ORIGINAL ARTICLE

Detection of sulfated glycoproteins in intestinal metaplasia: a comparison of traditional mucin staining with immunohistochemistry for the sulfo-Lewis^a carbohydrate epitope

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Background: Premalignant Barrett's oesophagus (BO) and gastric intestinal metaplasia (IM) show phenotypic variability. Incompletely differentiated sulfomucin rich gastric IM (type III) may have increased malignant potential. The types of sulfated oligosaccharide structures present in IM, BO, and colon have not been fully characterised.

Aims: To compare sulfo-Lewis^a epitope tissue distribution with high iron diamine (HID) positive sulfomucin in metaplastic, dysplastic, and neoplastic tissues from oesophagus and stomach.

Methods: Sections containing gastric IM or BO (some associated with dysplasia or adenocarcinoma) were stained by the HID/alcian blue (AB) method and immunohistochemically (antibody 91.9H) to detect sulfo-Lewis^a. Based on HID/AB staining, IM was subtyped into type I (complete) or types II and III (incomplete).

Results: In total, 125 sections from 38 subjects were studied. Normal squamous oesophagus, normal gastric epithelium, and type I IM were negative for sulfomucin and sulfo-Lewis^a. In type II IM, occasional goblet cells were HID and sulfo-Lewis^a positive, but sialomucin secreting (AB positive) columnar cells were sulfo-Lewis^a negative. Type III IM was always sulfo-Lewis^a positive. Sulfomucin staining in dysplasia and cancer was variable, but HID positive areas were always sulfo-Lewis^a positive.

Conclusions: Sulfo-Le^a, which is expressed on colonic mucin, is invariably present on sulfomucins in gastric IM and BO. Its presence in incomplete variants of IM and its absence from type I IM emphasises the phenotypic differences between complete and incomplete forms of metaplasia. 91.9H immunostaining is useful in IM subtyping. Characterising the molecular basis of sulfo-Lewis^a expression may help understand the process of aberrant differentiation.

Intestinal metaplasia (IM), whether in the stomach or distal oesophagus (Barrett's oesophagus), is generally considered to be a precursor of adenocarcinoma.^{1–3} However, its relatively common occurrence and presence in benign conditions limits its value as a marker of those at risk for developing cancer. Histologically, IM is defined by the presence of goblet cells, but it exhibits considerable heterogeneity in the degree of cellular differentiation and crypt architecture. The diversity of IM has been further recognised by studies of ultrastructure,⁴ enzyme profile,⁵ mucin secretion, and expression of specific mucin antigens.^{6–7}

In an attempt to improve specificity for predicting cancer risk, mucin histochemistry was used to identify specific subtypes of IM based on the pattern of cell differentiation and mucin secretion.^{8–10} A classification of IM was proposed based on sequential staining with high iron diamine (HID), which stains sulfomucins brown, followed by alcian blue (AB) at pH 2.5, which stains sialomucins blue.^{8–10}

Using these simple cationic dyes, several studies raised the possibility that different types of IM may have different malignant potential. In the stomach, a variant of IM characterised by incomplete cell differentiation and HID positive sulfomucin secretion (designated type IIB or III) was associated with intestinal-type gastric cancer.^{9–15} Some authors have argued in favour of histochemical subtyping of gastric IM as a means of identifying higher risk patients who may benefit from closer surveillance,¹¹ but others have highlighted the relatively low sensitivity and specificity of the sulfomucin rich IM variant for predicting cancer progression.^{16–18}

An association between a sulfomucin rich type of IM and adenocarcinoma developing in Barrett's oesophagus has also been reported,¹⁹ although other studies have suggested that such variants of IM are common in Barrett's mucosa and of limited prognostic relevance.²⁰ Interpreting the various studies is made difficult by the fact that the recognition of type III IM is not straightforward and the HID/AB method requires very careful handling to produce consistent results.^{21–22}

"Given the important functions of sulfated oligosaccharide structures as ligands in various recognition processes, alterations in the sulfation of metaplastic cells could have important effects on differentiation and malignant progression"

