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Opposing effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and hydrocortisone on growth and differentiation of cultured malignant human keratinocytes

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The human keratinocyte line SCC-13, derived from a squamous cell carcinoma of epidermis, was examined for effects on growth and differentiation upon treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Inhibition of growth was observable at 0.1 nM and maximal in the concentration range of 1 – 100 nM TCDD, but was completely antagonized by addition of hydrocortisone to the growth medium. TCDD was found to inhibit several aspects of keratinocyte differentiation that are stimulated by hydrocortisone. In confluent cultures, accumulation of keratin protein and transglutaminase activity were suppressed as well as spontaneous envelope formation and envelope competence. This phenomenon occurred without significant effect of TCDD on depletion of hydrocortisone from the medium. We conclude that the response of SCC-13 cells to TCDD depends upon hormonal conditions in culture and that this agent can interfere with cellular responses to normal physiological conditions, thereby altering the differentiation program ordinarily observed.

Introduction

Among the halogenated aromatic hydrocarbons in the environment to which humans are exposed at low but potentially significant levels, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)* has received particular attention as a useful model compound. This highly toxic agent, like other polychlorinated polycyclic compounds, produces a variety of effects in target tissues, including chloracne in human skin (1). In rodents, it has been reported to be a potent carcinogen (2) and an extraordinarily effective promoter of rat hepatocellular (3) and mouse skin tumors (4). However, the species specificity of its lethal effects and of the distinctive metaplasia and proliferation occurring in target epithelia has been puzzling. Since TCDD appears poorly metabolized and little evidence of macromolecular adduct formation is demonstrable (5), this agent presumably produces toxicity by altering cellular gene expression (6).

TCDD has been shown to bind reversibly and with high affinity to a soluble cytoplasmic receptor protein (7). The receptor-ligand complex translocates to the cell nucleus (8,9) and, by analogy with steroid receptor action, is believed consequently to induce the coordinate expression of aryl hydrocarbon hydroxylase and a battery of other enzymes primarily involved in drug metabolism. Binding to the receptor is stereospecific for those agents which produce toxic effects (10). The *Ah* genetic locus in the mouse appears responsible for expression of the receptor (11) and thus inducibility of both enzyme activities and toxicity (12,13). However, a

distinct locus, designated *hr*, has been found to control toxic effects without altering the enzyme inducibility (14).

To complement the emerging genetic model of TCDD action, a cell culture model for studying biochemical effects and their mechanisms is highly desirable. Many cell lines have been found in which TCDD induces aryl hydrocarbon hydroxylase but not toxicity (15,16). None of these lines were keratinocytes, however, which comprise the principal cell type of epidermis and thus are likely candidates to model the hyperkeratosis observed in sensitive species including primates. Indeed, the keratinocyte cell line XB derived from a mouse teratoma has been shown to respond with increased stratification when treated for extended periods with TCDD (17). Considerable information has now accumulated on the differentiation program exhibited by cultivated keratinocytes (18), permitting further analysis of TCDD effects.

Several continuous lines of human keratinocytes have recently been derived from squamous cell carcinomas of the face and oral cavity (19), and shown to express TCDD receptor and inducible ethoxycoumarin-O-deethylase activities (20). The present work explores effects of TCDD on growth and differentiation of such cells and the influence of hydrocortisone and retinyl acetate. Hydrocortisone and vitamin A have been shown to influence the differentiation of many epithelia *in vivo* (21,22) as well as keratinocytes in culture (23,24). Indeed, in malignant human keratinocyte lines, the ability of the cells to form cross-linked envelopes at confluence is stimulated by this steroid and antagonized by vitamin A (25,26). These findings suggested that proper evaluation of TCDD toxic effects might depend critically upon culture conditions chosen.

