Developmental changes in mucosubstances revealed by immunostaining with antimucus monoclonal antibodies and lectin staining in the epithelium lining the segment from gizzard to duodenum of the chick embryo

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ABSTRACT

The mucosubstances in the epithelium lining the segment from gizzard to duodenum during development of the chick embryo was studied histochemically using monoclonal antibodies against gizzard mucus and lectins, with attention to the regional differentiation of the epithelium in this segment. The anterior limit of epithelial CdxA mRNA expression detected by in situ hybridisation, which served as the position of the gizzard-duodenal boundary, was clearly found from d 3. Granules positive for some antibodies or lectins were found in the region ranging from the posterior part of the gizzard to the duodenum at d 3, which was followed by an increase in the number of granules and a gradual enlargement of the granule-positive area to the anterior part of the gizzard over 4–6 d. From d 4, the epithelia of the gizzard body and of the pyloric or duodenal region came to be differently stained with some antibodies or lectins. From d 10, each region showed a specific pattern of staining. The epithelia of the gizzard body and pyloric region contained abundant mucus granules with a different staining pattern. In the duodenum the number of stained granules was low except in occasional goblet cells. Thus the epithelia of the gizzard body, pyloric region and duodenum may produce different mucosubstances and the regional differentiation in these epithelia may start at rather early stages soon after the formation of digestive tube.

Key words: Glycoconjugates; gastrointestinal tract; gizzard-duodenal boundary.

INTRODUCTION

The digestive tract consists of several regions (organs) with specific digestive and absorptive functions aligned anteroposteriorly, which poses an interesting question as to the anteroposterior regional specification in this internal organ system during organogenesis. The differentiation of each organ rudiment during normal development has been studied extensively in which the interaction between the endoderm and mesoderm plays an important role (reviewed by Haffen et al. 1987; Mizuno & Yasugi, 1990; Yasugi & Mizuno, 1990; Yasugi, 1993, 1995). However, the development of the junctional region between 2 neighbouring organs has so far attracted less interest. Such a study is essential for understanding the mode of regional specification in the digestive tract, because regional differentiation into the 2 organs occurs in this region.

The gizzard and intestine (duodenum), neighbouring digestive organs in the chicken, manifest quite different patterns of differentiation: the former develops tubular glands secreting mucus (Romanoff, 1960; Toner, 1966; Hodges, 1974; Sgambati et al. 1996) while the latter forms villi covered by a simple columnar epithelium developing a brush-border with its enzymes, including sucrase (Hijmans & McCarty, 1966; Brown, 1971; Hodges, 1974; Matsushita, 1985, 1991). The morphological boundary between the gizzard and duodenum appears...
during development from d 10 (Matsushita, 1991) and the intestinal epithelium and the stomach-type mucous epithelium meet with each other at this boundary (Aitken, 1958; Hodges, 1974; Matsushita, 1991). However, the pyloric region near the boundary is devoid of tubular glands (Aitken, 1958; Hodges, 1974; Matsushita, 1991) and differs from the gizzard body, which raises the possibility that the nature of the mucus in these 2 regions may differ. At d 6–8, both the duodenal and gizzard epithelia contain possible mucus granules (Matsushita, 1991). Thus it is essential to characterise the nature of mucosubstances in the epithelium lining these regions to know the mode of regional specification of the endoderm in these regions. Although mucus histochemistry in the chick embryonic gizzard or intestine some time after its formation has been reported (Van Alten & Fennell, 1957; Gheri et al. 1994; Sgambati et al. 1996), the mucosubstances in their junctional region during development has not been examined in detail. Thus the present study was devised to elucidate the appearance and distribution of mucosubstances during development of the chick embryo in the epithelium lining the segment from the gizzard to duodenum soon after its formation, using antigizzard mucus monoclonal antibodies and lectins. Detection of epithelial CdxA mRNA-expression, recently reported to be apparent in the epithelium of intestine soon after its formation and closely related to intestinal differentiation (Ishii et al. 1997), could help in precise regional identification of the gizzard (pyloric region)-duodenal boundary from the early stages of development.

