Morphogenesis of Early 1,2-Dimethylhydrazine-induced Lesions and Latent Period Reduction of Colon Carcinogenesis in Mice by a Variant of Citrobacter freundii

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SUMMARY

The morphogenesis of 1,2-dimethylhydrazine (DMH)-induced lesions in the colon of outbred NIH Swiss mice was determined for up to 5 months of treatment. The effect of hyperplasia on DMH carcinogenesis was also evaluated by introducing a transient hyperplastic stimulus to the colon during the chronic weekly treatment regimen of DMH. The hyperplastic stimulus was a naturally occurring disease of mice, transmissible murine colonic hyperplasia, which is caused by a variant of Citrobacter freundii. In control mice, those not receiving the bacterium, weekly injections of the carcinogen induced neoplastic changes first detectable at two months of treatment in all segments of the colon and in both sexes. The changes increased in frequency and severity with time. Diffuse mucosal hyperplasia and chronic inflammatory and degenerative changes were also associated with DMH after prolonged treatment. The hyperplastic stimulus of C. freundii reduced the latent period for appearance of early DMH tumors, but it had no influence on already established DMH tumors.

INTRODUCTION

Hyperplasia is believed to precede neoplasia of the colon. Evidence for this observation is based on the higher incidence and earlier age of onset of colonic cancer in patients with diseases of the bowel with exaggerated proliferative activity, such as Crohn's disease, ulcerative colitis, and familial polyposis (5, 12, 16, 20, 34), and the increased proliferative activity seen in the flat mucosa between existing tumors of the colon (7, 18). Recently, it has been shown experimentally that nonspecific mucosal injury to the large bowel of rats causes mucosal proliferation that enhances DMH-induced neoplasia at the site of injury (26).

A unique opportunity exists to explore the interaction of a hyperplastic stimulus and chemical carcinogenesis in the mouse. Mice are susceptible to a natural infectious disease, TMCH, which is caused by a specific variant of Citrobacter freundii (1). In TMCH there is severe although transient mucosal hyperplasia of the distal colon (2), which is also the site of predilection for DMH-induced neoplasia (13, 31, 33). Colonic neoplasms induced by DMH or related compounds in mice have been described by several authors (6, 8-10, 13, 24, 31, 37). A study of the interaction of TMCH and DMH would provide insight into the role of a temporary although strong hyperplastic stimulus on DMH carcinogenesis.

The present experiment was designed to introduce TMCH at different times during a chronic weekly regimen of DMH. Since only a few reports (6, 13, 31, 32) have incompletely described early neoplastic events in the mouse colon, this study also sought to define the early DMH-induced neoplastic and nonneoplastic lesions in the colon of outbred NIH Swiss mice.

MATERIALS AND METHODS

Outbred NIH Swiss mice were obtained from a barrier-maintained production colony in the Division of Animal Care, Yale University School of Medicine, New Haven, Conn. Mice were 21 to 35 days of age at the onset of the experiments and were fed Purina laboratory chow and water ad libitum. They were housed by sex in groups of 6 in plastic boxes with pine chip bedding.

Experiment 1. Four hundred seventy-one mice were divided into 6 experimental groups (A to E and X), each consisting of one-half males and one-half females. Each experimental group was further divided into 2 subgroups. One subgroup was treated with weekly injections of 20 mg DMH (1,2-dimethylhydrazine dihydrochloride, Matheson, Coleman & Bell, Norwood, Ohio) per kg s.c. in 0.1 ml 0.001 m EDTA, and the other subgroup was treated similarly with EDTA alone. Treatments continued until the animals were killed for necropsy. Experimental Groups A to E were also inoculated with 2 to 3 drops p.o. of a thioglycolate broth culture of C. freundii (1) at different monthly intervals during the weekly DMH-EDTA or EDTA treatment regimen. Experimental Group X was not exposed to C. freundii and served as DMH-EDTA- and EDTA-treated controls. Although they received simultaneous and similar DMH and EDTA treatments, Groups X and A to E were maintained in separate rooms to avoid inadvertent exposure to C. freundii. Table 1 illustrates the design of the experiment, the number of mice, and the time of C. freundii inoculation. The groups were actually established in staggered monthly intervals so that Groups A to E were inoculated simultaneously with the same broth culture of C. freundii. In addition to these groups, 20 mice were given an equal volume of sterile water weekly; 10 of these mice were killed at 1 month of treatment, and 10 were killed at 2 months of...
Morphogenesis and Latency Reduction of DMH Carcinogenesis

