Abnormal fucosylation of ileal mucus in cystic fibrosis: I A histochemical study using peroxidase labelled lectins

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Abstract

Peroxidase conjugated lectins were used to analyse the glycoproteins of small intestinal mucins in normal infants and those with cystic fibrosis to ascertain whether there are any detectable histochemical differences in saccharide composition. A significant decrease in *Lotus tetragonolobus* (LTG) binding fucose was shown in normal small intestinal mucin starting around 36 weeks' gestation with total absence of staining at term and beyond. In contrast, the age matched patients with cystic fibrosis showed persistent and intense LTG binding of fucose. These results provide the first clear histochemical evidence that cystic fibrosis mucin is abnormal and confirm the findings of previous biochemical studies.

The basic biochemical defect in cystic fibrosis remains obscure, although the pathology has been well documented.^{1,2} In search of qualitative differences from normal mucus much attention has been given to the biochemical analysis of the mucus produced in cystic fibrosis. Reports have shown that there are changes in the carbohydrate composition of mucus glycoproteins, including an increased fucose content in the meconium of these patients.^{3,4} More recently the fucose content of membrane glycoproteins from cystic fibrosis fibroblasts has been shown to be increased.⁵

The diagnosis of cystic fibrosis in the neonatal period, when the sweat test is ineffective, can be extremely difficult and is often based on the morphological abnormalities caused by the viscid mucus in various organs, especially the pancreas and ileum. These secondary manifestations are non-specific, however, and recently one of us reported a series of six premature infants with inspissated meconium syndrome, none of whom had a family history or subsequent clinical history of cystic fibrosis.⁶ This difficulty in establishing a diagnosis in the neonatal period⁷ when the sweat test is impractical led us to a lectin histochemical study of the ileum.

Lectins are proteins, derived from plants and animals, which bind to carbohydrate residues of specific structures and configuration,⁸ and have been shown to localise differentially glycoproteins in the intestine.⁹ The aim of this study was to analyse small intestinal mucins using horseradish peroxi-

dase (HRP) conjugated lectins to ascertain whether there are any detectable histochemical differences in saccharide composition between the mucin glycoproteins in patients with cystic fibrosis and normal, age matched controls.

Methods

Formalin fixed, paraffin wax embedded specimens of small intestine from necropsies and surgical procedures were obtained from Addenbrooke's Hospital, Cambridge, and other centres. The group with cystic fibrosis comprised 24 cases diagnosed by clinical symptoms or positive sweat tests, or both. Most of the specimens were from surgical procedures. The ages of these cases ranged from 30 weeks' gestation to 4 months post term, and except for one case none had received any antibiotics. The group with cystic fibrosis also included two fetuses which had been therapeutically aborted at 17 and 20 weeks' gestation because of a family history of cystic fibrosis and at necropsy had histological changes consistent with cystic fibrosis in the pancreas and small intestine. The control group consisted of 48 subjects ranging from 19 weeks' gestation to three months post term. These cases had no family history, clinical or histopathological evidence of cystic fibrosis, and none had been treated with antibiotics.

The horseradish peroxidase conjugated lectins were purchased from EY Laboratories, San Mateo, California, USA. The lectins used and their sugar specificities are given in the table. The HRP conjugated lectins were tested on surgical cases of gut from known cases of cystic fibrosis and non-cystic fibrosis cases. Each lectin was diluted in 0.05 M TRIS buffered saline (TBS), pH 7.6, to give concentrations of 1 in 25, 1 in 50, 1 in 100 and 1 in 200 and incubated at room temperature for 30, 45, and 60 minutes, using the standard direct immunoperoxidase method described below. The sections were examined microscopically and the combination which gave the

Lectins used and their sugar specificities

Lectin	Abbreviation	Sugar specificity
Concanavalin A	Con A	a-D-Mannose a-D-Glucose
Glycin max-soybean	SBA	N-acetyl galactosamine Galactose
<i>Ricinus communis</i> <i>Triticum vulgaris</i>	RCA-1 WGA	Galactose N-acetyl-glucosamine N-acetylneuraminic acid
<i>Ulex europaeus</i>	UEA	a-L-fucose
<i>Lotus tetragonolobus</i>	LTG	a-L-fucose

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Figure 1 Ileum from term control. Negative staining of goblet cells with LTG.

best staining of the mucins with minimum background was chosen—that is, in *Triticum vulgaris* 1 in 25, Glycin max-soy bean 1 in 50, *Ricinus communis* 1 in 100, and LTG 1 in 50, all incubated for 45 minutes. Concanavalin A did not give an acceptable result and so was tried at a stronger concentration (1 in 10). An incubation time of 60 minutes gave the best result.

