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Lectin-Resistant Mutants of Polarized Epithelial Cells

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Two lectin-resistant mutants derived from Madin Darby canine kidney cells, with constitutive alterations in the asparagine-linked carbohydrate moieties, retained the characteristic structural and functional epithelial polarity of the parental cells. A ricin-resistant cell line was unable to incorporate galactose-sialic acid into glycoproteins and, from the pattern of cross-resistance to other lectins, appears to be different from previously described lines resistant to this lectin; the mutation in a concanavalin A-resistant line results, probably, in the production of defective carbohydrate cores of glycoproteins. In spite of glycosylation defects which result in an increased electrophoretic mobility of many cellular glycoproteins, both mutants retained the typical asymmetric structure of the plasma membrane (microvilli on the apical surface, junctional elements on the basolateral surface), functional tight junctions, and unidirectional active transport of electrolytes and water. These results suggest that glycoproteins with terminal galactose-sialic acid moieties are not critically involved in the development and maintenance of polarity in epithelial cells. The mutant cells, particularly the ricin-resistant line, exhibited, however, morphological and electrophysiological changes which suggest a quantitative effect of the mutations on intracellular traffic of membranes and tight junction formation. The cell lines described in this paper, the first lectin-resistant mutants of epithelial lineage, should prove useful tools for studying the peculiarities of glycosylating pathways in polarized cells.

The ability of epithelial membranes to function vectorially depends on the polarity of the epithelial cells, observable both in the asymmetric distribution of organelles in the cytoplasm and in the segregation of different sets of proteins into two opposite plasma membrane domains, apical and basolateral, separated by tight junctions. External signals, such as the contact with other cells or with the basal lamina through interaction with specific surface molecules, may be important determinants in establishing and maintaining epithelial polarity (E. Rodriguez-Boulan, *in* B. Satir, ed., *Modern Cell Biology Reviews*, in press). Given the well-documented involvement of the carbohydrates of glycoproteins and glycolipids in surface recognition processes, we became interested in studying the effect of constitutive alterations of glycosylation on the polarity of epithelial cells.

In recent years, epithelial cell lines which retain in culture polarity properties of natural epithelia have become available. The dog kidney cell line Madin Darby canine kidney (MDCK) has been the object of the largest number of studies. As confluent monolayers, MDCK cells become morphologically and functionally polarized. They develop transmonolayer electrical resistances of 100 $\Omega \cdot cm^2$, a small electrical potential (about 1 mV, positive on the basal side), and cationic selective permeability (Na^+ is preferred ten times over Cl^-) (1, 2, 12). The cells transport water and ions unidirectionally to the basolateral side. When the monolayers are grown on impermeable substrata, this results in the formation of domes. Like natural epithelia, MDCK cells generate two domains in the plasma membrane, apical and basolateral, separated by tight junctions (1, 2, 8, 12). Plasma membrane proteins are asymmetrically distributed between the apical and basolateral surfaces (10, 16). This is dramatically demonstrated by the asymmetric budding of enveloped viruses from only one plasma membrane domain, which results from the accumulation of viral envelope glycoproteins in the appropriate plasma membrane region (19, 20). Influenza virus, Sendai virus, and simian virus 5 bud from the apical surface of MDCK cells, but vesicular stomatitis virus (VSV) is assembled only from basolateral plasma membrane regions.

In this paper, we report the selection and partial characterization of mutant cell lines resistant to concanavalin A (ConA) and to *Ricinus communis* agglutinin (RCA), derived from

MDCK cells and designated ConA^r and RCA^r cells, respectively. In spite of constitutive alterations in the carbohydrate moieties of glycoproteins, which in the case of the RCA^r cell line amount to the loss of at least the galactose-sialic acid residues, the lectin-resistant mutant cell lines retain ultrastructural and physiological polarity and the ability to develop functional tight junctions. This is in agreement with our previous demonstration that both mutants exhibit polarized budding of enveloped viruses and an asymmetric distribution of viral glycoproteins in the plasma membrane (6).

