Metabolic and Ultrastructural Aspects of the \textit{in Vitro} Lysis of Chronic Lymphocytic Leukemia Cells by Glucocorticoids

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ABSTRACT

Human chronic lymphocytic leukemia (CLL) cells like prothymocytes and immunoactivated T-lymphocytes are readily lysed \textit{in vitro} by pharmacological concentrations of glucocorticoids such as cortisol, whereas peripheral blood lymphocytes and thyocytes are unaffected by the hormone. In this study, metabolic and ultrastructural aspects of the cortisol-induced killing process of CLL cells are recorded. \textit{In vitro} lysis was found to be temperature dependent and was detected only after 6 to 8 hr incubation with cortisol by means of the trypan blue exclusion test. However, 30 min of incubation with cortisol at either 37° or 4° followed by the removal of the hormone was still sufficient to induce the lytic process. Ultrastructural studies demonstrated sequential changes in the cytoplasm, including swelling of mitochondria and cytoplasmic decompartmentalization, followed by loss of surface microvilli with the appearance of "holes" in the cell membrane, and subsequent condensation of nuclear chromatin. The large holes in the membrane appearing after 6 hr of incubation with the hormone may be the cause for the penetration of the viable stain into the dead cells, as seen by light microscopy. Addition of metabolic inhibitors including actinomycin D, puromycin, and cycloheximide following administration of cortisol resulted in inhibition of the cell lysis. An excess of an antagonist such as cortexolone was found to inhibit the cortisol-induced cytolysis of the CLL cells. It is suggested that the glucocorticoid-induced lysis of human CLL cells is similar to the phenomenon observed in rat or murine lymphocytes and is mediated by interaction of the steroid molecule with the cytoplasmic receptor. The result complex appears to activate specific gene(s) the products of which eventually cause cytolysis.

INTRODUCTION

GC\textsuperscript{3} induce an \textit{in vitro} and \textit{in vivo} cytophilic effect on murine rats and lymphocytes and peripheral lymphocytes. However, these hormones do not affect viability of the same human cell lines \textit{in vitro} (2, 3, 26). Recently, we have described the sensitivity of subpopulations of lymphoid cells including human prothymocytes and activated T-lymphocytes (8-11) to GC-induced cytolysis. Several types of leukemic lymphocytes were also readily lysed \textit{in vitro} by pharmacological concentrations of cortisol. In particular, CLL and some ALL cells were sensitive, whereas myeloid leukemia cells and other ALL cell populations as well as normal lymphocytes and thyocytes were resistant to the lytic effect of the hormone \textit{in vitro} (12).

Studies on the mechanism of GC-induced lysis of rodent thymocytes or lymphoma cells have shown that specific cytoplasmic receptors are involved in the process of killing (6, 18, 21, 25, 27). It appears that the steroid molecule rapidly penetrates through the cell membrane and interacts with specific cytoplasmic receptors. Thereafter, the complex penetrates into the nucleus, binds to the chromatin, and activates certain genes. The subsequent gene product(s) synthesized eventually causes cell death (15, 22).

The purpose of the present study was to record various aspects of the \textit{in vitro} GC-induced cytolysis of human leukemic cells. For this purpose, CLL cells were used as targets, and the metabolic and morphological events occurring in the course of lysis were studied. The data obtained from these experiments suggest that GC-induced lysis of CLL cells is mediated by intracellular events similar to those occurring in GC-sensitive rodent lymphoid cell populations. Furthermore, ultrastructural observations show sequential changes in the cytoplasm and subsequently on the cell surface, which precede actual cell death by several hr. All studies for analyzing cell lysis were performed at the pharmacological concentration \(2 \times 10^{-5} \text{ M} \) cortisol (3).

MATERIALS AND METHODS

\textbf{CLL Lymphocytes.} Cells were obtained from the peripheral blood of CLL patients with leukocyte counts of above 50,000/cu mm (range, 50,000 to 350,000 cells/cu mm). The lymphocytes were separated from the blood by gradient centrifugation using Ficoll-Hypaque, washed, and resuspended in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum, and brought to a final concentration of \(2 \times 10^6 \text{ cells/ml} \).

