Avian-to-Mammal Transmission of an Avian Rotavirus: Analysis of Its Pathogenicity in a Heterologous Mouse Model

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INTRODUCTION

Rotaviruses are recognized as the most important cause of acute gastroenteritis in infants and young animals of many mammalian and avian species (Estes and Cohen, 1989). Group A avian rotaviruses (avian rotaviruses) have been isolated from pigeons (Minamoto et al., 1988), turkeys, chickens (McNulty et al., 1980), pheasants (Legrottaglie et al., 1997), and scoters (Takehara et al., 1991). Avian rotaviruses have been shown to be closely related to each other in terms of antigenic and genetic properties (Ito et al., 1995, 1997; Minamoto et al., 1988, 1993). Some studies have suggested that avian rotaviruses had separated early from group A mammalian rotaviruses (mammalian rotaviruses) during evolution (Ito et al., 1997, 2001; Rohwedder et al., 1995, 1997). A comparison of the deduced amino acid sequence of the inner capsid protein, VP6, which is a very conservative protein among mammalian rotaviruses sharing more than 90% homology (Gorzgiglia et al., 1988; Lopez and Arias, 1993; Palombo and Bishop, 1994), showed that there is a low degree of only about 70% homology among avian and mammalian rotaviruses (Ito et al., 1997).

Recently, several investigators have reported natural cases in which a mammalian rotavirus could have been transmitted from the original hosts to heterologous mammals (Nakagomi et al., 1990; Pongsuwanna et al., 1996; Urasawa et al., 1992). In Germany, rotavirus 993/83 was isolated from a calf with diarrhea (Brüssow et al., 1992a). It has been shown by RNA–RNA hybridization, nucleotide sequencing, and antigenic examination that this strain is more similar to avian rotaviruses than to mammalian rotaviruses (Brüssow et al., 1992a,b). Sequence analysis of VP6, VP7, and VP8 from 993/83 showed high-level identities with those of the Japanese pigeon rotavirus PO-13 (Ito et al., 1997; Rohwedder et al., 1995, 1997). These facts strongly suggest that 993/83 is an avian rotavirus that had been transmitted to calves. However, there is no direct evidence that avian rotaviruses induce a diarrheal illness in calves or other mammalian species.

There have been no experimental studies on infection of avian rotaviruses in mammalian species. On the other hand, interspecies infection of mammalian rotaviruses has been studied in several animals, including dogs (Schwers et al., 1983), calves (Castrucci et al., 1984), rats (Guerin-Danan et al., 1998), rabbits (Castrucci et al., 1984; Ciarlet et al., 1998a,b, 2000; Conner et al., 1988), and mice (Bell et al., 1987; Offit et al., 1984; Ramig, 1988). Among animal models, the physiopathology of rotaviruses in rabbit and mouse models is the most well understood since these models have been most often used. However, few heterologous rotaviruses were able to induce diarrhea in the rabbit model (Ciarlet et al., 1998a, 2000). Furthermore, rabbits are more troublesome to maintain and require a larger volume of rotavirus for studying its infectivity than do mice. Therefore, we considered the mouse model to be the best model for studying interspecies transmissions of avian rotaviruses.

This paper describes the infectivity and pathogenicity...
of avian rotaviruses in the mouse model and demonstrates that PO-13 has a potential to infect and induce disease in mammalian species.

RESULTS

Clinical signs of diarrhea

Three-day-old mice inoculated with $2.2 \times 10^6$ focus-forming units (FFU) of PO-13 showed watery-yellow diarrhea between 1 and 2 days postinoculation but not other symptoms, such as dehydration, decrease in weight gain, and death. The diarrhea persisted for only 2 to 4 days and most of the mice had recovered by the fifth day after inoculation. None of the noninoculated mice in the same cage developed diarrhea. The diarrhea was indistinguishable from that induced by SA-11.

The 50% diarrhea-inducing dose of avian and mammalian rotaviruses

To compare the degree of virulence of two avian and one mammalian rotaviruses in suckling mice, we determined the 50% diarrhea-inducing dose (DD$_{50}$) of these rotaviruses in 3-day-old mice. The DD$_{50}$ values are shown in Table 1. The DD$_{50}$ of PO-13 was $8.1 \times 10^3$ FFU, which is a similar dose to that of SA-11. On the other hand, the turkey rotavirus, Ty-3, was not virulent in suckling mice, even when the maximum dose, $5.6 \times 10^7$ FFU, was inoculated. Therefore, the virulence of Ty-3 in suckling mice was at least $10^3$-fold lower than that of PO-13.