MATERIALS AND METHODS

Cell lines, viruses, and chemicals. Wild-type (WT) MDCK cells were obtained from the American Type Culture Collection, Rockville, Md. A hypoxanthine-guanine-phosphoribosyltransferase-negative derivative was obtained without mutagenesis by using 5 μg of 6-thioguanine per ml. The cells were grown on plastic tissue culture dishes in Dulbecco modified Eagle minimum essential medium, supplemented with 5% fetal calf serum and 5% donor calf serum, in humidified incubators, with 10% CO_2 at 37°C. Sources and conditions for growth and infection with VSV (Indiana serotype) were as previously described (6; see also E. Rodriguez-Boulan, Methods Enzymol., in press). ConA, RCA II, phytohemagglutinin L (PHA), and wheat germ agglutinin (WGA) were purchased from Vector Laboratories, Burlingame, Calif. Tunicamycin was a generous gift from John Douros of the National Institutes of Health, Bethesda, Md.

Selection of lectin-resistant mutants. The parental lines, WT MDCK and its hypoxanthine-guanine-phosphoribosyltransferase-negative derivative, after mutagenesis with nitrosoguanidine (0.5 μg/ml) and intermediate cultivation (11), were incubated with 0.75 μg of RCA or 50 μg of ConA per ml, doses which allowed no WT survivors. The putative mutants were purified by recloning in the presence of lectins and were subsequently maintained in the absence of lectins.

Lectin dosage. Cells to be tested were plated in 60-mm plastic petri dishes so that they would give about 200 colonies in a control medium to which no lectin had been added. The next day, the lectins were added at a variety of concentrations. After about 12 days, the plates were fixed and stained with Giemsa stain, and colonies which had more than 40 cells were scored. The dose of lectin which allowed 10% survival of clones relative to the number of clones obtained in control medium was recorded.

Isotopes and cell labeling. Sources of [^{3}H]glucosamine and [^{35}S]methionine and conditions for cell labeling were as previously described (6). D-[^{3}H]galactose (2,147 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass., and added to the labeling medium (Earle minimum essential medium containing one-third of the normal amount of glucose and 2% dialyzed fetal calf serum) at concentrations of 50 μCi/ml. Additional details are provided below and in the figure legends.

Electrical tests. Cells were plated at high density on collagen-coated nylon disks, and the transepithelial electrical resistance was measured as previously described (2). Briefly, MDCK cells were plated at high density (250,000 cells per ml per well) onto collagen-coated nylon disks (diameter, 13 mm) in 24-well tissue culture dishes. The disks were transferred to a new chamber after 90 min, and the transmonolayer resistance was determined in an Ussing-type chamber with a Keithley DC electrometer, a Simpson microamperemeter, and a DC variable power supply.

Electron microscopy. Monolayers on plastic culture dishes were washed with cold 0.1 M cacodylate buffer, pH 7.4, and fixed for 1 to 20 h at 4°C with 2% (wt/vol) glutaraldehyde in the same buffer. After three buffer rinses, samples were postfixed with 2% OsO_4 in cacodylate buffer, block stained with 1% aqueous uranyl acetate, and dehydrated in alcohols. The monolayers were removed from the petri dishes by using amyl acetate (15), washed extensively in 100% ethanol, and embedded in Epon 812. Thin sections were cut with diamond knives, picked up on Parlodion-coated carbon-stabilized copper grids, and examined in a Philips 300 electron microscope.

RESULTS

Isolation and genetic characterization of lectin-resistant mutants of MDCK cells. RCA^r and ConA^r mutants were isolated from WT MDCK cells by incubation in the presence of 0.75 and 50 μg of the respective lectins per ml as described above. The frequency of spontaneous mutation to the RCA^r phenotype was 6×10^{-6}, whereas the frequency of mutation to ConA^r was 3.3×10^{-6}. Treatment with the mutagen nitrosoguanidine increased the mutation frequencies by approximately 5- and 10-fold, respectively. The lectin-resistant cell lines are quite stable since they have retained their lectin-resistant phenotype after over 6 months of culture in the absence of the lectins. Furthermore, different clones of each mutant behaved similarly with respect to the phenotypic properties examined; these phenotypes are the only ones we observed among the survivors of three independent selections with both lectins.