The precise nature of the sulfated glycoproteins responsible for HID positivity in type III IM is not known, and it is not clear whether the composition of the sulfomucins expressed in gastric IM is the same as that in Barrett's mucosa. Increased expression of sulfated sugars may be simply a phenotypic marker of a colonic type of cellular glycosylation, without serving a direct role in carcinogenesis. However, given the important functions of sulfated oligosaccharide structures as ligands in various recognition processes,²³ alterations in the

Abbreviations: AB, alcian blue; BO, Barrett's oesophagus; HID, high iron diamine; IM, intestinal metaplasia; mAb, monoclonal antibody

Table 1 Prevalence of different subtypes of intestinal metaplasia (IM) based on high iron diamine (HID)/alcian blue (pH 2.5) mucin histochemistry

Diagnostic group	Number of patients evaluated (number of sections)	Type I IM (no. patients)	Type II IM (no. patients)	Type III IM (no. patients)
Barrett's oesophagus	13 (38)	11/13	13/13	8/13
Barrett's oesophagus with dysplasia	8 (35)	8/8	7/8	3/8
Barrett's adenocarcinoma	3 (7)	2/3	3/3	3/3
Gastric IM	7 (21)	6/7	3/7	2/7
Gastric IM with dysplasia	4 (12)	1/4	3/4	2/4
Gastric adenocarcinoma (distal)	3 (12)	1/3	3/3	1/3

Type I IM: alcian blue positive goblet cells and non-secretory "absorptive-type" cells with no HID or sulfo-Lewis^a staining (fig 1A,B). A few isolated mature goblet cells may show HID positivity with corresponding immunoreactivity for sulfo-Lewis^a in serial section (100% agreement). Type II IM: alcian blue positive goblet cells and incompletely differentiated "intermediate-type" cells having alcian blue positive apical secretory granules, without HID or sulfo-Lewis^a staining (fig 1C,D). The deeper glands, and occasional surface goblet cells, may show HID positivity and corresponding sulfo-Lewis^a expression (100% agreement). Type III IM: HID positive goblet cells and incompletely differentiated intermediate-type cells having HID positive apical secretory granules with corresponding sulfo-Lewis^a expression (100% agreement). Dysplasia: the dysplastic cells themselves exhibited variable HID staining (mainly localised to the apical membrane) and there was occasional discordance between mucin histochemistry and the sulfo-Lewis^a staining (that is, HID negative but sulfo-Lewis^a positive; see Results). Adenocarcinoma: the cancer cells themselves exhibited variable HID staining and there was some discordance between mucin histochemistry and the sulfo-Lewis^a staining as in dysplastic epithelium (see Results).

sulfation of metaplastic cells could have important effects on differentiation and malignant progression.

Further definition of the structures present in sulfomucin rich variants of IM could provide important insights into the mechanisms of altered glycoprotein expression in IM and lead to a better definition of cellular phenotypes with high malignant potential. Progress in this field has previously been hampered by the lack of immunohistochemical tools of defined specificity for studying the expression of specific sulfated residues. Irimura *et al* have reported a monoclonal antibody (mAb 91.9H) raised against partially purified colonic mucin, which has specificity for the oligosaccharide sequence, HSO₃-3Galβ1-3(Fucα1-4)GlcNAc-R (sulfo-Lewis^a).²⁴⁻²⁶ The aim of our present study was to compare, using mAb 91.9H, the tissue distribution of the carbohydrate epitope, sulfo-Lewis^a, with that of HID positive mucous glycoproteins in intestinal metaplasia of the stomach and oesophagus.

METHODS

Archival cases of intestinal metaplasia (including examples with dysplasia and adenocarcinoma) were selected from the department of pathology database, Royal Liverpool University Hospital, UK. In the case of Barrett's oesophagus, only sections containing specialised (goblet cell containing) mucosa were selected. Serial 5 µm sections were obtained from paraffin wax embedded, formalin fixed tissues (endoscopic biopsies and surgical resections). Traditional mucin histochemistry using the HID/AB (pH 2.5) method was performed as described previously, using fresh 60% ferric chloride solution.^{27,28} Normal colon and ileum served as control tissue and all sections were stained in a single batch using fresh reagents. Briefly, sections were dewaxed and rehydrated, then immersed overnight (18 hours) in a solution of *N,N*-dimethylmetaphenylene diamine dihydrochloride, *N,N*-dimethylparaphenylene diamine dihydrochloride, and fresh ferric chloride (60% wt/vol) solution, as described previously.^{27,28} After washing in running tap water, sections were counterstained with 1% AB in 3% acetic acid (pH 2.5) for 30 minutes and then dehydrated, passed through xylene, and mounted.

