Insoluble Glucan Synthesis by Mutansucrase as a Determinant of the Cariogenicity of *Streptococcus mutans*

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Five strains of *Streptococcus mutans* were grown in continuous culture with either a limited supply or an excess of glucose. Proteins secreted into the extracellular fluid by strains C67-1, 3209 and K1 rapidly catalysed the synthesis of insoluble glucan from sucrose (mutansucrase activity). The culture fluid from strains Ingbritt or C67-25 catalysed the synthesis of soluble glucan (dextransucrase activity) and fructan, but little or no mutansucrase activity was detected. The strains which secreted active mutansucrase readily colonized a smooth hard surface during growth in batch culture and were more cariogenic in pathogen-free rats than those which secreted little mutansucrase activity. There was no similar correlation between fructosyltransferase, dextransucrase or total glucosyltransferase activity and either adherence or cariogenicity. We conclude that the ability to catalyse insoluble glucan synthesis is a major determinant of the cariogenicity of *S. mutans* strains.

INTRODUCTION

The synthesis of extracellular polysaccharides from sucrose is believed to be a major determinant in the induction of tooth decay by *Streptococcus mutans*. Evidence for this includes the demonstration that mutants which produce more polysaccharide than the wild type are more adherent (Schachtele *et al.*, 1975) and those which produce less are less cariogenic (De Stoppelaar *et al.*, 1971; Tanzer *et al.*, 1974; Koga & Inoue, 1978). Furthermore, experimental animals which had been injected with enzymes involved in polysaccharide synthesis became less susceptible to caries induced by *S. mutans* (Smith *et al.*, 1979). Also, fewer lesions developed when plaque was treated with glucanases (Guggenheim *et al.*, 1972, 1980). Apparently conflicting results include the isolation of a non-cariogenic mutant which forms normal quantities of polysaccharide that is unable to bind to the bacterial surface (Freedman & Tanzer, 1974). It was suggested that the binding of glucans to surface receptors is more important than the quantity of glucan formed in determining the cariogenicity of a particular strain. Furthermore, Dummer & Green (1980) recently reported that there was no correlation between the extracellular glucosyltransferase activity secreted by a strain and its cariogenicity.

At least three different types of polysaccharide are synthesized from sucrose by extracellular proteins of *S. mutans*. They are a water-soluble glucan ('dextran', formed by dextransucrase) in which 1,6- α glycosidic bonds predominate, an insoluble glucan ('mutan', formed by mutansucrase) with a majority of 1,3- α and a minority of 1,6- α glycosidic bonds and a fructan with either 2,1- β or 2,6- β linkages (formed by fructosyltransferase) (Baird *et al.*, 1973; Ellwood *et al.*, 1976). The most significant polysaccharide in the disease process is believed to be the insoluble glucan which both binds bacteria to the tooth surface and forms a permeability barrier within which acidic fermentation products accumulate and

demineralization occurs (Hojo *et al.*, 1976). Although qualitative differences between the amounts of glucan or fructan formed by different strains have been reported, few authors have attempted to determine the relative quantities of mutan and dextran formed by strains of differing cariogenicity. Only Wenham *et al.* (1979) have reported rates of synthesis of dextransucrase, mutansucrase and fructosyltransferase during growth of *S. mutans* strains under rigorously controlled conditions. The aim of the work to be described was to determine whether there is a correlation between the mutansucrase activity secreted by a particular strain (rather than the total rate of extracellular polysaccharide or glucan synthesis) and its cariogenicity or ability to colonize a smooth surface.

METHODS

Organisms, media and culture conditions. *Streptococcus mutans* strains Ingbritt, C67-1 and C67-25 (all serotype *c*) were obtained as freeze-dried cultures from J. M. Hardie, The London Hospital Medical College, London E1. These strains were used because Ingbritt was originally isolated as a cariogenic strain from a human (Krasse, 1966), and C67-25 is a non-cariogenic derivative of the cariogenic C67-1 which differs from its parent only in its inability to form insoluble glucan (De Stoppelaar *et al.*, 1971, 1973; Wenham, 1979). Strain K1 (serotype *d*) was obtained as colonies on nutrient agar from R. R. B. Russell, Royal College of Surgeons of England, Downe, Kent. Strains 3209 and 3450 (both serotype *c*) were isolated from the carious dentine of a permanent tooth and a deciduous tooth, respectively. They were received as colonies on blood agar from D. A. Sidaway, The Dental Hospital, St Chad's Queensway, Birmingham. Strain D282 (serotype *c*) was obtained as a freeze-dried culture from ICI Pharmaceuticals Division, Alderley Edge, Cheshire.

