^35\) Therefore, the reason for reduced fucose incorporation into major cell surface glycoproteins in the RCS rat RPE was likely to be the second and/or third possibility.

In this study, the second possibility was explored further by comparing the sensitivity of normal and dystrophic rat RPE to the toxicity of plant lectins. Lectin toxicity assays have been used both to isolate and characterize mammalian cell lines which possess mutations in oligosaccharide-processing pathways.\(^9\)–\(^11\) The degree of lethality of a particular lectin to a cell correlates with the amount or accessibility of its target carbohydrate on the cell surface. Using this very sensitive assay, other investigators detected subtle changes in carbohydrate on the surface of mutant cells that could not be seen by electron microscopic or radiochemical methods of quantitating lectin binding. Other advantages of this assay are that it requires few cells (compared with biochemical analysis) and provides some information about the effect of altered carbohydrate on the functioning of glycoproteins and glycolipids in intact membranes. It has been generally found that disruption of early glycoprotein-processing steps results in large differences in the sensitivities of control and mutant cells to the toxicity of lectins which recognize the sugars involved.\(^13\) Mutations that affect later processing enzymes (many of which can be tissue specific\(^36\)) can result in a small but reproducible difference in the sensitivity of the mutant and control cells to the toxic effect of appropriate lectins.\(^12\) The limitation of the assay is that information is inferred and must be confirmed by further biochemical analysis.

The assays of relative toxicity of lectins to normal and dystrophic rat RPE showed that the dystrophic cells were more sensitive to lectins that bind the core sugars, mannose, and GlcNAc. One possible reason for greater sensitivity to mannose-binding lectins is that the dystrophic RPE cells lack a mannosidase activity for the processing of N-linked oligosaccharides and accumulate an increased number of high mannose, N-linked oligosaccharides. However, the results of other experiments show that while the major cell-surface glycoproteins of the dystrophic rat RPE con-
tain a great deal of N-linked oligosaccharide, they are resistant to Endo H, and incorporate normal amounts of 3H-mannose. This result argues against mannosidase deficiency. An alternative explanation for increased sensitivity of the dystrophic RPE to mannose-binding lectins is that core structures are more accessible to lectin binding due to failure of the dystrophic RPE to add certain terminally located sugars.

Interpretation of the WGA results is ambiguous, due to the fact that WGA binds to both sialic acid and GlcNAc. Therefore, the WGA results could be consistent with either greater exposure of core GlcNAc or increased content of sialic acid or GlcNAc in the RCS RPE. Tests of sialic acid-binding lectins were inconclusive. Normal and dystrophic RPE displayed similar (variable) sensitivities to LPA and EBL. Other GlcNAc-specific lectins, which may have helped to resolve this question, were nontoxic to the RPE. However, greater exposure of GlcNAc in dystrophic RPE is the explanation most consistent with results obtained with RCA120 and SJA.

The dystrophic RPE cells were less sensitive than normal cells to SJA and RCA120, lectins that bind to the more terminally located sugars, Gal and GalNAc. If oligosaccharide structures of the dystrophic RPE were simply lacking in terminal fucose, it is expected that the dystrophic RPE would show increased sensitivity to all Gal- and GalNAc-binding lectins, due to exposure of terminal Gal and GalNAc. Since this was not the case, the results suggest that oligosaccharide structures of the surface of dystrophic RPE cells contain reduced quantities of Gal and/or GalNAc residues compared with normal cells. Failure of the dystrophic rat RPE to add certain Gal or GalNAc residues to oligosaccharides could result in diminished subsequent addition of fucose, since fucose is often added to Gal.

The binding requirements of SJA suggests that Gal or GalNAc, in β linkage specifically, is altered in dystrophic RPE. The lectin SJA binds to β-linked Gal or GalNAc in both N- and O-linked oligosaccharides, and Gal has been found in β-linkage to Glc, GlcNAc, and GalNAc via the number 3, 4 or 6 carbons. To a lesser extent GalNAc is found in β-linkage in oligosaccharides of glycoproteins. Defects in any of these number of residues could cause the altered sensitivity of the dystrophic RPE to ricin and SJA. Unfortunately, other Gal- and GalNAc-binding lectins of greater specificity were not toxic to the rat RPE (Table 1).

The results of PHA-E toxicity assays excluded one class of Gal-containing residues as reduced in the RCS RPE. The lectin PHA-E recognizes a particular type of N-linked complex oligosaccharide: a bianten-
been acquired. In this case, the RCS rat would possess a mutation in a gene which controls the developmental expression of glycosyltransferases.6 This possibility will be further explored by assaying normal and dystrophic RPE for galactosyl-, N-acetylgalactosaminyl-, and fucosyltransferase activities. Lectin toxicity assays, although relatively nonspecific, have been an aid in narrowing down the choice of enzyme classes to assay.

The expression of reduced glycosylation of cell-surface molecules may not be the direct cause of failure of the RCS RPE to phagocytize ROS. Reports by Hall et al19 suggest that terminal processing of N-linked oligosaccharides in glycoproteins is not required for phagocytic capabilities in normal RPE. However, these studies do not address the possible contribution of O-linked oligosaccharides or glycolipids, many of which are processed by the same terminal processing enzymes.37,48 O-linked oligosaccharides have been found to be important in conferring stability on cell-surface glycoprotein receptors.49-51 Neutral glycosphingolipids and gangliosides have been found to play important roles in development and signal transduction.52-54

Finally, the lectin toxicity assays have shed some light on the third possible explanation for reduced fucose incorporation into glycoproteins of the dystrophic rat RPE, ie, the possibility of a lack of fucosyltransferanse activity. There have been reports of at least 11 types of fucosyltransferase activities in mammalian tissues.33 One of these is the N-acetylgalactosaminidase α1-6 fucosyltransferase, which catalyzes the addition of fucose to the asparagine-linked GlcNAc of N-linked oligosaccharides. The results of the tests of PSA and LeH show that the dystrophic RPE cells do not lack this enzyme, since these mannose-binding lectins have an absolute requirement for this particular fucose linkage for binding to occur. Since the dystrophic RPE cells displayed sensitivity to PSA and LeH, they must express this particular fucosyltransferase activity. The remaining fucosyltransferases add fucose to terminal positions in oligosaccharide structures of both glycoproteins and glycolipids. The activities of these enzymes in normal and dystrophic RPE are currently being studied.

**Key words:** inherited retinal dystrophy, RCS rat, retinal pigment epithelium, cell surface carbohydrate, lectins

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