Materials and methods

Cell culture

Keratinocytes were cultivated with support from a feeder layer of lethally irradiated 3T3 cells (19) in Dulbecco-Vogt Eagle's medium supplemented with 5% fetal bovine serum depleted of steroids by charcoal-dextran treatment at 55°C (27). TCDD, hydrocortisone and retinyl acetate were added to cultures one day after inoculation and dimethylsulfoxide (solvent for TCDD and retinyl acetate) in treated and control cultures was 0.05 to 0.1%. Each condition was examined in duplicate or triplicate cultures (of which representative dishes are pictured) in repeated experiments. Measures of relative cell number and viability and colony formation in treated and untreated cultures are presented as mean ± standard deviation (n = 4 or 5). Medium (4 ml/60 mm dish) was changed at 4 day intervals except in experiments employing retinyl acetate (at 2-day intervals). Cultures were fixed in 10% formalin in isotonic

*Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene.

phosphate buffer (0.15 M NaCl − 0.01 M Na_2H PO_4, pH 7.2) and stained with Rhodanile blue (28).

Differentiation markers

To measure envelope competence, confluent cultures were trypsinized, treated with the ionophore X537A (50 μg/ml of serum free medium) for 2 h at 37°C and then with sodium dodecyl sulfate and dithioerythritol (29). Envelopes were counted by phase contrast optics after the cellular contents were dissolved (~ 15 min).

Spontaneously cross-linked envelopes were measured after dissolving the cultures in 2% sodium dodecyl sulfate − 10 mM Tris buffer − 5 mM dithioerythritol. Viscosity from cellular DNA was reduced by mild sonication or passage through a 26 gauge syringe needle and the envelopes were recovered by centrifugation. The pellets were rinsed twice in 0.1% sodium dodecyl sulfate and resuspended in 1 ml of this solution for measurement of light attenuation at 340 nm (29).

For analysis of keratins, cultures were rinsed three times in isotonic saline and extracted three times in 1% Triton X-100 − 10 mM Tris-HCl (pH 7.4) − 1 mM EDTA (30). The pelleted keratins recovered by centrifugation at 8000 g for 2 min were dissolved by boiling in 10 mM Tris-HCl − 2% sodium dodecyl sulfate − 2 mM dithioerythritol. Protein concentrations in the Triton-soluble and keratin fractions were measured with the Folin reagent (31) in the presence of 0.1% sodium dodecyl sulfate and were corrected where appropriate for the low color yield of dithioerythritol.

For measurement of transglutaminase activity (29,32), cultures were vigorously rinsed twice with isotonic phosphate buffer, sonicated in 2 ml of 10 mM Tris-HCl (pH 8.0) − 1 mM EDTA − 0.3% Emulgen 911 nonionic detergent, and centrifuged 2 min at 8000 g at 4°C. Aliquots (5 − 20 μl) of the supernatant extract (1 − 2 mg protein/ml) were assayed in the linear range in 0.25 ml final volumes containing 0.5 mg of dimethyl casein, 0.5 μCi of [^{3}H]-putrescine (125 Ci/mol), 50 μmol of Tris-HCl (pH 8.0), 2.5 μmol of $CaCl_2$, 0.25 μmol of EDTA and 0.4 μmol of dithioerythritol. Protein-bound radioactivity was measured after 20 min incubation at 35°C by precipitation and rinsing with trichloroacetic acid. Specific activities, radioactivity incorporated per unit of extract protein (~ 100 c.p.m./μg in cultures without added hydrocortisone), were used to compare effects of culture conditions.

Corticosteroid chromatography

Confluent cultures were incubated in fresh medium containing [^{3}H]hydrocortisone (0.5 μCi/ml) with added unlabeled hydrocortisone (1 μM) and TCDD (10 nM). After 2 and 4 days, 0.25 ml aliquots of medium were removed in duplicate and each extracted with 2.5 ml of CH_2Cl_2. The aqueous (upper) phase was carefully removed and discarded and 2 ml of the organic phase were dried at 35°C under a stream of nitrogen gas. Recovery of label was 80% (uncorrected for ~ 20% loss in sampling) and was unaffected by adjusting the medium to 0.2 N in NaOH prior to extraction. Samples were redissolved in 0.25 ml of 25% acetonitrile and 25 μl aliquots were submitted to reverse phase liquid chromatography with 25% acetonitrile (33) or 50% methanol (34) as solvents using a 30 cm μBondapak C_{18} column and a Waters 6000A solvent delivery system (88% recovery of label). Corticosteroid markers were detected by light absorption at 254 or 210 nm. When [^{3}H]hydrocortisone was chromatographed in acetonitrile either directly or after 4-day incubation in medium (no cells) with subsequent extraction, a single peak of radioactivity was detected in the elution position of unlabeled hydrocortisone which accounted for 95 − 100% of the applied label.

