17. The animal was transferred in its home cage to a different cabinet and exposed to a saturating light pulse that consisted of a 6-hour exposure to 36-W 4-foot fluorescent "cool white" (General Electric) light 25 cm above the cage lid (300 to 350 lux). The exposure time was chosen to achieve the maximal responses to light pulses.
18. Period in DD was measured by a ψ^2 periodogram [P. G. Sokolova and W. N. Bushel, *Fed. Proc.* **38**, 2589 (1978)] from 10 consecutive days when no manipulations were performed: days 5 to 15 in DD and several weeks later after three light pulses had been given. Data were analyzed by a Generalized Model (GLM) analysis of variance (ANOVA) by using NCSS, with Scheffe's post-hoc tests for pairwise comparisons.
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Induction and Evasion of Host Defenses by Type 1–Piliated Uropathogenic *Escherichia coli*

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Virtually all uropathogenic strains of *Escherichia coli* encode filamentous surface adhesive organelles called type 1 pili. High-resolution electron microscopy of infected mouse bladders revealed that type 1 pilus tips interacted directly with the lumenal surface of the bladder, which is embedded with hexagonal arrays of integral membrane glycoproteins known as uroplakins. Attached pili were shortened and facilitated intimate contact of the bacteria with the uroplakin-coated host cells. Bacterial attachment resulted in exfoliation of host bladder epithelial cells as part of an innate host defense system. Exfoliation occurred through a rapid apoptosis-like mechanism involving caspase activation and host DNA fragmentation. Bacteria resisted clearance in the face of host defenses within the bladder by invading into the epithelium.

Urinary tract infections (UTIs) are among the most common infectious diseases acquired by humans, affecting over 7 million people annually in the United States alone and accounting for substantial morbidity (*1*). *Escherichia coli*, the primary causative agent of UTIs, including cystitis, encodes surface adhesive organelles called type 1 pili (*1*, *2*). These fibrous extensions consist of a 7-nm-thick helical rod composed of repeating FimA subunits joined to a 3-nm-wide distal tip structure containing the adhesin FimH (*3*). Binding of the FimH adhesin to mannosylated host receptors present on the bladder epithelium (urothelium) is critical to the ability of *E. coli* to colonize the bladder and cause cystitis (*2*, *4*, *5*). The lumenal surface of the bladder is lined by a layer of superficial umbrella cells that deposit on their apical surfaces a quasi-crystalline array of hexagonal complexes made up of four integral membrane glycoproteins known as uroplakins (*6*). In vitro binding assays have shown that two of the uroplakins, UPIa and UPIb, can specifically bind to *E. coli* expressing type 1 pili (*7*).

Scanning and high-resolution transmission electron microscopy (EM) techniques were used in a murine cystitis model to investigate the structural basis and consequences of the in vivo interactions between type 1–piliated *E. coli* and the uroplakin-coated host superficial bladder cells. C57BL/6 mice were infected by transurethral inoculation with the *E. coli* cystitis isolate NU14 grown in static broth (*8*), a growth condition that specifically induces the expression of type 1 pili by this strain. The type 1 pili produced by NU14 have been well characterized (*2*, *5*, *9*). Two hours after infection, mouse bladders were removed and processed for EM (*10*). As determined by scanning EM (SEM), numerous bacteria adhered randomly across the bladder lumenal surface, both singly and in large, biofilmlike microcolonies, some of which contained several hundred bacteria (Fig. 1A). The bacteria were often situated in grooves and niches formed by the apical membrane of the superficial cells (Fig. 1B). This uroplakin-embedded membrane has been termed the asymmetric unit membrane (AUM) because of its appearance in cross section (*6*). In contrast to NU14-infected bladders, almost no bacteria were found attached to bladders taken from mice infected with the isogenic *fimH*$^-$ mutant NU14-1 (*2*, *11*), confirming the role of the FimH adhesin in colonization of the bladder.