Indirect immunohistochemistry was performed as described previously using a streptavidin-biotin complex method (VectaStain Elite ABC kit; Vector Laboratories, Burlingame, California, USA).^{24,25} After dewaxing, sections were rehydrated and endogenous peroxidase was blocked with hydrogen peroxide in methanol. After blocking with normal serum buffer, slides were incubated with mAb 91.9H (1/1000 dilution of stock solution in phosphate buffered saline, a generous gift from Professor T Irimura, University of Tokyo, Japan) for two hours at room temperature, washed in phosphate buffered saline, and then developed using sequential streptavidin-biotin-peroxidase

technique with diaminobenzidine as the final substrate. Sections were finally lightly counterstained with Mayer's haematoxylin, dehydrated, passed through xylene, and mounted.

RESULTS

In total, 125 formalin fixed, paraffin wax embedded sections from 38 patients were studied (table 1). Two of the Barrett's adenocarcinomas and one of the gastric adenocarcinomas were surgical resections, the remainder were endoscopic biopsy specimens (one to seven biopsies for each case). The three oesophageal cancers were all moderately differentiated intestinal-type tumours, whereas the gastric cancers included one case each of signet ring-type, well differentiated-type, and moderate to poorly differentiated-type tumours. Table 1 summarises the prevalences of the different subtypes of IM within each diagnostic group, based on the results of HID/AB histochemistry. Staining patterns for HID/AB and sulfo-Lewis^a in IM and in dysplasia/cancer are described separately below. Mucin staining and immunostaining were negative in normal squamous oesophagus and gastric-type mucosa, but control sections of colonic mucosa showed positive goblet cell sulfo-Lewis^a immunoreactivity, in agreement with the localisation of HID positivity.

Intestinal metaplasia

Tissue distribution of HID/AB staining versus sulfo-Lewis^a

Type I (complete) intestinal metaplasia

In areas of complete IM (type I), mature goblet cells were either unstained or HID negative/AB positive, and non-secretory (absorptive-type) columnar cells were unstained. In every case (29 patients) staining for sulfo-Lewis^a was absent from areas of complete IM (fig 1A,B).

Type II (incomplete) intestinal metaplasia

Foci of incomplete IM of type II are characterised by the presence of mature goblet cells (mainly AB positive, occasionally HID positive) at the surface and within crypts that are interspersed by secretory columnar cells with predominantly AB positive apical mucin granules. Again, such areas showed complete absence of sulfo-Lewis^a expression (32 of 32 patients), except in the occasional mature goblet cell, in agreement with the HID mucin histochemistry (fig 1C,D). Deeper glands within areas of IM are frequently positive for sulfomucin by HID/AB, and such areas were invariably also sulfo-Lewis^a positive (fig 1C,D).

Type III (incomplete) intestinal metaplasia

Type III IM is characterised by the presence of columnar mucous secreting (non-goblet) cells with HID positive apical

Figure 1 Subtypes of intestinal metaplasia. Comparison between (A,C,E) immunohistochemistry for the sulfo-Lewis^x epitope and (B,D,F) high iron diamine/alcan blue mucin histochemistry. (A,B) Complete intestinal metaplasia (type I) of the stomach; (B) shows well differentiated sialomucin secreting goblet cells (GC, stained blue) interspersed with non-secretory absorptive cells (AC). (B) Note the absence of sulfomucin staining, in agreement with (A) the negative immunostaining for sulfo-Lewis^x. Original magnification, $\times 40$. (C,D) Incomplete intestinal metaplasia (type II) in Barrett's oesophagus. (D) Well differentiated goblet cells (GC) interspersed with incompletely differentiated columnar cells (IC), with predominantly sialomucin positive apical mucin granules (stained blue). (D) Note the positive staining for sulfomucin (purple/brown) in occasional goblet cells and in the deeper glands, in agreement with (C) the staining pattern for sulfo-Lewis^x. Original magnification, $\times 25$. (E,F) Incomplete intestinal metaplasia (type III) in Barrett's oesophagus. (F) Well differentiated goblet cells (GC) interspersed with incompletely differentiated columnar cells (IC) with sulfomucin positive apical mucin granules (stained purple/brown in the centre). (E) Note the close agreement between (E) areas positive for sulfo-Lewis^x staining and (F) those positive for sulfomucin staining. At a subcellular level, immunostaining is more extensive within the cytoplasm and at the apical membrane, particularly in deeper glands. Original magnification, $\times 40$.

secretory granules. Nineteen patients had one or more foci of type III IM identified by HID/AB, all of which were found to be sulfo-Lewis^x positive (fig 1E,F), with immunostaining of both mature goblet cells and of secretory columnar cells.

