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Cytotoxic T cells and NK cells will acquire features of apoptosis when exposed to oxygen radicals, but the molecular mechanisms underlying this phenomenon are incompletely understood. We have investigated the role of two enzyme systems responsible for execution of cell death, caspases and the poly(ADP-ribose) polymerase (PARP). We report that although human cytotoxic lymphocytes were only marginally protected by caspase inhibitors, PARP inhibitors completely protected lymphocytes from radical-induced apoptosis and restored their cytotoxic function. The radical-induced, PARP-dependent cell death was accompanied by nuclear accumulation of apoptosis-inducing factor and a characteristic pattern of large-fragment DNA degradation. It is concluded that the PARP/apoptosis-inducing factor axis is critically involved in oxygen radical-induced apoptosis in cytotoxic lymphocytes. The Journal of Immunology, 2006, 176: 7301–7307.

I
n many forms of cancer, cytotoxic lymphocytes have been shown to be dysfunctional with, for example, a defective capacity to transduce activating signals and an increased propensity of undergoing apoptotic cell death (for review, see Ref. 1). This phenomenon, which is often referred to as cancer-related immunosuppression and a significant part of the larger entity of cancer escape mechanisms, may serve not only to explain why malignancies arise and progress, but also why immunotherapies, aimed at inducing immune-mediated destruction of tumor cells, are frequently ineffectual in several types of human cancer.

Oxygen radicals are produced by phagocytes as part of a defense strategy against intra- and extracellular pathogens. The radicals are formed by the assembly and activation of a membrane enzyme complex, the NADPH oxidase, which transforms molecular oxygen into superoxide anion, which in turn is converted to hydrogen peroxide, toxic halides, and other radical species. Extracellularly released oxygen radicals are toxic to a variety of cells and tissues (“oxidative stress”) (reviewed in Refs. 2 and 3). During the past decade, much attention has been directed toward the ability of mononuclear phagocytes to adversely affect cytotoxic lymphocyte function (4), with a focus on the immunosuppressive role of phagocyte-derived oxygen radicals (5–8). Thus, several investigators have demonstrated that phagocyte-derived oxygen radicals strongly inhibit the antitumor cytotoxicity and other functions of T cells and NK cells. Typically, these tumor-killing lymphocytes are functionally suppressed by relatively low concentrations of exogenous hydrogen peroxide. PARP inhibitors, on the other hand, were efficiently protective against phagocyte-derived oxygen radicals as well as exogenous hydrogen peroxide displayed apoptotic characteristics including depolarization of the mitochondrial transmembrane potential, caspase activation, and extracellular exposure of phosphatidyl serine. Pancaspase inhibitors did not protect cytotoxic lymphocytes against oxygen radicals. PARP inhibitors, on the other hand, were efficiently protective against phagocyte-derived oxygen radicals as well as exogenous hydrogen peroxide. PARP-dependent cell death was accompanied by a reduction of mitochondrial transmembrane potential in lymphocytes, nuclear accumulation of apoptosis-inducing factor (AIF), and large-scale DNA fragmentation. It is concluded that caspase activation is a late event during oxygen radical-induced lymphocyte apoptosis and that the role of caspases is rather in the execution phase than in the induction phase of apoptosis. In contrast, PARP/AIF axis may be critically involved

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3 Abbreviations used in this paper: PARP, poly(ADP-ribose) polymerase; AIF, apoptosis-inducing factor; FLICA, fluorochrome-labeled inhibitor of caspase; FAM-VAD.fmk, carboxyfluorescein-benzylxoycarbonyl-Val-Ala-Asp(Ome) fluoromethyl ketone; Z-VAD.fmk, benzylcarbonyl-Val-Ala-Asp(Ome) fluoromethylketone; Δψm, mitochondrial transmembrane potential; DPI, diphenyleneiodonium; TP3, To-Pro-3.
in initiating phagocyte-mediated, oxygen radical-induced lymphocyte apoptosis.