Sections 4.5 µm thick were cut from formalin fixed, paraffin wax embedded specimens, mounted on glass slides, and dried at 56°F for one hour. The sections were dewaxed in xylene and taken through graded alcohols to water. Endogenous peroxide activity was blocked by treating the sections with a 3% aqueous solution of hydrogen peroxide for 15 minutes. After rinsing in tap water the sections were warmed to 37°C in a water bath and placed in a solution of 0.01% trypsin (Sigma) containing 0.01 M calcium chloride, pH 7.8, at 37°C for 20 minutes.

Figure 2 Ileum from case of cystic fibrosis at term. Intense staining of goblet cells with LTG.

They were rinsed in cold running tap water to stop the trypsin activity and placed in a bath of TBS for at least 10 minutes. The slides were removed from the bath, excess TBS was wiped off, and then diluted lectin was applied to the sections which were incubated in a moist atmosphere at room temperature. The slides were rinsed in TBS and washed in TBS for a further 10 minutes. The HRP was visualised using 3.3' diaminobenzidine hydrochloride (DAB).¹⁰ The sections were counterstained with a weak haematoxylin, dehydrated, cleared and mounted in DPX.

Results

The sections were analysed for the intensity and distribution of lectin binding. The intensity was graded on a scale of 1+ for minimal staining to 3+ for very heavy staining, and negative staining was recorded as 0. Lectin binding was most prominent within the goblet cell mucin and the microvillous border of the epithelial cells. There was frequent binding to intracellular glycoproteins, which was either diffuse within the cytoplasm or localised to the supranuclear golgi region. In each section the intensity of epithelial microvillous border, goblet cell mucin, and intracellular staining was noted in the crypts, mid-villous, and superficial villous regions. To reduce observer bias the sections were analysed "blind" without knowledge of the gestation age or clinical state. Sections from random cases were stained on more than one occasion to ensure reproducibility.

Concanavalin A (Con A) Neither goblet cell mucin nor the villous border were labelled in any sections but intracellular staining in the superficial parts of the villi was observed in both cystic fibrosis patients and controls with no significant differences.

Glycin Max-Soy Bean (SBA) There was no difference in staining between controls and cases of cystic fibrosis. After birth the intensity of goblet cell mucin staining for all levels of the villus gradually seemed to decrease with crypt goblet cell mucin staining, being negative by about 4 months.

Ricinus communis (RCA) and Triticum vulgaris (WGA) No significant difference in staining between patients and controls or with age of patient was noted.

Ulex europaeus (UEA) There was very little goblet cell mucin, intracellular, or microvillous border staining and no difference was detected between patients and controls.

Lotus tetragonolobus (LTG) LTG produced moderate staining of the microvillous border, but very little intracellular staining, and the pattern was similar in both patients and controls. There was a striking difference, however, between the two groups in the staining of goblet cell mucin (figs 1–3). In the control group there was heavy 3+ staining of goblet cell mucin in infants of 19–32 weeks' gestation and negative staining in those 36 weeks or older. Intermediate 2+ staining was seen in infants of 32–36 weeks' gestation. In contrast, there was very heavy 3+ and equally

Figure 3 High power of figs 1 and 2. Control (A), cystic fibrosis (B).

intense goblet cell mucin staining in all cases of cystic fibrosis tested except one case, ranging in age from 17 weeks' gestation to 12 weeks post term. In the control group, therefore, there seemed to be a decline in LTG staining of goblet cell mucin with maturation of the infant; there was no such decline with gestational age in the cases of cystic fibrosis (figs 4 and 5). The single exception was a case aged 40 + 12 weeks, who had been treated with a variety of antibiotics (cephalosporin, amoxycillin, and ampicillin) for *Pseudomonas* septicaemia.