Table 1
Design of Experiment 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Weekly treatments</th>
<th>No. of mice at onset</th>
<th>C. freundii inoculation (mo.)</th>
<th>No. of mice killed for necropsy at time interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>DMH-EDTA</td>
<td>12</td>
<td>4</td>
<td>0 mo. 1 2 mos. 3 mos. 4 mos. 5 mos.</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>12</td>
<td></td>
<td>9 9 11 10 11 11</td>
</tr>
<tr>
<td>B</td>
<td>DMH-EDTA</td>
<td>24</td>
<td>3</td>
<td>24 24 24 24 24 24</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>24</td>
<td></td>
<td>9 9 11 10 11 11</td>
</tr>
<tr>
<td>C</td>
<td>DMH-EDTA</td>
<td>36</td>
<td>2</td>
<td>36 36 36 36 36 36</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>36</td>
<td></td>
<td>11 10 10 10 10 10</td>
</tr>
<tr>
<td>D</td>
<td>DMH-EDTA</td>
<td>48</td>
<td>1</td>
<td>48 48 48 48 48 48</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>48</td>
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<td>10 11 11 11 11 11</td>
</tr>
<tr>
<td>E</td>
<td>DMH-EDTA</td>
<td>60</td>
<td>0</td>
<td>60 60 60 60 60 60</td>
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<tr>
<td></td>
<td>EDTA</td>
<td>60</td>
<td></td>
<td>9 9 10 10 10 10</td>
</tr>
<tr>
<td>X</td>
<td>DMH-EDTA</td>
<td>53</td>
<td>Uninoculated</td>
<td>53 53 53 53 53 53</td>
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<tr>
<td></td>
<td>EDTA</td>
<td>58</td>
<td></td>
<td>10 10 10 10 10 10</td>
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</table>

Mice in Groups A to E and X were given weekly treatments with DMH-EDTA or EDTA, whereas only mice in Groups A to E were inoculated with C. freundii at different time intervals during treatment.

RESULTS

Results are based on changes found in the ascending, transverse, and descending colon (15). Since various laboratories use differing terminology, the normal colon morphology of adult NIH Swiss mice will be briefly described for reference. The ascending colon is approximately 3.5 cm long, exits the cecum in the lower left quadrant of the abdomen, traverses the pelvic abdominal cavity, and then ascends on the right side to the level of the pylorus. This segment has distinct oblique mucosal ridges, which are grossly visible through the serosal surface. The ridges are prominent histologically, since they do not disappear when the colon is opened and spread (Fig. 1). Crypts in this region have many goblet cells, which are most numerous at the base (Fig. 2). The transverse colon is short (approximately 1.5 cm long) and crosses the upper abdomen. When empty it has thick longitudinal mucosal ridges. The transverse colon has higher, more uniform crypts than the other regions, with an even distribution of goblet cells along the crypt columns (Fig. 3). The transverse colon turns caudad into the descending colon, which is approximately 3 cm long and lies along the left side of the abdomen, and turns to the midline one-half of the way along its course, continuing through the pelvic cavity and into the 1- to 2-mm-long rectum. The descending colon has fine longitudinal ridges that disappear when distended or spread. It has short crypts and relatively less goblet cells located primarily in the luminal half of the crypt (Fig. 4).