Materials and Methods
Separation of leukocytes
Peripheral venous blood was obtained as freshly prepared leukopacks from healthy blood donors at the Blood Centre, Sahlgrens University Hospital (Göteborg, Sweden). The blood (65 ml per donor) was mixed with 92.5 ml of IDMEM, 35 ml of 6% dextran, and 7.5 ml of acid citrate dextrose. After incubation for 15 min at room temperature, the supernatant was carefully layered on top of a Ficoll-Hypaque (Lymphoprep) density gradient. After centrifugation at 380 × g for 15 min, mononuclear cells were collected at the interface. The cells were washed and resuspended in IDMEM supplemented with 10% AB⁺ serum. Cells were further separated into lymphocytes and monocytes using a countercurrent centrifugal elutriation technique in which the sedimentation rate of cells in a spinning rotor is balanced by a counterdirected flow through the chamber. By slowly increasing the flow rate, fractions of cells of well-defined sizes can be collected. The mononuclear cells were resuspended in elutriation buffer, buffered NaCl supplemented with 0.5% BSA and 0.1% EDTA, and fed into a Beckman J2–21 ultracentrifuge with a JE-6B rotor (Beckman Coulter) at 2100 rpm. A fraction with >90% monocytes was obtained at a flow rate of ~19 ml/min. A lymphocyte fraction enriched for NK cells (CD3⁻/56⁺ phenotype) and T cells (CD3⁻/56⁻ phenotype) was recovered at flow rates of 14–15 ml/min. Within the population of elutriated lymphocytes, ~25–35% were CD56⁺ NK cells, 25–35% were CD8⁺ T cells, and the remaining 35–40% were CD4⁺ T cells. For the cytotoxicity assay, NK cells were further enriched using the IMag NK cell isolation kit (BD Biosciences) according to the instructions provided by the manufacturer.

Lymphocyte cell death
After overnight incubation with mononuclear phagocytes or hydrogen peroxide (H₂O₂), end-stage oxidant-induced cell death in lymphocytes was assayed using flow cytometry, based on the altered characteristics displayed by end-stage apoptotic cells, i.e., a reduced forward scatter and an increased right angle scatter. To ensure that cytotoxic lymphocytes were sensitive to induction of apoptosis, lymphocytes were sorted by use of flow cytometry into CD3⁻/CD56⁺ NK cells or CD8⁻/CD56⁻/CCR7⁻CD45RA⁺ CTLs (19). In accordance with previous studies (20), both of these cytotoxic lymphocyte cell subsets were highly sensitive to oxidative induction of apoptosis (data not shown).

Altered mitochondrial transmembrane potential
The Mitochondrial Membrane Sensor Kit (BD Clontech) was used to identify cells with altered mitochondrial transmembrane potential according to the manufacturer’s protocol. Lymphocytes with altered mitochondrial transmembrane potential (ΔΨm) displayed an increase in green fluorescence and a slight decrease in orange fluorescence, which could be detected using a FACSAria flow cytometer (BD Biosciences).

Extracellular exposure of phosphatidyl serine
FITC- or PE-labeled annexin V (BD Pharmingen) was used to identify lymphocytes that had lost the asymmetrical distribution of membrane phospholipids and thus were exposing phosphatidyl serine on the extracellular side of the plasma membrane.

Increased plasma membrane permeability
Loss of structural integrity of the plasma membrane was monitored by adding the cationic dye To-Pro-3 (TP3) (1 μM; Molecular Probes) right before the flow cytometry analysis.

Caspase-3 activation
Lymphocytes exposed to hydrogen peroxide or oxygen radical-producing phagocytes were assayed for caspase activation using a fluorochrome-labeled inhibitor of caspases (FLICA) assay (MP Biomedicals). Lymphocytes were incubated with the carboxyfluorescein-labeled caspase inhibitor carboxyfluorescein-benzylocarbonyl-Val-Ala-Asp(OMe) fluoromethylketone (FAM-VAD.fmkl) for 1 h according to the instructions provided by the manufacturer, and the percentage of cells with active caspase-3 was determined using flow cytometry.

Caspase-3 activation was also monitored using the fluorogenic caspase-3 substrate PhiPhiLux (OncoImmunin) according to the manufacturer’s instructions. The percentage of cell with activated caspases was determined using flow cytometry.

Immunoblotting
Nuclear extracts from lymphocytes were prepared using the NE-PER kit (Pierce) according to the instructions provided by the manufacturer. The protein content in the nuclear extracts was determined using a BCA Protein Assay (Pierce), and 2 μg of protein from each sample was loaded on the gel. After SDS-PAGE and Western blotting, blots were incubated with a polyclonal rabbit anti-AIF Ab (Santa Cruz Biotechnology) and a HRP-conjugated goat anti-rabbit Ab (DakoCytomation) at optimized dilutions.

Pulsed-field gel electrophoresis
Human lymphocytes were exposed to 250 μM H₂O₂ and incubated overnight at 37°C. After 16 h, the cells were washed twice with PBS and resuspended in PBS. Cells were mixed with an equal volume of 2% low-melting-point agarose and cast into agarose plugs. After solidifying, plugs were incubated overnight at 56°C in a buffer containing 0.2% sodium deoxycholate and 0.5% N-lauroyl sarcosine supplemented with 0.5 mg/ml protease K.