Discussion

The intense and persistent staining with LTG in the cases of cystic fibrosis indicates the presence of fucose in the goblet cell mucins. In contrast to the controls which had diminished staining for fucose at 32–36 weeks' gestation and absent staining from 36 weeks to 12 weeks post term, the cases of cystic fibrosis had persistent, intense staining for fucose up to term and beyond. These results provide the first clear *histochemical* evidence that cystic fibrosis mucin is abnormal and confirm the findings of previous biochemical studies. Dische reported an increased fucose to sialic acid ratio in the duodenal secretions from patients with cystic fibrosis and postulated that the increased fucose content changed the rheological properties.¹¹ Clamp and Gough analysed mucus glycoproteins in the meconium of three neonates with cystic fibrosis and showed a significant increase in fucose and decrease in sialic acid residues in the meconium

Figure 4 Intensity of LTG staining in controls (○) and cases of cystic fibrosis (●) at different gestational ages. 0 = negative; 1 = minimal; 2 = moderate; 3 = heavy (the case of cystic fibrosis with minimal staining at 12 weeks post term is patient BC).

Figure 5 Statistical analysis of LTG staining in cases of cystic fibrosis and controls showing (a) loss of staining with maturation of fetus in controls, (b) persistent and heavy staining with maturation of fetus in cystic fibrosis.

mucins.³ They postulated that the hydrophobic C6 methyl group of fucose increased the hydrophobic interactions between mucin polysaccharide side chains, resulting in an increase in the gelling properties of cystic fibrosis mucins. Wesley *et al* analysed the intestinal mucins from six cases of cystic fibrosis aged 9–23 years and showed an increase in fucose, galactose, and N-acetyl glucosamine residues in cystic fibrosis mucins.¹² They also showed lengthening of the polysaccharide chains in cystic fibrosis mucins and postulated that this would increase the hydrodynamic volume of the mucin molecules and enhance their viscosity and gelling properties. Ryley *et al* analysed and compared the carbohydrate composition of a meconium glycoprotein fraction from 20 healthy full term infants, 19 premature infants, and 19 infants with confirmed cystic fibrosis and showed that the mean total saccharide composition was significantly less in premature infants.¹³ This reduced saccharide composition was reflected in the average contents of all individual saccharides analysed, with the exception of fucose. They also showed that the meconium glycoprotein of a term infant with cystic fibrosis has a composition similar to that of the meconium glycoprotein from premature normal infants of 28–32 weeks' gestation and postulated that the basic defect in cystic fibrosis is a delay or an arrest in the maturation of epithelial secretions. Their study also showed that no noticeable differences were seen that could be related to blood group activity.

Conflicting results have been reported from biochemical studies of sputum and salivary mucus. Reid reported that there was no qualitative difference between cystic fibrosis and normal bronchial mucus and that cystic fibrosis mucus is excessively viscous because of bacterial infections.⁴ Gibson found no difference between normal and cystic fibrosis salivary mucins and postulated that the hyper-viscosity of cystic fibrosis mucins is due to direct hyperpermeability induced by the

increased calcium concentration present in cystic fibrosis secretions.¹⁴ Chace *et al* analysed tracheobronchial secretions from patients with cystic fibrosis and showed increased concentrations of the mucin content of the secretions, especially of a highly sulphated mucin component, with increasing severity of the disease.¹⁵ Cultured upper respiratory epithelial tissues from cases of cystic fibrosis have been shown to produce, compared with controls, mucous glycoproteins at increased rates, with higher levels of sulphation and greater acidity.¹⁶ Most of these studies were done on children with cystic fibrosis and adults who had a history of respiratory tract infections. The infections and the subsequent antibiotic treatment are important and uncontrolled variables may well explain these conflicting reports. It has been shown that antibiotics substantially change the faecal anaerobic bacterial flora and that commensal intestinal bacteria have an important role in the degradation of mucins.¹⁷ To avoid these conflicting problems of infections, antibiotics, and faecal flora we confined our study to small intestinal tissues from premature infants and neonates, most of whom had not had any antibiotics. By using these tissues we have tried to show primary abnormalities of the disease itself and not the secondary effects.

