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Oral Ingestion of Egg Yolk Immunoglobulin from Hens Immunized with an Enterotoxigenic *Escherichia coli* Strain Prevents Diarrhea in Rabbits Challenged with the Same Strain

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White Leghorn hens were immunized with enterotoxigenic *Escherichia coli* B16-4 with heat-labile enterotoxin and colonization factor antigen I in Freund's adjuvant. Specific antibodies were detected by an enzyme-linked immunosorbent assay in the serum after 8 days and in eggs after 10 days, with levels reaching peaks at 15 and 20 days after the first immunization, respectively. The protective effects of the egg yolk antibodies were tested in the rabbit reversible ileal tie model of diarrhea. Five control rabbits developed severe diarrhea within 72 h after inoculation with enterotoxigenic *E. coli* B16-4. Oral ingestion of egg yolks from immunized hens for 4 days prior to inoculation protected five rabbits from diarrhea after challenge with the same strain of *E. coli*. The rabbits showed no adverse effects from the ingestion of the egg yolks. Four rabbits fed control eggs were also afforded some protection in that three rabbits developed mild diarrhea and one rabbit remained entirely well. In vitro experiments showed that immunoglobulin from egg yolks interfered with the binding of *E. coli* to purified small bowel mucins; immunoglobulin from immunized hens reduced binding more than immunoglobulin from nonimmunized hens. These findings indicate that eggs from hens immunized with appropriate antigens have potential as a useful source of passive immunity.

The mammalian gastrointestinal (GI) tract processes large quantities of foreign material daily (19). It is important that the gut immunologically tolerates ingested dietary antigens; however, it is equally important that the gut retains the ability to recognize and respond to antigens presented by viral and bacterial pathogens that may be ingested with dietary substances. Diarrheal disease caused by these ingested pathogens remains the leading cause of morbidity and mortality in children in the developing world. The role of the GI tract-associated lymphoid tissue in the tolerance of dietary antigens and in the prevention of diarrheal disease is therefore important and complex (1). A primary function of the GI tract-associated lymphoid tissue is the production of specific immunoglobulin A (IgA), which is transported into the lumen of the gut (19). Although the precise protective mechanisms of secreted IgA are not clearly understood, there is a general consensus that it assists in protection against bacterial, viral, and parasitic infections by inhibiting binding, preventing colonization, and neutralizing toxins (26).

In certain circumstances, the secretory IgA system is absent or inadequate; increased colonization of the GI tract by bacterial or viral pathogens may be one of the sequelae of this condition. Even in the presence of normal levels of secretory IgA, overwhelming exposure to an intestinal pathogen can result in the colonization of the gut and the development of diarrheal disease. A particularly vulnerable period is in the weeks and months after birth, when the immune system is still immature (15). Nature has provided a perfect protective supplement for this period in the form of breast milk, itself a rich source of IgA (16). Numerous studies have documented the role of colostrum and breast milk in protecting the newborn against GI tract infections (6, 9, 25). It has also been shown that oral immunization of the lactating mother against potential infectious agents boosts the protective capability of milk by stimulating specific IgA production (6, 20). More recent studies have examined the possibility of using nonmaternal antibodies to passively immunize the GI tract. Oral ingestion of immunoglobulin from colostrum from immunized cows has been shown to protect adult humans against gastroenteritis (18), while infants supplemented with human IgA and IgG containing high titers of antibodies against a battery of viral and bacterial pathogens were protected from necrotizing enterocolitis (5).

The yolks of hen eggs are a rich source of immunoglobulin (10) which, while species specific, approximates human immunoglobulin. Egg yolks can easily be incorporated into animal or human diets. The specific humoral response seen in the serum of hens after immunization is mirrored in the egg yolks some days later and remains elevated for several weeks (21). In this study, we tested the ability of egg yolks from immunized hens to passively immunize the GI tract in an animal model of gastroenteritis.