Table 1 compares several properties of the ConA^r and RCA^r cell lines. As can be seen, the growth rate of the mutants was similar to that of

TABLE 1. Properties of lectin-resistant mutants of MDCK cells

Cell line	Growth rate[a]		LDS_{10}[b] (μg/ml)			
	With lectin	Without lectin	RCA	ConA	WGA	PHA
WT		21	0.01	10	0.5	2
RCA^r	19	19	0.15	10	0.5	10
ConA^r	19	19	0.05	50	1.0	10

[a] Measured as the time (in hours) required for doubling the number of cells during logarithmic growth.

[b] LDS_{10}, Dose of lectin allowing 10% survival of clones relative to the number of clones obtained in control.

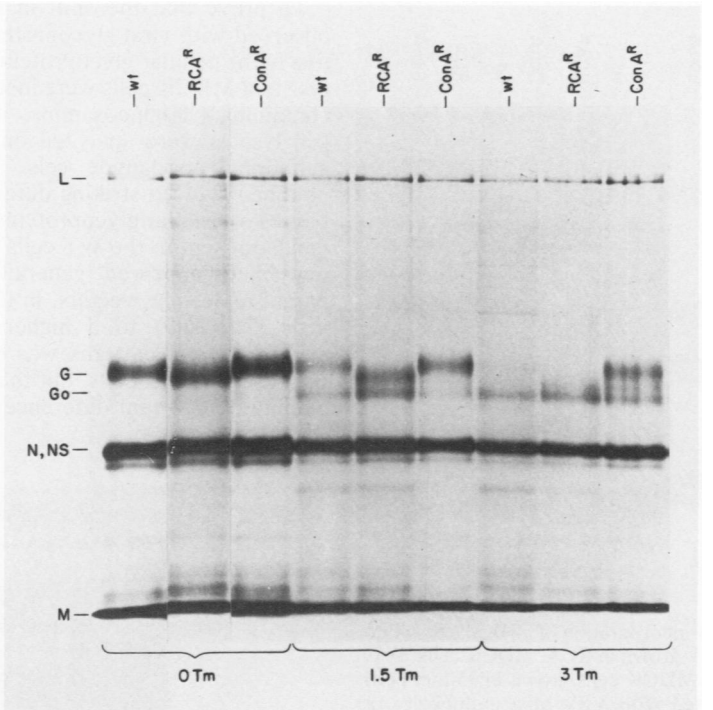


FIG. 1. Identical mobility of G protein from VSV grown in lectin-resistant MDCK cells in the presence of tunicamycin (Tm). WT, RCA^r, and ConA^r MDCK cells were infected with VSV at a multiplicity of infection of 10 after pretreatment for 2 h with 0, 1.5, and 3 μg of tunicamycin per ml. The same concentrations of tunicamycin were maintained during all stages of the experiment. At 3 h, labeling medium containing 10 μCi of [^{35}S]methionine per ml and one-tenth of the normal concentration of cold methionine was added, and the cells were incubated for 15 h. Supernatant viruses were collected by centrifugation through a 20% sucrose cushion, dissolved in gel sample buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were dried and exposed to X-ray film for autoradiography. Letters at left indicate bands for VSV proteins.

the WT MDCK cells (doubling time, about 19 h). Table 1 also illustrates the sensitivity of lectin-resistant cells to other lectins. RCA^r MDCK cells were similar to WT cells in their sensitivity to ConA and WGA but were fivefold more resistant to PHA. ConA^r MDCK cells, in contrast, became more resistant to RCA (fivefold) and WGA (twofold) as well. These phenotypes are distinct from that reported for an RCA^r mutant of Chinese hamster ovary (CHO) cells, found to be defective in glucosamine *N*-acetyltransferase I, which demonstrated increased cross-resistance to PHA and WGA and increased sensitivity to ConA (25). These data indicate that our RCA^r and ConA^r mutants are distinct from each other and from another, well-characterized, RCA^r cell line.