Effect of host age on induction of diarrhea

It is known that disease induced by group A rotaviruses is dependent on the host age (Ciarlet et al., 1998b; Guerin-Danan et al., 1998; Parashar et al., 1998; Ramig, 1988; Wolf et al., 1981). To confirm the effect of host age on induction of diarrhea, 2- to 13-day-old mice were inoculated with $2.2 \times 10^5$ FFU (272 DD$_{50}$ in 3-day-old mice) of PO-13. The percentages of diarrheal induction in each age group ($n = 10$) are shown in Fig. 1. All of the 2- to 4-day-old mice, except for one 2-day-old mouse, developed diarrhea. The percentages gradually decreased from 5-day-old mice, and none of 11- or 13-day-old mice developed diarrhea. This result therefore demonstrated that diarrhea induced by PO-13 is also dependent on the age of mice. Subsequently, 3- to 4-day-old mice were used in our experiments.

Replication of avian rotaviruses in intestines of mice

To confirm the replication of avian rotaviruses in intestines of suckling mice, we measured virus titers in the intestines of suckling mice inoculated with PO-13 or Ty-3. The results are shown in Fig. 2. From 4 to 36 h postinoculation, PO-13 showed a constant titer of about $10^5$ FFU. The titer of PO-13 decreased from 48 h postinoculation and could not be detected after 72 h postinoculation. However, PO-13 could not be detected at the levels above the input inoculum throughout the examination. On the other hand, Ty-3 could not be detected at any time after inoculation in any except two samples (at 12 and 48 h postinoculation). These results showed that PO-13, but not Ty-3, could limitedly (nonproductively) replicate in the intestine of suckling mice. It was reported that nonreplicating rotavirus particles caused diarrheal illness in suckling mice (Shaw et al., 1995). However, the replication of PO-13 in the intestine is assumed to be required for disease because suckling mice inoculated with UV-inactivated virus did not develop diarrhea (data not shown).

Histopathological examination of mice inoculated with PO-13

To evaluate how mice inoculated with PO-13 develop diarrhea despite the limited infection of PO-13, we histopathologically examined various intestine regions of mice inoculated with PO-13 at various times. By indirect fluorescent (IF) staining, VP6 antigen was dispersedly detected in absorptive epithelial cells on the ileum at 1 and 3 days postinoculation (Fig. 3 and Table 2). The...

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**TABLE 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>DD$_{50}$ (FFU)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO-13</td>
<td>$8.1 \times 10^3$</td>
</tr>
<tr>
<td>Ty-3</td>
<td>$5.6 \times 10^7$</td>
</tr>
<tr>
<td>SA-11</td>
<td>$2.6 \times 10^4$</td>
</tr>
</tbody>
</table>

* The DD$_{50}$ was measured in 3-day-old mice.
antigen was observed as granular fluorescences in cytoplasm. Most of cells in which the antigen was detected were located in the upper halves of villi. The finding of few epithelial cells containing rotavirus antigen supported the lack of productive replication of PO-13. On the other hand, lesions that appeared as ballooning degeneration of enterocytes were observed all over the small intestine except for the upper half of the duodenum (Fig. 4A and Table 2). Swelling and transparent cytoplasm and karyopyknosis were observed, and vacuoles were also recognized occasionally in these cells. These lesions appeared from 2 to 4 days postinoculation, with a delay of 1 day from the appearance of viral antigen in the ileum (Table 2). Most of the enterocytes located in the lower halves of villi and a few of the enterocytes located at the apices of villi had degenerated at 2 days postinoculation.

### TABLE 2
Detection of Symptoms, Viral Antigen, and Lesions in Suckling Mice Orally Inoculated with PO-13

<table>
<thead>
<tr>
<th>Item examined in mice</th>
<th>Region</th>
<th>Days postinoculation</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptom</td>
<td>Duodenum, upper</td>
<td>0/23</td>
<td>13/23</td>
<td>22/23</td>
<td>20/23</td>
<td>10/23</td>
<td>1/23</td>
<td></td>
</tr>
<tr>
<td>IF antigen</td>
<td>Duodenum, lower</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jejunum, upper</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jejunum, lower</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
<td>0/3</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colon</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Lesion</td>
<td>Duodenum, upper</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Duodenum, lower</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
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<td>NT</td>
<td>NT</td>
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</tr>
<tr>
<td></td>
<td>Jejunum, upper</td>
<td>0/3</td>
<td>0/3</td>
<td>2/3</td>
<td>3/3</td>
<td>0/3</td>
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<tr>
<td></td>
<td>Jejunum, lower</td>
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<td>1/3</td>
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</tr>
<tr>
<td></td>
<td>Ileum</td>
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<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colon</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

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* All mice were inoculated with $2.2 \times 10^5$ FFU of PO-13 at 3 days of age. Data are expressed as the number of positive mice/the number of tested mice. NT means that samples were not tested.

