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Lena Blomberg

Howard C. Krivan

*See next page for additional authors*

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Piglet Ileal Mucus Contains Protein and Glycolipid (Galactosylceramide) Receptors Specific for Escherichia coli K88 Fimbriae

LENA BLOMBERG, HOWARD C. KRIVAN, PAUL S. COHEN, AND PATRICIA L. CONWAY†∗

Department of General and Marine Microbiology, University of Göteborg, Carl Skottsbergs Gata 22, S-413 19, Göteborg, Sweden; MicroCarb Inc., Gaithersburg, Maryland 20879; and Department of Microbiology, University of Rhode Island, Kingston, Rhode Island 02881

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The aim of this study was to characterize the Escherichia coli K88-specific receptors in mucus from the small intestines of 35-day-old piglets with the isogenic strains E. coli K-12(pMK005) (K88+) and E. coli K-12(pMK002) (K88−). These strains differed only in that the latter one cannot produce intact K88 fimbriae because of a deletion in the gene coding for the major fimbrial subunit. Adhesion was studied by incubating 3H-labeled bacteria with crude mucus, pronase-treated whole mucus, mucus fractionated by gel filtration, delipidated mucus, or extracted lipids immobilized in microtiter wells. In addition, E. coli strains were tested for adhesion to glycolipids extracted from mucus by overlaying glycolipid chromatograms with 125I-labeled bacteria. The recently reported finding that K88 fimbriae bind to glycoproteins in mucus from the piglet small intestine was confirmed in two ways. Pronase treatment of immobilized mucus reduced adhesion by 82%, and adhesion to delipidated mucus was 14 times greater for the K88+ than for the K88− strain. E. coli K88+ adhered to several of the fractions collected after gel filtration of crude mucus, including the void volume (Mr > 250,000). Receptor activity specific for the K88 fimbriae was demonstrated in the lipids extracted from mucus, as the neutral lipids contained six times as much receptor activity as the acidic lipid fraction. Specificity was confirmed by demonstrating that adhesion to the total lipids could be inhibited by pretreatment of the immobilized lipids with K88 fimbriae. Relative to K-12 (K88−), the K-12 (K88+) bacterial cells bound more avidly to galactosylceramide when the neutral lipids were separated on thin-layer chromatography plates. No adhesion to lipids in the acidic fraction separated on thin-layer plates was detected. Relative to adhesion of K-12 (K88−), adhesion of K-12 (K88+) to commercially available galactosylceramide immobilized in microtiter wells confirmed the results with the thin-layer plates. It can be concluded that 35-day-old piglet mucus contains both protein and glycolipid receptors specific for K88 fimbriae, the latter being galactosylceramide.

Preweaned and immediately postweaned piglets are particularly susceptible to diarrhea induced by colonization of the small intestine by enterotoxigenic Escherichia coli strains which express K88 fimbriae (26, 27). Antigenic variants referred to as K88ab, -ac, and -ad fimbriae have been identified (8, 22). These fimbriae mediate adhesion of the E. coli K88 strains to the epithelial mucosa lining the small intestine (2) and thereby contribute to their virulence (10). Furthermore, piglets which are phenotypically resistant to E. coli K88-induced diarrhea lack K88-specific receptor in the epithelial cell brush borders (25). Initially, studies directed towards elucidating the receptor for the K88 fimbriae concentrated on the receptor involved in the hemagglutination of guinea pig erythrocytes by K88 (28). Gibbons et al. (7) succeeded in inhibiting hemagglutination with glycoproteins from colostrum and then destroyed the effect by removal of the terminal beta-D-galactose residue. This group of workers then inhibited hemagglutination with intestinal mucus glycoproteins but not serum glycoproteins. In addition, by chemical modification of these mucus glycoproteins, it was apparent that unsubstituted beta-D-galactosyl residues were an essential part of the receptor. Attention was then directed towards the brush border receptor for K88 fimbriae. Kearns and Gibbons (13) showed that brush border polar glycolipids varied for K88-sensitive and -resistant pigs. Because beta-D-galactose and periodate interfered with receptor activity, Sellwood (24) suggested that the receptor was either a glycoprotein or a glycolipid. Putative receptors for K88 fimbriae have been released into organ cultures of small intestinal explants and subsequently shown to be a 35-kDa glycoprotein or protein (32).