\textbf{In Vitro GC-induced Lysis.} This was performed as described in earlier studies (8-12). Briefly, aliquots of 0.2 ml CLL cells (\(2 \times 10^6 \text{ cells/ml} \)) were incubated with \(2 \times 10^{-5} \text{ M} \) cortisol (Ikapharm, Ramat Gan, Israel) in flat-bottomed microwells (Cooke Engineering Co., Alexandria, Va.) for various periods of time (1 to 20 hr) at 37° and at other temperatures when indicated. The proportion of lysed cells was assessed by determining the concentration of the remaining viable cells in a hemocytometer using the trypan blue exclusion test. The percentage of cytolysis was calculated by means of the formula

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(a - b)/a \times 100
\]

where \(a\) is the concentration of viable cells in control wells containing medium without cortisol and \(b\) is the concentration of viable cells in wells containing the hormone. The effect of other steroids was also studied.

For all electron microscopic studies, 10-ml aliquots of the lymphoid suspensions were incubated with \(2 \times 10^{-5} \text{ M} \) cortisol at 37° in 5%...
CO₂ humidified atmosphere. Cells were harvested at 1, 2, 4, and 6 hr of incubation, respectively, and processed for electron microscopy as described below.

**Metabolic Inhibitors.** Actinomycin D (0.01 µg/ml; Sigma Chemical Co., St. Louis, Mo.), an inhibitor of RNA synthesis, and cycloheximide (0.1 µg/ml; Sigma) and puromycin (0.1 µg/ml; Sigma), both inhibitors of protein synthesis, were used to determine whether transcriptional and translational events occur in the cells in the course of GC-induced cytolysis. These inhibitors were added to the cell suspensions at various time intervals after administration of cortisol. The degree of inhibition of cortisol-induced lysis in the presence of the inhibitor was measured after an additional 8-hr incubation. During this incubation period, the metabolic inhibitors alone did not affect the viability of the CLL cells. Mortality in the presence of the inhibitor alone never exceeded 5% of dead cells (trypan blue exclusion).

**TEM.** Cell pellets (6 to 8 × 10⁶ cells) were fixed with phosphate-buffered 1.25% glutaraldehyde (pH 7.3, 4°C) for at least 1 hr, rinsed with 0.2 M phosphate buffer, postfixed in osmium tetroxide for 1 hr at 4°C, dehydrated through a graded series of ethanol, embedded in low-viscosity epoxy resin embedding medium according to the method of Spurr (28), and sectioned with an MT-2 Porter-Blum microtome equipped with a diamond knife. Thin sections were mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and viewed with a Phillips EM-300 electron microscope.

**SEM.** Six million cells were fixed in suspension in 1% phosphate-buffered glutaraldehyde (pH 7.3, 310 mosmol) for at least 1 hr at room temperature and then collected onto glass coverslips covered by poly-L-lysine. Coverslips with monolayers of cells were fixed for 1 hr more at room temperature, then prepared for SEM by further dehydration in a graded series of ethanol and Freon 113, and critical point dried with Freon 113 as described in earlier studies (24). The specimens were then coated with a thin layer of gold-palladium and examined with a Jeol SM-35X scanning electron microscope at an accelerating voltage of 35 to 39 kV.

**RESULTS**

**Effect of Temperature on Cortisol-induced Cytolysis of CLL Cells.** The process of lysis is not rapid and can be detected by means of the vital staining technique only after 6 hr of incubation with the hormone at 37°C (Chart 1). Lysis peaks after 8 hr of incubation. No significant increase in lysis was detected after further incubation for periods of time up to 20 hr. The process of lysis is temperature dependent since after incubation at 24°C or 4°C the cells are unaffected. However, the penetration of the hormone into the cells followed by its binding to cytoplasmic receptors is a rapid process which is temperature independent. Thus, incubation of the cells for 30 min with 2 × 10⁻⁶ M cortisol at 37°C or 4°C, followed by the removal of the hormone from the medium and further incubation of the cells at 37°C, resulted in the same extent of cell lysis obtained in the suspensions containing the hormone for the entire incubation period (Chart 1).