* The intestine was divided into six regions and used for the histopathological examinations.

* Symptoms were examined in 23 mice.

* Three mice were examined for the presence of IF antigen and lesion. IF antigens were detected with biotinylated anti-VP6 monoclonal antibody and fluorescein-conjugated streptavidin. Lesions were observed by HE staining.
These cells had completely disappeared after 5 days postinoculation. No degenerative cells were detected in mice mock-inoculated (Fig. 4B). Interestingly, many degenerative cells were detected in the duodenum and the jejunum without viral antigen. Also, no antigen was detected in the degenerative cells in the ileum. These observations were confirmed by the result that PO-13 could not be recovered from the upper small intestine after 24 h postinoculation (data not shown). Contrary to the positional discrepancy, the time course of the antigen and the degenerative cells was consistent with that of the symptoms (Table 2).

**DISCUSSION**

A pigeon rotavirus, PO-13, induced watery-yellow diarrhea in suckling mice. The disease in mice induced by PO-13 had three points in common with those caused in suckling mice by mammalian rotaviruses. First, the symptoms, onset, and duration of disease induced by PO-13 were quite similar to those of disease induced by SA-11. The symptoms are also similar to those of other mammalian rotaviruses reported by other researchers, including G3 serotype human rotaviruses, WI77, WI78, and CC3-17, simian rotaviruses, SA-11 and RRV, and a
bovine rotavirus, B223 (Bell et al., 1987; Ramig, 1988). Second, the DD_{50} of PO-13 (8.1 \times 10^{4} PFU) was similar to that of SA-11 (2.6 \times 10^{4} PFU). The DD_{50} of SA-11 determined in this study was almost identical to those reported by other researchers: 1 \times 10^{4} PFU (Offit et al., 1986) and 1 \times 10^{5} PFU (Bell et al., 1987). It has been reported that SA-11 was more virulent in suckling mice than in other heterologous strains (Bell et al., 1987; Offit et al., 1986). Third, the diarrhea induced by PO-13 was dependent on host age. This age-dependent diarrhea is characteristic of group A rotaviruses. In humans, the incidence of clinical illness peaks in 4- to 36-month-old children (Parashar et al., 1998). In mice models, it was also found that homologous and heterologous rotaviruses did not induce disease in mice older than 15 days (Ramig, 1988; Wolf et al., 1981). Several studies have suggested that the cause of these restrictions is that there is no or little replication of rotaviruses in adult animals (Ramig, 1988; Riepenhoff-Talty et al., 1982; Wolf et al., 1981). The decrease in viral replication is thought to be due to the reduced expression of rotavirus receptors on mature enterocytes or to the maturation of the immune system or the pancreas correlated with intestinal maturation (Riepenhoff-Talty et al., 1982; Wolf et al., 1981). However, all of five 33-day-old mice orally inoculated with PO-13 seroconverted against PO-13 in an enzyme-linked immunosorbent assay (ELISA), while none of the mice inoculated with UV-inactivated PO-13 did (data not shown). This result supports the speculation that this virus can infect adult mice. It is known that adult humans occasionally develop disease caused by rotaviruses (Parashar et al., 1998). Moreover, other studies have shown that rotaviruses were replicated in adult animals as well as in young animals but did not cause disease (Burns et al., 1995; Ciarlet et al., 1998b; Ramig, 1988). It has been speculated that these cases are caused by differences between degrees of severity of histopathological changes, between the degrees of sensitivity of cells to rotavirus infection, or between the compensatory mechanisms of fluid absorption in suckling and adult animals (Ciarlet et al., 1998b). Recently, Ball et al. (1996) proposed that the nonstructural protein 4, NSP4, of simian rotavirus had worked as an enteroxin and that its function had been age-dependent. As described above, the sharing of similar pathogenic properties between PO-13 and mammalian rotaviruses, in spite of their being different genetically and antigenically, is interesting and useful for studying the pathogenicity of group A rotaviruses.