**MATERIALS AND METHODS**

**Tissue collection and preparation**

Anterior and posterior walls of the gizzard endodermal pocket, pyloric region and duodenum (Fig. 1) were taken from White Leghorn chick embryos at d 3, 4, 6, 10, 14 and 18 (stages 20–44, Hamburger & Hamilton, 1951) and young chicks after hatching. At least 3 animals were studied at each age period. Samples were fixed in ice-cold 95% ethanol for 4 h and embedded in paraffin for immunohistochemistry (Sainte-Marie, 1962) and for lectin histochemistry (Allison, 1987).

**In situ hybridisation**

The samples were fixed in 4% paraformaldehyde in PBS at 4 °C overnight and washed with 30% sucrose. In situ hybridisation for CdxA mRNA was performed on 12 μm cryosections as described previously (Ishii et al. 1997).

**Production of monoclonal antibodies and immunostaining**

Secreted gizzard mucus from 10 d embryos was homogenised in distilled water. The homogenate containing about 50 μg of protein as determined by Lowry’s method (Lowry et al. 1951) using bovine serum albumin as standard was injected i.p. into BALB/c mice, which were boosted after a 3 wk interval. Three days after boosting, spleen cells from the immunised mouse were fused with myeloma P3-X63-Ag8-U1 (ratio, 5:1) according to Galfre et al. (1977). The hybridoma supernatant was screened by the indirect immunofluorescence method as reported previously (Takiguchi-Hayasi & Yasugi, 1991). Hybridomas producing antibodies against gizzard mucus were selected and cloned twice by limiting dilution. Five hybridomas producing antigizzard mucus antibodies were obtained (GMA1–5). Immunoglobulin subclass, as identified by use of the mouse monoclonal subisotyping kit (Sigma Chemical Company, MO, USA), was IgG1 (GMA1, 2, 3, 5) or IgG3 (GMA4). The hybridomas were injected into the abdominal cavity of pristane-primed mice, and the abdominal fluid containing the monoclonal antibody was collected from these mice later.

For immunohistochemical analysis, 6 μm paraffin sections were subjected to indirect immunofluorescence (Matsushita, 1991, 1995, 1996) using the hybridoma culture medium or the abdominal fluid and FITC-conjugated goat antimouse IgG (Cooper Biochemical, PA, USA). Sections were mounted in buffered glycerol and observed with an Olympus epifluorescence microscope. Photographs were processed on a Macintosh computer using Adobe Photoshop software and were printed with Fujix Pictrography 3000 (Fuji Photo Film Co, Tokyo, Japan).

**Lectin histochemistry**

Sections (6 μm) were incubated after rehydration at room temperature for 1 h with FITC-conjugated WFA, VVA, MPA, BPA, STA (Sigma) or PWM (E-Y Laboratories, CA, USA) diluted in phosphate buffered saline (PBS) (Table 1). After washing with 3 changes of PBS each for 10 min, sections were mounted in glycerol-PBS (9:1) and rapidly observed with an Olympus epifluorescent microscope. Control
Table 1. Lectins and inhibitory sugars used

<table>
<thead>
<tr>
<th>Lectin (abbreviation)</th>
<th>Carbohydrate specificitya,b</th>
<th>Concentration of lectin</th>
<th>Inhibitory sugarb (concentration used)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistaria floribunda (WFA)</td>
<td>GalAc &gt; Gal</td>
<td>50 µg/ml</td>
<td>D-GalAc (0.2 m)</td>
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<tr>
<td>Vicia villosa (VVA)</td>
<td>α-GalβAc &gt; α-Gal</td>
<td>10 µg/ml</td>
<td>D-GalAc (0.2 m)</td>
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<tr>
<td>Maclura pomifera (MPA)</td>
<td>GalAc &gt; Gal</td>
<td>10 µg/ml</td>
<td>D-GalAc (1.0 m)</td>
</tr>
<tr>
<td>Bauchinia purpurea (BPA)</td>
<td>GalAc &gt; Gal</td>
<td>50 µg/ml</td>
<td>D-GalAc (0.2 m)</td>
</tr>
<tr>
<td>Solanum tuberosum (STA)</td>
<td>GlcAc (1,4GlcAc)β1-4GlcAc &gt; GlcAcβ1-4GlcAcβ1-4GlcAc &gt; GlcAcβ1-4GlcAc ≥ GlcAc</td>
<td>50 µg/ml</td>
<td>N,N',N'-triacetylchitotriose (0.05 m)</td>
</tr>
<tr>
<td>Phytolacca americana (PWM)</td>
<td>(1,4GlcAc)β1-4GlcAc ≥ (−3Galβ1-4GlcAc/1−)n</td>
<td>10 µg/ml</td>
<td>N,N',N'-triacetylchitotriose (0.05 m)</td>
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</tbody>
</table>

