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Levodopa Induces Apoptosis in Cultured Neuronal Cells—A Possible Accelerator of Nigrostriatal Degeneration in Parkinson's Disease?

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Summary: Apoptosis is an active, intrinsic cell suicide program. We recently suggested that it may have a role in the death of nigrostriatal dopaminergic neurons in Parkinson's disease (PD). We now report that levodopa, the current major therapy for PD, is a potent inducer of apoptosis in cultured postmitotic chick sympathetic neurons. Levodopa, in a concentration range of 0.01–0.3 m*M*, caused the characteristic apoptotic cascade of cell shrinkage, massive membrane blebbing, and nuclear fragmentation, as evident by nuclear flow cytometry and fluorescence microscopy. Levodopa-induced apoptosis was inhibited by antioxidants, indicating that it may be mediated by autooxidation-reactive species. Levodopa treatment for PD may therefore constitute an additional challenge for the defective apoptosis-inhibiting systems in the nigrostriatal neurons. Despite reassuring data from some, but not all, previous studies, these findings suggest that the possible in vivo toxic effects of levodopa on the survival of the remaining nigral neurons should be further explored. **Key Words:** Levodopa—Apoptosis—Oxidative stress—Parkinson's disease—Toxicity.

Enzymatic or autooxidation of levodopa can generate a variety of toxic free radical species including superoxide, H_2O_2, semiquinones, and quinones (1,2). Levodopa is toxic to neuronal as well as nonneuronal cell cultures in vitro (3–8). Therefore, although levodopa remains the most effective and widely used treatment for Parkinson's disease, there has been a persistent concern about its potential toxicity to the remaining dopaminergic nigrostriatal neurons in these patients (9). Several in vivo studies using long-term administration of large levodopa doses to rodents (10–13) and clinical investigations in chronically treated patients (14) did not show damage to nigrostriatal neurons or acceleration of the illness, respectively. By contrast, Blunt et al. (15) recently demonstrated that chronic levodopa had a further destructive effect on rat ventral tegmental dopaminergic neurons surviving previous exposure to 6-hydroxydopamine. Consequently, it is still not entirely ruled out that additional factors, including the basic unknown cause of Parkinson's disease, may make the surviving nigrostriatal neurons more vulnerable to the prolonged bombardment with exogenous levodopa.

Levodopa may exert its toxic effects by inducing cell membrane lipid peroxidation and rupture (16) and inhibition of mitochondrial electron-transport chains (17). We recently showed that dopamine, the endogenous neurotransmitter within nigrostriatal neurons, can trigger apoptosis or programmed cell death in several cell cultures, including postmitotic sympathetic neurons, thymocytes, and PC-12 cells (18,19). Dopamine-induced programmed cell death could be blocked by treatment with antioxidants and by vector-driven expression of the apoptosis-inhibiting protooncogene *bcl*-2 (18,19). We hypothesized that inappropriate activation of the intrinsic, dormant, genetically controlled cell-suicide program in nigral neurons by "unrestrained" dopamine and its toxic oxidation products may play a role in the pathogenesis of Parkinson's disease (18). We therefore examined whether exposure to levodopa can also trigger apoptosis in cultured chick sympathetic neurons.

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METHODS

Cell Culture and Treatments

Paravertebral sympathetic ganglia of chick embryo at embryonic day 9 (E9) were dissected out, and cells were dissociated by trypsinization (0.25% in EDTA for 30 min at 37°C). Cells were then plated on coverslips, fitted within polylysine-coated 24-well tissue-culture plates (Corning Ltd; cell density of 3×10^6 cells/well), and grown with serum-free medium DCCM-1, containing 0.5% horse serum, 20 μg/ml fluorodeoxyuridine (FDU) and 50 μg/ml uridine (to kill nonneuronal dividing cells), 2 m*M* glutamine, 10 ng/ml nerve growth factor (NGF), streptomycin, penicillin, and amphotericin-B. On the day 4 in culture, cells were treated with levodopa (0.01–1 m*M*) for 24 h. Plates maintained in the same conditions, but without exposure to levodopa, served as controls.

