Effect of Avitaminosis A and Hypervitaminosis A on Urinary Bladder Carcinogenicity of N-[4-(5-Nitro-2-furyl)-2-Thiazoyl]formamide

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SUMMARY

The effect of vitamin A deficiency and hypervitaminosis A on the urothelial carcinogenicity of N-[4-(5-nitro-2-furyl)-2-thiazoyl]formamide (FANFT) was determined in female weanling Sprague-Dawley rats. Vitamin A deficiency resulted in squamous metaplasia of the urinary bladder and high incidences of cystitis, ureteritis, and pyelonephritis. Administration of FANFT to vitamin A-deficient rats appeared to accelerate the carcinogenic process, with earlier appearance of urinary bladder tumors and the development of ureteral and renal pelvic carcinomas. Most of these tumors were squamous cell, occasionally with transitional cell foci. Hypervitaminosis A prevented the appearance of squamous metaplasia and squamous cell neoplasia in rats fed FANFT, but it did not inhibit the formation of transitional cell hyperplasia or neoplasia in comparison to rats receiving normal levels of vitamin A and FANFT.

INTRODUCTION

FANFT demonstrated mutagenic activity in bacterial test systems and strong carcinogenic activity for the urinary bladder of rats, mice, hamsters, and dogs, inducing transitional or squamous cell carcinomas or mixtures of the two. Squamous cell tumors usually produce keratin as a characteristic feature. Vitamin A deficiency, associated with squamous metaplasia of the rat bladder epithelium, was tested for its effects on FANFT tumor induction. Other epithelial structures, in particular the bronchus, also demonstrated squamous metaplasia as a feature of vitamin A deficiency and as a concomitant of tumor production, beginning with the normal ciliated columnar epithelium and progressing to squamous cell carcinoma. Respiratory tract squamous cell tumors were prevented in rodents by the administration of large doses of vitamin A, and the effect of such doses of vitamin A on FANFT bladder carcinogenicity was studied.

MATERIALS AND METHODS

FANFT was purchased from Saber Laboratories (Morton Grove, Ill.), RP was from Schwarz/Mann (Orangeburg, N.Y.), and vitamin A-deficient diet (Vitamin A test diet) was from Nutritional Biochemical Corp. (now ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio). This basic diet was (%): starch, 65; vitamin-free casein, 18; dried yeast vitamin D, 8; vegetable oil, 5; and salt mixture No. 2, 4. Viosterol, 0.5 g/100 lb of diet, was added. Salt Mixture No. 2 was (%): calcium lactate-5H2O, 32.7; KH2PO4, 23.98; MgSO4, 13.7; CaH2(PO4)2, 13.58; NaH2PO4, 2H2O, 8.7; NaCl, 4.35; and ferric citrate-5H2O, 2.97.

Experiment 1. Weanling female Sprague-Dawley rats (Sprague-Dawley, Madison, Wis.) weighing 44 to 76 g were divided into 7 groups of 40 (Table 1) and placed in raised, wire mesh cages with 4 rats/cage. Group 1 served as unmedicated controls and received a standard pellet rat diet (Wayne Lab Blox, Allied Mills, Inc., Chicago, Ill.). Groups 2 and 3 were fed the vitamin A-deficient diet without vitamin A supplementation except for the administration of 100 IU RP per rat via stomach tube during Weeks 9 and 16 of the experiment. Groups 4 and 5 were fed the vitamin A test diet supplemented with 5 IU RP per g of diet (approximately 100 IU/rat/day). Groups 6 and 7 received the test diet supplemented with RP, 250 IU/g of diet (approximately 5000 IU/rat/day), for the 1st 4 weeks, and then the vitamin A content was increased to 500 IU/g. After 1 week on their respective vitamin A diets, FANFT at a dose of 0.188% was added to the diets of Groups 3, 5, and 7 for 12 weeks, and then the dose was reduced to 0.1% for the following 8 weeks. Diets were prepared weekly and refrigerated until fed to the rats. At the end of 20 weeks of FANFT administration (21 weeks of the experiment), FANFT was removed from the diet of Groups 3, 5, and 7. Groups 1 and 4 to 7 were fed their respective vitamin A diets for an additional 10 weeks, at which time they were killed. Remaining rats in Groups 2 and 3 were killed at the end of Week 22 of the experiment.

Rats were weighed and food consumption was determined biweekly. Rats were killed from each group periodically for histological examination of the tissues and for vitamin A determinations of blood, liver, kidneys, and urinary bladder. An autopsy of all rats killed or dying was performed, and the tissues were processed as described for histological sections. Step serial sections of each bladder were prepared, and 6 sections from each inflated hemibladder were inspected as described previously.