Effect of the mutations on the carbohydrate moieties of glycoproteins. In a previous paper (6), we reported that infection of ConA^r and RCA^r MDCK cells with influenza resulted in increased electrophoretic mobility of the hemagglutinin in both cell lines, whereas VSV G protein only shifted to a lower apparent molecular weight upon the infection of RCA^r cells. This result is consistent with the existence of glycosylation defects which result in a reduction in the size of the carbohydrate moieties of the glycoproteins produced by the mutant cells. To prove this point, we carried out experiments with the antibiotic tunicamycin, which blocks the synthesis of mannose-rich cores from a dolichol phosphate intermediate, resulting in the synthesis of non-glycosylated glycoproteins (28). Treatment with tunicamycin abolished the difference in molecular weight observed for the glycosylated forms of the G protein of VSV grown in WT and RCA^r MDCK cells (Fig. 1). The unglycosylated forms of the G protein migrated with a lower electrophoretic mobility which was identical for the lectin-resistant mutants and the WT cells. The latter experiment also revealed a decreased sensitivity to tunicamycin generated by the mutation to the ConA^r phenotype since the highest dose of this drug which was utilized (3 μg/ml), though totally effective in blocking the glycosylation of G protein in WT and RCA^r cells, was only partially effective in ConA^r cells (Fig. 1).

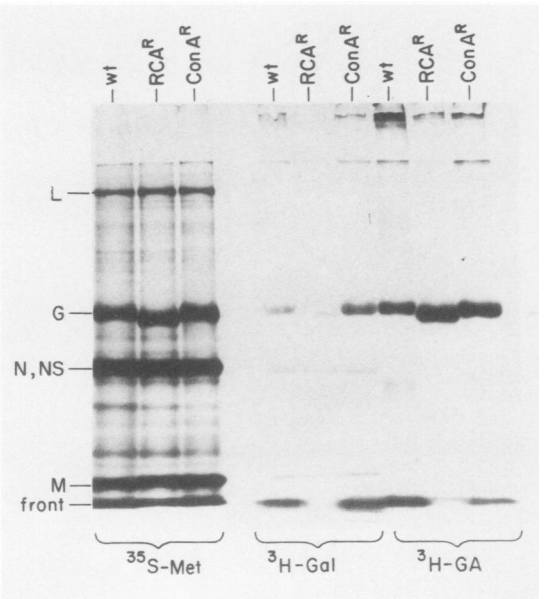


FIG. 2. Lack of incorporation of [^{3}H]galactose into G protein from VSV grown in RCA^r MDCK cells. WT, RCA^r, and ConA^r MDCK cells grown in 35-mm petri dishes were infected with VSV at a multiplicity of infection of 10 and were incubated in Earle minimum essential medium containing 2% fetal calf serum. At 3 h postinfection, the medium was changed to low-methionine (one-tenth of normal), low-glucose (one-third of normal) Earle minimum essential medium containing 5 mM pyruvate and 2% dialyzed fetal calf serum. One plate of each cell line received [^{35}S]methionine (20 μCi/ml, ^{35}S-Met), [^{3}H]galactose (50 μCi/ml, ^{3}H-Gal), [^{3}H]glucosamine (50 μCi/ml, ^{3}H-GA). The cultures were incubated for 15 h at 37°C, and on the next day the virions in the supernatant were collected by centrifugation through a 20% sucrose cushion. The viral pellets were dissolved in gel sample buffer, and the specific radioactivities were measured. The [^{35}S]methionine incorporation ratios between virus grown in RCA^r or ConA^r mutants and virus grown in WT MDCK cells were used to calculate the relative amounts of [^{3}H]galactose- and [^{3}H]glucosamine-labeled samples to be loaded onto a 10% acrylamide gel. The gel was treated with scintillation fluid, dried, and exposed over a week for autoradiography. Letters at left indicate bands for VSV proteins.

Incorporation of [^{3}H]galactose into glycoproteins of defined carbohydrate structure can serve as a direct indication of the synthesis of complex oligosaccharides. RCA^r MDCK cells do not incorporate detectable amounts of galactose into VSV G protein, in contrast with the WT and ConA^r cells (Fig. 2). In this, the RCA^r MDCK cell line is similar to the RCA^r mutant of CHO cells previously described (18), which showed a similar lack of [^{3}H]galactose incorporation into G protein.