a Carbohydrate specificities of lectins are according to Goldstein & Poretz (1986).

b Abbreviations used: Gal, galactose; GalAc, α-acetylgalactosamine; GlcAc, α-acetylgalactosamine.

sections were prepared with lectins that had been preincubated overnight at 4 °C with specific inhibitory sugar, α-acetylgalactosamine or N, N', N'-triacetylchitotriose (Sigma) (Table 1) in which the epithelial staining was inhibited.

RESULTS

Position of the gizzard (pyloric region)-duodenal boundary as revealed by in situ hybridisation for CdxA mRNA.

At d 3, the stomach primordium was seen as a swelling in the digestive tube lying posterior to the lung bud (Fig. 1). At the posterior part of the stomach primordium, the dorsal endodermal wall developed a slight bulge, which eventually forms an endodermal pocket in the middle of the gizzard body. CdxA mRNA expression was detected posteriorly from the position just caudal to this bulge, where the endodermal tube became constricted again (Fig. 2a); this point was regarded as the gizzard-duodenal boundary. At d 4, the endodermal bulge at the posterior stomach grew deeper to form a pocket. The anterior limit of CdxA expression was found in the constricted tube following the stomach, at a point where the lumen of the tube was slightly enlarged (Fig. 2b). The pyloric region of the gizzard could thus be clearly identified from d 4 as the anterior part of the constricted endodermal tube without CdxA mRNA. At d 6, the rostral limit of CdxA expression was found at a point where the endodermal tube left the mesenchymal mass of the gizzard body (Fig. 2c). From d 10, the morphological boundary between the gizzard (pyloric region) and duodenum became apparent as previously shown (Matsushita, 1991) and the duodenal epithelium up to this boundary was
A d 3, a considerable number of GMA3-positive granules and a few GMA2, 4 or 5-positive granules were already evident in the apical and middle parts of the epithelium of the gastroduodenal region, mainly in the segment from posterior gizzard to anterior duodenum that included the probable gizzard-duodenal boundary (Fig. 3a, b). During d 4–6, the granules positive for respective antibodies including GMA1 increased in amount and the anterior wall of the gizzard endodermal pocket gradually came to contain as many granules as the posterior gizzard. The granules stained with GMA1, 3 and 5 were abundant in the pyloric region and anterior duodenum (Fig. 3e), posterior to which they decreased. GMA2 or 4-positive granules were rare in the pyloric and duodenal regions (Fig. 3c, d).

During d 10–18, morphogenesis specific to the respective regions proceeded, and staining with the antibodies showed different patterns between these regions. In the gizzard, numerous granules mostly in the apical part and many lacunae in the middle or basal parts of the epithelium of d 10 or 14 embryos were stained with all antibodies (Fig. 3f, h). The cellular protrusions into the mesenchyme from the basal side of the d 14 epithelium, which were eventually to form the lower part of the tubular glands, contained some granules stained with GMA1, 3 and 5 (Fig. 3h). The outermost layer covering the almost-formed glands in d 18 gizzard contained numerous granules that stained with all antibodies (Fig. 3j). Cells at the upper part of tubular glands were rarely stained with GMA2 or 4 (Fig. 3j). The pyloric epithelium showed frequent GMA1, 3 or 5 staining in the apical part or in the less frequently present lacunae, and infrequent or rare GMA2 or 4 staining (Fig. 3g, i). The duodenal epithelium at d 10 showed an apparent decrease in the amount of stained granules as compared with those at d 6 (Fig. 3g), but from d 14 developed some punctate staining with occasional diffused appearances probably in immature goblet cells.