Sephadex LH-20 chromatography was performed using a 0.9 x 35 cm column maintained in CH_2Cl_2:CH_3OH (96:4). Samples with added internal markers (0.1 − 0.2 mg each) were dried under nitrogen gas and dissolved in this solvent for chromatography. Recovery of label was ~ 95%. The solvent proportions of 98:2 generally employed for corticosteroid separations (35) did not elute 20β-OH-hydrocortisone in a convenient time interval, while the proportions 95:5 did not fully resolve this reduced derivative from hydrocortisone.

Results

Effects on cell growth

Initial experiments showed that TCDD produced a marked inhibition of cell growth in five keratinocyte lines derived from human squamous cell carcinomas. This effect was especially obvious in SCC-13 (Figure 1), SCC-9 and SCC-12F; it was observed (but not as markedly) in SCC-12B and SCC-15 and was not apparent in SCC-4 even with 100 nM TCDD. On the basis of this survey and our previous findings on modulation of differentiated functions by hydrocortisone and retinyl acetate (25,26), SCC-13 was chosen for more
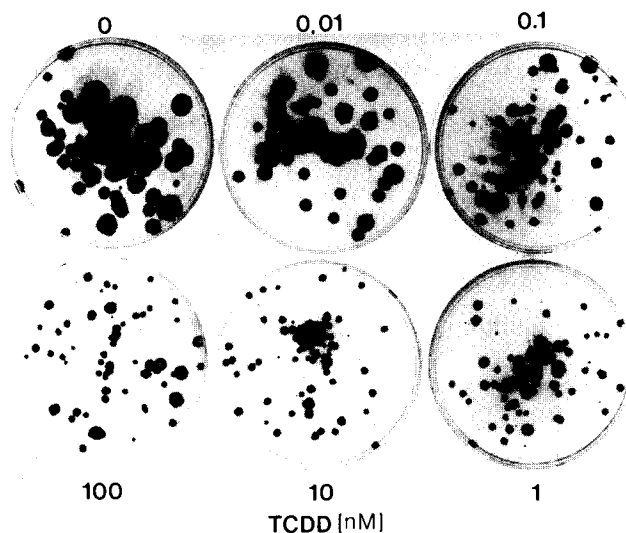

Fig. 1. Inhibition of growth of SCC-13 as a function of TCDD concentration. TCDD was added one day after the cells were inoculated (150 per dish), and the cultures were fixed and stained after 17 days.

detailed examination of TCDD effects on growth and differentiation.

As seen in Figure 1, TCDD visibly reduced colony expansion in SCC-13 at 0.1 nM and produced a maximal effect in the concentration range of 1 − 100 nM. (SCC-12F exhibited a nearly identical response to these concentrations of TCDD but grew relatively poorly in the absence of added hydrocortisone). This phenomenon appeared to result from a general suppression of cell growth. Smaller colonies in treated cultures (10 nM TCDD) were reflected in fewer cells per culture (44 ± 11% of controls on days 16 − 17) but not in any significant change in cell size (as measured by Coulter counter (36)) or in viability (judged by dye exclusion). In the growing cultures, 96 ± 2% of both control and TCDD treated cells excluded trypan blue (37).The ratio of colonies in treated to control cultures was not significantly different from unity (1.08 ± 0.31). The cells treated at the higher TCDD concentrations grew progressively and did reach confluence, though more slowly than control cultures. A second passage of the treated cells in 10 nM TCDD did not appear to decrease further the growth, while removal of the agent at second passage appeared to restore growth immediately to the same rate as control cells.

As shown in Figure 2A, addition of corticosteroids to the medium antagonized the inhibitory action of TCDD on cell growth. Hydrocortisone was partially effective at 10 nM, and at 1 μM the colonies expanded at the same rate as in the absence of added TCDD. Dexamethasone had a maximal effect at 10 nM, in keeping with its greater affinity for the corticosteroid receptor. In the absence of TCDD, hydrocortisone had no visible effect on colony expansion, while in the highest concentration of dexamethasone employed (1 μM) the colonies appeared slightly smaller than in controls (Figure 2B).