**Effect of Metabolic Inhibitors on Cortisol-induced Lysis.** From the results of this study, it appears that the cortisol-induced lytic process is dependent on the production of RNA and protein. The addition of actinomycin D together with the hormone resulted in a 70% inhibition of the induced lysis (Chart 2). Similarly, addition of the RNA translation inhibitors cycloheximide and puromycin, together with the cortisol, resulted in 80 to 90% inhibition of the lysis observed in cell suspensions containing the hormone alone. However, when the actinomycin D was added 10 min after the cortisol, the inhibition was less marked. No inhibitory effect was detected when actinomycin D was added to cells which had been incubated with cortisol for 20 min or more. Similarly, the protein synthesis inhibitors failed to inhibit cytolysis when added to cell suspensions which had been incubated with cortisol for 30 min at 37°C.

**Ultrastructural (TEM and SEM) Observations on GC-induced Cytolysis.** Generally, CLL lymphocytes are small round cells (7 to 8 µm in diameter) which contain a nucleus that comprises the large proportion of the cell. The nuclei show dense areas of margined heterochromatin and a distinct nuclear membrane. The cytoplasm shows a moderate number of round or elongated mitochondria, some strands of endoplasmic reticulum, and numerous ribosomes, some of which are clustered (Figs. 1 and 2). TEM and SEM show that the cell membrane is frequently villous (Figs. 1 to 4). CLL cells which were incubated with 2 × 10⁻⁶ M cortisol at 37°C for varying time periods were studied in order to document the ultrastructural changes occurring during cell death. Although visible lysis of the cells could be detected only after 6 hr of incubation with the hormone (Chart 1), early changes resulting from the cytolytic process were seen after 2 hr (Figs. 5 to 8). No distinct

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**Chart 1.** Cytolysis of CLL cells at various temperatures. The lymphocytes (2 × 10⁶ cells/ml) were incubated with 2 × 10⁻⁶ M cortisol for various time periods. Thereafter, the concentration of the viable cells was assessed by the trypan blue exclusion test. The percentage of lysis was calculated by as described in "Materials and Methods." O, cells incubated at 37°C; □, cells incubated at 24°C; Δ, cells incubated at 4°C; ◆, cells incubated at 37°C with the hormone for 30 min, after which the hormone was removed from the medium by washing and the cells were incubated further at 37°C; ▼, cells incubated with the hormone for 30 min at 4°C, after which hormone was removed, and cells were further incubated at 37°C. The data are of a representative experiment (one of 5 performed with similar results).

**Chart 2.** Inhibitory effect of actinomycin D (0.01 µg/ml) (○), cycloheximide (0.1 µg/ml) (□), and puromycin (0.1 µg/ml) (△) on cortisol-induced cytolysis of CLL cells. The metabolic inhibitors were added at various time intervals following administration of cortisol, and the cell suspensions were further incubated for 8 hr at 37°C. The percentage of inhibition of lysis was assessed by comparing the lysis in suspensions containing the hormone with that scored in suspensions containing the hormone and the inhibitor. The inhibitors alone were found not to cause cell death under these incubation conditions. Data are the means of 6 different experiments.
alterations were evident in the nucleus, but cytoplasmic changes were seen. The ribosomes lost their clustered appearance and were scattered homogeneously throughout the cytoplasm, suggesting that the cytoplasmic "compartmentalization" and organization were disturbed (Figs. 5 and 6). The mitochondria became more rounded and swollen, but their cristae were still preserved. The surface microvilli of many cells disappeared, and these cells showed smooth surfaces (Figs. 7 and 8).

After 4 hr of incubation with cortisol, cells showed more obvious changes. Distinct alterations were seen in the nuclei (Figs. 9 and 10; Table 1). Large areas of the nuclear chromatin became condensed and osmiophilic, but the nuclear membrane was still retained. The cytoplasm became more homogeneous, losing its former electron-dense appearance, and in many cells vacuoles were detected beneath the cell surface. These vacuoles corresponded to the numerous small pores seen in the membrane by means of SEM (Figs. 11 and 12). After 6 hr of incubation with the hormone, round condensed nuclei contained within the boundaries of their nuclear membranes were frequently seen in most of the cells (Figs. 13, 14, and 17). Large areas of nuclear chromatin had lost their osmiophilic features, and large vacuoles were seen beneath the cell membrane (Fig. 14), which corresponded to the "large holes" seen in the cell surface by means of SEM (Figs. 15 and 16; Table 1). Cell death, detected by viable staining, seems to be due to the formation of these holes in the membrane, which enables the trypan blue to penetrate into the cytoplasm. The surface membrane alterations seem to be specific for the affected cells since neither unaffected lymphocytes nor monocytes lost their characteristic surface structure after incubation with cortisol (Fig. 18).