Subcellular localisation of HID positive and sulfo-Lewis^x positive staining

Whereas the tissue distribution at a cellular level of HID and sulfo-Le^x staining showed complete agreement, the subcellular distribution of staining within areas of positivity differed somewhat between the two techniques (fig 1C–F). In general,

HID positivity was confined to secretory vacuoles with occasional, usually faint, staining of the apical/luminal surface membrane and intraluminal secretions. In contrast, sulfo-Lewis^x staining was more extensive. In addition to secretory vacuoles, sulfo-Lewis^x was expressed in the supranuclear region of secretory cells and also prominently at the cell membrane and in secreted mucous. Focal areas of surface membrane expression of sulfo-Lewis^x were occasionally seen without convincing HID positivity in the serial section (fig C–F), but the reverse was not seen (HID positive/sulfo-Lewis^x negative).

Figure 2 Dysplasia and cancer. (A,C,E) Comparison between immunohistochemistry for the sulfo-Lewis^x epitope and (B,D,F) high iron diamine/alcan blue mucin histochemistry. (A,B) Gastric dysplasia from a case of gastric cancer. High power view of dysplastic glands showing close agreement between sulfomucin staining and staining for sulfo-Lewis^x. (A) Note the more extensive cytoplasmic immunostaining of dysplastic cells. Infiltrating cancer cells from an adjacent adenocarcinoma (not shown) are seen in the lower part of the figure. Original magnification, $\times 40$. (C,D) Gastric adenocarcinoma. Cancer cells show (D) absent to low intensity sulfomucin staining and (C) low to moderate sulfo-Lewis^x staining. Original magnification, $\times 40$. (E,F) Barrett's adenocarcinoma infiltrating beneath squamous mucosa. Focal sulfomucin staining is mainly apparent in the luminal secretory material, with more extensive and intense staining for sulfo-Lewis^x in secretions. Original magnification, $\times 25$.

Dysplasia and adenocarcinoma

Tissue distribution of HID/AB staining versus sulfo-Lewis^a

Foci of epithelial dysplasia exhibited varying degrees of HID positivity. However, areas of HID positive dysplasia were always sulfo-Lewis^a positive on serial section (fig 2A,B). Of the eight patients with dysplastic areas within Barrett's oesophagus, seven demonstrated concordance for HID/sulfo-Lewis^a staining of dysplastic cells (weak or moderate HID positivity; moderate or strong sulfo-Lewis^a staining), and there was one discordant case (HID negative; sulfo-Lewis^a positive). Of four patients with gastric dysplasia, three were concordant (weak HID positivity; moderate or strong sulfo-Lewis^a staining), and one case was discordant (HID negative; sulfo-Lewis^a positive).

Within adenocarcinoma tissue, HID staining varied from absent (fig 2C,D), to focally strong (fig 2E,F), to strong. All HID positive areas were also sulfo-Lewis^a positive, but weak sulfo-Lewis^a positivity was also seen in apparently HID negative cancer tissue (fig 2E,F). In relation to the histological subtype, the signet ring gastric tumour exhibited focally strong HID and sulfo-Lewis^a staining (with the two stains showing close agreement), whereas both the well differentiated and moderate to poorly differentiated gastric tumours demonstrated weak or absent staining. Despite the fact that the three oesophageal tumours had similar histopathological features, the Barrett's cancers included one example of strong staining (fig 2E,F) and one example of very weak staining.

Subcellular localisation of HID positive and sulfo-Lewis^a positive staining

Within dysplastic cells, HID staining was generally localised to the apices/luminal pole, whereas sulfo-Lewis^a positivity was more widespread in the cytoplasm (fig 2A,B). Within sulfomucin positive adenocarcinoma tissue, staining of the secretory material was more pronounced for sulfo-Lewis^a than for HID (fig 2E,F).