In contrast, several agents which also exhibit anti-inflammatory activity (38 − 40) were inactive as TCDD antagonists up to the highest concentrations tested, above which they were toxic: indomethacin (0.2 μg/ml), 5,8,11,14-eicosatetraynoic acid (10 μg/ml), benoxaprofen (10 μg/ml), and BW 755c (2 μg/ml). While retinyl acetate suppresses the stimulation by hydrocortisone of differentiation in confluent SCC-13 cells

A

0   10^{-9} 10^{-8} 10^{-7} 10^{-6}

Corticosteroid (M)

B

0   10^{-7} 10^{-6}
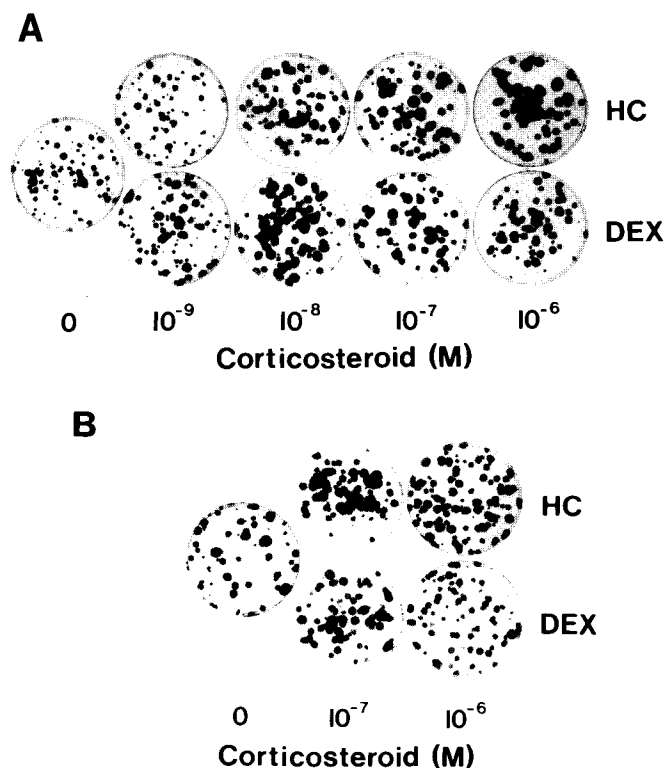
Corticosteroid (M)

Fig. 2. Corticosteroid antagonism of growth inhibition by TCDD in SCC-13. Hydrocortisone (HC) or dexamethasone (DEX) was added one day after the cells were inoculated (200 per dish), and the cultures were fixed and stained after 17 days. In **A**, the medium contained 10 nM TCDD; in **B**, no TCDD was added.

Table I. Effect of TCDD on ionophore-inducible envelopes

Medium additions[a]	Envelope competence (%)[b]	
	No TCDD	+ TCDD
None	31	26
HC	61	36
RA	8	10
HC + RA	29	8

[a]The cells were grown in medium containing hydrocortisone (HC, 1 μM), retinyl acetate (RA, 0.1 μM) and TCDD (10 nM) as indicated.
[b]Approximately 1 week after reaching confluence, envelope forming ability as a percentage of total cells in each culture was measured by treatment with X537A. Values presented are averages of two representative experiments.

(25,26), it had no effect on the growth inhibitory action of TCDD or its reversal by hydrocortisone. Moreover, when the cells were grown in medium supplemented with serum depleted of vitamin A by solvent extraction (41), 10 nM TCDD was inhibitory to the same extent as shown in Figure 1.