Specificity of GC Activity through Interaction of the Cytoplasmic Receptors. In order to demonstrate the specific interaction between cortisol and cytoplasmic receptors as one of the initial stages in the GC-induced lysis of the CLL cells, we tried to inhibit this interaction by competitive inhibitors of steroid nature which do not cause lysis. Such possible inhibitors are progesterone and cortexolone. The inhibition experiment could not be performed by studying lysis following prolonged incubation of cells with cortisol (2 × 10⁻⁶ M) and excess inhibitors (10⁻⁴ M), since such concentrations of progesterone and cortexolone lyse lymphocytes nonspecifically by alteration of the cell membrane. Thus, GC such as cortisol or dexamethasone specifically kill CLL cells without affecting normal peripheral blood lymphocytes whereas prolonged incubation with progesterone or cortexolone caused the lysis of normal peripheral blood lymphocytes as well (Chart 3). The steroids estradiol and 5α-dihydrotestosterone, which do not interact with the cytoplasmic receptors, fail also to affect the viability of the CLL cells at 10⁻⁶ M. Since progesterone was found to exert a high nonspecific lysis due to its strong lipophilic affinity, only cortexolone was utilized as a competitive inhibitor. Inhibitory competition experiments were performed by incubation of CLL cells with cortexolone (10⁻⁴ M) for 30 min and administration of cortisol (2 × 10⁻⁶ M) for additional 30 min incubation at 37°. Thereafter, the steroids were removed by washing in order to avoid a nonspecific lysis by cortexolone. The cells were further incubated for 6 hr at 37°. As can be seen in Table 2, in such conditions, coincubation of cells with cortisol and a 5-fold excess of cortexolone resulted in a 75% inhibition of cytolyis observed with cortisol alone.

DISCUSSION

Until now, most of the information concerning GC-induced
lysis of lymphoid cells has been obtained from studies on cells obtained from experimental animals, such as mouse and rat thymocytes and murine lymphoma cells (1, 6, 15, 22). The data obtained from the present study indicate that the mechanism of GC-induced lysis of CLL cells appears to be in accord with the general model of steroid action on animal cells. Incubation of CLL cells with cortisol at 4°C for 30 min with subsequent removal of the hormone from the medium resulted in the same extent of cell lysis observed in CLL cells which were incubated with the hormone at 37°C for 30 min followed by subsequent incubation for an additional 8 hr without the hormone. The rapid penetration of the hormone and the irreversibility of the lytic process are well recognized and have been demonstrated in our earlier studies (12), where 5 min of incubation of CLL cells with the hormone were sufficient to induce lysis of CLL cells.

In contrast to the binding of GC to the receptors and the formation of a complex which is temperature independent, the translocation of the complex into the nucleus was found to be temperature dependent in rat thymocytes and does not occur in the cold (22). The latter phenomenon may explain why cell lysis is inhibited at 4°C. However, the fact that cells incubated for 1 hr at 37°C and thereafter at 4°C (not shown in results) were not lysed indicates that there are additional stages in the lytic process which are in fact temperature dependent. It is also possible that these stages include transcription of the gene(s) activated by the steroid-receptor complex and the posttranscriptional events which may include the generation of “lethal” products or activation of enzymes causing autolysis. Unlike other systems involving the anabolic effects of steroids in which a variety of gene products including specific mRNA and proteins can be identified in exposed cells (7, 13), no such macromolecules have been isolated from the in vitro lytic systems in animal target cells. However, it is of interest to note that the use of metabolic inhibitors of RNA and protein synthesis did result in inhibition of GC-induced lysis in the rat thymocyte system (22). The inhibition of lysis of CLL cells obtained by the addition of actinomycin D, cycloheximide, or puromycin suggests that the killing of human lymphoid cells induced by GC is also mediated through the production of specific mRNA and proteins. The kinetics of the inhibition of cell lysis by these agents indicates that the inhibitors were effective only if they were added to the cells within 20 min after the addition of cortisol. From the above data, it can be assumed that the duration of the intracellular process, from the time of steroid penetration into the cell until the onset of the first posttranscriptional events, is no more than 30 min, despite the fact that cell death is not detected until 6 hr later. The biological effects of GC studied in a variety of anabolic systems such as those involved in the induction of aminotransferases in hepatocytes and hepatoma cells were also found to initial transcriptional and translational events rapidly (7, 13). The mRNAs or proteins synthesized in these hepatocytes as a result of exposure to the hormone could also be detected in the cells within 10 to 20 min after the administration of the hormone. Similarly, studies on the inhibition of GC-induced lysis of rat thymocytes indicate that RNA synthesis occurred within several min after administration of the hormone (22).