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MATERIALS AND METHODS

Bacteria. Enterotoxigenic *Escherichia coli* B16-4, originally isolated from a child with diarrhea in Brazil, was used. B16-4 is known to contain only the heat-labile enterotoxin, as determined by a rabbit ileal loop bioassay (14), by a CHO cell elongation assay (24), and with an oligonucleotide probe for heat-labile enterotoxin (11). Strain B16-4 also carries human colonization factor antigen (CFA) I, as determined by

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an enzyme-linked immunosorbent assay (ELISA) and hemagglutination with human type A erythrocytes. Bacteria were maintained on nutrient agar at 4°C and subcultured to CFA agar for immunization and the ELISA. For the intestinal inoculations, bacteria were grown in Casamino Acids-yeast extract broth at 37°C with shaking for 18 h.

One strain each of *Shigella sonnei*, *Shigella dysenteriae*, *Salmonella* sp., and *Campylobacter jejuni* isolated from patients with diarrhea were also used in the ELISA. *E. coli* K-12 from the Centers for Disease Control was used as a nontoxigenic, noncolonizing negative control. All strains were grown on nutrient agar for these studies.

Immunization. Adult egg-laying White Leghorn hens from The Biological Laboratories flock were immunized with enterotoxigenic *E. coli* B16-4. The organism was grown on CFA agar plates for 18 h at 37°C before immunization; 10^9 CFU of the organism was scraped from the plate, washed, suspended in 0.5 ml of sterile phosphate-buffered saline (PBS) (pH 7.4), boiled for 5 min, and emulsified with an equal volume of Freund's adjuvant. This 1-ml preparation was divided and injected into both upper thighs and three separate sites on a breast of each hen (for a total of five equal injections) on the initial day of immunization. Subsequently, 1-ml booster injections of *E. coli* prepared in the same fashion but emulsified in Freund's incomplete adjuvant were given in the same manner, three times at 10-day intervals. Blood samples were taken from the hens at 7-day intervals, and the eggs for the animal immunizations were collected for 12 to 20 weeks after the completion of hen immunization. The hens remained clinically well throughout this period.

ELISA for bacterium-specific egg yolk antibodies. Plastic plates (96 well; Nunc) were coated overnight with whole-cell cultures of enterotoxigenic *E. coli* B16-4 or other organisms as described below for individual experiments. The organisms were scraped from the surfaces of the CFA agar plates and suspended in carbonate-bicarbonate buffer (pH 9.6) at a concentration that yielded a spectrophotometric reading of 1.00 at 560 nm. Aliquots of 50 μl were used throughout. After the plates were washed three times with PBS-Tween, the wells were blocked with 1% bovine serum albumin in PBS-Tween for 30 min at room temperature. The plates were washed again, and the samples were applied in triplicate (serum or egg yolks were diluted 1:1,000 in PBS-Tween) and incubated at room temperature for 1 h. After the plates were washed again, alkaline phosphatase-labeled anti-chicken IgY (Zymed) was applied. The incubation and washing steps were repeated, and the amount of bound conjugate was visualized by use of an enzyme substrate, paranitrophenylphosphate, and an ELISA reader to measure the absorbance. When individual hens were being assessed for antibody production, three or more eggs from each hen were pooled for each ELISA point; for the remainder of the experiments, three or more eggs from three immunized hens were pooled, as were three or more eggs from three control (nonimmunized) hens.

Immunoglobulin preparation. Individual egg yolks from immunized or control hens were separated from egg whites, measured, and diluted 1:10 (vol/vol) in distilled water. The yolk solution was adjusted to pH 7.4 and frozen at −60°C for 60 min. This preparation was thawed and centrifuged at 6,000 rpm (Sorvall) for 30 min. The supernatant and lipids were discarded, and the immunoglobulins were precipitated with 4.1 M ammonium sulfate at a 2:3 ammonium sulfate/yolk ratio. This solution was stirred for 10 min at room temperature, allowed to stand for 30 min, and recentrifuged at 6,000 rpm for 30 min. The supernatant from this centrifugation was also discarded, and the pellet was dialyzed in distilled water for 24 h, after which the protein concentration was determined (10, 13).

