Compared Colonizing and Immunizing Efficiency of Toxinogenic (A⁺ B⁺) *Vibrio cholerae* and an A⁻ B⁺ Mutant (Texas Star-SR) Studied in Adult Rabbits

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Four strains of *Vibrio cholerae* O1 were compared for their ability to colonize and immunize adult rabbit intestine. Three were virulent, toxinogenic (A⁺ B⁺) isolates, and one, an A⁻ B⁺ mutant (Texas Star-SR), was derived by mutagenesis with nitrosoguanidine. When given orally to nonimmune rabbits, virulent strains colonized the small bowel with similar efficiency, whereas Texas Star-SR colonized poorly. Rabbits fed <50 CFU of an A⁺ B⁺ strain developed marked serotype-specific resistance to recolonization. In contrast, Texas Star-SR evoked resistance to reinfection less efficiently, with a minimum immunizing dose of 10⁵ CFU when given once or 10³ CFU when given twice. Oral inoculation with an A⁺ B⁺ strain also evoked vigorous, dose-dependent mucosal antitoxin responses; comparable inocula of Texas Star-SR were much less effective, causing antitoxin responses that were 90 to 95% smaller. Finally, rabbits inoculated once with 10⁴ CFU of an A⁺ B⁺ strain were markedly protected against experimental cholera or fecal shedding of *V. cholerae* when challenged with 10,000 times the 50% effective dose of a virulent strain by the RITARD technique. In contrast, an inoculum of 10⁴ CFU of Texas Star-SR was nonprotective, and 10¹⁰ CFU was only partially protective. These studies reveal the remarkable efficiency with which virulent *V. cholerae* evokes intestinal immunity to recolonization or experimental cholera and show that the A⁻ B⁺ mutant, Texas Star-SR, is substantially less effective.

Recent attempts to develop a safe and effective live oral cholera vaccine have focused on the creation of mutants of *Vibrio cholerae* O1 that cannot produce intact cholera toxin (CT). It has been hoped that such strains would colonize the intestine efficiently and evoke a protective mucosal immune response without causing diarrhea. Creation of mutants that produce the B subunit, but no A subunit, of CT has been of particular interest because the B subunit is antigenic, but not toxic, and thus might evoke antitoxic immunity as part of the response to enteric colonization. Several nontoxinogenic mutants have now been made, including both A⁻ B⁻ and A⁻ B⁺ types (5, 6b; J. B. Kaper, H. Lockman, M. M. Baldini, and M. M. Levine, manuscript in preparation).

Although the protective efficacy of such strains must ultimately be defined in humans, it is probable that detailed comparative studies of their ability to colonize and immunize the intestine can only be done in animals. This report describes a series of such studies which used recently described techniques for achieving either asymptomatic colonization (3) or cholera-like disease (11) in adult rabbits inoculated orally or intratestinally with live *V. cholerae* O1. The *V. cholerae* strains studied included virulent, toxinogenic isolates and an A⁻ B⁺ mutant, designated Texas Star-SR, derived from one of them (5). The results show that intestinal colonization with virulent, fully toxinogenic *V. cholerae* was easily achieved, and that rabbits were immunized very efficiently against attempted recolonization or the induction of cholera-like diarrhea. In contrast, the mutant strain colonized and immunized rabbit intestine inefficiently.

MATERIALS AND METHODS

Rabbits. Rabbits were locally supplied New Zealand white males weighing 2 to 2.5 kg (age, 9 to 11 weeks).

***V. cholerae* O1 strains.** Four strains were used: *V. cholerae* Ogawa 395 and Inaba B36237, which are of the classical biotype, fully toxinogenic and virulent (3, 8); *V. cholerae* Ogawa 3083, which is an El Tor biotype, fully toxinogenic and virulent for infant rabbits (5); and Texas Star-SR, which is a streptomycin-resistant, nitrosoguanidine-induced mutant of Ogawa 3083 that produces the B subunit, but not the A subunit, of CT (5). *V. cholerae* strains were stored and inocula prepared as previously described (3), except that the seed culture of Texas Star-SR was prepared in a medium containing, per liter, peptone (20 g; Difco Laboratories), NaCl (10 g), and yeast extract (5 g) (pH 7.5).