Actinomycin-D (AMD; 0.1 μg/ml) and cycloheximide (1 μg/ml) were used to assess effect of inhibition of RNA and protein synthesis, respectively, on the observed levodopa-induced death process. These agents in these doses have been shown to inhibit protein synthesis, and consequently, also to inhibit apoptosis induced by NGF deprivation in a similar model of cultured sympathetic neurons (20).

Effect of antioxidant treatment was tested by adding dithiothreitol (DTT, 0.5 m*M*) or *N*-acetyl-cysteine (NAC, 5 m*M*). Exposure to anti-NGF antibody (0.01%, 48 h) was used for comparison as a well-established model for apoptosis in sympathetic neurons. Reagents were all purchased from Sigma, Tel-Aviv, Israel.

Assessment of Cell Survival

The trypan-blue exclusion test is a widely used assay for cell survival, including that in neuronal cultures (21,22). Cells were incubated for 10 min in room temperature with trypan blue (0.1%) in phosphate-buffered saline (PBS) and then washed twice with PBS. Three randomly chosen fields, each containing ~500 cells, were then analyzed by phase-contrast and brightfield microscopy. The live, trypan-excluding (unstained), and dead (stained) cells were counted, and percentage of surviving cells was calculated by the formula:

$$\text{Cell survival (\%)} = \text{Number of unstained cells/Number of stained + unstained cells} \quad (1)$$

Mean and SD were calculated, and dose/viability and time/course of levodopa effect were characterized.

Characterization of the Mode of Cell Death as Apoptosis

Nuclear Flow Cytometry

Nuclear fragmentation, characteristic of apoptosis, was evaluated by flow-cytometric analysis of purified, propidium-iodide (PI)-stained cell nuclei. Nuclear purification and staining were performed by the method of Vindelov et al. (23). Flow cytometry was then performed on a FACStar (Becton Dickinson, Mountain View, CA) by using an argon ion laser (Coherent, Palo Alto, CA) adjusted to an excitation wavelength of 488 nm. FACStar software was used for data acquisition. Forward light scattering (FSC) and fluorescence signals (FL_2) were measured and stored in listmode data files; each measurement contained 10^4 cells, and flow rate was 60 events/s. Apoptotic nuclear changes were evaluated according to the criteria of Dive et al. (24) and Nicoletti et al. (25) [i.e., the evolution of a subdiploid, "apoptotic" peak on the FL_2 scale (reflecting emergence of nuclear particles with reduced DNA content)].

Fluorescence Microscopy of DAPI (4,6-diamidino-2-phenylindol)-stained Cell Nuclei

Cells were washed with PBS pH 7.4 and fixed for 10 min in 4% formaldehyde (in PBS). Neurons were then washed with PBS and stained for 5 min with DAPI. A drop of glycerol was then added to each well, and nuclei were observed and photographed in UV light by inverted microscope (×400, Nikon Diaphot).

Scanning Electron Microscopy (SEM)

Cells were plated on poly-L-lysine 13-mm coated Thermanox disks, fixed overnight with 2.5% glutaraldehyde in PBS pH 7.3, and dehydrated in graded alcohol (25–100%). Alcohol was thereafter replaced with CO_2, and cells were dried to their critical point of drying (CPD) and then coated with gold and examined with Jeol 840A scanning electron microscope. Cell structure was examined for the characteristic apoptotic alterations (i.e., cell shrinkage and extensive membrane blebbing). Results were replicated in three independent sets of experiments.

RESULTS

Levodopa was markedly toxic to the neuronal cells. Dose/viability curve, as assessed by the trypan-blue exclusion test after exposure to levodopa for 24 h (Fig. 1), revealed an LD_{50} (lethal dose for 50% of cells) of 50 μ*M*. However, this effect was not immediate; as shown in Fig. 2, $t_{1/2}$ (time required to kill 50% of cells) after exposure to 300 μ*M* of levodopa was 13 h, and ~20 h was required for this toxic effect to be expressed fully. Such a time course is frequently seen in apoptotic processes.