Experiment 1. The normal mucosal morphology of Group
X EDTA controls varied with the age of the mice. At the onset of the experiment, when mice were 21 to 35 days of age and had received no treatment, the average height of crypts of the descending and transverse colon was 28 cells. Crypt column height later increased in the transverse colon to a level higher than that of the descending colon, as illustrated in Charts 1 and 2. Ascending colon crypt column height did not vary with age or treatment and remained between 25 and 30 cells. No sex differences were detectable. EDTA produced no noticeable effect on the morphology of the colon compared to that produced by water treatment for up to 2 months.

Three general categories of changes occurred in the colon of DMH-treated Group X mice: neoplastic change, hyperplastic change, and degenerative-inflammatory change. No changes were detectable in the cecum.

Neoplastic changes occurred only in DMH-treated mice. The lesions were graded on a basis of 1 to 4. Grade 1 lesions were focal areas of epithelial proliferation in the luminal one-third to one-half of individual crypts. The bases of the affected crypts were normal. Proliferating cells lacked mucin droplets, were mitotically active, and became crowded and pseudostratified, but they maintained their orientation along sinuous basement membranes. These foci formed microscopic nodules in the superficial mucosa (Fig. 5). Grade 2 lesions has similar changes in groups of adjacent crypts, forming nodules in the superficial mucosa. These nodules appeared to grow by expansion deeper into the mucosa and outward into the colonic lumen (Fig. 6). When these nodules had extended the entire width of the mucosa and abutted the muscularis mucosa, they were graded as 3 (Fig. 7). Grade 4 lesions had invasion of neoplastic epithelium through the muscularis mucosa into the submucosa. Invading epithelium usually maintained a glandular appearance and formed cysts and papillary configurations (Fig. 8).

In Group X mice, most neoplastic lesions were found in the descending and transverse colons. The incidence, distribution, and grade of lesions found in both sexes combined are plotted in Chart 3. Chart 4 illustrates the distribution and incidence of neoplastic lesions in the different segments of the colon.
sexes. Lesions were often multiple, particularly after 3 months, and in severely affected mice coalesced to produce broad areas of neoplastic change. No positive correlation of lesions with lymphoid nodules was observed.

Elongation of crypts within the colonic mucosa, which were not involved in neoplastic change, was also seen in Group X DMH-treated mice (Charts 1 and 2). At 3 months or more of DMH treatment, mice of both sexes had higher crypt cell column heights in the descending and transverse colon although not in the ascending colon compared to those in control mice (p < 0.01).

Various inflammatory and degenerative changes in the mucosa and submucosa were seen after 2 months of DMH treatment. The incidence and distribution of these changes, since they occurred simultaneously, are illustrated in Chart 5. Edema, leukocytic infiltration, and hyperplasia of lymph nodules occurred. Crypts developed irregular increases in number and size of goblet cells, distension with mucin, tortuosity and, occasionally, branching of their bases. Other crypts had reduced numbers of goblet cells. These changes were most obvious at 5 months of treatment and were present in all mice at that time. They were most severe in the descending and transverse colon. The incidence decreased after 3 months in the ascending colon. In comparison, only 2 of the EDTA controls were found to have mild goblet cell increases and mucinous crypt distension at 4 and 5 months of treatment.

In Groups A to E, mucosal hyperplasia typical of TMCH was present in most of the mice examined at 1 month after C. freundii inoculation. Lesions of TMCH had regressed by 4 and 5 months of treatment. In contrast to the normal colon (Fig. 9), colons of TMCH-affected mice had varying degrees of mural thickening of the descending portion due to mucosal hyperplasia, edema, inflammation, and muscle contraction (Fig. 10). Neoplastic lesions typical of those induced by DMH could be readily distinguished from TMCH, even when the 2 entities were superimposed. DMH lesions remained after regression of TMCH. There were no detectable differences in incidence or severity of neoplasia or TMCH between sexes.