More recently, it has been established that receptors for the K88 fimbriae are detectable in the mucus overlaying the epithelial cells of the piglet small intestine (3, 5, 20, 31). The amount of K88ab receptor in the mucus was shown to be age related (3, 5, 31), as is susceptibility to E. coli K88-induced diarrhea, and it was therefore proposed that the presence of receptors in mucus may play a role in the pathogenesis of K88-bearing strains (4, 5).

Mucus consists of numerous glycoproteins, small proteins, lipids, and glycolipids (1) and is highly viscous in its native form because of mucin, which is a large glycoprotein (Mr, 2,000,000). Metcalfe et al. (20) showed that piglet small intestinal mucus contained a glycoprotein (Mr, 42,000) with specific affinity for K88ab fimbriae by adsorbing purified fimbriae with mucus and then analyzing them by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, it was shown that K88ab and -ac fimbriae bound to several small glycoproteins in the same size range by Western blotting (immunoblotting) mucus proteins separated by SDS-PAGE (31).
Glycolipids are frequently reported to function as receptors for a range of bacteria (12, 17) including enterotoxigenic E. coli carrying K99 fimbriae (18, 30). Both Metcalfe et al. (20) and Willemsen and de Graaf (31) have detected K88-specific glycoprotein receptors in piglet intestinal mucus; however, neither group detected glycolipid receptors. Our preliminary studies of K88 receptors in ileal mucus showed that components with a $M_t$ of $>250,000$ contained K88 receptor activity but little protein. The aim of this study, therefore, was to investigate the receptor activity of both the lipid- and the protein-containing fractions of piglet ileal mucus.

**MATERIALS AND METHODS**

**Bacterial cultures and culturing conditions.** The isogenic strains of E. coli K-12(pMK005) (K88$^+$) and E. coli K-12(pMK002) (K88$^-$) were obtained by electroporation of the pMK005 and pMK002 plasmids into E. coli K-12. These plasmids were very kindly provided by D. Pickard, Wellcome Foundation, Beckenham, United Kingdom, as described previously (14) and supplied in E. coli HB101. The pMK005 plasmid contained the pBR322 vector and the 6.5-kbp gene cluster encoding the K88 fimbriae. This 6.5-kbp gene cluster contained two EcoRI sites, and the pMK002 plasmid was constructed by removing the 1.75-kbp EcoRI fragment, which contains a large percentage of the faeD gene encoding the K88 fimbrial subunit. Although this plasmid contains the faeA, -B, and -C genes encoding the K88 fimbriae, it cannot produce functional K88 fimbriae, as confirmed with anti-K88ac serum. In addition, the Gyles strains of E. coli K88$^+$ and K88$^-$ refer to E. coli K-12 containing the entire plasmid on which K88 is located and E. coli K-12 without the plasmid, respectively. All strains were stored in 30% glycerol at $-70^\circ\mathrm{C}$ and then grown in Trypticase soy broth at $37^\circ\mathrm{C}$ overnight before use in the various adhesion assays.

For the adhesion assay, E. coli strains were inoculated (1%) in Trypticase soy broth and incubated for 5 h and then resuspended (1%) in Trypticase soy broth containing 1% [methyl-1,2-3H]thymidine (specific activity, 117 Ci mmol$^{-1}$; Amersham). Cultures were incubated statically overnight at $37^\circ\mathrm{C}$. The cells were harvested by centrifugation ($3,000 \times g$) for 7 min at $4^\circ\mathrm{C}$ prior to being washed and resuspended in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-Hanks buffer (pH 7.2) to yield an optical density of 0.5 (600 nm).