Examination of infected mouse bladders with high-resolution, freeze-fracture, deep-etch EM revealed the tips of type 1 pili making direct contact with the AUM (Fig. 1, C to H) (*12*). Type 1 pili spanned the distance between the bacterial outer membrane and the AUM directly, with seemingly little slack along their lengths (Fig. 1H). The molecular architecture of these pili was identical to the type 1 pili present on the inoculated NU14 bacteria (*3*, *9*), except that they were shorter. Of 70 pili measured from 23 individual bacteria, the average pilus length from the bacterial outer surface to the host cell membrane was 0.12 ± 0.07 μm. In contrast, type 1 pili present on bacteria in broth culture are typi-

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cally 1 to 2 μm long (*13*) but can vary from a few fractions of a micrometer to greater than 5 μm in length. It has been proposed that some types of pili expressed by *E. coli* and other species are able to retract (*14*). Thus, it is possible that the shorter type 1 pili observed in the electron micrographs are the result of pilus retraction, which could potentially pull the bacterium closer to the AUM after attachment to host receptors. In addition, contact of type 1 pilus tips with the AUM would potentially impede the growth of any nascent pili (*15*), and this could also account for some of the shortened pili observed. Either hindrance of pilus growth or a pilus retraction mechanism could result in a buildup of unassembled pilin subunits in the periplasm, a condition that is known to activate signal transduction cascades regulating gene expression (*16*). This could provide a means for the infecting bacteria to sense attachment and facilitate their survival.

At higher magnifications, the hexagonal array of uroplakin complexes embedded within the AUM was distinguishable (Fig. 1, E and G). FimH-containing tips were identified (Fig. 1E, arrow) and often appeared to be buried among the uroplakin complexes (Fig. 1G). Each hexagonal complex of uroplakins has a 3.7-nm-wide central cavity that is proposed, on the basis of cross-linking studies, to be lined by one or the other of the putative FimH receptors UP1a or UP1b (*17*). These cavities could conceivably accommodate the 3-nm-wide adhesive tips of type 1 pili during bacterial attachment. In some cases, the AUM appeared to be "zippering" around and enveloping the attached bacteria (Fig. 1F). Superficial bladder cells can internalize uropathogenic strains of *E. coli* (*18*), and the image shown in Fig. 1F may represent an initial step in this process.

One consequence of bacterial colonization of the bladder is the exfoliation and excretion of infected and damaged superficial cells (*18*, *19*). This process of cell elimination is proposed to be an innate host defense mechanism of the urinary tract. Terminally differentiated superficial cells have distinctive pentagonal or hexagonal outlines and a characteristic pattern of ridges on their apical surface and are among the largest cells found in mammals (Fig. 2A) (*20*). Two hours after inoculation with NU14, the bladder epithelium appeared mostly intact, with only a few areas showing signs of exfoliation (Fig. 2B). Six hours after NU14 inoculation, massive exfoliation of the superficial cells had occurred, exposing the underlying, less differentiated urothelial cells (Fig. 2C). Occasionally, regenerating host cells were seen advancing across ulcerated areas where the basement membrane had been exposed as a consequence of infection (Fig. 2D). Such ulcerated areas could also be detected at 12, 24, and 48 hours after infection. However, at these later time points, urothelial cells that were more rounded and substantially smaller than the terminally differentiated superficial cells covered most of the lumenal surface of the bladder (Fig. 2E). In many instances, the small exposed cells appeared to be differentiating, obtaining the outline, flattened profile, and ridged surface pattern associated with mature superficial cells (Fig. 2, E and F). The recombinant strain AAEC185/pSH2 (type 1^+), which is derived from a laboratory K-12 strain MM294 (*21*), also caused massive exfoliation of urothelial cells within 6 hours after inoculation (*11*). In contrast to NU14 and AAEC185/pSH2, the *fimH*$^-$ mutants NU14-1 and AAEC185/pUT2002 caused no obvious alteration of the bladder urothelium. In addition, AAEC185 expressing P pili (*21*), which are adhesive organelles more closely associated with upper UTIs, caused no exfoliation of the bladder epithelium. These results indicate that bacterial attachment mediated by the type 1 pilus FimH adhesin is a critical step leading to exfoliation