Enteric colonization with *V. cholerae*. The technique used is described in detail elsewhere (3). In brief, rabbits were fasted overnight and sedated. Gastric acid was neutralized with a combination of intravenous cimetidine and intragastric NaHCO₃, after which the bacterial inoculum, in 15 ml of Casamino Acids-yeast extract medium, was given by gastric intubation. After 30 min, 2 ml of tincture of opium USP was given intraperitoneally.

Quantitation of intestinal colonization. The technique for quantitation of intestinal colonization has also been described elsewhere (3). In the present study, all counts of viable *V. cholerae* were determined on washed, homogenized segments of small intestine obtained 18 h after oral inoculation (or reinoculation). Because Texas Star-SR grew relatively slowly in vitro, cultures of this organism were incubated for 24 h, rather than 18 h, before being read.

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Challenge by the RITARD technique. The RITARD challenge technique was used as described previously (3, 11) to assess protection against cholera-like diarrhea in rabbits that were previously colonized with viable *V. cholerae*. Age-matched controls, not previously inoculated, were included in each challenge. Rabbits were observed for 5 days after challenge, and the challenge outcome was graded as none, mild, severe, or lethal diarrhea as described elsewhere (3). All challenges were with 10^{11} *V. cholerae* Ogawa 395, which is 10,000 times the dose required to cause severe or lethal diarrhea in 50% of nonimmune control rabbits (3).

Assay of mucosal antitoxin-containing cells. Antitoxin-containing plasma cells (ACC) were enumerated in the lamina propria of intestinal biopsies by a described fluorescent antibody technique (9). This method detects ACC evoked either by intact CT or its B subunit (7, 9). ACC counts in biopsies are expressed as geometric means ($\times$ standard error) per cubic millimeter in the crypt region of intestinal lamina propria.

Statistics. Statistical analysis was carried out by Student's *t* test applied to geometric mean counts of bacteria or ACC, or to diarrhea attack rates in rabbits challenged by the RITARD technique.

RESULTS

Relative colonizing efficacy of strains Ogawa 395, Ogawa 3083, and Texas Star-SR. The relative colonizing efficacy of these strains was determined in rabbits not previously colonized with *V. cholerae*. Groups of three to eight rabbits were inoculated with 10^4 , 10^7 , or 10^{10} living bacteria of each strain; none of the rabbits developed diarrhea. After 18 h, viable *V. cholerae* were enumerated in washed, homogenized segments of proximal jejunum and distal ileum. *V. cholerae* Ogawa 395 and Ogawa 3083 colonized with similar efficiency (Fig. 1). The magnitude of colonization was dose dependent and consistently greater in the ileum than jejunum; these results are in accord with previous studies of rabbits colonized with strain Ogawa 395 (3). In contrast, Texas Star-SR colonized very inefficiently. For each size of inoculum, jejunal and ileal colonization with Texas Star-SR was markedly poorer than for either Ogawa 395 or the parent strain Ogawa 3083. With inocula of 10^{10} CFU, for example, jejunal and ileal counts of Texas Star-SR were about 5 log₁₀ units lower per gram of tissue than those of Ogawa 3083.

Resistance to recolonization after primary colonization with different strains of *V. cholerae*. (i) **Effect of primary inoculum size and serotype.** A previous study showed that *V. cholerae* Ogawa 395 and Inaba B36237 colonized nonimmune rabbits with similar efficiency in a dose-dependent manner and that a single large inoculum (i.e., 10^{10} CFU) of either strain evoked nearly complete protection against attempted recolonization with 10^{10} *V. cholerae* Ogawa 395 (3). In the present study, we sought to more precisely define the immunizing efficiency of a single inoculation against recolonization with the homologous serotype and to compare this with the immunizing efficiency of the heterologous serotype. Accordingly, rabbits were given a primary inoculation with varying numbers of either Ogawa 395 or Inaba B36237, after which none developed diarrhea; recolonization with 10^{10} Ogawa 395 was attempted 19 to 23 days later.

Primary inoculation with 13 viable Ogawa 395 organisms did not protect against subsequent recolonization, whereas primary inoculation with only 36 viable bacteria evoked marked, nearly complete, protection (Fig. 2). In contrast, primary inoculation with 370 CFU of Inaba B36237 was only modestly protective; marked resistance to recolonization

occurred, however, when the primary inoculum of this strain was 10^4 CFU or greater.