For adhesion to thin-layer chromatography (TLC) plates, E. coli strains were grown without shaking for 18 h at $37^\circ\mathrm{C}$ in Trypticase soy broth without added glucose. Broth cultures were centrifuged at $4^\circ\mathrm{C}$ and $10,000 \times g$ for 20 min, and the pellets were washed three times in $0.01 \mathrm{M}$ sodium phosphate (pH 7.2) containing 0.15 M sodium chloride. Bacteria were radioiodinated as described previously (16). Briefly, approximately $10^9$ cells were transferred to a tube (10 by 75 mm) coated previously with 100 $\mu$g of Iodogen and reacted with 0.5 mCi of Na$^{251}$ at $4^\circ\mathrm{C}$. The iodination was terminated after 5 min by removal of the cells from the reaction tube, centrifugation, and three washes in 0.05 M Tris-HCl (pH 7.8) containing 0.15 M sodium chloride–1% bovine serum albumin (TBS-BSA). The labeled bacteria were resuspended to $2 \times 10^9$ cpm ml$^{-1}$ in TBS-BSA.

**Mucus collection and fractionation.** Mucus was collected from the ileal region of the small intestines of 35-day-old piglets by scraping with a rubber spatula and using HEPES-Hanks buffer. The collected material was centrifuged twice at $27,000 \times g$ for 20 min to remove bacterial and epithelial cells prior to being stored in small aliquots at $-85^\circ\mathrm{C}$. Some of the supernatant was immediately freeze-dried and used for lipid extraction studies. Mucus was collected from three piglets and tested separately.

Epithelial cells were also collected from the ileal region of the three piglets and stored frozen as described previously (5). These epithelial cells were used to ensure that the three piglets were of the K88-susceptible phenotype (25). This was determined by studying the interaction between K88-bearing E. coli cells and ileal epithelial cells by direct microscopy, as described by Wilson and Hohmann (33). Several bacteria bound to the brush border membranes, and very few, if any, bound to the remainder of the epithelial cells.

Lipids were extracted from the freeze-dried mucus with chloroform-methanol (2:1) (16) and separated into neutral and acidic lipids by high-performance liquid chromatography (HPLC) on silica Iatrobeads and by anion-exchange chromatography on DEAE-cellulose, respectively, as described previously (15). Lipid fractions were dried under nitrogen and stored until use. Crude mucus was also fractionated by gel filtration, using an XK 26/40 column packed with Sephadex 200 SF (Pharmacia), and eluted with HEPES-Hanks at a flow rate of $1.1 \mathrm{ml} \ h^{-1}$ and with HEPES-Hanks as the mobile phase. The crude eluate was monitored by measuring the $A_{340}$. The various fractions were immobilized in the microtiter wells and tested for receptor activity.

**Adhesion to surface-immobilized mucus and components.** Adhesion of E. coli to immobilized mucus was largely performed as described by Laux et al. (19). Briefly, the mucus was diluted in HEPES-Hanks buffer (pH 7.2) to yield 0.5 mg of protein ml$^{-1}$ and immobilized on polystyrene tissue culture plates (Nunc; Delta) overnight at $4^\circ\mathrm{C}$. Immobilized BSA was used as a control. The wells were rinsed twice with 0.5 ml of HEPES-Hanks buffer prior to the addition of $^3$H-labeled E. coli. After incubation at $37^\circ\mathrm{C}$ for 1 h, the wells were rinsed twice with 0.5 ml of buffer, and then 0.5 ml of 5% SDS was added to release the irreversibly bound bacteria by incubating at $60^\circ\mathrm{C}$ for 1 h. The radioactivity of the SDS sample was enumerated with an Aquascore scintillation fluid and a liquid scintillation spectrophotometer (L6000LL; Beckman).

**Characterization of classes of K88 receptors.** To determine whether there were proteinaceous and/or lipid-containing receptors for K88 fimbriae in the mucus, the following experiments were carried out.

(i) Crude mucus was diluted to 1 mg of protein ml$^{-1}$ and treated with pronase (0.5-mg ml$^{-1}$ final concentration; Calbiochem) for 1 h at $37^\circ\mathrm{C}$. Controls included BSA (1 mg ml$^{-1}$) treated in the same way as well as mucus incubated at $37^\circ\mathrm{C}$ without pronase addition. After the treatment, samples were immobilized and the adhesion was carried out as described above, except that the wells containing the bacteria were incubated on ice to avoid further activity of the pronase. In addition, the residual mucus components after extraction of the lipids were solubilized in HEPES-Hanks buffer and immobilized in microtiter wells. The presence of receptor was tested with the $^3$H-labeled E. coli cells as described before.