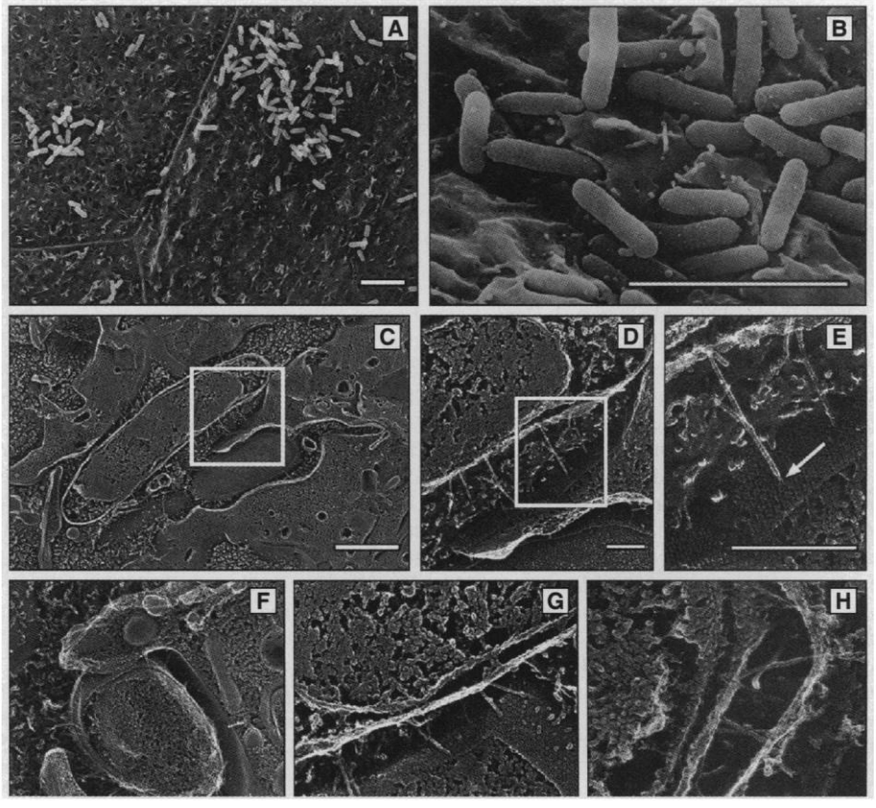

Fig. 1. Type 1 pilus-mediated bacterial adherence to the bladder epithelium. Mouse bladders were processed for (**A** and **B**) SEM (*10*) or for (**C** to **H**) high-resolution EM (*12*) at 2 hours after infection with NU14. The boxed areas in (C) and (D) are shown magnified in (D) and (E), respectively. The arrow in (E) indicates a FimH-containing tip. In (H), type 1 pili span from the host cell membrane on the right to the bacterium on the left. Scale bars indicate 5 μm (A and B), 0.5 μm (C and F), and 0.1 μm (D, E, G, and H).

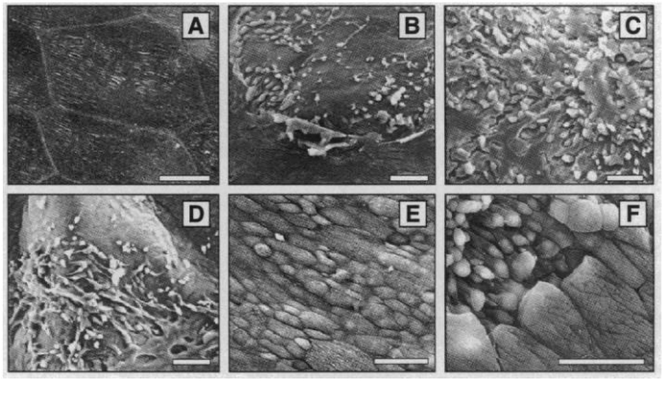

Fig. 2. Progressive exfoliation and regeneration of the urothelium after FimH-mediated bacterial attachment. SEM images were taken of the apical surface of bladders at 48 hours after inoculation with PBS (mock infected) (**A**) and at 2 (**B**), 6 (**C** and **D**), 12, 24, and 48 hours (**E** and **F**) after inoculation with NU14 (*8*, *10*). Scale bars, 50 μm.

of bladder cells during UTIs.