On the other hand, it is also interesting that the turkey rotavirus Ty-3, which is genetically related to PO-13, did not induce diarrhea in suckling mice. We speculate that the avirulence of Ty-3 in suckling mice was due to its absent or small efficiency of replication in enterocytes of mice, as well as the cases of some heterologous rotaviruses reported by other researchers (Bell et al., 1987; Ramig, 1988). Previous studies of the pathogenicity of rotavirus in heterologous species showed that host range restrictions were determined by VP4, VP7, and NSP1 (Broome et al., 1993; Ciarlet et al., 1998a; Offit et al., 1986). VP4 may be the major determinant of host range, because this gene clusters along host species (Lopez et al., 1991).

The infectivity of PO-13 to mice was also reduced in comparison with that of homologous murine rotaviruses. The DD_{50} of PO-13 was at least 10^{4}- to 10^{5}-fold lower than those of murine rotaviruses (Burns et al., 1995). It has been reported that homologous murine rotavirus infections spread readily to noninoculated suckling mice in the same cage (Burns et al., 1995). Although the rotaviral RNA from stools of all of six suckling mice infected with 2.2 \times 10^{6} FFU of PO-13 was detected between 1 and 6 days postinoculation by the reverse transcription–polymerase chain reaction (data not shown), the horizontal transmission of PO-13 was never observed by detection of diarrhea. This may be due to much weaker replication of PO-13 in the intestine of mice than that of homologous rotaviruses and suggests that PO-13 cannot form an infection cycle among the murine group. In nature, however, it has been shown that many other mammalian species might be infected with avian rotaviruses; more than 70% of German cattle older than 1 year showed serum neutralizing antibodies to 993/83 and PO-13 (Brüssow et al., 1992b), and 35.6% of Japanese people were found to have serum hemagglutination-inhibiting antibodies to PO-13 (Minamoto et al., 1988). However, 993/83 is only one avian-like rotavirus isolated from mammalian species (Brüssow et al., 1992a). Most infections of avian rotaviruses in mammalian species may pass unnoticed, and it may be difficult to isolate viruses due to the low rate of replication, like that of PO-13 in the mouse model, even if the viruses induce diarrhea. Further studies are needed to elucidate the epidemiology and the infectivity of avian rotaviruses in mammalian species.

In previous histopathological examinations, group A rotaviruses induced lesions characterized by vacuolation and detachment from their basement membranes of absorptive cells at the tips of villi, blunting and fusion of villi, crypt hyperplasia, and lymphoreticular hyperplasia (Bell et al., 1987; Chang et al., 1999; Johnson et al., 1986; Offit et al., 1984). In the present study, histopathological changes associated with infection of PO-13 were limited to ballooning degeneration, which was a kind of vacuolation, of absorptive cells. Some studies have also showed that vacuolation of enterocytes was the only lesion in heterologous rotaviruses (Bell et al., 1987; Guerin-Danan et al., 1998; Offit et al., 1984). However, as far as we know, the finding in this study that the lesions were mainly located at the lower halves of villi is the first case of rotavirus infection. Furthermore, we did not observe the viral antigen in these degenerative cells and failed to recover the virus at time points in which there is
disease. These results suggest that viral infection and replication were not directly responsible for the degeneration of enterocytes. A similar discordance of infection and lesion in rats inoculated with SA-11 (Guerin-Danan et al., 1998) and in mice inoculated with Wa (Ramig, 1988) has been reported. These observations may be explained by the hypothesis of Ball et al. (1996), namely, that rotavirus bind to and penetrate enterocytes, and they replicate in these cells. Then, NSP4 expressed in these cells may be released in the lumen of the intestine and interact with surrounding enterocytes that have NSP4-specific receptors, resulting in a facilitation of the endogenous secretory pathway and diarrhea. However, this hypothesis has some problems in the case of PO-13. First, NSP4 of PO-13 might not have the same entero-toxin function as those of other mammalian rotaviruses, because the homologies of amino acid sequences of NSP4 in PO-13 and mammalian rotaviruses ranged from only 32 to 36% (Ito et al., 2001). Second, enterocytes with rotaviral antigens were detected only in the ileum, but degenerative cells were observed even in the duodenum, which is upstream of the ileum. It is difficult to consider that NSP4 released into the lumen of the ileum is transferred to enterocytes in the duodenum. However, this observation might be explained by the mechanism proposed by Lundgren et al. (2000). Rotavirus replication may directly or indirectly stimulate dendrites or free nerve endings located underneath the epithelial layer, thereby activating secretory nervous reflexes over a wide region. Lundgren et al. suggested that NSP4 might be an activator of endocrine cells, inducing stimulation of the nervous system.