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Vitamin A and FANFT Bladder Carcinogenesis

Table 1

Experimental design and results of Experiment 1

Groups of 40 Sprague-Dawley rats were fed diets containing normal, high, and deficient levels of vitamin A with and without FANFT. The rats were weighed and food consumption was determined biweekly. Rats were sacrificed from each group periodically for histological examination of tissues and for vitamin A determinations of blood, liver, kidneys, and urinary bladder. At autopsy, all tissues were stained with hematoxylin and eosin. Step serial sections of each bladder were prepared, and 6 sections from each inflated hemibladder were inspected.

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Wk fed diet</th>
<th>Wk 5</th>
<th>Wk 10</th>
<th>Wk 15</th>
<th>Wk 18</th>
<th>Wk 22</th>
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<tr>
<td>1a</td>
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<td>0-31</td>
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<td>N' 4</td>
<td>N' 3</td>
<td>N 4</td>
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<td>N 2</td>
<td>N 1</td>
<td>Sqh 7</td>
<td>Sqh 5</td>
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<td></td>
<td>Trh 1</td>
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<td>Trh 3</td>
<td>TrSqh 1</td>
<td>TrSqh 2</td>
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<td>Trh 4</td>
<td>Trc 1</td>
<td>Sqh 1</td>
<td>Trc 1</td>
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<td></td>
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<td>Sqc 2</td>
<td>Sqc 4</td>
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<td>Trh 4</td>
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<td>N 4</td>
<td>N 4</td>
<td>Trh 4</td>
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<td>RP 250 IU/g diet</td>
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<td>500 IU/g diet</td>
<td>0-31</td>
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<td>Trc 1</td>
<td>Trc 1</td>
<td>Trc 1</td>
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</table>

* Time was measured from beginning of feeding various vitamin A regimens.
* One rat had a single breast adenofibroma at 31 weeks.
* Bladder histology abbreviations: N, normal; Sqc, squamous cell hyperplasia; Trh, transitional cell hyperplasia; TrSqc, transitional and squamous cell hyperplasia; Trc, transitional cell carcinoma; Sqc, squamous cell carcinoma; TrSqc, transitional and squamous cell carcinoma.
* Rats were given 100 IU of RP by stomach tube at Weeks 9 and 16.
* Additional tumors in this group were: 1 rat with Wilm's tumor of kidney at 22 weeks; 2 Trc of renal pelvis, 1 each at Weeks 15 and 22; 3 Sqc of renal pelvis, 2 at Week 15 and 1 at Week 22; 2 unilateral ureteral Sqc, 1 each at Weeks 15 and 22; 1 rat with bilateral ureteral Sqc at Week 22; and 1 forestomach Sqc at 22 weeks.

All tissue sections were stained with hematoxylin and eosin. Vitamin A levels of blood and tissues of 4 rats from each group at each time period were determined by the micro-method described by McLaren et al. (20).

Experiment 2. A 2nd experiment was performed after the results of the 1st study were known, since the rats receiving the hypervitamin A diet never developed clinical signs of marked toxicity characteristic of hypervitaminosis A. Four groups of 36 weanling female rats each were fed ground Wayne Lab Blox diet. Groups 3 and 4 had their diet supplemented with RP at the levels given in Table 2. After 1 week on their diets Groups 2 and 4 had FANFT added at a dose of 0.1% for 20 weeks, after which the rats were again fed their respective diets. All rats remaining alive at 35 weeks were killed at that time.

RESULTS

Experiment 1. All groups receiving the synthetic vitamin A test diet gained weight at noticeably slower rates than Group 1 which received the standard pellet rat chow (Chart 1). The rats receiving FANFT (Groups 3, 5, and 7) and their respective control groups (Groups 2, 4, and 6) grew at the same rate, and the "hypervitaminosis" A rats (Groups 6 and 7) and the "normal" vitamin A rats (Groups 4 and 5) grew at the same rate. The avitaminosis A rats (Groups 2 and 3) grew more slowly than the rats receiving "normal" or hypervitamin A levels. The cumulative average consumption of FANFT per rat per 21 weeks of feeding was 3.4, 4.2, and 3.9 g for Groups 3, 5, and 7, respectively. This is a maximum estimate since diet spillage was not measured.

The histological changes in the urinary bladder of the different groups are summarized in Table 1. Some rats from each group died unexpectedly, were cannibalized, or exhibited tissue autolysis and were not evaluated.