Host eukaryotic cells may undergo apoptosis as a means of hindering the progression of an invading pathogen (*22*). To test the hypothesis that bladder cell exfoliation might be part of a bacterially induced apoptotic mechanism, we performed terminal deoxytransferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays with frozen infected mouse bladder sections to probe for fragmentation of host bladder cell DNA, a hallmark of apoptosis (*23*) (Fig. 3, A to F). Two hours after infection with NU14, many of the epithelial cells lining the lumenal surface of the bladder reacted positively in TUNEL assays (Fig. 3, B and C), in contrast to mock-infected controls (Fig. 3A). At this time point, only a small amount of exfoliation is evident, as determined by SEM, suggesting that mechanisms resulting in DNA fragmentation are initiated before or concurrent with exfoliation. Like NU14, the type 1–piliated recombinant strain AAEC185/pSH2 also induced fragmentation of host cell DNA within 2 hours after infection (Fig. 3E), whereas the *fimH*$^-$ mutants NU14-1 and AAEC185/pUT2002 did not (Fig. 3, D and F). The speed with which DNA fragmentation occurred after inoculation suggests that urothelial cells may be sensitized to undergo rapid programmed cell death in response to infection by type 1–piliated pathogens.

Accumulating evidence indicates that many forms of programmed cell death require the activation of proteolytic enzymes known as caspases (cysteine-containing aspartate-specific proteases) (*23*). We found that treatment of mice with the cell-permeable pan-caspase inhibitor Boc-aspartyl(OMe)-fluormethylketone (BAF) inhibited exfoliation of urothelial cells in response to infection (*24*). At 6 hours after infection, the bladder epithelium from BAF-treated mice remained mostly intact, as determined by SEM, with superficial umbrella cells covering most of the lumenal surface (*11*), in contrast to controls (similar to Fig. 2, C and D). Massive exfoliation of superficial cells eventually occurred in the BAF-treated mice by 24 hours after infection (*11*). In addition, at 12 hours after infection with NU14, control mouse bladders had an average of 85% fewer bacteria in comparison with BAF-treated mice (Fig. 3G). These results support a role for exfoliation in clearance of bacteria and further implicate a caspase-mediated apoptosis-like mechanism in the shedding of urothelial cells in response to bladder infections.

Increased exfoliation of the bladder epithelium correlated temporally with an initial 16-fold decrease (an average 94% reduction) in bacterial titers between 2 and 6 hours after infection with NU14 (Figs. 2 and 4A) (*25*). However, despite bladder host defense mechanisms, substantial numbers of bacteria persisted within the bladder up to 48 hours after infection (Fig. 4A), although few, if any, bacteria were detected by SEM on the bladder surface at or beyond 6 hours after infection. SEM of bladders at 2 hours after infection showed signs of bacterial invasion. The AUM enveloped some bacteria (Fig. 4, B to E), perhaps through contact points with type 1 pili as seen in Fig. 1F. These bacteria were often situated at the center of distinct star-shaped distortions formed by the AUM (Fig. 4B), suggesting substantial cytoskeletal alterations in areas surrounding bacterial internalization. In in vitro assays, we have found that FimH$^+$, but not FimH$^-$, type 1–piliated bacteria can invade and survive within cultured human bladder carcinoma epithelial cells (*11*). To address whether invasion of the urothelium is advantageous to the survival of NU14 in whole bladders, we determined the number of intracellular bacteria at various time points after infection by incubating bladders ex vivo with the host membrane–impermeable antibiotic gentamicin so as to kill all extracellular bacteria (*25*). The number of bacteria protected from gentamicin killing amounted to less than 0.5% of the total number of bacteria within the bladder at 2 hours after infection (Fig. 4A). However, by 48 hours after infection, the number of gentamicin-protected bacteria often accounted for the vast majority of the total number of bacteria remaining in the bladder.