(ii) **Compared immunizing efficiency of Ogawa 3083 and Texas Star-SR.** A similar approach was used to compare the immunizing efficiency of the parent-mutant pair, Ogawa 3083 and Texas Star-SR. In this instance, rabbits were inoculated once with varying numbers of either strain; 19 to 23 days later, recolonization was attempted with 10^{10} *V. cholerae* Ogawa 395. Primary colonization with Ogawa 3083 immunized at least as efficiently against recolonization as did Ogawa 395 (Fig. 3; cf. Fig. 2 and 3). Thus, marked protection against recolonization occurred after a single oral inoculation with only 12 viable bacteria. In contrast, 10^3 CFU of Texas Star-SR were not significantly protective. Marked protection did occur, however, with inocula of 10^5 CFU or larger.

Additional studies were done to determine whether small inocula of Texas Star-SR were more immunogenic when given repeatedly. Rabbits were given 10^3 viable Texas Star-SR organisms twice, with a 19-day interval between inoculations, and recolonization with 10^{10} CFU of Ogawa 395 was attempted on day 38. In this instance, significant resistance to recolonization was observed. Mean jejunal and ileal counts of viable *V. cholerae* after 18 h were reduced by 2.5 and 3.8 log₁₀ CFU per gram, respectively, of washed, homogenized intestine when compared with rabbits immunized with only a single oral inoculum of 10^3 Texas Star-SR bacteria ($P < 0.001$ for each comparison).

Mucosal ACC responses after oral inoculation with toxigenic (A⁺ B⁺) or mutant (A⁻ B⁺) *V. cholerae*. ACC responses

FIG. 1. Intestinal colonization by *V. cholerae* Ogawa 395, Ogawa 3083, and Texas Star-SR. Rabbits were fed the indicated inoculum of each strain. Bacterial counts on washed, homogenized segments of intestine were determined 18 h later. Each mean value represents data from three to eight rabbits. SE, Standard error.

FIG. 2. Resistance to colonization with *V. cholerae* Ogawa 395 in previously colonized rabbits: effect of primary inoculum size and serotype. The first oral inoculation was with the indicated number of *V. cholerae* Ogawa 35 or Inaba B36237. After 19 to 23 days, rabbits were reinoculated with 10^{10} living *V. cholerae* Ogawa 395. Counts of viable *V. cholerae* on washed, homogenized segments of jejunum and ileum were determined 18 h later. Each mean value represents data from two to six rabbits.

in jejunal and ileal lamina propria were determined at varying intervals after oral inoculation (or reinoculation) with Ogawa 395 ($A^+ B^+$) or Texas Star-SR ($A^- B^+$). Results in Table 1 indicate that jejunal ACC responses arose slowly after a single oral inoculation with 10^{10} CFU of Ogawa 395; repeating the inoculation on day 19 had a rapid, but transient, booster effect ($P < 0.01$, comparing responses on day 24 in rabbits immunized one and two times). Immunization with a smaller inoculum (10^4 CFU) evoked a significantly smaller primary ACC response on day 24 ($P < 0.02$, compared with rabbits given one dose of 10^{10} CFU), and repeat inoculation on day 19 had no booster effect. In contrast, inoculation once with 10^{10} CFU of Texas Star-SR caused a mucosal ACC response on day 24 which was 90% smaller than that evoked by Ogawa 395; moreover, the response to reinoculation with Texas Star-SR revealed no significant booster effect ($P > 0.40$, comparing rabbits immunized once

with those given a second inoculum on day 19), and was 96% less than the response in rabbits inoculated twice with Ogawa 395. Ileal ACC responses in the preceding studies (data not shown) paralleled those in the jejunum, but they were 50 to 65% smaller.