Pure cultures of each strain fermented mannitol, sorbitol and raffinose, were catalase-negative and formed a characteristic range of colony types on mitis-salivarius agar (Difco) and horse blood agar (Shklair & Keene, 1974). Stock cultures were maintained on horse blood agar and were subcultured every 14 d. Fresh cultures were generated from freeze-dried stocks every 3 months. To ensure that the origin of each strain was known, fresh cultures were always used to prepare inocula for continuous cultures, for colonization studies and for cariogenicity experiments.

Bacteria were grown in continuous culture as described by Wenham *et al.* (1979). The pH, temperature and dilution rate were 6.6 ± 0.1 , 37 °C and 0.12 h^{-1} , respectively. The volume was 840 ml in a 1.5 l fermentation vessel and N_2/CO_2 (95:5, by vol.) was passed through the culture at 500 ml h^{-1} . The growth rate was within the range estimated for streptococci in the mouth (as quoted by Hamilton *et al.*, 1979). Bacteria in the deepest layers of dental plaque in contact with the tooth enamel are believed to be carbon-limited, but excess fermentable carbohydrate is supplied to bacteria in surface layers during feeding (Carlsson & Griffith, 1974; Geddes, 1975; van der Hoeven, 1976). Enzyme activities were therefore determined for steady-state cultures (after at least five culture volumes had passed through the fermenter and when the cell yield was constant) in which glucose was either the growth-limiting nutrient or available in excess (6 or 36 g l^{-1} , respectively). In the latter case, growth was limited by one or more organic nitrogen compounds (other than cysteine) in yeast extract, Casamino acids or peptone. Hamilton *et al.* (1979) have presented evidence that growth might be limited by the supply of arginine and asparagine.

Colonization studies. The method was based on that described by McCabe *et al.* (1967). Each strain was inoculated into 5 ml brain heart infusion broth (Oxoid) supplemented with 5% (w/v) of either glucose or sucrose in a $10 \times 1 \text{ cm}$ test tube. A sterile nichrome wire was inserted into each tube and supported by a rubber bung. Tubes were agitated gently for 24 h at 37 °C and each wire was then transferred to fresh broth for a further 24 h. The extent of bacterial adherence and colonization was recorded photographically.

Enzyme assays and chemical determinations. Extracellular glucosyl- and fructosyltransferase assays were exactly as described by Wenham *et al.* (1979). Briefly, 1.0 ml of culture supernatant and sucrose were incubated at 37 °C in multiple sets of tubes for 0.5 to 2.0 h with or without $60 \mu\text{g dextran ml}^{-1}$ (mol. wt range 60 000 to 90 000; BDH). Insoluble glucan was sedimented by centrifugation, washed three times with water, dissolved in 1.0 M-NaOH and the quantities of ketohexose and total polysaccharide in the polymer were determined. The total polysaccharide (mutan, soluble glucan and fructan) formed in some of these tubes was precipitated at 4 °C with 80% (v/v) methanol. After three cycles of centrifugation, redissolution in 1 ml water and precipitation at 4 °C with 80% (v/v) methanol, the pellet was dissolved in 1.0 M-NaOH and the quantities of fructan and total polysaccharide were determined. Thus, the mutansucrase and fructosyltransferase activities were determined by direct assay and dextransucrase activity was calculated from the formula given by Wenham *et al.* (1979).

Cell-associated enzyme activities were assayed by incubating 1 ml of a washed cell suspension (resuspended in acetate buffer to the same turbidity as in the original culture) with sucrose and precipitating the polysaccharides formed with methanol. Other details were the same as for extracellular enzyme assays and were described by

Wenham *et al.* (1979). Activities are expressed as μg glucan or fructan formed h^{-1} (ml suspension) $^{-1}$. Chemical determinations were as described by Wenham *et al.* (1979).