Rabbit model of gastroenteritis. The reversible ileal tie model was used as previously described (14, 23). In brief, 1.5-kg New Zealand White rabbits (Milbrook Farms, Amherst, Mass.) were anesthetized with intramuscular ketamine and xylazine. A midline abdominal incision was made, and the mid-distal ileum was mobilized through the peritoneum. A length of sterile umbilical tape was placed around the ileum approximately 20 cm from the ileal-cecal junction, gently tightened, and held in place with surgical clamps. Twenty milliliters of whole bacteria in a broth culture at a concentration of 8.0×10^8 CFU/ml was introduced into the proximal small bowel with a small-gauge needle. The peritoneal and abdominal incisions were closed, with the surgical clamps exterior, holding the umbilical tape firmly around the ileum. The animals were monitored under anesthesia for 5 h, at which time the surgical clamps were released and the umbilical tape was gently removed from the abdominal incision. The animals were allowed to eat and drink ad libitum after the procedure and were monitored clinically twice daily for 7 days for evidence of diarrhea (brown bottom, loose stools in the cage, or clinically debilitating disease) or surgical wound infection.

Feeding protocol. Eggs were collected from control hens and immunized hens more than 10 days after the final immunization. The yolks were separated from the whites, mixed 1:1 with 0.05 M carbonate-bicarbonate buffer (pH 9.6), and stored at 4°C. Ten test rabbits were each fed 15 ml (a one-yolk volume) of pooled yolk mixture through an orogastric tube on the morning of each day for 4 days. Six rabbits received pooled *E. coli* antibody-containing egg yolks, while four received pooled yolks from nonimmunized hen eggs. Throughout the experimental period, the animals were allowed water and chow ad libitum. On the fifth day, the reversible ileal tie procedure was carried out as described above.

In vitro binding assays. Small bowel mucin glycoprotein was purified from New Zealand White rabbits as previously described (3) and used in an in vitro mucin binding assay also as previously described (22). In brief, 10 μg of iodinated mucin glycoprotein was incubated with 1 ml of washed *E. coli* B16-4 resuspended in PBS at a concentration of 1.0×10^9 CFU/ml for 3 h with or without the addition of 10 μg of ammonium sulfate-precipitated immunoglobulin (protein weight) from immune and control egg yolks (10, 13). Bacteria with any adherent labelled mucin were pelleted, washed, and gamma counted. The assay was done in triplicate and repeated three times; results are given as the mean ± the standard deviation of the amount of mucin glycoprotein adherent to 1.0×10^9 CFU of *E. coli* B16-4.

Statistics. Statistical analyses were done by the unpaired Student's *t* test or the Wilcoxon rank sum test.

RESULTS

***E. coli*-specific antibody in hen serum and eggs.** An increase in *E. coli* B16-4-specific immunoglobulin was detected in the serum of immunized hens 10 days after the initial immunization by the ELISA (data not shown). This humoral response reached a peak at about day 20. The antibody response was mirrored in the eggs of immunized hens (Fig. 1). The antibody response had increased by the end of the first week after immunization and was significantly greater than that in the control at 3 weeks after immunization ($P <$

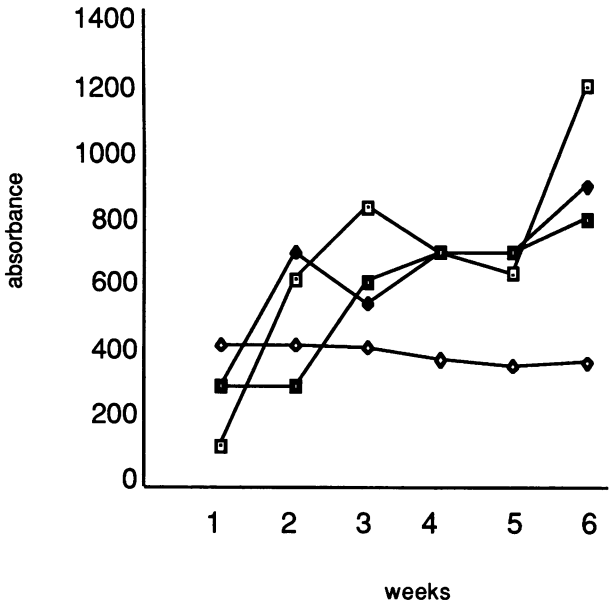

FIG. 1. Results of the quantitative ELISA demonstrating the level of antibody response in three pooled egg yolks from each of three hens immunized with enterotoxigenic *E. coli* B16-4 (⊡, ◆, and ◘) and in three pooled egg yolks from each of three hens that received no immunization (◇) to *E. coli* B16-4. The antibody response was detectable by 2 to 3 weeks after immunization and persisted through 12 to 20 weeks, although data are shown only for the first 6 weeks.