This is the first report of direct evidence that an avian rotavirus is transmissible to mammalian species. Furthermore, the mouse model established in this study will be useful for analyzing the mechanisms of pathogenicity in mammalian animals and the host range restriction of avian rotaviruses. We are planning to investigate the enterotoxic effect of NSP4 of PO-13 using this model in a future study.

MATERIALS AND METHODS

Viruses

A pigeon rotavirus, PO-13 strain, a turkey rotavirus, Ty-3 strain, and a simian rotavirus, SA-11 strain, were grown in MA104 in the presence of trypsin as described previously (Minamoto et al., 1988). To prepare the stock virus of Ty-3 with a high titer, a culture fluid of MA104 cells infected with Ty-3 was centrifuged at 96,300 g for 2 h at 4°C, and the resulting precipitate was resuspended in a 1/100 volume of starting fluid with Eagle’s minimal essential medium (E-MEM). Virus titers were determined by a fluorescent focus assay in MA104 cells as follows. Briefly, after reaction with an equal volume of 20 μg/ml of trypsin, the stock viruses were serially diluted 10-fold and were inoculated onto a monolayer of MA104 cells grown in 24-well plates. After incubation at 37°C for 1 h, a monolayer was washed with Hank’s solution and cultured in E-MEM containing 10% fetal bovine serum, 0.5% methylcellulose, and antibiotics. After incubation for 2 or 3 days, cells were fixed with 2% buffered paraformaldehyde and methanol and stained with anti-PO-13 rabbit serum (Minamoto et al., 1988) and fluorescein-conjugated anti-rabbit IgG goat serum (Miles Lab., USA). The numbers of fluorescent foci were counted, and titers are expressed as FFU per milliliter.

Animals

Pregnant ddY mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). The mice were housed individually in cages. After delivery, blood samples were taken from each dam, and anti-PO-13 serum antibody was checked by ELISA as described previously (Burns et al., 1995). All the dams checked were seronegative (titer, <200). The litters were kept with their dams throughout the course of the experiments.

Animal inoculation

Litters of 2- to 13-day-old suckling mice were given 20 μl of virus by oral gavage. Inoculated mice were observed for clinical signs of disease, such as diarrhea, dehydration, decrease in weight gain, and death, at various times postinoculation. Diarrhea was diagnosed by gentle abdominal palpation. The state of stool was classified into three categories; watery diarrhea, loose yellow stool, and ordinary stool. Only water diarrhea was considered diarrhea, but loose yellow stool was not. Based on the results obtained from a series of virus dilutions, the DD50 was calculated by the method of Reed and Muench (1938).

Viral growth curves in the intestines of suckling mice

At various times postinoculation, three mice that had been inoculated with 5.2 × 10^5 FFU of PO-13 (642 DD50) or 5.6 × 10^5 FFU of Ty-3 were sacrificed under diethyl ether anesthesia, and intestine samples were collected. The intestines were weighed and stored at −80°C until use. The intestines were homogenized, treated with AK-225 (Asahi Glass, Japan), and centrifuged for 10 min at 750 g at 4°C. The resulting aqueous layers were collected as an inoculum for MA104 cells. Virus titers were determined by the fluorescent focus assay and expressed as FFU per intestine.

Histopathological examination

Litters of 3-day-old mice were inoculated with 2.2 × 10^6 FFU of PO-13, and their intestines were collected at various times postinoculation. The intestine samples were divided into five pieces of the small intestine (upper
and lower halves of the duodenum, upper and lower halves of the jejunum, and the entire ileum) and one piece of the large intestine (the entire colon). After the pieces had been fixed in 4% buffered paraformaldehyde for 1 day and equilibrated with 30% sucrose in PBS, they were embedded with the Tissue-Tek O.C.T. compound (Sakura, Japan) and frozen. Cryosections 10 μm in thickness were prepared and used for IF or hematoxylin and eosin (HE) staining. IF staining was performed as follows. Briefly, after the Tissue-Tek O.C.T. compound had been removed with phosphate-buffered saline (PBS), the intestinal sections were fixed with acetone, and nonspecific reactions were blocked with 5% skimmed milk solution in PBS. The sections were then incubated with a biotinylated monoclonal antibody against VP6 of PO-13 (P3-29) (Minamoto et al., 1993) at 4°C overnight. The sections were then washed with PBS and incubated with fluorescein-conjugated streptavidin (Zymed, U.S.A.) for 60 min at 37°C. After the final washing with PBS, the sections were observed under a UV light microscope (Olympus, Japan). HE staining was performed by the standard technique, and the resulting sections were observed under a light microscope (Akiokskop 2, Carl Zeiss, Germany).

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