Cariogenicity of S. mutans in pathogen-free rats. Albino rats were bred specifically pathogen-free in the Animal Breeding Unit, ICI Pharmaceuticals Division, Alderley Edge, Cheshire, according to category 4 of the Medical Research Council accreditation scheme. Pups from seven litters of six were distributed into pairs of similar weight when 20 d old. Bacteria from a freeze-dried culture were propagated on blood agar plus 1% (w/v) glucose and transferred to 30 ml brain heart infusion broth supplemented with 1% (w/v) glucose. After 16 h at 37 °C, cells were harvested by centrifugation for 5 min at 3000 *g* and resuspended in 30 ml sterile distilled water. The absorbance at 650 nm of each suspension was adjusted to 1.0 with sterile water and rats were inoculated with 0.2 ml via the tip of a blunt needle attached to a syringe. This inoculation procedure was repeated with fresh cultures of each strain for each of the following 4 d. In addition, 25 ml of the bacterial suspension was added to 1 l of sterile tap water and used as drinking water for the first 5 d of the study. The cariogenic diet containing 66% (w/w) icing sugar, 32% (w/w) skimmed milk powder and 2% (w/w) liver extract was available *ad libitum*.

Cross-infection was reduced by keeping the same food and water containers in each cage throughout the study. Three cages containing uninfected rats were interspersed between cages of infected rats: if there had been extensive transfer of any of the test strains of *S. mutans* to other groups of rats, these uninfected rats would also become contaminated early in the trial and the caries score would be comparable to that of other groups. The caries score of this group therefore provides an indication of the extent of cross-contamination between cages.

The establishment of *S. mutans* was confirmed by taking oral swabs from two rats in each group 2 weeks after the first inoculation. A sterile, moistened swab was passed over the molar teeth and placed in 10 ml of 0.9% (w/v) saline. The swab was agitated in the saline on a vortex mixer. Duplicate 0.1 ml samples of a 10^{-2} dilution were spread on mitis-salivarius agar and growth was recorded after 48 h at 37 °C. Many (20 to 500) colonies of *S. mutans* were observed on plates infected with samples from every rat exposed to the test *S. mutans* strains. It was impossible, however, to obtain quantitative estimates of the relative numbers of each *S. mutans* strain on the rats' teeth. No *S. mutans* colonies were obtained from samples taken before the rats were infected or from the uninfected control group of rats during the following 14 d. Specifically pathogen-free rats therefore provide an adequate model for cariogenicity assays despite the preference by some research workers for gnotobiotic rats.

Rats were killed 4 weeks after the first inoculation. The heads were autoclaved, defleshed and the lower jaws were stored in alcohol prior to scoring for smooth surface caries by the following modification of the method of Keyes (1958). A low-power binocular microscope was used to score the extent of a lesion in one plane. Only lesions which had destroyed the surface integrity of the enamel and penetrated the dentine were recorded. The number of molars with lesions was first recorded. The number of carious areas was then assessed by dividing the buccal and lingual surfaces of the first molars (which were the largest) into three equal areas and of the second and third molars into two areas and then recording the number of areas with lesions. The smooth surface caries score was determined by assessing out of three the extent of the lesion in each of these areas. The maximum possible scores for the first or second and third molars are 18 and 12, respectively; the maximum possible score for an entire mandible is 84.

RESULTS

Rates of glucosyltransferase and fructosyltransferase synthesis by different strains of S. mutans

Various strains of *S. mutans* were grown in continuous culture with either a limited supply or an excess of glucose, and extracellular and cell-associated glucosyl- and fructosyl-transferase activities were determined (Tables 1 and 2).

Mutansucrase was extremely active in cultures of C67-1, the serotype *d* strain K1 and the two serotype *c* strains which had recently been isolated from carious dentine (3209 and 3450). Although little or no mutansucrase was detected in cultures of strains Ingbritt or C67-25, dextransucrase activity was readily detected. With the exception of the non-cariogenic mutant C67-25, both dextransucrase and mutansucrase were more active in the extracellular fluid from cultures grown with excess glucose than in glucose-limited cultures. The concentrations of acid-precipitable protein in culture supernatants were in the range 80–100 $\mu\text{g ml}^{-1}$ and 150–200 $\mu\text{g ml}^{-1}$ for cultures grown with a limited supply or excess glucose, respectively. The higher extracellular glucosyltransferase activities during growth of strains C67-1, Ingbritt and 3209 with excess glucose were therefore the result of a higher specific rate of enzyme synthesis rather than due to a constant rate of enzyme synthesis by a more dense culture. The same conclusion applies to the dextransucrase activity of strain 3450.