DISCUSSION

The specificity of monoclonal antibody 91.9H for the sulfo-Lewis^a structure (HSO₃-3Galβ1-3(Fucα1-4)GlcNAc-R) is well established,²⁶ and its use as a specific immunohistochemical reagent has been described.^{24,25} Irimura's group has demonstrated immunostaining for sulfo-Lewis^a in both metaplastic and cancer tissues from the oesophagus and stomach,²⁹⁻³¹ but our present study is the first detailed comparison of the tissue distribution of this epitope with that of sulfomucins identified by traditional mucin histochemistry. Our results suggest that, in metaplastic mucosa within the oesophagus and stomach, sulfo-Lewis^a is invariably present in areas expressing HID positivity.

It has been reported that in normal colonic mucosa the pattern of staining with mAb 91.9H differed between subjects depending on the Lewis genotype, with strong staining seen in Lewis positive individuals, but only limited staining seen in Lewis a and b negative subjects.²⁵ Although we did not establish the Lewis status of our subjects, we found no cases of reduced or absent 91.9H immunostaining in areas containing HID positive sulfomucin. This suggests that in sulfomucin rich metaplastic tissues, the expression of sulfo-Lewis^a may occur irrespective of Lewis genotype, with aberrant expression of the sulfated type I structure in Lewis negative individuals. Lewis^a expression in gastric IM has been reported in Lewis b positive subjects,³² and neosynthesis of Lewis^a in Barrett's columnar epithelium has also been described.³³ Augmentation of Lewis fucosyltransferase expression in gastric IM has been documented using a specific antibody against the enzyme.³⁴

The use of immunohistochemistry, rather than the HID method, for the identification of sulfomucins within metaplastic mucosa has obvious advantages, including the avoidance of potentially toxic diamine reagents,³⁵ easier reproducibility, more rapid staining technique, and better definition of

tissue architecture (with haematoxylin counterstaining). The overall distribution of staining of IM for the two techniques showed very close agreement. There was concordance of staining both for mature sulfomucin secreting goblet cell vesicles and for the apical secretory vesicles of incompletely differentiated columnar cells (type III IM).

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Within sulfomucin secreting metaplastic and dysplastic cells, positive staining for sulfo-Lewis^a was generally more widely distributed and/or more intense than HID positivity, particularly in the supranuclear region, at the apical cell membrane, and in luminal surface material. There are several possible interpretations for this finding. First, the immunohistochemical technique may be more sensitive than the HID stain, detecting sulfate groups at a lower concentration. It has been suggested that the absence of HID staining does not preclude the presence of sulfate in small amounts, which may not be detected by histochemical methods.³⁶ The more extensive cytoplasmic immunostaining for sulfo-Lewis^a may represent the presence of the epitope on sulfomucins during their processing within the Golgi and/or transport to secretory vesicles. The concentration of sulfomucin within these areas may be too low to produce HID staining, which was generally localised to secretory goblets and apical mucin granules, where sulfomucins are presumably at their highest concentration.

Second, the sulfo-Lewis^a epitope may be expressed on non-mucin glycoproteins, which fail to give rise to HID staining. In an earlier report, Ohe *et al* reported a discrepancy between the expression of the 91.9H epitope and that of the core proteins, MUC1 and MUC2, in gastric cancer tissues, suggesting that the epitope may be expressed on other proteins.²⁹ Many surface membrane glycoproteins are heavily O-glycosylated, including the adhesion molecule, CD-44, which exhibits various cancer associated glycosylation abnormalities.³⁷ Preliminary data using CD-44 immunoprecipitation of surface membrane proteins from a sulfomucin secreting cancer cell line suggest that the sulfo-Lewis^a epitope may be expressed on CD-44.³⁸ If these findings are confirmed for metaplastic tissues, it would raise the possibility that there may be a more generalised change in cellular sulfation in "high risk" sulfomucin rich IM. The biological importance of this requires further study. A variety of adhesion molecules, including several selectins, recognise sulfated carbohydrate epitopes, suggesting a role in cell adhesive behaviour.^{39,40} The expression of sulfo-Lewis^a on glycoproteins in IM may simply reflect a colonic type of glycosylation (that is, a phenotypic marker), but a direct role in cell-cell interactions and malignant progression cannot be excluded.