Pseudomonas aeruginosa, which is non-virulent in an immunocompetent host, often causes severe respiratory infections in patients with cystic fibrosis even though they are not immunocompromised. Invasion by pathogenic micro-organisms is thought to be initiated by their adhesion to appropriate target cells, and there is evidence to suggest that bacterial surface lectins are involved in this adhesion.¹⁸ There has been a recent report of pulmonary pathogenic bacteria binding specifically to carbohydrate sequences found in glycosphingolipids of cell-surface receptors.¹⁹ One of these glycolipids contains terminal fucosyl residues. It is therefore tempting to speculate that, as in the ileum, cystic fibrosis mucins of the respiratory tract have a *de novo* abnormality of carbohydrate structure which could be a major factor in determining the increased incidence of infections from *Ps aeruginosa*. In particular, the increased fucose content of ileal mucins shown in our study, if true in the case of respiratory mucins, may facilitate the attachment of sufficient numbers of microbes to the surface of the bronchial epithelium to produce the bronchitis-bronchiolitis to which patients with cystic fibrosis are susceptible. It would be interesting to repeat our study on the respiratory mucosa from normal controls and patients. Unfortunately, a major drawback is the availability of obtaining tissue, especially from cases of cystic fibrosis who have not had respiratory infections or been treated with antibiotics.

We have shown that as the fetus matures there is a significant decrease in LTG binding fucose in normal small intestinal mucin; the decline starts around 34–36 weeks' gestation, with total absence of staining at term and beyond (tested up to 12 weeks post term). In contrast, in cystic fibrosis there is no such

decline, with persistent and intense LTG binding of fucose even at term and beyond. This implies certain possibilities which do not have to be mutually exclusive: (a) a delay or arrest in maturation of epithelial secretions as suggested by the biochemical studies of Ryley *et al*; (b) aberrant function of any enzyme involved in the fucosylation of glycoproteins.

In connection with the latter possibility it is of interest that certain enzymes in the second trimester amniotic fluid of fetuses with cystic fibrosis are significantly depressed when compared with normal controls.²⁰ The enzymes concerned include peptides and disaccharidases which are localised in the membrane of epithelial cell microvilli.

There has been only one previous report of lectin histochemistry in cystic fibrosis.²¹ This study used fluorescein conjugated lectins to fucose, galactose, N-acetyl galactosamine and N-acetyl glucosamine and showed no qualitative differences between cases of cystic fibrosis aged 8–18 years and non-age matched controls of 7 months–11 years. The fucose binding lectin used was UEA and not LTG. Lectins can bind to terminal sugar groups or to those present in side chains, or following partial digestion, even to sugars embedded in the backbone of glycoproteins. They bind best, however, to terminal sugars and cannot be used to analyse all sugar residues within the oligosaccharide chain. This may explain why, unlike the biochemical study of Wesley *et al*,¹² we were unable to show any difference in the binding of lectin to galactose (RCA) and N-acetyl glucosamine (WGA). We also did not show any significant differences between patient and control mucins using a second fucose binding lectin UEA. An explanation for this could be that lectins considered "identical" in terms of monosaccharide specificity possess the ability to recognise fine differences in more complex structures, and although LTG and UEA have the same major sugar specificity they exhibit different binding affinities for fucosaccharide chains.²² This is shown by the fact that although UEA is a very good marker of vascular endothelium which has a high concentration of fucose, LTG does not identify vascular endothelium.²³ Moreover, LTG is strongly specific for blood group antigens with difucosylated type 2 chains but does not react with fucose residues of type 1 chains.^{24 25} UEA, on the other hand, does react with fucosyl oligosaccharides of both type 1 and type 2 chains. To understand these subtle variations in composition of the cytoplasmic glycoconjugates, in addition to the lectins with the broader specificities, a more sensitive method of identifying the oligosaccharide sequences is needed. There are now a number of monoclonal antibodies which are directed against fucose containing oligosaccharide chains, based on type 1 or type 2 blood group chains²⁶ and we have expanded the present study by using a range of such monospecific reagents.

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