Rats fed the control pellet rat chow (Group 1) demonstrated no macroscopic or microscopic changes in the urothelium of the kidney pelvis, ureter, or urinary bladder. No tumors were present in the rats receiving synthetic diet with "normal" levels of vitamin A (Group 4), and the urothelium was normal except for mild, focal transitional cell hyperpla-
Table 2
Experimental design and results for Experiment 2
Groups of 36 weanling rats were fed ground Wayne Lab Blox diet alone and supplemented with FANFT, vitamin A, or vitamin A plus FANFT. All rats alive at 35 weeks were killed, an autopsy was performed, and tissues were processed for histological sections.

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Dose of chemical (per g diet)</th>
<th>Time fed given dose (wk)</th>
<th>Urinary bladder histology</th>
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<td>1</td>
<td>Wayne Lab Blox</td>
<td></td>
<td>0-35</td>
<td>Normal 34</td>
</tr>
<tr>
<td>2</td>
<td>Wayne Lab Blox</td>
<td></td>
<td>0-35</td>
<td>Hyperplasia 26</td>
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<tr>
<td>3</td>
<td>Wayne Lab Blox + FANFT</td>
<td>1 mg</td>
<td>2-21</td>
<td>Carcinoma 2</td>
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<tr>
<td>4</td>
<td>Wayne Lab Blox + RP</td>
<td>930 IU</td>
<td>0-2</td>
<td>Normal 11</td>
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<tr>
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<td>1600 IU</td>
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<td>2500 IU</td>
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<td>1100 IU</td>
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<td>Wayne Lab Blox + FANFT</td>
<td>1 mg</td>
<td>0-35</td>
<td>Normal 24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0-35</td>
<td>Hyperplasia 12</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0-35</td>
<td>Carcinoma 0</td>
</tr>
</tbody>
</table>

The results of the experiment showed that the urinary bladder histology varied depending on the dietary supplementation. Rats receiving normal diet (Group 1) showed no histological changes. Rats on vitamin A-deficient diet (Group 2) exhibited hyperplasia, while those on vitamin A-deficient diet with FANFT (Group 3) showed a similar pattern. Rats on vitamin A plus FANFT (Group 4) demonstrated progressive changes in the urinary bladder epithelium, with mild hyperplasia at Week 5, severe hyperplasia at Weeks 10 and 15, and microinvasive transitional cell carcinomas in 1 of 4 rats at 22 weeks and in 16 of 20 rats at the end of the experiment. The remaining 7 rats living 22 or more weeks had severe transitional cell hyperplasia. No evidence of squamous metaplasia was seen in any of the hyperplastic or neoplastic lesions, and the ureters and kidney pelves were normal. The serum levels of vitamin A (retinol plus RP) in the “normal” vitamin A rats with and without FANFT (Groups 4 and 5) were similar and initially ranged from 20 to 60 μg/100 ml. By Week 4 and thereafter, they ranged from 50 to 150 μg/100 ml. At all time periods kidney and bladder vitamin A levels were 20 to 40 μg/g of tissue (wet weight), while hepatic levels were 200 to 800 μg/g of tissue. Similar quantities were present in the serum and tissues of rats fed the pellet chow (Group 1).

At the end of Week 1, rats receiving the vitamin A-deficient diet (Groups 2 and 3) had serum levels of vitamin A comparable to those fed “normal” vitamin A (Group 4), but the levels in the livers were 40 to 160 μg/g, one-fifth that of normals. By the end of the 10th week of the experiment, undetectable amounts of vitamin A were present in serum, liver, kidney, and bladder of the rats receiving the deficient diets. The rats fed the deficient diet plus FANFT (Group 3) had the same vitamin A levels as rats receiving the deficient diet without FANFT (Group 2), and both groups demonstrated signs of vitamin A deficiency beginning at Week 10, which became progressively more severe as the experiment continued. The signs of deficiency included anorexia, growth retardation, rough and untidy fur, xerophthalmia, and eventually death. All of the surviving rats in the deficient groups (Groups 2 and 3) were nearly moribund by the end of Week 22 and were killed.