To prove that the shift in molecular weight observed with viral glycoproteins has a correlative in the cellular glycoproteins, WT and lectin-resistant MDCK cells were incubated in medium containing [^{3}H]glucosamine, and the resulting cell lysates were analyzed on sodium dodecyl sulfate-polyacrylamide gels. The fluorograms obtained showed striking differences in the mobilities of cellular glycoproteins (Fig. 3). Several bands present in the WT cells disappeared, and new bands appeared, generally with lower apparent molecular weights, in the lectin-resistant cells. The shift to a higher mobility in the electrophoretic patterns was more dramatic in the case of RCA^r cells, but the ConA^r cells also exhibited important differences.

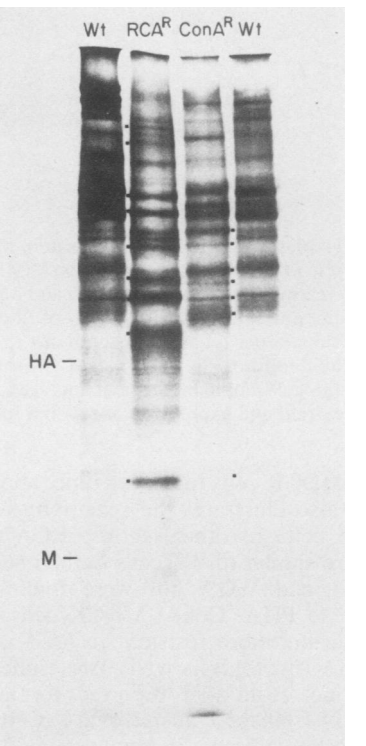


FIG. 3. Altered electrophoretic mobilities of glycoproteins synthesized by lectin-resistant MDCK cells. WT and lectin-resistant (RCA^r and ConA^r) MDCK cells were incubated overnight with 50 μCi of [^{3}H]glucosamine in minimum essential medium containing 2% fetal calf serum, after which postnuclear supernatants were prepared and electrophoresed as previously described (6). Some of the proteins with altered electrophoretic mobility in the lectin-resistant mutants are indicated by dots. The mobilities of two influenza proteins, hemagglutinin (HA; molecular weight, 75,000) and M (molecular weight, 25,000), electrophoresed in the same gel, are included as molecular weight markers.

Taken as a whole, the above data suggest that the mutation in the RCA^r MDCK cells results in the production of truncated carbohydrate side chains, possibly lacking terminal galactose-sialic acid residues. The ConA^r mutation is clearly different and might tentatively be localized in the carbohydrate cores. We have reported a differential effect of the ConA^r mutation on the synthesis of carbohydrates of VSV G and influenza HA proteins and suggested that this might result from a lesion in the synthesis of high-mannose residues which left the synthesis of complex chains unaffected (6). However, studies on the glycopeptides of G protein in ConA^r cells demonstrate the existence of an alteration (unpublished data with John Hakimi).

Ultrastructure of the lectin-resistant mutants of MDCK cells. Lectin-resistant MDCK cells were examined by electron microscopy to reveal the presence or absence of characteristic morphological features of WT cells. Both ConA^r and RCA^r cells appeared similar to parental cells in that microvilli were present at the apical free surface and tight junctions and desmosomes were present at the lateral face (Fig. 4). RCA^r cells exhibited a large number of dilated smooth-surfaced cytoplasmic vacuoles which ranged in size from 0.1 to 2 μm. ConA^r cells also exhibited a slightly increased number of vacuoles smaller than those observed in RCA^r cells. Preliminary freeze-fracture analysis of ConA^r and RCA^r cell lines demonstrated the existence of tight junctions similar in complexity to those found in parental MDCK cells (data not shown).