Inhibition of cell differentiation

Judging by involucrin content and competence in forming ionophore-inducible cross-linked envelopes, preconfluent SCC-13 cells exhibit minimal differentiation (25). Envelope competence was not affected by TCDD at this stage. Hence, the effect of TCDD on these and other keratinocyte markers was examined in confluent cultures. As shown in Table I and consistent with earlier findings (25), hydrocortisone stimulated envelope competence while retinyl acetate suppressed it. TCDD had little effect in the absence of hydro-

Table II. Effect of TCDD on differentiation markers

Medium additions[a]	Keratin content (%)	Transglutaminase activity[b] (normalized)	Spontaneous envelopes[c] (A^{340})
None	11	1.0	0.02
TCDD	7	0.8	0.06
HC	46	6.3	0.27
TCDD + HC	11	2.3	0.04

[a]Cultures were grown in medium containing hydrocortisone (HC, 1 μM) and TCDD (10 nM) as indicated and harvested ~2 weeks after reaching confluence. Values presented are averages of two representative experiments.
[b]Specific activities normalized to those in untreated cultures (0.6 pmol of putrescine incorporated per μg of soluble protein).
[c]Actual cell counts in parallel dishes gave estimates of envelopes in trypsinized cultures without hydrocortisone of <0.5% and with hydrocortisone of up to 20%. The latter were difficult to count accurately, however, due to incomplete disaggregation of the cells with envelopes by trypsin or detergent treatment, necessitating this alternate method of quantitation (29).
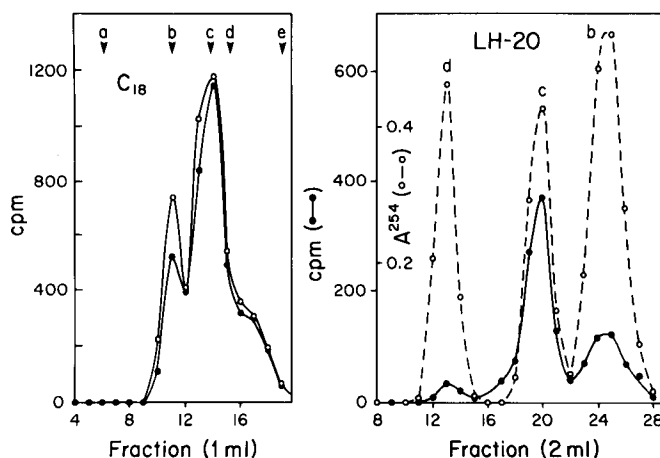

Fig. 3. Biotransformation of hydrocortisone in keratinocyte cultures. In the left panel, medium containing [^{3}H]hydrocortisone was sampled after 4 day exposure to cultures treated with (○——○) or without (●——●) 10 nM TCDD. The radioactivity in the CH_2Cl_2 extract was fractionated by reverse phase C_{18} h.p.l.c. In the right panel, radioactivity in the CH_2Cl_2 extract of 4 day medium from cultures treated with TCDD was chromatographed on Sephadex LH-20 (●——●). Marker steroids were a, 6β-hydroxycortisone; b, 4-pregnene-11β,17α,20β,21-tetrol-3-one (20β-OH-hydrocortisone); c, hydrocortisone; b, cortisone; and e, 5α-pregnene-11β,17α,21-triol-3,20-dione (dihydrohydrocortisone). In the left panel, tetrahydrohydrocortisone (5α-pregnene-3α,11β,17α,21-tetrol-20-one) eluted ~1 ml after dihydrohydrocortisone.

cortisone but markedly suppressed competence in the presence of the steroid, even when retinyl acetate was also present.

Three other keratinocyte markers in these cells were also examined: keratin content, transglutaminase activity, and spontaneous envelope formation. As seen in Table II, hydrocortisone considerably stimulated the expression of each marker. TCDD had little effect alone, but largely or completely antagonized the action of hydrocortisone. Since transglutaminase activity is required for envelope formation (42), its reduction is consistent with concomitant reduction of spontaneous and ionophore-inducible envelopes. However, among the four markers examined, it is clear that at least two independent ones (keratins, transglutaminase) were similarly affected.