0.04). The antibody response persisted in the egg yolks throughout the period that the eggs were monitored (12 to 20 weeks); data are shown only for weeks 1 to 6, as the antibody level remained constant thereafter.

Antibodies to other bacterial species and strains. The antibody response to additional enteric pathogens in the immune eggs is shown in Fig. 2. Levels of antibodies to *Shigella* spp., a *Salmonella* sp., and *E. coli* K-12 were also increased in the yolks of the eggs from hens that had been immunized with *E. coli* B16-4, as determined by the ELISA. At 21 days, these antibody levels were obviously increased but were significantly lower than the levels of antibodies to *E. coli* B16-4 ($P < 0.01$). Levels of antibodies to *C. jejuni* in the immune eggs were not above the baseline and were significantly lower than the levels of antibodies to *Shigella* spp., the *Salmonella* sp., and *E. coli* K-12 and B16-4 ($P < 0.01$). Antibodies to *E. coli* K-12, the *Salmonella* sp., and *Shigella* spp. were also detectable in nonimmune eggs, to a lesser degree but above the baseline A_{350} readings. Antibodies to *C. jejuni* were not detected in these eggs by this ELISA.

Protective effect of egg yolks in an animal model of gastroenteritis. Rabbits fed immune and control eggs all tolerated the egg yolk mixture without any adverse effects. No animal developed diarrhea or any other sign of clinical illness after being fed either control or immune eggs.

A total of 16 animals were challenged with *E. coli* B16-4; 6 of these had received no yolk mixture and served as positive controls. Six animals had received the pooled yolk mixture from immunized hens, and four had received the pooled yolk mixture from nonimmunized hens. Two animals, one from the control group and one that had received the pooled yolk mixture from an immunized hen, died during anesthesia and were excluded from the analysis; a total of 14 rabbits remained for analysis. The remaining five positive control animals all developed frank, watery diarrhea (brown bottom and loose, watery stools in the cage) and had ruffled fur and clinical evidence of illness between 24 and 36 h after surgical inoculation. They became ill enough, with severe and unremitting diarrhea, that all were sacrificed by 72 h postprocedure. The remaining five animals that had received the pooled yolk mixture from immunized hens did well postoperatively and were well 5 to 7 days postinoculation, when they were sacrificed. The fur of these animals remained unstained by feces, and the animals acted and appeared well and had only formed stools in their cages. Three of the four animals that had received the pooled yolk mixture from nonimmunized hens developed mild diarrhea at 24 h that lasted 12 to 18 h and resolved spontaneously. These animals had some brown staining of fur with feces and soft, less formed stools in their cages, although all appeared clinically well. The fourth animal given the pooled yolk mixture from nonimmunized hens remained completely well, without any sign of diarrhea. The animals were all sacrificed at various times postinoculation, depending on symptoms, so quantitative culturing of the small bowel at the time of sacrifice was not done.

An additional four rabbits were fed 200 mg (protein weight; a one-yolk equivalent) of immunoglobulin precipitate daily for 4 days in carbonate-bicarbonate buffer. Two animals received immunoglobulin from pooled immune egg yolks, and two received immunoglobulin from pooled nonimmune egg yolks. The rabbits tolerated the immunoglobulin without any difficulty. Rabbits given the immune egg yolk immunoglobulin were protected from diarrheal illness when challenged with enterotoxigenic *E. coli* B16-4 and remained completely well, with unstained fur and formed stools. One of the two rabbits given immunoglobulin from nonimmune egg yolk developed mild diarrhea, with brown staining of fur and loose stools in the cage for 12 h beginning 24 h after challenge with enterotoxigenic *E. coli* B16-4. The second rabbit given immunoglobulin from nonimmune egg yolk remained completely well after challenge with the enterotoxigenic strain.