Protection against RITARD challenge by prior colonization with virulent or mutant *V. cholerae*. A previous study showed that a single oral inoculation with 10^{10} virulent *V. cholerae* evoked nearly complete protection against subsequent challenge with up to 10,000 times the 50% effective dose of the homologous or heterologous serotype of *V. cholerae* by the RITARD technique (3). Additional studies were performed to determine whether similar protection could be stimulated by much smaller inocula and to compare the protective efficiency of the $A^- B^+$ mutant, Texas Star-SR, with that of fully virulent strains. Rabbits were inoculated once with 10^4 CFU of a virulent strain (Ogawa 395, Inaba

FIG. 3. Resistance to recolonization with *V. cholerae* Ogawa 395 after primary colonization with Ogawa 3083 or Texas Star-SR. The first oral inoculation was with the indicated number of *V. cholerae* Ogawa 3083 or Texas Star-SR. After 19 to 23 days, rabbits were reinoculated with 10^{10} living Ogawa 395. Counts of viable *V. cholerae* on washed, homogenized segments of jejunum and ileum were determined 18 h later. Each mean value represents data from four to five rabbits.

TABLE 1. Compared jejunal ACC responses evoked by oral inoculation with *V. cholerae* Ogawa 395 or Texas Star-SR

Immunizing strain and inoculum, log ₁₀ CFU	Day(s) of inoculum administration	Jejunal ACC response (mean $\pm$ SE) on day ^a			
		5	19	24	40
Ogawa 395, 10 ¹⁰	1	120 ^a (1.1)	2,530 (1.5)	3,930 (1.2)	
Ogawa 395, 10 ¹⁰	1, 19			12,400 (1.3)	1,910 (1.9)
Ogawa 395, 10 ⁴	1			1,070 (1.7)	
Ogawa 395, 10 ⁴	1, 19			960 (1.8)	
Texas Star-SR, 10 ¹⁰	1			390 (1.7)	
Texas Star-SR, 10 ¹⁰	1, 19			690 (1.9)	

^a Geometric mean ACC response per cubic millimeter of jejunal lamina propria on indicated day after first oral inoculation; n = five to nine rabbits for each mean value.

B36237, or Ogawa 3083) or 10⁴ to 10¹⁰ CFU of Texas Star-SR. Between 19 and 23 days later, these rabbits and unimmunized controls were challenged with 10¹¹ CFU of Ogawa 395 by the RITARD technique.

Results in Tables 2 and 3 show that inoculation with 10⁴ Ogawa 395 or Inaba B36237 caused 100% protection against severe or lethal diarrhea after RITARD challenge and 71 to 75% protection against detectable fecal shedding of *V. cholerae* after the challenge. Inoculation with Ogawa 3083 was slightly less protective, giving 75% protection against severe or lethal diarrhea and 37% protection against fecal shedding of *V. cholerae* after RITARD challenge. In contrast, 10⁴ CFU of Texas Star-SR evoked no significant protection against disease or passage of *V. cholerae* in feces. Only with an initial inoculum of 10¹⁰ CFU was 50% protection against disease achieved, and that reflected mostly an elimination of lethal disease, without a reduction in disease classified as severe. Even this large inoculum provided only modest (25%) protection against fecal shedding of the challenge organism.

DISCUSSION

Enteric colonization of humans with virulent *V. cholerae* causes marked protection against reinfection (2, 6). An obvious goal in developing a live oral cholera vaccine is to obtain mutant strains that are safe but that reproduce this immunizing effect as nearly as possible. This study shows that the immunizing efficiency of mutant and virulent strains can be quantitatively compared in a convenient animal model. By using two experimental methods in adult rabbits, it was possible to compare the ability of test strains to colonize the small bowel, immunize against recolonization, evoke a specific mucosal immune response, and immunize

against cholera-like disease and fecal shedding of the challenge inoculum. The results describe basic features of immunization by virulent strains and show that the mutant studied, Texas Star-SR, is a relatively inefficient immunogen.

A previous study has shown that classical strains Ogawa 395 and Inaba B36237 colonize rabbit intestine in a dose-dependent fashion and with similar efficiency (3). In the present study, Ogawa 3083, the El Tor parent of Texas Star-SR, colonized at least as well. In contrast, Texas Star-SR colonized poorly; for example, inocula of 10¹⁰ CFU yielded jejunal and ileal counts 4 to 5 log₁₀ CFU lower than were achieved with the parental strain. The poor colonization by Texas Star-SR is unexplained but may be related to its slow rate of growth in vitro (10), its inability to produce active CT, which may aid colonization, or other unrecognized defects produced during mutagenesis with nitrosoguanidine. Studies in infant mice have also shown that Texas Star-SR colonizes the proximal intestine less well than does Ogawa 3083, an effect attributed to a diminished capacity for mucosal adherence (10). Evidence that large inocula of Texas Star-SR (i.e., >10⁸ CFU) cause modest jejunal colonization in volunteers suggests that this organism may colonize humans somewhat more efficiently than rabbits (6a). However, studies strictly comparing enteric colonization by virulent strains and Texas Star-SR in humans have not been performed.