Typical apoptotic morphologic alterations were observed in the SEM studies after exposure to levodopa (Fig. 3). Cells underwent shrinkage and massive mem-

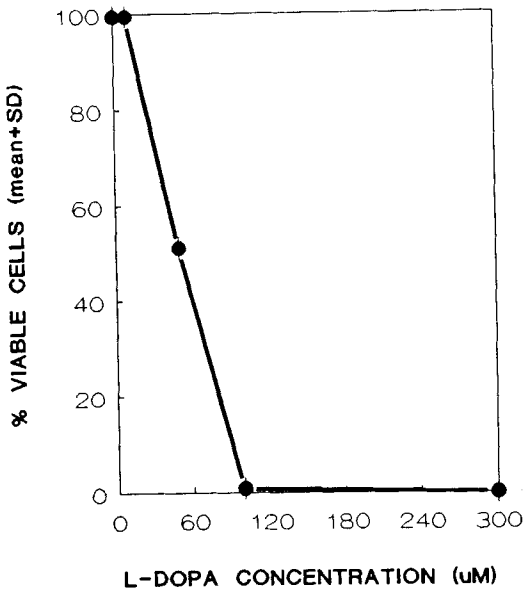

FIG. 1. Effect of levodopa on survival of cultured, postmitotic chick sympathetic neurons (measured by trypan-blue exclusion test). Dose–effect curve, 24-h exposure. Co, control; LD, levodopa (mean ± SD).

brane blebbing. Normal healthy cells (Fig. 3A) were actually converted to clusters of apoptotic bodies (Fig. 3B).

Fluorescence microscopy of DAPI-stained cell nuclei (Fig. 4) revealed the emergence, after treatment with levodopa, of condensed and fragmented nuclear particles, characteristic of apoptosis. These nuclear alterations were further corroborated by the flow-cytometric analysis of purified, PI-stained cell nuclei (Fig. 5), in which levodopa treatment caused the emergence of a distinct, subdiploid peak, reflecting a population of nuclear particles with reduced DNA content, typical of an apoptotic process (13,14). Messenger RNA (mRNA) and protein-synthesis inhibitors did not affect levodopa toxicity. In contrast, both antioxidants DTT and NAC markedly inhibited this levodopa-induced death process (Fig. 6).

DISCUSSION

This study showed that levodopa toxicity to cultured, postmitotic chick sympathetic neurons is mediated by the induction of apoptosis. Cells exposed to levodopa manifested the apoptotic hallmarks (26) of cellular shrinkage, membrane blebbing, and nuclear fragmentation. In accordance with the recent report by Walkinshaw and Waters (27), levodopa-induced apoptosis could be blocked by concomitant treatment with thiol-containing antioxidants, suggesting a role for oxygen free radicals in its execution.

In this study, we used only one concentration of each antioxidant (DTT, 0.5 m*M*, or NAC, 5 m*M*). It was performed to test the possible role of oxidative metabolites of levodopa in its apoptosis-triggering effect, at the conceptual level, and in a qualitative manner. However, we later confirmed and expanded this observation, comparing various types of antioxidants in a wide dose range (D. Offen et al., unpublished observations, 1996).

We did not find levodopa-triggered apoptosis to require de novo protein synthesis. Contrary to previous concepts, apoptosis is not necessarily prevented by protein-synthesis inhibitors. Moreover, in some models, these agents may even induce the death program (28,29). This differential response may indicate the existence of several pathways of apoptotic death, some of which do not require additional expression of mediators. Alternatively, protein-synthesis inhibitors may also block the induction of vital cellular protective (e.g., antioxidant) mechanisms, thus augmenting the cytotoxic insult.

The mechanism responsible for activation of apoptosis by levodopa is still unknown. The analogy between this process and our previous finding of apoptosis induced by dopamine (10), including the inhibitory effect of antioxidant treatment, suggests a common pathway, probably mediated by the products of enzymatic or autooxidation or both of these catecholamines. Apoptosis is frequently triggered by DNA damage. Possible genotoxicity of levodopa and its derivates is supported by the recent demonstration of 8-hydroxyguanosine accumulation after exposure to these agents (30). Wick (31) showed that levodopa and dopamine can inhibit several vital enzymatic pathways of DNA repair. DNA damage, leading to initiation of apoptosis, is the mechanism of action of many anticancer drugs (32) and may therefore also explain the antitumor activity of levodopa (7,8). Taken together, these data indicate that in addition to the cell membrane (16) and mitochondria (17), the cell nucleus is also a major scene of events where cellular toxicity of levodopa is exerted.