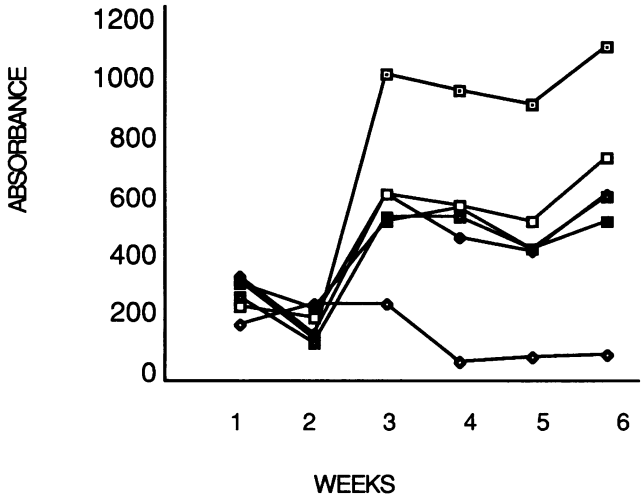

FIG. 2. Results of the quantitative ELISA demonstrating the level of antibody response in egg yolks pooled from three hens immunized with enterotoxigenic *E. coli* B16-4 to *E. coli* B16-4 (⊡), *E. coli* K-12 (nonpathogenic strain) (□), a *Salmonella* sp. (◘), *S. dysenteriae* (■), and *C. jejuni* (◇). See the text for details.

In vitro mucin glycoprotein binding studies. The yolk mixture from immunized hens was able to significantly decrease the binding of *E. coli* B16-4 to small bowel mucins: with B16-4 alone, 0.130 ± 0.001 (mean ± standard deviation) μg of mucin bound to 1.0×10^9 CFU of *E. coli*, and immune egg yolk immunoglobulin reduced this value to 0.115 ± 0.001 ($P < 0.02$). The yolk mixture from nonimmunized hens did not change bacterial binding in the binding assay from that which was seen with bacteria and mucins without the addition of any yolk mixture: 0.128 ± 0.002 μg of mucin bound to 1.0×10^9 CFU of *E. coli* when nonimmune egg yolk immunoglobulin was used.

DISCUSSION

Hen eggs contain as much as 200 mg of immunoglobulin, which is found almost exclusively in the yolk (13). The immunoglobulin, which is not only readily produced but also easily purified (12), is described as being like mammalian IgG, although recently, evidence has emerged to suggest that this avian immunoglobulin, called either IgG and IgY, is antigenically similar to mammalian IgA (7). In this study, we have confirmed the previous finding that the specific humoral response to an injected antigen in the serum of an immunized hen is mirrored in the egg yolk (2, 17, 21). Increased levels of serum antibodies to immunizing enterotoxigenic *E. coli* B16-4 could be detected 10 days after immunization and reached a peak 5 days later.

Egg yolks from hens immunized with killed enterotoxigenic *E. coli* B16-4, when fed to rabbits for 4 days, protected these animals from developing diarrhea when challenged with the same enterotoxigenic organism. Immunoglobulin precipitate from the immune egg yolks appeared equally protective in this model, suggesting that the immunoglobulin rather than some other component of the egg yolk was providing the protection. As expected, since the antibody response in the egg yolks persisted for 12 to 20 weeks, the protective effect persisted when the egg yolks were collected from eggs laid by hens up to 12 to 20 weeks postimmunization. The animals in our study were sacrificed at different times after challenge, so it was not possible to compare the numbers of luminal small bowel *E. coli* at the time of sacrifice to determine the effects of the egg yolk mixture on small bowel colonization by the organism. The in vitro binding studies suggest that the egg yolk immunoglobulin interferes with the binding of enterotoxigenic *E. coli* to small bowel mucins, a step that may be necessary for this organism to colonize the small bowel (22).

It is known that bovine milk immunoglobulins resist proteolysis and retain some specific antibody activity after passage through the GI tract of infants (4, 11). Other studies have demonstrated that in adults, bovine immunoglobulin resists proteolysis and inactivation by gastric acid when antacid is given after meals (18). In this study, we used carbonate-bicarbonate buffer to suspend the egg yolks during oral inoculation to protect the egg yolk immunoglobulin in its passage through the stomach so that it could reach the small intestine. However, in vitro experiments need to be carried out to determine the survival of hen egg antibodies in the mammalian GI tract. Further work is also needed to determine whether cooking eggs denatures or otherwise functionally inactivates the immunoglobulin. Previous work by Yolken et al. demonstrated that antibodies to rotaviruses persisted in commercially pooled egg preparations that had been pasteurized (28). Indeed, immunoglobulin is stable at 60°C, the maximum temperature reached in the yolk of a boiled egg. While both immunoglobulin and whole egg yolk appear to have the same protective effect, various theoretical and practical concerns may alter the usefulness of each preparation. While the immunoglobulin precipitate may be more stable for long periods of storage, it is more time-consuming and expensive to prepare. However, egg yolk preparations carry the concern of transmission of salmonellae or other bacterial contamination and, in the developed world, the concern of cholesterol content.