Taken together, these data suggest that type 1–piliated organisms induce programmed cell death and exfoliation of bladder epithelial cells but that they can resist this innate host defense mechanism by invading into deeper tissue. This may account for the high amount of disease recurrence, despite antibiotic treatment, in many patients with UTIs. Recurrent UTIs are a common problem, frequently affecting women with anatomically and functionally normal urinary tracts (*1*). Studies have shown that 32 to 68% of recurrent UTIs are caused by uropatho-

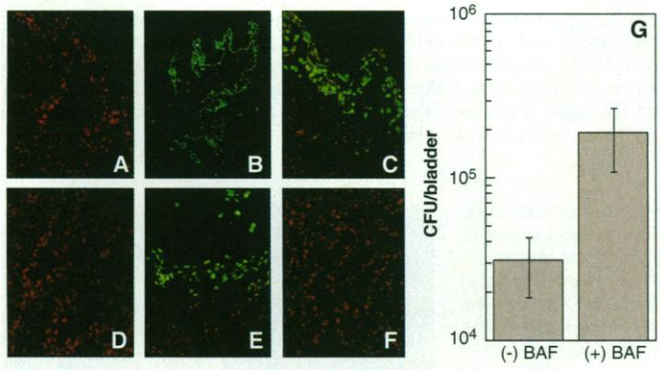

Fig. 3. Bacterially induced exfoliation of urothelial cells occurs through an apoptosis-like mechanism. Mice were mock infected with (**A**) 50 μl of PBS or infected with (**B** and **C**) NU14, (**D**) NU14-1 (type 1$^+$/*fimH*$^-$), (**E**) AAEC185/pSH2 (type 1$^+$), or (**F**) AAEC185/pUT2002 (type 1$^+$/*fimH*$^-$) (*8*). At 2 hours after inoculation, bladders were removed and frozen in Tissue-Tek O.C.T. embedding medium. Five-micrometer-thick frozen cross sections were stained with the Apoptag Fluorescein Kit (Intergen, Purchase, NY), counterstained with propidium iodide, and observed by immunofluorescence microscopy with a ×4 (B) or ×20 (A and C to F) objective lens. Random sections from two or more mice were examined qualitatively for each type of sample. (**G**) Inhibition of bacterial clearance from bladders by BAF treatment. Groups of four mice were inoculated with NU14 with or without administration of BAF (*24*). The mean titers (±SD) recovered from BAF-treated versus control bladders at 12 hours after infection are shown.

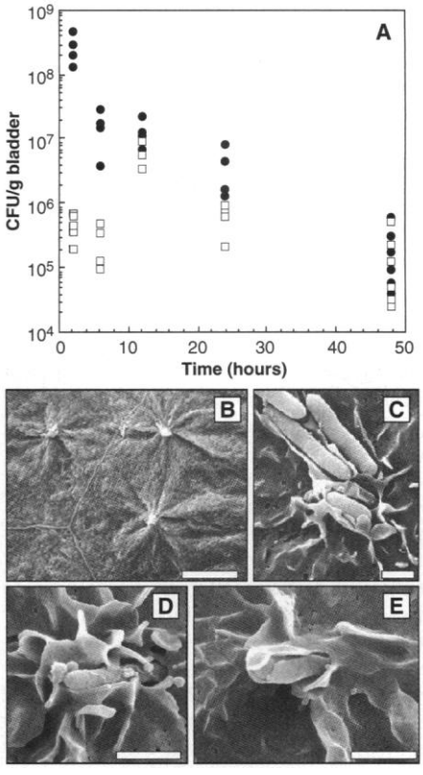

Fig. 4. Kinetics of bacterial reduction in NU14-infected bladders and the persistence of intracellular bacteria. (**A**) The total numbers of bacteria per gram of mouse bladder (black circles) and the numbers of gentamicin-protected bacteria (open boxes) were determined at various times after infection (*25*). At least four bladders were examined per time point. (**B** to **E**) Bacteria in various stages of internalization into superficial cells at 2 hours after infection with NU14 were detected by SEM. Scale bars indicate 10 μm (B) and 1 μm (C to E).

genic strains previously identified in the patient (*26*). The ability of type 1–piliated uropathogens to invade the urothelium suggests that recurrent UTIs may, in some cases, be a manifestation of a lingering chronic infection and not necessarily a reinoculation of the urinary tract.