Introduction of a transient hyperplastic stimulus (TMCH) at different intervals during the weekly DMH-EDTA treatment regimen of Groups A to E caused 2 notable effects compared to those produced in Group X. (a) TMCH introduced early in the DMH treatment (Groups D and E) reduced the latent period for appearance of DMH neoplastic lesions. (b) TMCH had no effect on established DMH neoplastic lesions when introduced late in the treatment regimen (Groups A and B). The incidence and mean severity of DMH neoplastic lesions are summarized for all treatments and statistically compared to EDTA controls within each group (Table 2). The latent period reduction was most pronounced in Group E mice, which had a high incidence (78%) of Grades 1 and 2 DMH lesions at 1 month, which was significantly higher than Group X (0%) at that interval (p < 0.01). A proportionately high incidence (70%) was seen in Group X mice only after 3 months of DMH treatment. In DMH-treated Group E mice at 1 month, typical lesions of Grades 1 and 2 had evolved in a background of diffuse mucosal hyperplasia of TMCH (Fig. 11). TMCH did not influence already established DMH lesions when introduced late in the course of DMH treatment, as illustrated in Groups A and B.

**Experiment 2.** This experiment confirmed the results seen in Experiment 1, Group E mice at 1 month of DMH treatment and also showed that a single injection of DMH at Time 0 or 1 week was sufficient to induce DMH lesions at 1 month (Table 3). Group E had significantly more and greater DMH lesions than did Group X (p < 0.01). A similar high incidence of DMH lesions occurred in Groups E0 (41.7%) and E1 (50.0%).

**DISCUSSION**

The present study provides information on the sequential morphogenesis of neoplastic and nonneoplastic lesions in the colon of mice treated with DMH. It also reveals that a hyperplastic stimulus such as TMCH can profoundly influence the course of tumor development.

DMH-induced colonic neoplasms appear to start as focal superficial epithelial proliferation in individual crypts and then to grow into adjacent crypts, with an eventual expansive growth that encompasses the entire width of the mucosa and with coalescence of adjacent foci. This sequence is in agreement with early tumor morphogenesis studies in the rat (25). The earliest neoplastic lesions resembled those seen by others (6, 13, 31, 32). Neoplastic lesions occurred most frequently in the descending and transverse colons, with a lower incidence in the ascending colon. The cecum remained uninvolved. Despite differences in anatomic terminology for the colon, other workers (13, 31, 33) have shown a similar distribution for late, well-developed tumors.

DMH also induced diffuse mucosal hyperplasia in the descending and transverse colons. This effect was found only after comparison with matched untreated controls. A significant increase in crypt cell column height was seen at 3 or more months of DMH treatment. Mucosal hyperplasia has been described in the rat after 3 months of DMH treatment, but it was localized to mucosal folds (36). Other investigators (6, 19, 22, 25, 29–31) have found diffuse cell kinetic changes that support these findings, although in-
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Table 2

Experiment 1
The percentage incidence and mean severity of DMH neoplastic lesions, Grades 1 to 4, are given for mice inoculated with *C. freundii* at different intervals during treatment with 20 mg DMH per kg (Groups A to E), compared to mice (Group X) uninoculated with *C. freundii* although receiving DMH treatment.

<table>
<thead>
<tr>
<th>Mos. of weekly DMH treatment</th>
<th>% inci-</th>
<th>sever-</th>
<th>% inci-</th>
<th>sever-</th>
<th>% inci-</th>
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<tr>
<td>Groups*</td>
<td>% inci-</td>
<td>severity</td>
<td>% inci-</td>
<td>severity</td>
<td>% inci-</td>
<td>severity</td>
<td>% inci-</td>
<td>severity</td>
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</tr>
<tr>
<td>E</td>
<td>78</td>
<td>1.1^a</td>
<td>70</td>
<td>0.8^c</td>
<td>70</td>
<td>0.8^c</td>
<td>70</td>
<td>0.8^c</td>
<td>70</td>
<td>0.8^c</td>
</tr>
<tr>
<td>X</td>
<td>0</td>
<td>0^c</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

* Time of *C. freundii* inoculation varied for each group: A, 4 months; B, 3 months; C, 2 months; D, 1 month; E, 0 month; X, uninoculated.