Although rabbits given a single large inoculum of virulent *V. cholerae* (i.e., 10¹⁰ CFU) develop marked protection against recolonization or RITARD challenge (3), the minimum inoculum required for this effect has not previously been determined. The present study shows that this method of immunization is extremely efficient. Thus, an inoculum of fewer than 50 bacteria caused nearly complete protection against recolonization with 10¹⁰ *V. cholerae* of the same serotype, and an inoculum of 10⁴ organisms was fully

TABLE 2. Protection against RITARD challenge afforded by prior colonization with virulent and mutant *V. cholerae*

Immunizing strain and inoculum, log ₁₀ CFU	Outcome of challenge						
	Immunized ^a			Control ^a			% Protection ^b
	None/mild	Severe	Lethal	None/mild	Severe	Lethal	
Ogawa 395, 10 ⁴	7	0	0	0	0	3	100
Inaba B36237, 10 ⁴	8	0	0	1	1	4	100
Ogawa 3083, 10 ⁴	6	1	1	0	0	4	75
Texas Star-SR, 10 ⁴	1	1	5	1	1	4	0
Texas Star-SR, 10 ⁷	5	2	2	2	2	3	38
Texas Star-SR, 10 ¹⁰	5	3	0	1	1	2	50

^a Number of rabbits with disease of indicated severity. RITARD challenge was with 10¹¹ *V. cholerae* Ogawa 395 19 to 23 days after oral immunization with the indicated strain.

^b Protection against the combined incidence of severe or lethal diarrhea compared with concurrently challenged controls.

^c Compared with combined incidence of severe and lethal diarrhea in immunized rabbits and concurrently challenged (or all challenged) controls. NS, Not significant.

TABLE 3. Effect of previous colonization with virulent or mutant strains on fecal shedding of *V. cholerae* after RITARD challenge

Immunizing strain and inoculum, log ₁₀ CFU	No. positive/no. challenged ^a		% Protection ^b	<i>P</i> ^c
	Immunized	Unimmunized		
Ogawa 395, 10 ⁴	2/7	3/3	71	<0.001
Inaba B36237, 10 ⁴	2/8	6/6	75	<0.001
Ogawa 3083, 10 ⁴	5/8	4/4	37	<0.05
Texas Star-SR, 10 ⁴	6/7	5/5	14	NS
Texas Star-SR, 10 ⁷	5/9	6/7	35	<0.05
Texas Star-SR, 10 ¹⁰	6/8	4/4	25	NS

^a Challenge was with 10¹¹ CFU of *V. cholerae* Ogawa 395 19 to 23 days after a single oral inoculation as indicated. Results express the number of rabbits with positive fecal culture 1 day after challenge per the number of rabbits challenged.

^b Protection against detectable fecal shedding of *V. cholerae* in immunized rabbits compared with concurrently challenged controls.

^c Compared with cumulative outcome in all challenged controls (28 positive per 29 challenged). NS, Not significant.

protective against RITARD challenge with 10,000 times the 50% effective dose of a virulent strain. Such protection apparently reflects efficient stimulation of mucosal lymphoid tissue by a small population of colonizing bacteria, since small inocula of *V. cholerae* multiply only about 100-fold in rabbit small intestine (3). This contrasts with the reported inefficiency of enteric immunization by repeatedly feeding nonliving bacterial antigens to rabbits (4). Possibly, living *V. cholerae* are especially immunogenic because antigen sampling mechanisms in rabbit intestine efficiently transport them from the lumen into underlying lymphoid tissue, in which the local immune response arises (R. L. Owen, N. F. Pierce, R. T. Apple, and W. C. Cray, Jr., manuscript in preparation).