(ii) The total, acidic, and neutral lipids extracted from the mucus were solubilized in HEPES-Hanks buffer and immobilized in microtiter wells. The presence of receptor was tested with the $^3$H-labeled E. coli cells as described before.
\(^3\)H-labeled \(E.\ coli\) cells to treated wells was performed as described above. In addition, twofold serially diluted lipids (six steps) were immobilized, and the adhesion of \(E.\ coli\) was similarly evaluated.

(iii) The specificity of the receptors in the lipids was evaluated by pretreating the immobilized lipids with crude K88 fimbrial preparation from \(E.\ coli(pMK005)\). The fimbrial extracts were prepared by heat treating the bacterial cells at 60°C for 20 min to release the fimbriae and then centrifuging twice at about 10,000 × g for 20 min to remove the bacterial cells. Immobilized mucus containing K88-specific receptors was pretreated with the K88 fimbrial preparation. Because the heat treatment used to release the K88 fimbriae from the cell may also release other components, heat extracts of \(E.\ coli(pMK002)\) were used as controls.

Adhesion to TLC plates. Lipids were chromatographed on aluminum-backed silica gel HPTLC plates developed in chloroform–methanol–0.25% aqueous KCl (5:4:1). The plates were coated with polyisobutylmethacrylate (0.1% in hexane), soaked in TBS-BSA, and incubated for 2 h at room temperature with \(^3\)H-labeled \(E.\ coli\) cells (2 \times 10^6 cpm ml\(^{-1}\)) suspended in TBS-BSA. The plates were gently washed to remove unbound bacteria, dried, and exposed for 24 h to XAR-5 X-ray film (Eastman Kodak, Rochester, N.Y.). Alternatively, the plates were sprayed with orcinol reagent to identify glycolipids (16).

Receptor activity of surface-immobilized galactosylceramide. Pure galactosylceramide (galactocerebroside; C 4905, Sigma Chemical Co.) was diluted in methanol (500 µg ml\(^{-1}\)) and then dried in microtiter wells as described above. After BSA treatment, \(^3\)H-labeled \(E.\ coli\) cells were added; after a 10-min incubation, the extent of adhesion was determined as described above.

**RESULTS**

Mucus samples from all piglets yielded comparable results, and so the results of one mucus batch are presented as typical. Gel filtration of crude mucus revealed that the K88 fimbriae had affinity for the void volume (\(M_r > 250,000\)) as well as several other fractions (Fig. 1).

Evidence of proteinaceous receptor activity in mucus. \(E.\ coli\) K-12(pMK005) (K88\(^*\)) only differs from \(E.\ coli\) K-12(pMK002) (K88\(^-\)) in that the pMK005 plasmid contains a functional K88 gene cluster while the latter does not. Consequently, enhanced adhesive characteristics of the strain containing plasmid pMK005 relative to the strain containing pMK002 are attributable to the K88 fimbriae. As illustrated in Fig. 2, \(E.\ coli(pMK005)\) adhered to mucus approximately 12 times more than \(E.\ coli(pMK002)\), whereas adhesion of the two strains to the control surface of BSA was equivalent. This adhesion mediated by the K88 fimbriae was reduced by 82% ± 4% (mean ± standard deviation) after pronase treatment (data not shown). Consistently, the adhesion of the \(E.\ coli(pMK005)\) K88\(^*\) strain to immobilized delipidated mucus was 14 times greater than the adhesion of the \(E.\ coli(pMK002)\) K88\(^-\) strain to the lipid-free mucus (data not shown).