^ Significant difference (P < 0.01).

^ Significant difference (P < 0.05).

^ Significant difference (P < 0.01).

^ Difference not significant.

Table 3

Experiment 2
The percentage incidence and mean severity of DMH lesions at 1 month is given for mice inoculated with *C. freundii* and given different treatments of DMH (20 mg/kg), compared to uninoculated mice.

<table>
<thead>
<tr>
<th>DMH lesions</th>
<th>% inci-</th>
<th>mean</th>
<th>Time of DMH treatment (wk)</th>
<th>Time of <em>C. freundii</em> inoculation</th>
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<tbody>
<tr>
<td>Groups</td>
<td>% inci-</td>
<td>severity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>50</td>
<td>0.8</td>
<td>0, 1, 2, 3</td>
<td>0</td>
</tr>
<tr>
<td>E0</td>
<td>42</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E1</td>
<td>50</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E2</td>
<td>9</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>X</td>
<td>8</td>
<td>0.1</td>
<td>0, 1, 2, 3</td>
<td>Uninoculated</td>
</tr>
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</table>

Concurrent with the hyperplastic effect of DMH, a variety of inflammatory and degenerative changes occurred in the colon of mice treated with DMH for 2 or more months. Edema, dilation of crypts, and leukocyte infiltrates resembled the acute effects of DMH or methylazoxymethanol acetate (21, 38). Other architectural changes suggest more chronic effects described in rats treated with DMH (25).

Imposition of the hyperplastic stimulus of *C. freundii* reduced the latent period of DMH neoplasia. DMH induced a 70% incidence of early neoplastic lesions in the colon of mice after 3 months of treatment. With the hyperplastic stimulus a comparable incidence and severity of lesions were detectable at only 1 month of treatment. Single doses of DMH at different weekly intervals revealed that the DMH treatments given at Time 0 or 1 week after *C. freundii* inoculation were responsible for the tumors seen at 1 month. Since hyperplasia is minimal or absent until at least 1 week after *C. freundii* inoculation (2) and DMH is known to be cleared rapidly after injection (29), the temporal sequence suggests that hyperplasia promotes cells initiated already by the carcinogenic action of DMH and thereby reduces the latent period for tumor appearance. This study also revealed that established DMH lesions are not promoted to greater severity by hyperplasia, thereby substantiating the autonomous, neoplastic nature of Grade 1 to 4 DMH lesions.

TMCH is probably not unique in its effect on carcinogenesis. On the contrary there is more than coincidental homology between factors involved in TMCH-DMH cocarcinogenesis and the complex of factors involved in the pathogenesis of natural and experimental colorectal cancer. Interrelationships between mucosal cell proliferative activity, diet, microflora, genetics, and carcinogens are important in the epidemiology of large bowel cancer (4, 14, 17, 19, 20, 27, 35). The pathogenesis of TMCH is equally complex. Increased mucosal proliferation in TMCH, necessary for DMH cocarcinogenesis, is the result of the interaction of a specific bacterium, a predisposing diet, and host genetic background (3).