Interestingly, animals given egg yolks or immunoglobulin from the control hens, which were not immunized, also appeared to be protected from diarrhea when challenged with enterotoxigenic *E. coli* B16-4. It is possible that some of the protection afforded by the egg yolks or immunoglobulin occurs by some nonspecific, non-antibody-mediated mechanism, such as physical coating and protection of the intestinal mucus layer. However, it is most likely that the intestines of our hens had been previously colonized with *E. coli*, and these hens may have developed natural antibodies against *E. coli* that were expressed in the egg yolks. Yolken et al. found in commercially available eggs natural antibodies to rotaviruses that were considered a result of naturally occurring infections with avian rotaviruses that cross-reacted with the antigenic determinants of human rotavirus strains (28). Alternatively, hens may have natural antibodies that are polyspecific and successfully cross-protect against challenge with enterotoxigenic *E. coli*. It is likely that the more complete protection provided by the immune egg yolk and immunoglobulin reflects the presence of antibodies to the specific virulence factors of strain B16-4, including antibodies to CFA I and heat-labile enterotoxin. Interestingly, after immunization with enterotoxigenic *E. coli* B16-4, levels of antibodies to other bacteria, including nonpathogenic *E. coli* K-12, a *Salmonella* sp., and two *Shigella* spp., also increased in all three immunized hens. We have not established whether these detected antibodies were directed against common antigens or whether immunization caused polyclonal B cell activation; however, as *E. coli*, *Shigella* spp., and *Salmonella* spp. are all members of the family *Enterobacteriaciae*, it is not surprising that they would share some surface antigenic determinants that could exhibit the type of booster phenomenon seen in the ELISA detection of antibodies. *C. jejuni*, as a member of the family *Spirillacaeae*, is unrelated to the members of the family *Enterobacteriaciae*, so the antigenic determinants would be expected to differ. We hypothesize that the antibody response seen was a booster phenomenon from residual antigens to which the hens had been previously, naturally exposed. Although poultry is often colonized by *Campylobacter* spp., we did not see any antigenic evidence of such colonization in the hens in our study, a result that may reflect the relatively sheltered existence of the flock. Further work to characterize the nature of the precise antibody response in the immune and nonimmune egg yolks and to delineate which of the components is most protective in this model is necessary.

In conclusion, we have demonstrated passive protection of the gut by xenogenic immunoglobulin. Antibody-containing egg yolks protected rabbits from enterotoxigenic *E. coli* B16-4-induced gastroenteritis; moreover, egg yolk immunoglobulin inhibited the binding of *E. coli* to rabbit small bowel mucin glycoprotein in vitro, suggesting that it may prevent colonization by the bacteria in vivo. The principle of passive immunization of the gut is well illustrated by the role of maternal milk in preventing GI tract infections (6, 9, 25), and previous studies have suggested that xenogenic immuno-

globulin may also be protective, as successful treatment of enterotoxigenic *E. coli* diarrhea with immune bovine colostrum was reported for infants (8) and oral ingestion of immunoglobulin from immunized cows protected against gastroenteritis in adults (18). Previous work has demonstrated in an in vivo neutralization assay for rotavirus that avian immunoglobulins are effective in mammalian systems (28) and that antifimbrial antibodies raised in hen eggs are protective in a porcine model of diarrhea (27), and our work confirms that hen eggs are a rich source of xenogenic immunoglobulin that can be used to passively immunize the mammalian GI tract and effectively prevent clinical diarrheal disease. Because hen eggs are such a rich source of immunoglobulin that is easily purified, these results have exciting implications for the prevention and perhaps treatment of common infectious diarrhea of humans and animals.

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