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8. NU14 and recombinant bacteria were grown in static Luria-Bertani broth at 37°C for 48 hours to induce expression of type 1 pili (*2*). Expression was verified by mannose-sensitive agglutination of a 3% solution of guinea pig erythrocytes, by EM, and by immunogold labeling. Ten- to fifteen-week-old female C57BL/6 mice were anesthetized with methoxyfluorane and inoculated transurethrally with 50 μl of bacterial suspension (~1×10^8 colony-forming units) in phosphate-buffered saline (PBS) (*2*). Mice were killed, and their bladders were aseptically removed at the indicated time points.
9. NU14 type 1 pili were purified and characterized biochemically, genetically [S. J. Hultgren, J. L. Duncan, A. J. Schaeffer, S. K. Amundsen, *Mol. Microbiol.* **4**, 1311 (1990)], by immunogold EM, and by high-resolution EM.
10. Bladders were bisected, splayed, and pinned down lumenal sides up under NHC buffer [100 mM NaCl, 30 mM Hepes (pH 7.4), and 2 mM $CaCl_2$]. After rinsing gently, bladders were fixed in 2% glutaraldehyde in NHC for 1 to 2 hours at room temperature. For SEM, bladders were postfixed with 1% OsO_4/NHC, rinsed, dehydrated in ascending concentrations of ethyl alcohol, critical point dried from liquid CO_2, coated with ~150 Å of gold, and examined with a Hitachi S-4500 FEG Scanning Electron Microscope. Two or more bladders were examined for each type of sample.
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25. Bladders were recovered and bisected at 2, 6, 12, 24, and 48 hours after infection (*8*). Each bladder half was splayed under warm Ringer solution [155 mM NaCl, 3 mM HCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 10 mM glucose, and 5 mM Hepes (pH 7.4)] and gently rinsed. One-half of each bladder was incubated with, and one half without, Ringer solution supplemented with gentamicin (100 μg/ml) for 90 min at room temperature. This incubation with gentamicin was sufficient to kill any external bacteria. Bladder halves were washed with PBS, weighed, and homogenized in 1 ml of 0.025% Triton X-100/PBS, and surviving bacteria were plated (*2*).
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Requirement for p53 and p21 to Sustain G_2 Arrest After DNA Damage

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After DNA damage, many cells appear to enter a sustained arrest in the G_2 phase of the cell cycle. It is shown here that this arrest could be sustained only when p53 was present in the cell and capable of transcriptionally activating the cyclin-dependent kinase inhibitor p21. After disruption of either the p53 or the p21 gene, γ radiated cells progressed into mitosis and exhibited a G_2 DNA content only because of a failure of cytokinesis. Thus, p53 and p21 appear to be essential for maintaining the G_2 checkpoint in human cells.

DNA damaging agents in the form of γ radiation and chemotherapeutic drugs are the mainstays of most current cancer treatment regimens. This has stimulated much research to understand the cellular responses to DNA damage. After DNA damage, cells arrest at the transition from G_1 to S phase (G_1-S) or from G_2 to M phase (G_2-M) of the cell cycle, with DNA complements of $2n$ or $4n$, respectively (*1*). Arrest at these checkpoints prevents DNA replication and mitosis in the presence of unrepaired chromosomal alterations. The proportion of cells that arrest at G_1-S or G_2-M depends on cell type, growth conditions, and the checkpoint controls operative in the cell (*2*). The G_1-S arrest results, at least in part, from p53-regulated synthesis of the cell cycle inhibitor $p21^{WAF1/CIP1}$ (*3–5*), which leads to inhibition of the cyclin-cdk complexes required for the transition from G_1 to S phase. However, arrest in G_2 after DNA damage occurs in both murine and human cells in the absence of p21 or p53 (*3–6*). This arrest is thought to result from activation of a protein kinase, Chk1, that phosphorylates and inhibits the function of the protein phosphatase Cdc25C (*7*). Inhibition of Cdc25C prevents the removal of inhibitory phosphates from Cdc2, a protein kinase that complexes with mitotic cyclins and is required for mitotic entry (*8*).

The integrity of the Chk1-Cdc25C-Cdc2 pathway in p21- or p53-mutant cells would appear to explain the prolonged G_2-M arrest that occurs in such cells (*3–7*). However,

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