Table 1. *Extracellular glucosyl- and fructosyltransferase activities of various strains of S. mutans after growth in continuous culture*

Bacteria were grown in continuous culture at a dilution rate of 0.12 h^{-1} with either a limited supply (6 g l^{-1}) or an excess (36 g l^{-1}) of glucose and were sedimented by centrifugation. The clarified culture supernatant was used for enzyme assays. Each experiment was repeated with at least two, and in some cases from up to six, independent cultures which gave essentially identical results. Activities are expressed as μg glucose or fructose from sucrose polymerized h^{-1} (ml culture supernatant) $^{-1}$.

Strain	Glucose in feed	Dextran in assay	Cell density (g l^{-1})	Dextranucrase	Mutansucrase	Fructosyltransferase
C67-1	Limiting	+	0.84	260	50	190
		—		59	46	180
	Excess	+	2.50	1100	450	190
		—		690	590	180
C67-25	Limiting	+	0.83	240	<5	190
		—		170	<5	190
	Excess	+	2.40	140	<5	90
		—		80	<5	70
Ingbritt	Limiting	+	1.05	70	4	720
		—		40	4	770
	Excess	+	2.15	430	18	320
		—		40	18	250
3209	Limiting	+	0.86	150	200	160
		—		125	165	190
	Excess	+	1.53	2100	4200	540
		—		250	4450	240
3450	Limiting	+	0.75	270	79	380
		—		135	83	320
	Excess	+	2.30	720	160	77
		—		220	290	75
K1	Limiting	+	0.80	170	280	<10
		—		40	270	<10
	Excess	+	1.50	200	540	<10
		—		<40	490	<10

Glucosyltransferase synthesis by these serotype *c* strains is not, therefore, regulated by catabolite repression (see Carlsson & Elander, 1973; Ellwood & Hunter, 1976). Dextran almost invariably stimulated the rate of soluble glucan synthesis and the dextranucrase activities of Ingbritt and K1 were totally dextran-dependent.

Fructosyltransferase was extremely active in the extracellular fluid from cultures of serotype *c* strains but was not detected with the serotype *d* strain, K1 (Table 1). Small quantities of insoluble fructan were formed with the culture fluid from strains 3450 and 3209 but this was less than 10% of the insoluble glucan formed and it might therefore be water-soluble fructan fortuitously trapped in the insoluble glucan matrix.

Cell-associated glucosyltransferase activities were detected with all of the strains examined (Table 2) but they were normally less, or far less, active than the corresponding extracellular enzymes. The glucosyltransferase activity in the extracellular fluid therefore provides a reliable indication of the relative quantities of enzyme formed by different strains (see also Janda & Kuramitsu, 1976). The failure of dextran to stimulate the cell-associated activity (except when strain 3209 was grown with excess glucose) is consistent with the role postulated for dextran in binding glucosyltransferase to the cell surface. Small quantities of dextran may have been formed from traces of sucrose which contaminates the components of complex media.

Colonization of S. mutans on nichrome wires

The ability of cultures of *S. mutans* to colonize smooth, hard surfaces was investigated by assessing the quantity of bacteria bound to the surface of a nichrome wire suspended for 2 d

Table 2. Cell-associated glucosyl- and fructosyltransferase activities of various strains of *S. mutans* after growth in continuous culture

Bacteria were harvested by centrifugation, washed with 0.1 M-sodium acetate pH 6.0 and resuspended in acetate buffer to the same turbidity as in the original culture. Other details are as for Table 1. Activities are expressed as μg glucan or fructan formed h^{-1} (ml suspension) $^{-1}$.