Three rats receiving the deficient diet without FANFT (Group 2) had normal urothelium at the end of 5 weeks, but one had a mild, focal transitional cell hyperplasia of the urinary bladder without evidence of squamous metaplasia (Table 1). By the end of 10 and 15 weeks, mild transitional cell hyperplasia was apparent in 6 rats, with focal squamous metaplasia. By 18 and 22 weeks, all rats had hyperplasia of the urinary bladder, either pure transitional cell, pure squamous cell with keratinization, or a mixture of the 2 cellular components. Several rats dying after 15 weeks had moderate to severe cystitis with focal ulceration of the mucosa and extensive polymorphonuclear infiltrate. Ureteritis and pyelitis were present in 6 rats and pyelonephritis with abscess formation developed in 2 rats. Mild transitional cell hyperplasia of the renal pelvis with extensive squamous...
metaplasia of the hyperplastic epithelium was present in 13 rats. Other tissues in the vitamin A-deficient rats showed few signs of chronic avitaminosis A. The forestomach became hyperkeratotic; the bronchi showed mild, focal squamous metaplasia; and 3 rats had squamous metaplasia of the endometrium. Xerophthalmia was apparent at 15 weeks in a few rats but was present in all vitamin A-deficient rats surviving longer than 17 weeks of the experiment.

Rats given FANFT with the vitamin A-deficient diet (Group 3) demonstrated mild transitional cell hyperplasia of the urinary bladder after 5 weeks (Table 1) identical to that seen in the "normal" vitamin A rats given FANFT (Group 5). By 10 weeks, the lesions of the urinary bladder were considerably more severe in the vitamin A-deficient rats than in the "normal" vitamin A rats, with microinvasive carcinomas in 4 of 4 rats. By 22 weeks, 12 of 13 vitamin A-deficient rats fed FANFT had invasive carcinomas.

As with the vitamin A-deficient rats without FANFT, cystitis, ureteritis, pyelitis, and pyelonephritis with abscesses were common findings after 10 weeks in vitamin A-deficient rats given FANFT (Group 3). Similarly, forestomach hyperkeratosis, bronchial squamous metaplasia, and endometrial squamous metaplasia were present in the vitamin A-deficient rats given FANFT. Thirteen rats had renal pelvic transitional cell hyperplasia, and 9 of these had squamous metaplasia. Unlike the other groups, neoplasms of the ureters and kidneys occurred in this group (Table 1).

Rats receiving high doses of RP (Group 6) developed no tumors and the urothelium was normal except for 4 of 17 rats with mild, focal transitional cell hyperplasia of the urinary bladder. By the end of Week 5, the level of vitamin A in the serum, urinary bladder, and kidneys was 2 to 3 times greater than in "normal" vitamin A rats, and the levels in the liver were 20 to 40 times "normal" levels. By 10 weeks and later, the vitamin A levels in the liver were 80 to 200 times the normal levels, but levels in the serum, urinary bladder, and kidneys remained 2 to 3 times normal. Levels of vitamin A were the same in groups receiving high doses of RP with or without FANFT. Despite the high doses of RP in these groups, clinical evidence of hypervitaminosis A did not develop. Rats fed high doses of RP and FANFT (Group 7) had lesions in the urinary bladder similar to those in the "normal" group fed FANFT (Group 5), beginning with mild transitional cell hyperplasia at 5 weeks and progressing to microinvasive transitional cell carcinomas in 16 of 19 rats at 31 weeks [p > 0.5 as calculated by the exact method for 2 x 2 tables (11)]. None of these rats had bladder squamous metaplasia, and the ureters and kidney pelves remained normal.

**Experiment 2.** The growth rate of rats supplemented with vitamin A (Groups 3 and 4) was retarded compared with the unmedicated control Group 1 (Chart 2). Despite impaired growth, nearly all rats survived until the designated experimental termination period of 35 weeks. The cumulative maximal average consumption of FANFT per rat per 20 weeks of feeding was 2.4 g for Group 2 and 2.2 g for Group 4.

Signs of hypervitaminosis A appeared in rats receiving the high doses of RP (Table 2) by 11 weeks of the experiment and included limb paralysis, loss of hair, and growth retardation. Limb paralysis continued and eventually resulted in multiple fractures of long bones. To permit greater longevity of the rats, the dose of RP was reduced to 1100 IU/g diet after 13 weeks of the experiment. The rats receiving control diet (Group 1) had no epithelial changes in the urinary bladder except for 2 instances of mild, focal transitional cell hyperplasia. Twelve of 36 rats receiving high doses of RP (Group 3) had similar changes in the urinary bladder, but ureters and renal pelves were normal. Rats receiving FANFT had similar incidences and types of lesions whether fed normal vitamin A diets or high doses of RP (Table 2). Two rats in each of these 2 groups had microinvasive urinary bladder transitional cell carcinomas. Twenty-six of 35 (Group 2) and 21 of 34 rats (Group 4), respectively, had moderate to severe transitional cell hyperplasia, and the bladders of the remainder appeared normal (p > 0.2). There was no evidence of squamous metaplasia in the normal bladders or those with lesions, and the ureters and renal pelves appeared normal.