Evidence of lipid receptor activity. Although the amount of receptor varied slightly from one mucus preparation to the next, all three mucus preparations had adhesion profiles similar to that of total lipids. Neutral and acidic lipids were extracted from two different mucus preparations from different pigs, and the K88\(^+\) and K88\(^-\) strains \([E.\ coli\ K-12 (pMK005)\) and \(E.\ coli\ K-12(pMK002),\) respectively] exhibited a consistent profile of adhesion to both preparations. It was found that \(E.\ coli\ K-12(pMK005)\) (K88\(^*\)) adhered significantly better to crude mucus and total and neutral lipids than \(E.\ coli\ K-12(pMK002)\) (K88\(^-\)) (Fig. 2). In addition, the adhesion of the K88\(^*\) strain (pMK005) to the acidic lipid fraction was only one-sixth of the adhesion to the neutral fraction. Nonspecific adhesion of the K88\(^*\) strain (pMK005) to the control surface (BSA) was very low compared with the adhesion to mucus and total and neutral lipids (Fig. 2). Serial dilution of the various lipid fractions showed that the 500-µg ml\(^{-1}\) concentration was appropriate for studies directed to understanding the nature of this lipid receptor (Fig. 3) and that adhesion was measurable with as little as 125 µg of lipid ml\(^{-1}\) for the total lipids. While the extent of adhesion decreased rapidly with decreasing concentration of total and neutral lipids, neutral lipids maintained their adhesiveness to a much greater extent than their total lipid counterparts.
neutral lipids, K88-mediated adhesion to the acidic lipids was low with the 500-μg/ml concentration and decreased slowly with decreasing concentration of lipid (Fig. 3).

The specificity of the adhesion of E. coli K-12(pMK005) to the total lipids was tested with crude K88 fimbrial preparations. Immobilized lipids were pretreated with the heat extracts from E. coli K-12(pMK005) (K88+) or E. coli K-12(pMK002) (K88−) to the total lipids as well as to the control mucus and BSA was unaffected by pretreatment of the mucus and mucus fractions with the heat extracts of pMK005 and pMK002 (Fig. 4a). As can be seen in Fig. 4b, this pretreatment of the mucus with K88-containing extracts resulted in a 70% reduction in adhesion (relative to the HEPES-Hanks-treated control) of E. coli K-12 (pMK005), while extracts from the strain containing plasmid pMK002 (K88−) had little effect on adhesion of K-12 (pMK005) to the mucus (Fig. 4b). Similarly, adhesion of K-12(pMK005) to total lipids was inhibited by pretreatment of the lipids with the K88 fimbriae but not by the heat extracts from the K88− strain (pMK002). The nonspecific adhesion of K-12(pMK005) to the control surface BSA was not affected by the K88 fimbriae or the pMK002 heat extracts.

Identification of the lipid receptor. The specific lipid(s) with affinity for the K88 fimbriae was identified by overlaying the radioactively labeled E. coli cells on the various lipid fractions after separation by TLC. The results of these studies are presented in Fig. 5. The total and neutral lipids contained many bands, whereas the acidic fraction contained a very limited number (Fig. 5A). E. coli (pMK005) K88+ cells adhered strongly to one band in both the total and neutral lipids (lanes 2 and 3, respectively, Fig. 5B) and very weakly to a second band. The band to which the K88+ strain adhered strongly corresponded in mobility to the authentic standard. In addition, these K88+ cells adhered to the standard galactosylceramide as well as to pure galactosylceramide (lanes 1 and 4, respectively, Fig. 5B). Adhesion of the K88− (pMK002) cells to the galactosylceramide was very low, as was adhesion of both K88+ and K88− cells to the bands from the acidic lipid fraction. The K88− cells also adhered to a band in the same position as the one for which the K88+ cells had a weak affinity.

The affinity of the E. coli cells for galactosylceramide was also tested by immobilizing the pure lipid to polystyrene microtiter wells. As can be seen in Fig. 6, the E. coli (pMK005) K88+ cells were three times more adhesive to the galactosylceramide than the K88− cells, while both of these E. coli strains adhered equally poorly to the BSA control.

DISCUSSION

The isogenic strains of E. coli K-12 containing plasmids pMK005 and pMK002 only differed in that the former contained a functional K88 fimbrial gene cluster and the latter did not. Any detected difference in receptor activity between the two could therefore be directly attributable to the K88 fimbriae. Previously, workers (5, 20, 31) have utilized the isogenic strains E. coli K-12 (K88+) and E. coli K-12 (K88−) originally constructed by insertion into K-12 of the entire wild plasmid on which the K88 genome was located. The K88− strain therefore did not contain any of the genes encoded by that plasmid, for example, raffinose utilization. For all subsequent studies, results are presented for only E. coli K-12(pMK005) and E. coli K-12(pMK002).