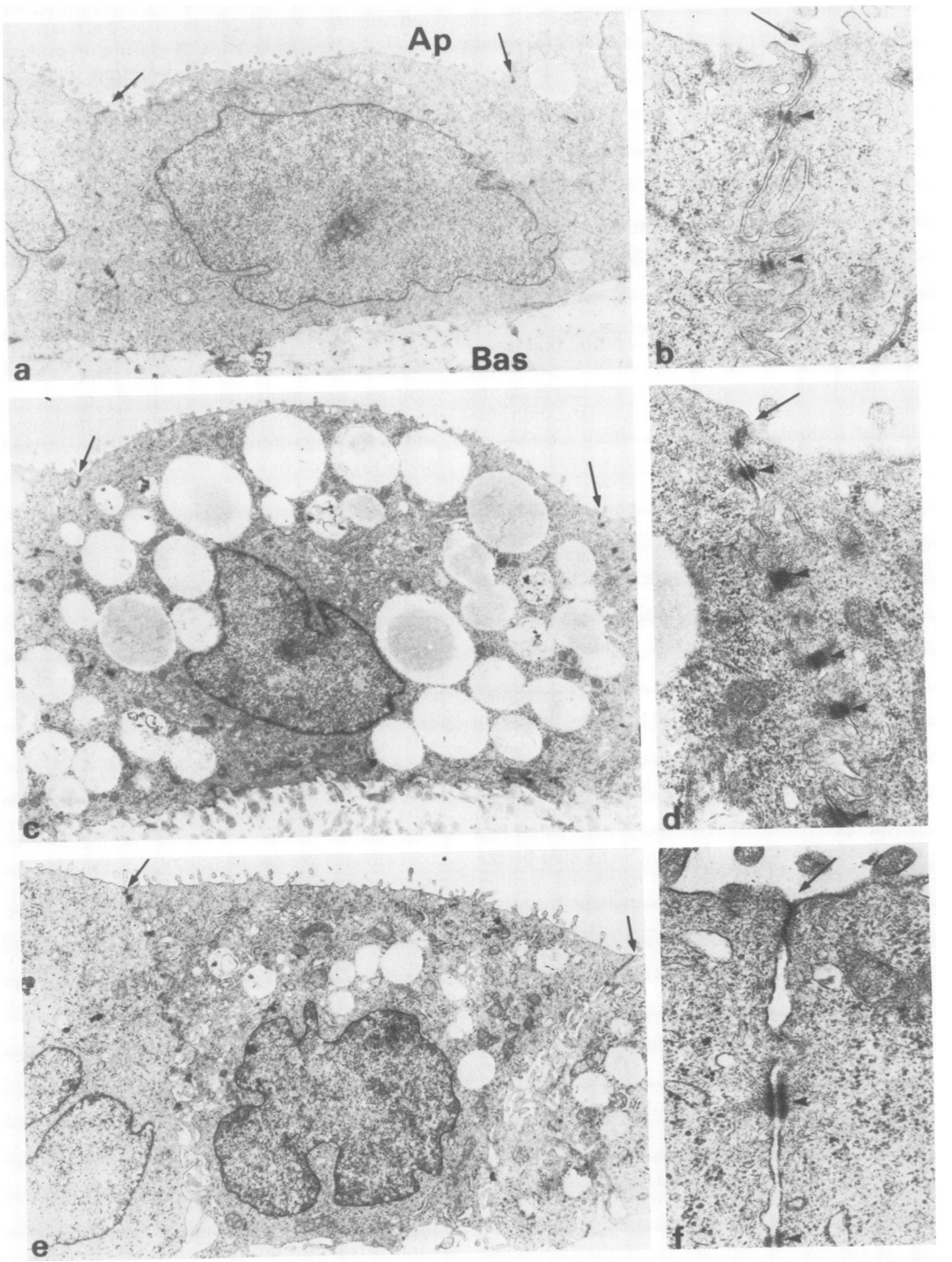
Dome formation by lectin-resistant mutants of MDCK cells. ConA^r and RCA^r mutants of MDCK cells, when grown to confluency on plastic petri dishes, developed epithelium-like monolayers that resembled those formed by the parental cell line MDCK. An increased number of cytoplasmic vacuoles was evident by phase microscopy in RCA^r MDCK cells. At 3 or 4 days after reaching confluency, the mutant cells, like the parental cell line, developed blisters, or domelike formations, which apparently result from transepithelial active transport and accumulation of fluid and electrolytes between the monolayer and the substratum (data not shown; see reference 8). As in the parental cell line, the formation of domes was inhibited by the addition of 10^{-5} M ouabain to the culture medium. The fact that dome formation is conserved in lectin-resistant mutants of MDCK cells suggests that these mutants, like the parental cells, possess functional tight junctions and are capable of carrying out unidirectional transport of salts and water.