By using very small inocula to immunize against recolonization, it was shown that the evoked protection was partly serotype specific. Evidence for this was the 300-fold larger inoculum of Inaba B36237 than of Ogawa 395 required to immunize against colonization with the latter strain, despite the equal ability of these strains to colonize nonimmune intestine (3). Serotype-specific protection against experimental cholera has also been described in orally immunized dogs (8). In contrast, a similarly designed experiment revealed no evidence of biotype-specific immunity. Thus, very small inocula of Ogawa 3083 (El Tor biotype) prevented recolonization with Ogawa 395 (classical biotype) as efficiently as did primary colonization with Ogawa 395 (cf. Fig. 2 and 3).

Although they caused marked protection against recolonization or RITARD challenge, small inocula (i.e., <10⁴ CFU) of virulent *V. cholerae* did not optimally stimulate mucosal immune responses. By using the antitoxin response as an example, it was shown that repeated inoculation and the use of larger inocula each contributed to enhanced immune responses. Thus, the peak antitoxin response to 10⁴ CFU of Ogawa 395 given once was only 8% of that evoked by two doses of 10¹⁰ CFU. These results suggest that optimal immunization with viable *V. cholerae* may require repeated administration of relatively large inocula. This view is supported by evidence that prolonged protection against recolonization in rabbits is best evoked by repeated large inocula of *V. cholerae* (3).

In contrast with virulent *V. cholerae*, including its parental strain, Texas Star-SR inefficiently immunized rabbit intes-

tine against recolonization or RITARD challenge. The explanation for this is speculative. Possibly, this reflects the diminished ability of Texas Star-SR to colonize and grow in rabbit intestine and thus deliver a sufficient antigenic stimulus to mucosal lymphoid tissue. Alternatively, Texas Star-SR may fail to produce in vivo normal amounts of certain antigens required for optimal immunization. Both possibilities are in accord with evidence that larger, or repeated, inocula were more protective. Limited protection against RITARD challenge, even after an inoculum of 10¹⁰ Texas Star-SR, may also have been due, in part, to the relatively poor antitoxin response which was evoked. Failure to achieve an adequate bacterial mass as a source of immunizing antigens was not a factor, however, because this large inoculum far exceeded the gut population of *V. cholerae* achieved when rabbits were inoculated (and efficiently immunized) with 10⁴ CFU of the virulent strains.

These results are compatible with reports that oral inoculation with Texas Star-SR significantly protected volunteers (6a) and rabbits (1) against subsequent challenge with virulent *V. cholerae*. In those studies, the demonstration of protection was probably favored by immunizing with relatively large inocula (10⁸ to 10¹⁰ CFU given one or more times) and by less severe challenge techniques. Nevertheless, even vigorous immunization was not fully protective in volunteers (6a). Moreover, both in volunteers and in the present study, vigorous immunization with Texas Star-SR protected only modestly against fecal shedding of *V. cholerae* at the time of challenge, whereas volunteers and rabbits previously colonized with smaller inocula of virulent strains were highly protected (2, 3).

It has been hoped that an A⁻ B⁺ mutant of *V. cholerae* used as an oral vaccine would evoke significant antitoxic immunity, protective against disease caused by *V. cholerae* and, possibly, heat-labile enterotoxin-producing *Escherichia coli* (1, 5). However, no direct evidence has been presented that such strains will efficiently stimulate mucosal antitoxin responses. In the present study, the A⁻ B⁺ mutant Texas Star-SR evoked antitoxin responses inefficiently, even when large inocula were given repeatedly. Although modest antitoxin responses of the sort evoked by Texas Star-SR might contribute to protection, they are not likely to be as effective as the 10- to 20-fold-larger responses caused by A⁺ B⁺ strains. The poor antitoxin responses evoked by large inocula of Texas Star-SR are unexplained but could reflect limited elaboration of the B subunit under in vivo growth conditions, the inherently weaker immunogenicity of B subunit than CT (7, 8), or both.

This study does not necessarily indicate that Texas Star-SR is insufficiently protective for practical use as a vaccine in humans. Studies in volunteers have already revealed partial efficacy (6a), and more vigorous immunizing regimens are possible. This study does show, however, that Texas Star-SR is considerably less immunogenic than fully virulent strains, probably due to uncharacterized defects in the mutant. Whether avirulent mutants with defined gene deletions or substitutions can be developed that colonize and immunize with an efficiency more like fully virulent *V. cholerae* remains to be determined.

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