Strain	Glucose in feed	Dextran in assay	Cell density (g l^{-1})	Glucosyltransferase	Fructosyltransferase
C67-1	Limiting	+	0.84	65	20
		—		55	20
	Excess	+	2.50	140	95
		—		160	85
C67-25	Limiting	+	0.83	ND	40
		—		40	30
	Excess	+	2.40	ND	120
		—		140	85
Ingbritt	Limiting	+	1.05	ND	ND
		—		77	80
	Excess	+	2.15	ND	ND
		—		65	140
3209	Limiting	+	0.86	100	140
		—		140	130
	Excess	+	1.53	115	105
		—		35	40
3450	Limiting	+	0.75	44	75
		—		43	72
	Excess	+	2.30	77	22
		—		52	49
K1	Limiting	+	0.80	ND	ND
		—		155	50
	Excess	+	1.50	ND	ND
		—		210	80

ND, Not determined.

in a batch culture growing with glucose or sucrose (McCabe *et al.*, 1967). Bacteria only formed large clumps on wires from cultures of strains 3209, K1 or C67-1 growing with sucrose (Fig. 1). The fluid around these wires was less turbid than the bulk culture. In contrast, strains C67-25 and Ingbritt formed clumps in the sucrose medium but did not adhere significantly to the wires (Fig. 1). These experiments demonstrated that sucrose enhances the ability of some but not all strains of *S. mutans* to colonize a smooth hard surface and to be retained on the wire during agitation and washing procedures.

Comparison of the cariogenicity of different strains in rats

Five of the strains which had been grown in continuous culture were used to determine whether there is a correlation between dextransucrase, mutansucrase or fructosyltransferase activities in such cultures and their cariogenicity in pathogen-free rats. Rats were orally infected with the organisms and fed a sucrose-rich diet. Strain D282, known to be cariogenic in rats, was included as a positive control, and uninfected rats provided a negative control. After 4 weeks, the mandibular jaw was scored for smooth surface caries on both the buccal and lingual surfaces of each of the six molars in each rat. The weight gain of each rat over the 4 weeks was measured and values for individuals within each group were comparable. Differences in caries scores between groups were not, therefore, the result of the consumption of more or less food. The total caries score, the total number of carious areas and the total number of carious molars were determined (Table 3).

Due to the nature and the absolute values of the data, a non-parametric statistical test was applied (Mann & Whitney, 1947). There were no significant differences at the 95%

Fig. 1. Adherence of *S. mutans* strains to a sterile nichrome wire. A sterile nichrome wire was inserted into a freshly inoculated 5 ml culture of brain heart infusion broth containing sucrose. After 24 h the wire was transferred to a second tube of sterile broth and photographed after a further 24 h.

Table 3. *Relative cariogenicities of six strains of S. mutans in pathogen-free rats*

Groups of six rats were orally infected and fed a cariogenic diet rich in sucrose. After 28 d the mandibles were scored for smooth surface caries (see Methods). Values are expressed as the mean for the six rats $\pm$ standard error of the mean.

Strain	Cariou molars	Cariou areas	Caries score
Uninfected control	0.5 ± 0.2	1.0 ± 0.4	1.2 ± 0.4
D282	4.0 ± 0.5	11.1 ± 2.2	15.5 ± 3.7
Ingbritt	1.5 ± 0.4	2.5 ± 0.8	2.5 ± 0.8
3209	3.8 ± 0.7	11.7 ± 3.2	19.2 ± 6.5
C67-1	3.2 ± 0.6	7.8 ± 1.9	9.2 ± 2.3
C67-25	1.0 ± 0.5	1.8 ± 0.9	1.8 ± 0.9
K1	4.7 ± 0.4	14.5 ± 2.6	22.0 ± 5.3

confidence level in caries scores between rats which had been infected with strains K1, 3209, D282 or C67-1, all of which were highly cariogenic. Similarly, there were no significant differences between the caries incidence in groups infected with Ingbritt, C67-25 or in the uninfected control group. However, there was a highly significant difference in cariogenicity between strains K1, 3209, D282 or C67-1 and strains Ingbritt or C67-25. Identical conclusions were obtained from the statistical analysis of results from all three methods used to assess the incidence of caries.

DISCUSSION

The many factors known to influence the induction of dental caries include the age and susceptibility of the host, the diet (especially the availability of sucrose) and the oral flora. There is also substantial evidence that some strains of *S. mutans* are especially active in

inducing smooth surface caries in man and rodents (Krasse *et al.*, 1967; Loesche *et al.*, 1975; Michalek *et al.*, 1975). Essential steps in the disease process are the initial adherence of bacteria to a clean or saliva-coated tooth surface, growth of bacteria to form dental plaque and modification of the soft initial plaque to form a sticky, impermeable matrix within which acidic fermentation products accumulate. Mutan is an important structural component of this impermeable matrix, so the mutansucrase activity secreted by a strain might be a major determinant of its cariogenicity and its ability to form clumps on a smooth hard surface.