Development of transepithelial electrical resistance in lectin-resistant mutants of MDCK cells. The time course of the development of electrical resistance in monolayers of ConA^r and RCA^r mutants of MDCK cells, as well as in the parental cell line, was determined as previously described (2) after heavy cell plating on collagen-coated nylon disks. The mutant cell lines showed kinetics of development of transmonolayer resistance similar to WT MDCK cells, with detectable resistance at about 4 to 6 h and a peak at 24 h after plating (Fig. 5). The peak values of resistance were similar for MDCK and ConA^r cells (180 to 200 $\Omega \cdot cm^2$); RCA^r cells showed somewhat lower peak values (120 $\Omega \cdot cm^2$). These results further indicate that the mutations responsible for lectin resistance did not prevent the development of tight junctions. However, the fact that monolayers of RCA^r cells consistently yielded lower values of transmonolayer electrical resistance suggests that the process of tight junction formation is partially impaired in these cells and that the carbohydrate moieties of surface glycoproteins may be directly or indirectly involved in it.

DISCUSSION

The carbohydrates in plasma membrane glycoproteins and glycolipids participate in the interactions of cells with each other as well as with viruses, hormones, growth factors, and other ligands (9, 13, 21, 22). Much information has been gained in recent years in the area of the biosynthesis of the carbohydrate moieties of glycoproteins (see references 14 and 26 for recent reviews). Lectin-resistant cell lines have proved particularly valuable in this regard, since they exhibit characteristic defects in specific steps in the glycosylation pathway (see reference 24 for a review). In addition, lectin-resistant cell lines are excellent tools for studying the effects upon cells of possessing a large number of specifically altered membrane glycoproteins. In fact, the lectin-resistant phenotype has been correlated with increased susceptibility to various drugs (29), loss of Thy 1 determinants from the cell surface (7), and restricted production of some enveloped viruses (23).

The potential of this approach, however, has not been fully exploited. All lectin-resistant lines to date have been isolated from cells having relatively undifferentiated or highly transformed properties (i.e., CHO, baby hamster kidney, HeLa, and lymphoma cells). The RCA^r and ConA^r MDCK cells described here are the first lectin-resistant mutants derived from a polarized epithelial cell line. Since the alterations in carbohydrate structure in these cells appeared to be stable and could be identified biochemically, it was of interest to study the effects of a given change in carbohydrate on cellular functions characteristic of epithelial cells. In this paper,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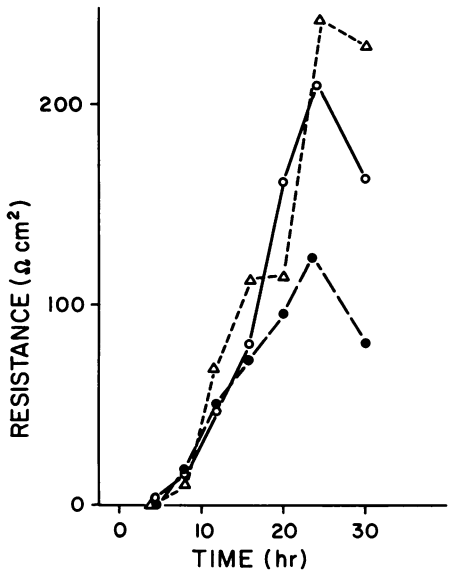

FIG. 5. Development of transmonolayer electrical resistance by lectin-resistant MDCK cells. WT (○——○), RCA^r (●- -●), and ConA^r (△- - -△) MDCK cells were plated on 1-cm^2 collagen-coated nylon disks at a density of 2.5 × 10^5 cells per disk in 24-well petri dishes. Each point represents the mean of six values of transmonolayer resistance measured in six separate disks as described in the text. Standard deviations ranged between 10 and 25% of the mean values.

we report that both mutant cell lines show clear lesions in carbohydrate synthesis but retain several differentiated functions such as tight junction assembly, dome formation, and development of transepithelial electrical resistance. Ultrastructural features of epithelial cell polarity are preserved, in agreement with previous findings that the viral glycoproteins of VSV and influenza virus retain their polarized distribution in the plasma membranes of infected lectin-resistant MDCK cells (6).