After hatching, specific morphogenesis was completed. The gizzard, which had lost the covering layer over tubular glands, was devoid of GMA2 or 4 staining. The stainability with the antibodies in the pyloric or duodenal region was similar to that of d 18 embryos.

**Lectin staining**

The results of lectin histochemical study are summarised in Table 3. Positively stained intraepithelial structures were almost the same as those found to be
Table 2. Staining with antigizzard mucus monoclonal antibodies in the epithelium lining the segment from the gizzard to duodenum of chick embryos and hatched chicks

<table>
<thead>
<tr>
<th>Age (days of incubation)</th>
<th>Antibodies</th>
<th>Anterior gizzard</th>
<th>Posterior gizzard</th>
<th>Pyloric region</th>
<th>Anterior duodenum</th>
<th>Posterior duodenum</th>
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a The number of epithelial cells positive for respective antibodies is shown: –, no positive cells; ±, a few positive cells; +, positive cells often found; ++, almost whole of the apical part of the epithelium, or of the luminal surface of the mucosa (in case of the gizzard or pyloric region of d 18 embryos or hatched chicks), is positive. b See Fig. 1. c Pyloric region in d 3 embryos was not analysed as it could not be identified clearly. d Occasional punctate staining probably including the mucus of some or most goblet cells. e Cells in the lower part of plicae were often negative. f Only the cells at the upper part of tubular glands were positive.

positive on immunohistochemistry, except for fine lectin-positive granules and perinuclear staining in the duodenum from d 14 and Golgi regions.

At d 3, a small number of granules weakly positive for some lectins were found in some embryos around the junctional region of the stomach and duodenum. During d 4–6, the intensity and the number of the granules positive for lectins other than PWM increased. The regional distribution of the positive granules was similar to the result of the immuno-staining study (Fig. 4a,b). However, the amount of VVA or WFA-positive cells or of BPA, STA or MPA-stained granules in most cells was still smaller in the anterior gizzard of d 6 embryos.

During d 10–18, respective regions showed different pattern of staining. The anterior and posterior gizzard epithelia at these stages showed abundant staining with lectins other than PWM, mostly with moderate or strong intensity, with the same staining pattern in both epithelia. Some granules in basal cellular protrusions at d 14 were stained only with STA or MPA, and PWM stained weakly only a few cells at the outermost surface of the d 14 or 18 gizzard epithelium (Fig. 4e). The pyloric epithelium showed weak or no staining with VVA, and the prominent PWM staining found at d 14 and 18 was the characteristic feature (Fig. 4c,d,f). The duodenal epithelium showed low degree of lectin staining at d 10, which was followed by the appearance from d 14 of punctate staining with some lectins, with a diffuse appearance for STA.
After hatching, the mucus in cells in the upper part of the gizzard tubular glands (Fig. 4g) lost MPA staining partly and VVA staining totally. The mucus in the apical part of the pyloric epithelium (Fig. 4h) and in goblet cells in the duodenal epithelium showed stainability with lectins similar to that in d 14–18.
Fig. 3. Immunostaining in the segment from gizzard to duodenum of embryonic and hatched chicks. ×153. Panels a–c show the section cut sagittally, with the ventral and dorsal sides facing the top and bottom, respectively. e, epithelium; m, mesenchyme; c, covering layer; S, secreted substances. Other abbreviations are as in Fig. 2. Brackets in g and i indicate the fold at the morphological boundary between gizzard (pyloric region) and duodenum. Bright round spots in the mesenchyme are red blood cells with autofluorescence. (a) Day 3 stomach region stained with GMA3. An arrowhead denotes the dorsal endodermal bulge. The stomach endoderm posterior to the level of dorsal endodermal bulge (arrowhead) and the ventral endoderm anterior to this level contained a considerable amount of positive granules. (b) Day 3 duodenal region stained with GMA3. An arrow denotes the approximate position of the rostral limit of CdxA expression. (c) The segment from posterior gizzard to anterior duodenum of a d 4 embryo. GMA2 staining. A considerable number of positive granules are seen in the posterior gizzard, while positive granules are rare in pyloric and duodenal regions. An arrowhead denotes the dorsal endodermal bulge growing deeper to form the endodermal pocket of gizzard. An arrow indicates the approximate position of the rostral limit of CdxA expression, possible junction of pyloric and duodenal epithelia. (d) Day 6 gizzard stained with GMA2. The epithelium near the pyloric region is devoid of staining. (e) Day 6 anterior duodenum stained with GMA5. (f) Day 10 posterior gizzard stained with GMA3. (g) Junctional region between pyloric region and anterior duodenum of a d 10 embryo. GMA3 staining. (h) Day 14 posterior gizzard stained with GMA1. Cellular processes with some positive granules (arrowheads), protruding from the epithelial basal surface are penetrating into the underlying mesenchyme. (i) Day 14 junctional region between pyloric region and anterior duodenum stained with GMA2. (j) Day 18 anterior gizzard stained with GMA2. Cells in the covering layer are mainly stained, while cells in the tubular glands underneath are rarely stained.