The posttranscriptional processes leading to GC-induced cell death are still not clearly understood. However, the ultrastructural studies of the lytic process provide some clues for the understanding of these events. The initial changes, detected after 2 hr of incubation of the affected cells with the hormone, were found in the cytoplasm and cell membrane, while the nuclei still retained their original shape and form. The homogeneous appearance of the cytoplasm and distribution of the ribosomes may be indicative of an early disruption of the cytoplasmic compartmentalization, which may also result in the penetration of water into the mitochondria, causing them to become swollen. At the same time, the cell surface loses its villous structure, indicating that the surface membrane structure is affected. The cytoplasmic changes appear to precede membrane alterations since they were also evident in cells which had retained their surface microvilli. It is also worthwhile recording that no changes were detected in the cells after 1 hr of incubation with cortisol. The condensation of the nuclear chromatin seen after 4 hr of incubation with GC seems to be secondary to structural alterations in the cytoplasm and cell surface and may reflect pyknosis due to activation of nucleases, as reported in the murine thymocyte system (30). Unlike the GC-induced killing of murine thymocytes, which is characterized by bursting of the nuclear chromatin into the cytoplasm (4), the nuclear membrane in the dying human CLL cell seems to remain intact even in cells showing large holes in their surface membrane. The ultrastructural studies suggest that cytoplasmic changes followed by surface membrane changes are among the first posttranscriptional events of GC-induced lysis, while the typical nuclear pyknosis seems to be a secondary change occurring at a later stage. A detailed study of enzyme activities initiated in the first 30 to 120 min of incubation with cortisol may be useful in the clearer understanding of the morphological changes reported here. In light of the fact that transcriptional and translational processes seem to be involved in GC-induced cytolysis, it is indeed possible that the administration of drugs known to block such metabolic events may exert an antagonistic effect when given to patients together with GC in a variety of therapeutic regimens.

It could be argued that the lysis observed in presence of $2 \times 10^{-5}$ M cortisol may be a result of a nonspecific in vitro membranal effect of the hormone on the cells studied (21). Our previous studies have indicated that cortisol-induced cytolysis is a specific effect which can be demonstrated in distinct normal lymphoid subsets. Human prothymocytes and immune activated T-cells are readily killed by such cortisol concentrations, whereas the human thymocytes or mature T-cells are completely resistant to lysis even at a concentration of $10^{-4}$ M cortisol (8–12). These studies suggested also that the GC sensitivity of the various stages in the normal T-cell ontogeny is dependent on certain characteristics of the particular differentiation stage rather than the level of cytoplasmic steroid receptors and may reflect a regulatory mechanism controlling the extent of immune response through alternated levels of cortisol secreted by the adrenal.