The main question is whether such apoptosis-triggering effect of levodopa also occurs in vivo and adversely affects the remaining nigrostriatal neurons of levodopa-treated patients with Parkinson's disease. Intriguing are the findings of Yahr (9) and Fahn and Bressman (33), showing that the adverse effects associated with long-term levodopa therapy correlate with length of levodopa treatment rather than with the severity of the disease. However, these are not necessarily caused by accelerated death of the nigrostriatal neurons. Steece-Collier et al. (34) showed that chronic injections of levodopa impaired the development of grafted embryonic dopaminergic neurons in rats. In contrast, Blunt et al. (15) showed that 27-week administration of levodopa to

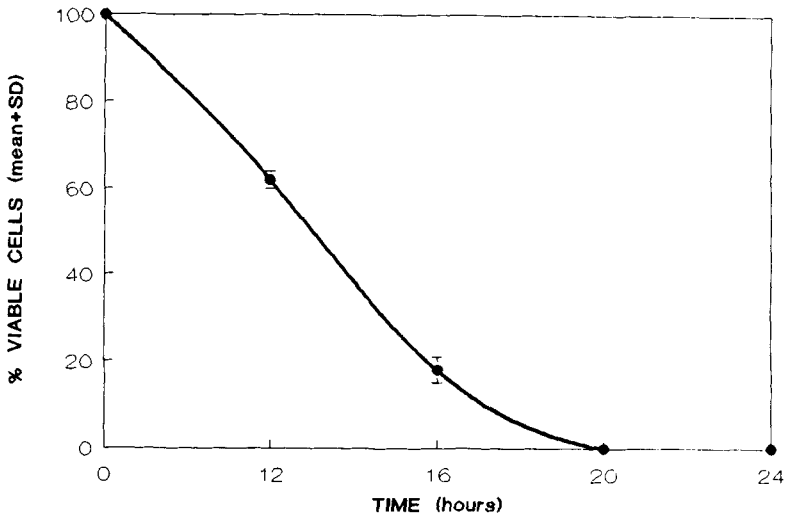

FIG. 2. Effect of levodopa (0.3 m*M*) on survival of cultured, postmitotic chick sympathetic neurons (measured by trypan-blue exclusion test). Time-course curve (mean ± SD).

rats, transplanted with fetal mesencephalic neurons after 6-hydroxydopamine nigrostriatal lesion, did not affect graft survival. We have shown that treatment of pregnant mice with large doses of levodopa did not damage fetal nigrostriatal neurons and did not affect their normal postnatal development (35). In addition, a recent autopsy finding in a patient with parkinsonism indicated that his transplanted fetal nigral graft survived and showed intense growth despite continuous postoperative treatment with levodopa (36).

These findings are in accordance with previous animal studies (10,11) in which chronic levodopa treatment in mice was not associated with damage to nigral neurons and also with the studies of Cotzias et al. (12), who observed beneficial effects of levodopa on the longevity of rodents. Quinn et al. (14) reported preservation of substantia nigra neurons in a nonparkinsonian patient receiving large doses of levodopa for >5 years.

One explanation for this discrepancy between the marked in vitro toxicity of levodopa and its absence in vivo effect might be the level of nigral neuronal exposure to the drug. The LD_{50} of levodopa for cultured sympathetic neurons observed in our study was ~50 μ*M*. The exact levodopa concentrations achieved in brain tissues including the nigra during long-term oral treatment are not known, but drug CSF levels were reported to be 10-fold lower (37). In addition, it is conceivable that potent defense mechanisms, including various antioxidant pathways, which are absent in the tissue-culture model, act to prevent in vivo toxicity of this drug. It is not impossible that long-term administration of levodopa acts to amplify the potency of such protective safeguards.