DISCUSSION

The present study has shown that the development of the gastroduodenal regions of the chick embryo as revealed by the production and accumulation of mucosubstances may roughly be divided into 2 phases: before and after about d 10.

The first phase is characterised by the initial accumulation of mucus granules over entire gastroduodenal segment and the gradual attainment of the region-specific staining pattern as observed in the later phase. Since CdxA mRNA expression in the duodenal epithelium with clear anterior border was apparent from d 3 and the pyloric and duodenal epithelia with rare GMA2 or 4 staining was apparent from d 4, the regional identity of the gizzard body, pyloric region and duodenum may have been established from the early stages of this phase soon after the formation of the digestive tube. At present, it is not known why the young duodenal epithelium accumulates many mucus granules. Protection from the proteolytic action possibly commencing from d 13 when the proventriculus with abundant pepsinogen-antigen expression (Takiguchi et al. 1986) begins to secrete HCl (Toner, 1965), may be the explanation for the mucus accumulation in the apical epithelial part of the gizzard body or the pyloric region as suggested by Gheri et al. (1994), although this may not be the case as the duodenal epithelium loses most of the initial granules by d 10. In this respect, the d 6 duodenal endoderm, which is already specified as intestine, is reported to have the potential to differentiate also into mucous epithelium resembling gizzard epithelium (Matsushita, 1995). During d 3–6, the mucus granule-positive area gradually enlarged anteriorly in the gizzard and the identical staining pattern in the anterior and posterior parts of the gizzard body was attained by d 10, suggesting that the differentiation of the epithelium in the gizzard body may proceed posteroanteriorly.

During the later phase after d 10, the epithelia in the gastroduodenal regions underwent a remarkable region-specific morphogenesis (Romanoff, 1960; Matsushita, 1991) and showed a specific pattern of staining. The epithelium of the gizzard body contained abundant mucus granules that stained with most antibodies and lectins. They showed a specific intra-epithelial distribution pattern characteristic of each stage of tubular gland formation, in accordance with the changes in secretory activity reported by Gheri et al. (1994) and Sgambati et al. (1996). The pyloric region underwent plicae formation and contained abundant mucus with a different staining pattern. The duodenal epithelium formed villi and had a low
content of mucus granules except in immature goblet cells. It is possible that the mesenchyme underlying the epithelium of each region may play a role in determining the region-specific epithelial differentiation with the production of region-specific type of mucosubstances, since the mesenchyme at least of the gizzard body and duodenum is known to have the potential to elicit mesenchyme-dependent morphogenesis and differentiation in the endoderm of these regions (Ishizuya-Oka & Mizuno, 1984, 1985, 1992; Matsushita, 1995). Such mesenchymal inductive activities may act from early stages to establish the initial regional difference observed at d 3–4. It has been reported that the d 3 intestinal mesenchyme may already have an inductive activity (Matsushita, 1996). After hatching only a slight change in the staining pattern was noted in the mucus of the cells at the top of the gizzard tubular glands, with a reduction of