No strain-specific differences were detected in the growth rates, rates of glucose, fructose or sucrose fermentation or in the identity of the fermentation products of any of the strains used (Wenham *et al.*, 1979; Wenham, 1979). Furthermore, no significant differences have been detected in the initial rate of adherence of these strains to hydroxyapatite beads (Adshead, Davies and Cole, unpublished) or in their cell-associated glucosyltransferase activities (Table 2). Differences in the cariogenicity of these strains were probably due to subsequent, sucrose-dependent steps in the colonization process, such as differences in the rate of insoluble glucan synthesis from sucrose.

Glucosyltransferase from bacteria growing with sucrose is first excreted into the extracellular fluid where it catalyses the synthesis of glucans which then bind the enzymes to the bacterial surface (Janda & Kuramitsu, 1976). Subsequently, little glucosyltransferase activity can be detected, presumably because it is inaccessible to its substrate. It is therefore impossible to determine the rate of mutansucrase synthesis in the rat mouth during the cariogenicity experiments. Similarly, during growth in continuous culture with excess sucrose, glucans formed in the culture fluid promote adhesion of bacteria to each other and to surfaces of the fermenter, so steady-state growth cannot be achieved. To avoid these difficulties, we first established, as far as was possible, that rates of glucosyltransferase synthesis were similar during growth with glucose, fructose, sucrose or a mixture of glucose and fructose (Wenham *et al.*, 1979; Wenham, 1979). Glucose was therefore used for continuous culture experiments in order to determine relative rates of glucosyltransferase synthesis by the different strains. Furthermore, there is now extensive evidence that the extracellular activity of glucosyltransferase correlates closely with the quantity of glucosyltransferase protein(s) synthesized during growth with glucose, but not during growth with sucrose (Wenham *et al.*, 1979; Newman *et al.*, 1980; Asem, Kenney & Cole, unpublished).

In view of the role postulated for insoluble glucan in the development of smooth surface caries, it was at first surprising that the Ingbritt strain, which was originally isolated from a carious lesion (Krasse, 1966), produced little mutansucrase activity (Wenham *et al.*, 1979). However, although this strain could readily be isolated from rat teeth 2 weeks after the initial infection, these rats developed significantly fewer lesions than those infected with four other strains of *S. mutans*. Although Ellwood *et al.* (1976) have documented differences in extracellular polysaccharides produced by cultures of the Ingbritt strain obtained from different laboratories, identical results were obtained from continuous culture experiments with Ingbritt cultures received from J. M. Hardie or D. C. Ellwood (Wenham *et al.*, 1979). Presumably, both cultures are non-cariogenic derivatives of the original isolate which has been sub-cultured repeatedly on laboratory media. Significantly, the two recent clinical isolates were both prolific sources of mutansucrase activity, and strain 3209 was both cariogenic and formed dense deposits on nichrome wires. These results illustrate the importance of checking the cariogenicity of strains used before factors involved in the disease process are investigated.

Strains C67-1 and C67-25 were included in this study because it had already been shown that C67-25, unlike its parent, was neither cariogenic nor able to synthesize insoluble glucans from sucrose (De Stoppelaar *et al.*, 1971, 1973). These results were readily confirmed, but were the only differences detected between the two strains (Table 1). The serotype *d* strain, K1, produced no fructosyltransferase and little dextransucrase activity, but was both cariogenic and able to stick to nichrome wires.

We conclude that previous confusion in the literature about the role, if any, of glucosyltransferase in the disease process has largely resulted from the failure of many authors to distinguish between the dextransucrase, mutansucrase and fructosyltransferase activities secreted by the strains under investigation. Incorrect conclusions have also been drawn from experiments in which enzymes have been assayed under sub-optimal conditions. When these pit-falls are avoided, it is apparent that strains of *S. mutans* with a high mutansucrase activity are significantly more cariogenic than those which secrete little mutansucrase activity. There are no similar correlations between the total glucosyltransferase activity, the dextransucrase activity or the fructosyltransferase activity and cariogenicity.

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