Specificity of cortisol-induced lysis has also been found with leukemic cells. CLL cells and malignant cells from some of the ALL patients were lysed by such cortisol concentrations, whereas myeloid leukemic cells and ALL cells from almost one-half of the patients studied were not affected by the hormone even at the superpharmacological concentration of $10^{-4}$ M (12). It should be noted that the synthetic GC dexamethasone elicits cytolysis to a degree not significantly different from that of cortisol. Thus, it seems that the factors conferring dexamethasone sensitivity are the same as those of cortisol-sensitivity.
methasone with its high clinical potency (high affinity to cytoplasmic receptors and prolonged retention in the circulation) do not affect the capacity of the hormone to induce cytolysis in the in vitro system. The specificity of the cortisol effect was demonstrated also morphologically utilizing SEM. While the CLL lymphocytes were lysed within 6 hr of incubation with cortisol, no effect on the membranal features of the GC-resistant monocytes and lymphocytes was observed (Fig. 18).

The electron microscopic studies demonstrating the cytoplasmic changes in cells which have not yet lost the surface microvilli following 2 hr of incubation with cortisol also support the assumption that the GC-induced cytolysis is not a result of a nonspecific membranal effect of the hormone but rather an intracellular specific chain of events.

The irreversibility of the lytic process following pulse exposure of the sensitive cells to cortisol at 4° or 37° for 30 min suggests the involvement of cytoplasmic steroid receptors. The involvement of such receptors in lysis was demonstrated by inhibition of the killing as a result of the administration of a 5-fold excess of cortexolone. This steroid is known also to compete with the cortisol in binding to the cytoplasmic receptors and to react as antagonist in the GC-induced lysis in rodents such as mice or rats (5, 16, 17, 29). It is not clear as yet if the cytolysis exerted by another known antagonist, progesterone, is merely a result of its high lipophilic affinity which alters the cell membrane surface or whether this hormone acts in the human system as an agonist. Such a possibility was suggested previously by Homo et al. (16).

Works on the effect of GC on the in vitro viability of human leukemic lymphoid cells have been performed recently utilizing the human lymphoblastoid T-cell line CEM (14, 23). These studies indicated that incubation with 10^-6 M dexamethasone results in inhibition of growth and formation of colonies in agarose and loss in cell viability after 18 to 24 hr, due to arrest of cells in G1. It seems that this phenomenon is different from the GC-induced lysis described in the present work, since the GC-sensitive CLL cells are mostly in G0 and do not proliferate or clone in vitro. Moreover, the lysis of CLL cells is already apparent within 6 hr of incubation with the hormone, whereas in the system utilizing CEM cells cell death was observed following 18 hr of incubation.

The method described here and in our previous study (12) thus enables the evaluation of the GC sensitivity of freshly obtained leukemic cells, which do not necessarily proliferate in in vitro conditions as a lymphoblastoid cell line.

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REFERENCES

Figs. 1 to 4. TEM and SEM of CLL lymphocytes. Note the marginated nuclear heterochromatin, elongated mitochondria, clustered ribosomes, and villous surfaces of the cells. Fig. 1, x 6,400; Fig. 2, x 9,500; Fig. 3, x 5,300; Fig. 4, x 15,000.

Figs. 5 to 8. TEM and SEM of CLL lymphocytes incubated for 2 hr with $2 \times 10^{-5}$ M cortisol at 37°. Note the swollen mitochondria, homogeneous distribution of ribosomes, and smooth surfaces of some of the cells. Fig. 5, x 8,600; Fig. 6, x 14,700; Fig. 7, x 7,000; Fig. 8, x 13,000.

Figs. 9 to 12. TEM and SEM of CLL lymphocytes incubated for 4 hr with $2 \times 10^{-5}$ M cortisol at 37°. Note the smooth but porous cell membranes, peripheral cytoplasmic vacuoles, and condensation of the nuclear chromatin in some of the cells. Fig. 9, x 5,300; Fig. 10, x 11,700; Fig. 11, x 6,200; Fig. 12, x 13,300.
GC Cytolysis of Leukemic Cells

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Figs. 13 to 18. TEM and SEM of CLL lymphocytes incubated for 6 hr with $2 \times 10^{-5}$ M cortisol at 37°C. Note the large holes in the cell membrane. The condensed chromatin is preserved within the nuclear membrane (Fig. 17). Some of the unaffected lymphocytes (L) and monocytes (M) still retain their original surface features (Fig. 18). Fig. 13, x 9,400; Fig. 14, x 14,700; Fig. 15, x 5,800; Fig. 16, x 10,000; Fig. 17, x 11,700; Fig. 18, x 4,000.
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