The association of hyperplasia with colonic neoplasia is known, but hyperplasia is not a required antecedent for colonic neoplasia. In fact, DMH-induced tumors can arise de novo without morphological evidence of prior hyperplasia (25). More correctly stated, mucosal proliferative activity in both health and disease influences neoplasia. Pozharis-ski (26) has shown that the distribution of DMH-induced tumors in the rat intestine correlates with the kinetic characteristics of the mucosa in different segments of bowel.
The descending colon, which has the largest stem cell population and shortest life cycle, is the site of greatest predilection for tumors. When proliferative activity was increased by nonspecific injury in a site of low tumor incidence (occur), tumors developed in high frequency. Similarly, increasing the proliferative activity of the distal colon mucosa in the present study reduced the latent period for tumorigenesis. The high incidence and early age of onset of colon cancer in humans with hyperplastic mucosal diseases may be explained on this basis. The colonic mucosa of mice with TMCH has mitotic activity along the entire crypt column (including the surface mucosa) similar to ulcerative colitis, familial polyposis, flat mucosa between tumors, and adenomatous and villous polyps, as well as colonic neoplasms in humans and mice (2, 19).

Two other factors, diet and microflora, are integrally related since one modifies the other. There is strong evidence that bacteria metabolize bile acids, bile salts, and other chemical compounds (including DMH) into carcino- genetic or cocarcinogenic products that act on the intestinal epithelium (14, 27). These factors also have an important effect on mucosal proliferative kinetics. The intestinal epithelial replacement rate is modified by gut microflora. The sluggish cell turnover rate of neonates increases with the establishment of gut microflora, but it remains sluggish if the animal is maintained germ free (23). The lower incidence of DMH tumors in germ-free versus conventional rats, which has been attributed to the bacterial deconjugation of carcinogens (28), could also be explained by direct microfloral effects on mucosal proliferation rates. Diet significantly affects colon crypt cell column heights in mice exposed to C. freundii as well as in normal, unexposed mice (3).

The genetic association to human large bowel cancer has been reviewed (4). A genetic factor is also involved in colon carcinogenesis with DMH. Certain inbred strains of mice (3). The genetic association to human large bowel cancer has been reviewed (4). A genetic factor is also involved in colon carcinogenesis with DMH. Certain inbred strains of mice (3).

REFERENCES

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Fig. 1. Ascending colon, normal mouse. Note the prominent mucosal ridges that remain when the colon is opened and spread. Goblet cells are prominent at the base of the crypts. H & E, × 32.

Fig. 2. Ascending colon, normal mouse. Higher magnification of mucosa is shown in Fig. 1. H & E, × 95.

Fig. 3. Transverse colon, normal mouse. Crypts are highest in this segment of colon, and goblet cells are evenly distributed along the crypt columns. H & E, × 95.

Fig. 4. Descending colon, normal mouse. Crypts are shorter and goblet cells are fewer than in the transverse colon. Goblet cells are located in the top half of the crypts. H & E, × 95.

Fig. 5. Grade 1 DMH-induced neoplastic lesion, transverse colon. The mucosa contains a nodule of epithelial cell proliferation apparently arising from a single crypt. Cells lack mucin droplets and are crowded and pseudostratified. H & E, × 95.

Fig. 6. Grade 2 DMH-induced neoplastic lesion, descending colon. The nodular growth within the superficial mucosa is composed of several adjacent abnormal crypts. H & E, × 95.

Fig. 7. Grade 3 DMH-induced neoplastic lesion, descending colon. The nodule encompasses the entire width of the mucosa, abuts the muscularis mucosa, and protrudes into the colonic lumen. H & E, × 95.

Fig. 8. Grade 4 DMH-induced neoplastic lesion, descending colon. The base of the neoplasm within the mucosa has infiltrating glandular cords extending downward through the muscularis mucosa into the submucosa. H & E, × 95.

Fig. 9. Cross-section of the descending colon from a normal mouse. H & E, × 12.5.

Fig. 10. Cross-section of the descending colon from a mouse with well-developed TMCH. The diameter is increased due to marked mucosal epithelial hyperplasia, inflammation, and muscular contraction. H & E, × 12.5.

Fig. 11. Descending colon from a mouse 1 month after C. freundii inoculation and onset of weekly DMH treatments (Group E). The mucosa is mildly and diffusely hyperplastic with 2 focal DMH neoplastic lesions superimposed. H & E, × 95.
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