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<th>Table 3. Lectin staining in the epithelium lining the segment from the gizzard to duodenum of chick embryos and hatched chicks</th>
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* The number of epithelial cells positive for respective lectins is shown: –, no positive cells; ±, a few positive cells; +, positive cells often found; +++, almost whole of the apical part of the epithelium, or of the luminal surface of the mucosa (in case of the gizzard or pyloric region of d 18 embryos or hatched chicks), is positive. b See Fig. 1. c Pyloric region in d 3 embryos was not analysed because it could not be identified clearly. d Cells containing small amount of weakly-positive granules. e Occasional punctate staining probably including the mucus of some or most goblet cells. f Cells in the lower part of plicae were negative. g Weakly-positive perinuclear cytoplasmic staining was also found. h Only the cells at the upper part of tubular glands are positive. i Probable Golgi regions also showed weak staining.
Fig. 4. Lectin staining in the segment from gizzard to duodenum of embryonic and hatched chicks ×153. Panel a shows the section cut sagittally, with the ventral and dorsal sides facing the top and bottom, respectively. Abbreviations are the same as in Fig. 3. Brackets in c, d and f indicate the fold at the morphological boundary between gizzard (pyloric region) and duodenum. Bright round spots in the mesenchyme are red blood cells with autofluorescence. (a) The stomach and pyloric region of a d 4 embryo stained with MPA. An arrowhead shows the dorsal endodermal bulge and an arrow indicates the approximate position of the rostral limit of CdxA expression. (b) Day 4 anterior duodenum stained with MPA. (c) Day 10 junctional region between pyloric region and anterior duodenum. No staining with VVA. (d) Day 14 junctional region between pyloric region and anterior duodenum stained with WFA. (e) Day 18 anterior gizzard stained with PWM. A few cells in the covering layer (arrowheads) and the luminal substances in tubular glands were weakly positive. (f) Day 18 junctional region between pyloric region and anterior duodenum stained with PWM. (g) Posterior gizzard of a 1-d-old hatched chick stained with STA. Cells at the upper part of tubular glands are strongly positive. (h) Pyloric region of a 1-d-old hatched chick stained with STA.
VVA and MPA staining. This may be related to the secretion and formation of a hard inner lining covering the mucosa and serving as a grinding plate for ingested food (Romanoff, 1960).

The results of lectin histochemistry suggested the presence of some sugar residues which are responsible for the regional and developmental changes of the mucosubstances. The gizzard epithelium from d 4 showed staining with WFA, VVA, MPA and BPA that bind to α-acetyl-d-galactosamine (Goldstein & Poretz, 1986), whose presence in the gizzard from d 7 was reported by Gheri et al. (1994). Among these lectins, MPA and BPA have a high affinity for galactosamine-(ß1–3)-α-acetylgalactosamine residue, while WFA or VVA have a high affinity for α-acetylgalactosamine-(ß1–3)-galactose and α-acetylgalactosamine-(ß1–3)-N-acetylgalactosamine residues or N-acetylgalactosamine-(ß1–3)-galactose residue and α-acetylgalactosamine linked to serine or threonine, respectively (Wu et al. 1994, 1996). Differential staining with these lectins found in the pyloric and duodenal regions of d 10–18 embryos may indicate the presence or absence of these sugar residues. STA and PWM bind to the oligomer of α-acetylgalactosamine (Goldstein & Poretz, 1986), which is rich in the gizzard that has also been reported (Gheri et al. 1994). STA may have an affinity for poly-α-acetylgalactosamine (Callaghan et al. 1990), and PWM binds to the highly-branched poly-α-acetylgalactosamine structure but not to the linear poly-α-acetylgalactosamine chains (Goldstein & Poretz, 1986). Thus differential PWM staining in the gizzard and pyloric region may be attributable to the differential distribution of linear and branched forms of poly-α-acetylgalactosamine. The functional role of these carbohydrates with a region-specific distribution remains to be elucidated.

In conclusion, the present study has revealed that the epithelia of the gizzard body, pyloric region and duodenum are quite different as revealed by the presence of mucosubstances with different histochemical characteristics; and that the regional differentiation in the epithelia of these regions is already apparent at d 3–4 of incubation, soon after the formation of the digestive tract and at a stage much earlier than the onset of morphogenesis specific to these regions.

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REFERENCES


MATSUISHITA S (1996) Chronological changes in the sucrase antigen-

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