On the other hand, all of these do not rule out a "susceptibility factor," unique to patients with parkinsonism, that may render them vulnerable to levodopa toxicity. Responsible mechanisms may include enhanced dopamine turnover in the remaining nigrostriatal neurons in patients with PD with enhanced associated oxidative stress or increased nigral iron levels in these patients (16). The importance of such possible susceptibility fac-

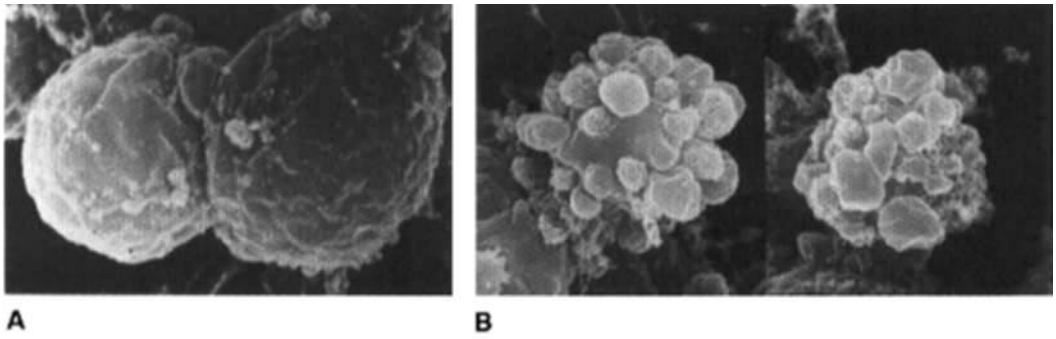

FIG. 3. Scanning electron microscopy: apoptotic morphologic alterations in cultured, postmitotic chick sympathetic neurons, induced by exposure to levodopa. **A:** Control. **B:** After exposure to levodopa (0.3 m*M* for 24 h): cell shrinkage and marked apoptotic membrane blebbing (×6,000).

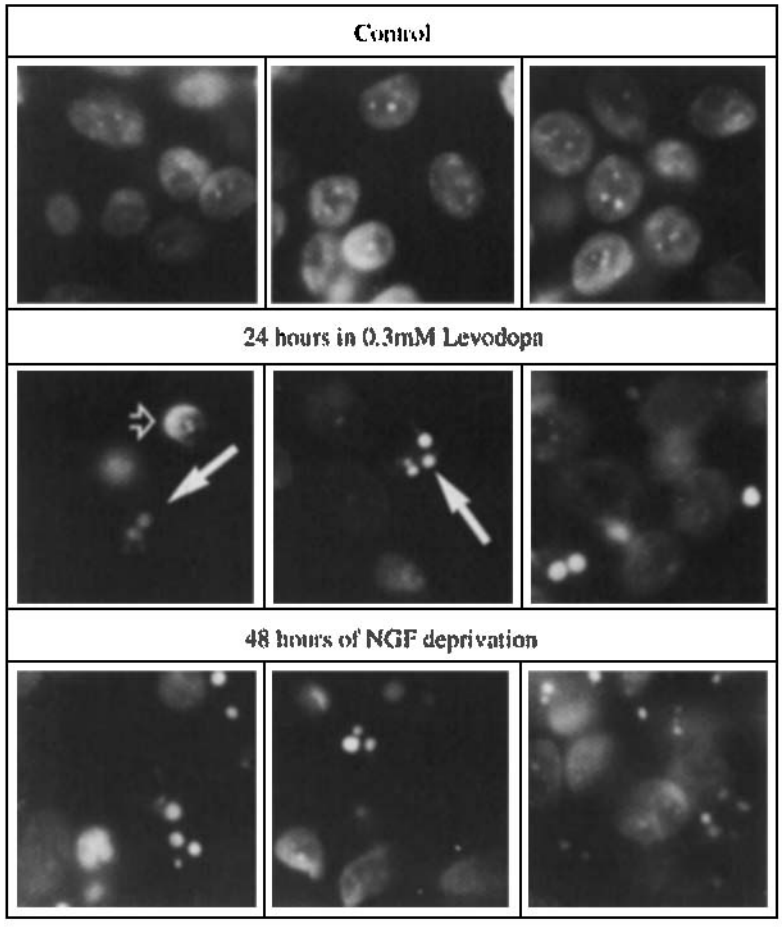

FIG. 4. Levodopa-induced apoptotic nuclear alterations. Fluorescence microscopy of DAPI (4,6-diamidino-2-phenylindol)-stained nuclei of cultured chick sympathetic neurons. **A:** Control cells. **B:** Effect of levodopa treatment (0.3 m*M*, 24 h): nuclear condensation and fragmentation, characteristic of apoptosis. **C:** Effect of nerve-growth factor (NGF) deprivation for 48 h. Levodopa caused chromatin margination (open arrow), condensation, and fragmentation (bold arrows), similar to the effect of NGF deprivation.