The mutants also bear interest in regard to the details of oligosaccharide synthesis. The RCA^r MDCK mutant appears to be different from the CHO-derived mutants in complementation group 1 described by Stanley et al. (25) since it does not show increased sensitivity to ConA or increased resistance to WGA and the increase in resistance to PHA is much smaller (5-fold against 1,000-fold) than that in group 1 mutants. It is also different from the mutants in Stanley's complementation group 4, which show highly increased resistance to PHA but no cross-resistance to RCA. Like both groups of CHO mutants, however, the RCA^r MDCK cells exhibit deficient incorporation of galactose into the G protein of VSV, which correlates with prematurely terminated carbohydrate branches. Preliminary analysis of glycopeptides derived from VSV and influenza virus grown in WT and RCA^r MDCK cells fully supports this conclusion.

One candidate for the mutant protein in RCA^r cells would be a galactosyltransferase, which would determine the production of glucosamine-*N*-acetyl-terminated branched structures. This is supported by preliminary evidence (data not shown) that the G protein in VSV-infected RCA^r MDCK cells is resistant to endoglycosidase H digestion, which indicates that significant post-microsomal processing of mannose-rich core oligosaccharides has occurred (17, 27). This would also explain the lectin cross-resistance phenotype of this mutant.

The ConA^r mutant is quite different in behavior from the RCA^r mutant. ConA^r cells have a different cross-resistance phenotype, and [^{3}H]galactose is incorporated efficiently into G protein produced by ConA^r cells infected with VSV. We have previously reported (6) that VSV G protein synthesized by ConA^r cells has an electrophoretic mobility similar to that of G protein synthesized in WT cells, whereas the mobility of influenza hemagglutinin is clearly increased when produced by the same mutant line. We correlated this disparity with the reported differences in the structures of the carbohydrate moieties of G and hemagglutinin proteins (mature hemagglutinin contains high-mannose as well as complex side chains) and concluded that the ConA^r mutation might affect only a subclass of core high-mannose oligosaccharides, possibly those not destined for processing into complex structures. Such a differential effect on high-mannose chains has been described in the case of class E Thy 1-negative lymphoma cells, which are also ConA^r (3, 4). However, analysis of glycopeptides derived from VSV-infected ConA^r cells indicates the existence of changes in the glycoprotein cores (unpublished data with John Hakimi).

It should be emphasized that although obvious indications of polarity are preserved in lectin-resistant MDCK cells, subtle effects of carbohydrate alterations on epithelial properties may still exist. RCA^r cells exhibit lower peak values of transmonolayer resistance than do WT cells,

FIG. 4. Ultrastructure of lectin-resistant MDCK cells. WT (a,b), RCA^r (c,d), and ConA^r (e,f) MDCK cells were grown on plastic petri dishes until confluency, fixed with 2% glutaraldehyde, and processed for electron microscopy as described in the text. Note the presence of microvilli in the apical (Ap) surface and of tight junctions (arrows) and desmosomes (arrow heads) in the basolateral face (Bas). Note also the existence of large vacuoles in the cytoplasm of RCA^r cells. Magnification, ×8,100 (a,c,e) and ×32,500 (b,d,f).

which may indicate a quantitative effect of the lectin resistance mutation on the tight junction integrity. Also intriguing in these cells is the presence of increased numbers of smooth-surfaced vesicles. Since the addition of ConA to macrophages prevents the fusion of phagosomes and primary lysosomes (5), it is possible that this accumulation of vesicles is caused by a defect in a carbohydrate-mediated fusion event involving two normally existing classes of intracellular vesicles. We are currently studying the effect of the mutations on the rates of endocytosis and lysosomal degradation of proteins.

All lectin-resistant mutants isolated to date have been derived from nonpolarized cell lines. The availability of lectin-resistant mutants selected from epithelial cells should help in studying the peculiarities of glycosylation pathways and mechanisms in polarized cells.

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