In a recent report, Silva and colleagues used a different monoclonal antibody to examine the expression of sulfated Lewis type I structures in gastric IM.⁴¹ Interestingly, immunostaining was reported to be present to some extent in all forms of IM, including complete variants. This is at odds with our findings. There are two possible explanations for this apparent disparity. First, the F2 antibody is less specific than mAb 91.9H. F2 reacts with both fucosylated and non-fucosylated sulfated type I structures (sulfo-Le a/c),⁴¹ whereas mAb 91.9H is specific for sulfo-Le^a alone.²⁶ Hence, any disparity in staining might relate to the presence of non-fucosylated type I chains. Second, the F2 antibody (using Silva's immunohistochemical protocol) may be more sensitive than mAb 91.9H (using our protocol) for detecting low level sulfated type I structures. We

Figure 3 Incomplete intestinal metaplasia. Comparison between (A) immunohistochemistry for sulfated type I structures using the F2 antibody and (B) high iron diamine (HID)/alcian blue mucin histochemistry. (B) Note that HID positive sulfomucin is detected in scattered goblet cells only (arrows), whereas F2 immunostaining suggests the presence of abundant sulfated type I structures in areas that are HID negative. Original magnification, $\times 25$.

have stained a limited number of sections of Barrett's oesophagus using the F2 antibody and have demonstrated the presence of F2 immunostaining in areas that are negative for sulfomucins by our HID method (fig 3A,B), a phenomenon not seen using mAb 91.9H.

The primary purpose of our present study was not to compare the frequency of different metaplasia subtypes between benign and malignant conditions. However, consistent with previous reports, the prevalence of type III IM in "benign" cases was higher in Barrett's oesophagus^{20,42} than in gastric IM,^{11,12} and type III IM was frequently seen in dysplasia/cancer cases. We examined a relatively small number of cases of adenocarcinoma, and found varying degrees of sulfation within cancer tissue, as described previously.²⁹ However, there was close agreement between HID staining and immunohistochemistry for sulfo-Lewis^x.

Recent analysis of core apomucin peptide expression in gastric IM has suggested that the complete form of IM (type I) contains the intestinal-type apomucin, MUC-2, whereas incomplete variants (types II and III) contain a mixture of both MUC-2 and gastric-type apomucins (MUC-5AC and/or MUC-6).⁴³ This suggests that incomplete variants of IM share a common differentiation pathway that is unlike normal adult epithelial phenotypes.⁴³ Our observation that sulfo-Lewis^x staining was absent from type I IM, but present to some extent in both types II and III IM (fig 1C,D), further emphasises the phenotypic difference between complete and incomplete variants of metaplasia.

In conclusion, our present study suggests that the sulfo-Lewis^x epitope is invariably expressed in areas of gastric and oesophageal intestinal metaplasia that show HID positivity. Hence, immunohistochemistry using mAb 91.9H offers an alternative to traditional HID staining for the identification of sulfated IM variants. The identification of the sulfo-Lewis^x structure, which is expressed by normal colonic mucin,²⁵ within type III IM confirms that this variant of intestinal metaplasia shares phenotypic features with the colon. The ability to study the expression of a specific carbohydrate

Take home messages

- The sulfo-Lewis^x epitope is invariably expressed in areas of gastric and oesophageal intestinal metaplasia (IM) that show high iron diamine (HID) positivity, so that immunohistochemistry using 91.9H provides an alternative to traditional HID staining for the identification of sulfated IM variants
- Sulfo-Lewis^x is expressed by normal colonic mucin and is also found within type III intestinal metaplasia, confirming that this variant of intestinal metaplasia shares phenotypic features with the colon
- The presence of sulfo-Lewis^x in the incomplete variants of IM and its absence from type I IM emphasises the phenotypic differences between complete and incomplete forms of metaplasia
- The ability to study the expression of a specific carbohydrate structure within premalignant epithelia will facilitate further work aimed at establishing the molecular basis for this colonic-type pattern of differentiation

structure within premalignant epithelia will facilitate further work aimed at establishing the molecular basis for this colonic-type pattern of differentiation.

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