tor was exemplified by Blunt et al. (15), who found that previous 6-hydroxydopamine–induced neurotoxic ventral tegmental lesion in rats was associated with enhanced vulnerability to the toxic effect of long-term levodopa treatment. In contrast, we were unable to show damage to nigrostriatal neurons in vivo in mice given long-term levodopa, even if these neurons were rendered susceptible by cotreatment with the neurotoxin MPTP or their firing rates enhanced by coadministration of haloperidol (13). It remains to be investigated whether, if

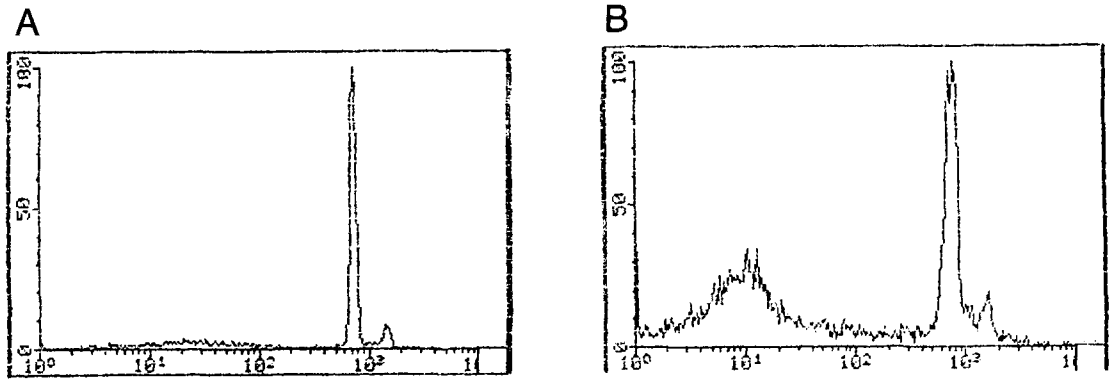

FIG. 5. Levodopa-induced apoptotic nuclear alterations. Flow-cytometric analysis of isolated cell nuclei with propidium-iodide (PI) fluorescence (FL_2). **A:** Control cells. **B:** Levodopa treatment (0.3 m*M*, 24 h). Levodopa caused the emergence of a distinct, subdiploid apoptotic peak, reflecting apoptotic nuclear fragmentation.

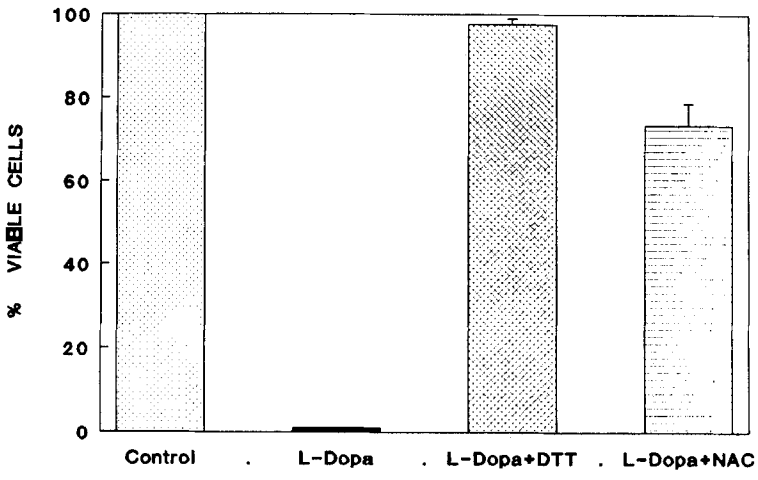

FIG. 6. Inhibition of levodopa (0.3 m*M*, 24 h)-induced death process by concomitant treatment with the antioxidants *N*-acetyl-cysteine (NAC, 5 m*M*) or dithiotreitol (DTT, 0.5 m*M*). Trypan-blue-exclusion survival test (mean ± SD).

there is a defect in nigral apoptosis–control systems in Parkinson's disease, levodopa treatment may constitute an additional challenge for the competence of these mechanisms, further lower the apoptotic threshold, and accelerate the neuronal degenerative process.

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