**DISCUSSION**

FANFT demonstrated urinary bladder carcinogenicity for several species (6-10) and induced occasional renal pelvic carcinomas in Sprague-Dawley rats (9, 10). Transitional cell and squamous cell carcinomas of the bladder mucosa were found as well as tumors with mixtures of the 2 cell components; glandular areas were present rarely. Vitamin A deficiency produced squamous metaplasia of the urothelium as...
well as of other epithelia (14, 15, 29), and keratinizing squamous metaplasia was found in the present experiment. Squamous metaplasia of the urothelium was accompanied by severe cystitis, ureteritis, and pylonephritis in high incidences. The combination of FANFT and vitamin A deficiency produced striking metaplastic and neoplastic responses. Tumors of the urothelium occurred in higher incidence, invaded submucosal tissues earlier, and involved ureters and renal pelves several months before tumors appeared in normal rats fed grain and FANFT. The bladder tumors were nearly all squamous cell carcinomas with keratin production, and the ureteral and pelvic tumors usually had considerable squamous components. Vitamin A deficiency clearly had an effect on the cellular differentiation of the tumors as well as an accelerating effect on the neoplastic process. What role the intense inflammation produced by vitamin A deficiency had on this process can only be conjectured at present. Of interest was the increased incidence of tumor implants when tumor was injected into bladders that had undergone a severe inflammatory reaction due to N-methyl-N-nitrosourea (28). There also was a severe, ulcerating, acute inflammatory reaction preceding the implantation and growth of tumor (28).

Since vitamin A deficiency accelerated the malignant response to FANFT, vitamin A excess might be expected to suppress the bladder carcinogenicity of FANFT. That was not the case. In Experiment 1, large doses of RP were administered with FANFT (Group 7). The tumor incidence, latency period, and incidence of bladder epithelial hyperplasia were similar for Group 7 compared to Group 5 receiving "normal" vitamin A levels and FANFT. Several possible explanations could account for this lack of epithelial suppression: (a) the dose of FANFT may have been excessive, overwhelming any effect of vitamin A; (b) the dose of vitamin A may not have been adequate as there was no clinical evidence of hypervitaminosis A toxicity; or (c) the vitamin A-deficient diet used may also have been deficient in several inorganic substances, particularly zinc, and absence of these may have had an effect on FANFT and/or vitamin A (12). Thus, a 2nd experiment was performed using a smaller dose of FANFT, a greater dose of vitamin A (sufficient to produce clinical toxicity), and a standard pellet diet rather than the deficient diet. Nevertheless, again, no epithelial suppressive effect by the excess vitamin A was found.

Other possible explanations might be offered for the lack of suppression by excess vitamin A. Although storage of vitamin A in the liver reflected the high doses of vitamin A, the levels in serum, kidneys, and bladder were only 2 to 3 times that in the rats fed "normal" vitamin A levels. Thus, the level in the affected tissue may not have been sufficient to suppress the effect of FANFT. Related to this are differences in the chemical form in which vitamin A is administered. In our experiments, RP was administered and it is possible that the acetate form or an analog of vitamin A may have greater effects on the urinary bladder than we found in the present experiment. Wide differences in biological activity have been demonstrated for analogs of vitamin A in various test systems (16, 17, 26).

Although excess vitamin A did not suppress the carcinogenic response to FANFT, squamous metaplasia was completely suppressed. A similar suppression of squamous metaplasia by vitamin A in a rat urinary bladder tumor in meniscus gradient culture has been demonstrated recently (27). Interestingly, in the in vitro system vitamin A did not suppress the proliferative ability of the tumor, suppressing only the appearance of squamous metaplasia.

The role of vitamin A in tumorigenesis and its possible role as a chemotherapeutic agent appear to be quite complicated. Several studies on squamous cell tumors of lung (4, 5, 23), skin (1, 21), and forestomach (2) have demonstrated that vitamin A, or an analog, suppresses the appearance of tumors, and similar results have been found with other, nonsquamous tumors (17). Others have found that vitamin A stimulates tumor production (18, 19, 22, 24, 25) and that differences between experiments may be related to, among other variables, the dose of vitamin A.

It is not surprising in view of these apparent contradictions that we found accelerated tumorigenesis with vitamin A deficiency but no effect on epithelial hyperplasia or tumor incidence with excess vitamin A.

ACKNOWLEDGMENTS

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REFERENCES


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