Pronase treatment of mucus decreased adhesion of the K88+ (K-12[pMK005]) strain by 82%. Furthermore, because adhesion to delipidated mucus of the K88+ (pMK005) strain was 14 times greater than that of the K88− (K-12[pMK002]) strain, one can suggest that a proteinaceous receptor is present in the mucus. This is consistent with the findings of Metcalfe et al. (20) and Willemsen and de Graaf (31), who reported affinity of purified K88 fimbriae for glycoproteins. Although both of these studies failed to detect lipid receptors for K88 in piglet ileal mucus, it appears that our mucus preparations do contain lipid receptors. Relative to the K88− (K-12[pMK002]) strain, the K88+ (K-12[pMK005]) strain adheres extremely well to both total lipids and neutral lipids (Fig. 7), while nonspecific adhesion of this K88− strain to the immobilized BSA control was very low. One can therefore

FIG. 3. Adhesion of E. coli (pMK005) to various concentrations of total (closed circles), neutral (open squares), and acidic (open diamonds) lipids. Adhesion is expressed as the amount of radioactivity remaining on the surface. Lipids were diluted stepwise from an initial concentration of 500 μg ml−1.

FIG. 4. Adhesion of E. coli K-12(pMK002) (K88+) (a) and E. coli K-12(pMK005) (K88−) (b) to immobilized crude mucus, total lipids, and BSA after pretreatment of the immobilized material with heat extracts (i.e., crude fimbrial preparations) from either the K88+ strain (hatched bars) or the K88− strain (open bars) or with HEPES-Hanks buffer (stippled bars). Results are expressed as percentage of the total number of bacterial cells which adhered (mean ± standard deviation of three individual assays) when bacteria were incubated on the immobilized mucus for 30 min.
suggest that the adhesion to lipids is due to specific binding of the K88 fimbriae to a lipid and, in particular, to a neutral lipid. Dilution of the total and neutral lipids immobilized in the microtiter wells resulted in a rapid decrease in adhesive activity of the K88⁺ (pMK005) strain, consistent with the suggestion that mucus contains a lipid receptor (Fig. 3). Adhesion of the K88⁺ [K-12(pMK005)] strain to the acidic fraction was only one-sixth of the adhesion to the neutral fraction.

Consistently, adhesion of these K88⁺ cells to total lipids was inhibited by the crude K88 fimbrial preparation but not by the extract from the K88⁻ [K-12(pMK005)] strain, and the adhesion to BSA was unaffected by these crude fimbrial preparations (Fig. 4b). The results from the TLC studies support the findings presented in Fig. 2 and 4 that mucus contains a K88-specific neutral lipid receptor. The K88⁺ (pMK005) strain did not adhere to the acidic lipid fraction on the TLC plates, consistent with the data presented in Fig. 2 for adhesion to this fraction upon immobilization in the microtiter wells.

From the TLC studies, it appears that the lipid receptor is galactosylceramide (Fig. 5). Although the K88⁺ (pMK005) cells had a weak affinity for a second band on the TLC plates (Fig. 5), the K88⁻ cells also adhered weakly to a band of the same size. Consequently, this adhesion is not mediated by the K88 fimbriae. When commercially available galactosylceramide was immobilized in the plastic microtiter wells, adhesion of the K88⁺ [K-12(pMK005)] strain was three times greater than that of the K88⁻ [K-12(pMK002)] strain (Fig. 6), consistent with the TLC data. This difference is not as great as that noted for adhesion to the neutral lipids from the mucus and could be because the galactosylceramide in the mucus probably differs in fatty acid composition relative to the commercial bovine galactosylceramide. The fatty acid residue present in the amide linkage of ceramides can vary in chain length, can be saturated or unsaturated, and can exist as hydroxylated and nonhydroxylated residues (23). Such chemical differences can influence conformation and thus reactivity of the glycolipid. Monoclonal antibodies have been used to discriminate structural differences (21) and to illustrate the altered reactivity of various ceramides (6, 9, 11, 29). One can therefore hypothesize that the affinity of ceramide for the K88 fimbriae will vary for ceramides of different origins.

In summary, piglet ileal mucus contains both protein and glycolipid receptors for K88. The lipid receptor was detectable in both the total and the neutral lipids, and K88 fimbrial preparations inhibited this adhesion. It is suggested that the lipid receptor is galactosylceramide.

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