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Lack of TCDD effect on hydrocrotisone depletion

Aliquots of medium were removed from confluent cultures 2 days and 4 days after feeding and analyzed chromatographically for degradation of added tracer [^{3}H]hydrocortisone. As seen in Figure 3 (left panel), the radioactive profiles obtained by reverse phase h.p.l.c. with 25% acetonitrile as solvent differed little whether or not the cultures were treated with TCDD. Approximately 80% and 60% of the applied radioactivity eluted in the position of authentic hydrocortisone after 2 and 4 days, respectively, while ~15% (2 days) and 30% (4 days) eluted in the position of the inactive derivative 20β-OH-hydrocortisone. No peaks of radioactivity were evident in the location of the markers 6β-hydroxycortisone, cortisone, dihydrohydrocortisone or tetrahydrohydrocortisone. When radioactive samples from TCDD-treated cultures were chromatographed on the same column with 50% methanol as solvent (not shown), one peak of labeled material was observed. Hydrocortisone and 20β-OH-hydrocortisone eluted together at this same location (9.5 ml), while 6β-hydroxycortisone (4.5 ml), Δ^4-pregnene-17α,20α/β, 21-triol-3,11-dione (8.0 ml) and cortisone (8.5 ml) eluted earlier.

Radioactive samples from cultures treated with TCDD for four days were also chromatographed on Sephadex LH-20 (35). With the CH$_2$Cl$_2$:CH$_3$OH (96:4) solvent employed, the markers cortisone, hydrocortisone and 20β-OH-hydrocortisone were cleanly separated (Figure 3, right panel). Approximately 60% of the label coeluted with hydrocortisone and 30% with 20β-OH-hydrocortisone, consistent with identifications obtained h.p.l.c. Thus, in SCC-13 cultures hydrocortisone was partially inactivated by reduction at carbon 20 with little conversion to cortisone, both of which reactions have been observed in cultured cells (43,44), but this metabolism was not significantly altered by TCDD treatment.

Discussion

The various effects of TCDD among tested animal species generally involve either tissue degeneration or altered differentiation. Elucidation of the molecular basis of this toxicity in target cells has been hampered by lack of appropriate culture systems exhibiting such effects. In the present work with keratinocytes of human tumor origin, TCDD inhibits cell growth in sparse culture and alters differentiated character depending upon culture conditions. Further investigation of the alterations in cell physiology responsible for these responses may help rationalize the highly conspicuous difference in interspecies sensitivities in target tissues. Moreover, these findings emphasize the critical importance of culture conditions and cell physiology in testing agents for chronic toxicity. Previous reports with keratinocytes *in vivo* or with cultured XB cells (17) have emphasized enhanced stratification produced by TCDD. Consistent with these findings, recent experiments with cultures held at confluence for extended periods show that hydrocortisone and to a lesser extent TCDD stimulate stratification in SCC-13 and SCC-9, an effect prevented by retinyl acetate (45).

With little effect on biochemical markers of keratinocyte differentiation in the absence of hydrocortisone, TCDD produced its major effect in confluent SCC-13 by antagonizing the action of this steroid. Since hydrocortisone was not depleted from the medium (nor is corticosterone depleted from the plasma of TCDD-treated rats (46)), the cells appeared unable to respond to its presence. Reduction of corticosteroid receptor levels, recently reported upon phorbol

ester treatment of mouse skin (47), could account for this phenomenon. Alternatively, TCDD could interfere with binding or processing of steroid receptor complexes at nuclear acceptor sites, as found with aflatoxin and other hepatocarcinogens in rat liver (48,49) and possibly cultured rat hepatocytes (50). However, the ability of hydrocortisone to antagonize TCDD action in sparse cultures, where keratinocyte differentiation is minimal, suggests these mechanisms are not applicable to the cells in their growth phase. Since TCDD has been reported to decrease significantly membrane receptors for epidermal growth factor in SCC-12F (51), further investigation of response of treated cells to growth factors in the medium appears warranted. Although membrane perturbation leading to production of arachidonic acid metabolites has been proposed to help explain toxic effects of a variety of hydrophobic agents (52), evidence for this hypothesis regarding TCDD was not obtained with the inhibitors of cyclooxygenase and lipoxygenase employed.

Imbalances of hormones, including corticosteroids (53), have been implicated or suggested as causative factors in development of a variety of carcinomas for many years (54). The striking effects of TCDD on certain organ systems have suggested an endocrine basis, particularly involving glucocorticoids (46), for its toxicity, which could plausibly bestow promoter activity (55). Thus, interfering with the response of target cells to hormones responsible for proper differentiated function could serve as selective stimulation. The influence of glucocorticoids on growth and differentiation of many cell types suggests the present findings may have some general applicability.

Acknowledgements

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