NK cell cytotoxicity assay
A cytotoxicity assay using PKH-26-labeled K562 cells was used for studying NK cell cytotoxic function (21, 22). NK cells pretreated with PJ34 (50 μM) or medium were exposed to mononuclear phagocytes (phagocyte/NK ratio, 1:2) or hydrogen peroxide (60 μM) overnight. Thereafter, K562 cells were labeled with the membrane dye PKH-26 (5 μM; Sigma-Aldrich) according to the protocol provided by the manufacturer. The labeled K562 cells were incubated for 4 h with NK cells at an E:T ratio of 5:1 in 96-well microplates (Nunc). Immediately before the flow cytometry analysis, the live cell-impermeant stain To-Pro-3 (TP3) was added (1 μM). Lysed target cells were identified as double-positive, PKH-26⁻/TP3⁺ cells, while live K562 cells displayed PKH-26-staining, but remained unaffected by TP3.

Compounds
The following compounds were used: diphenylenediiodonitride (DP), DPQ, PJ34, PKH, and benzoyloxy carbonyl-Val-Ala-Asp(OMe) fluoromethylketone (Z-VAD.fmkl) (Sigma-Aldrich); Catalase (Boehringer Mannheim); Q-VAD-OPh (EMD Biosciences); dextran (Kabi Pharmacia); acid citrate dextrose (Baxter); BSA (ICN Biomedicals); EDTA and hydrogen peroxide (VWR); Ficoll-Hypaque and Lymphoprep (Nycomed); and To-Pro-3 (Molecular Probes). Annexin V reagents and pure, FITC-, PE-, PerCP- and allophycocyanin-conjugated mAbs against various surface markers were all purchased from BD Biosciences.

Results
Radicals released from mononuclear phagocytes or exogenous hydrogen peroxide induce cell death in PBLs
In accordance with earlier studies (5, 23), human mononuclear phagocytes triggered cell death in PBLs after overnight incubation. This process was mimicked by exogenously added hydrogen peroxide and was most likely mediated by spontaneously released oxygen radicals derived from the phagocytosed NADPH oxidase as lymphocytes were protected from phagocyte-induced cell death by antioxidative substances, such as catalase, histamine, and the NADPH oxidase inhibitor DPI (Fig. 1A). To investigate the sensitivity of different lymphocyte subsets to oxygen radicals, lymphocytes exposed to hydrogen peroxide were stained and analyzed using flow cytometry. As shown in Fig. 1B, these phenotypic analyses revealed that T cells as well as NK cells were clearly prone to radical-induced apoptosis. NK cells were by far the most sensitive cells, and CD3⁺/8⁺ T cells were more sensitive than CD3⁻/4⁺ T cells.

To study the role of caspases for oxygen radical-induced cell death in cytotoxic lymphocytes, these cells were exposed to phagocytes or hydrogen peroxide and assayed for caspase activation using two methods, a substrate assay (PhiPhiLux) and a FLICA assay. The PhiPhiLux reagent becomes fluorescent on cleavage by caspases, whereas the FLICA reagent is a fluorochrome-conjugated caspase-3 inhibitor that traverses the membranes of all cells and binds to the active site of activated caspase-3. Thus, only cells with activated caspases will retain the reagent to become fluorescent (24). As shown in Fig. 2, lymphocytes fatally exposed to...
Oxygen radicals bound the fluorochrome-conjugated caspase-3 inhibitor, suggesting that caspase-3 became activated during phagocyte-induced cell death. However, despite caspase activation pretreatment with pancaspase inhibitors, such as Z-VAD.fmK and Q-VD.OPh (data not shown) failed to protect lymphocytes from oxidant-induced cell death (Fig. 2B). The inability of pancaspase inhibitors to protect radical-exposed lymphocytes incited us to investigate when caspase activation occurs during the apoptotic process. Lymphocytes were subjected to phagocytes or H2O2 and assayed for caspase activation at different time points. As shown in Fig. 3, caspase activation was a late event in the apoptotic process.

The nuclear enzyme PARP was recently identified as a mediator of cell death in neural tissue after ischemia-reperfusion injury and glutamate excitotoxicity. PARP-dependent cell death is reportedly accompanied with a perturbation of mitochondria, resulting in the release of AIF into the cytosol (25). To investigate whether the PARP/AIF axis was of importance in oxidant-induced cell death in lymphocytes, we treated lymphocytes with the PARP inhibitor PJ34 (500 nM) before exposing these cells to phagocytes or H2O2.

As shown in Fig. 4A, lymphocytes pretreated with PARP inhibitors resisted the oxidative stress imposed by phagocytes or exogenously added hydrogen peroxide. Similar results were obtained in experiments in which PJ34 was replaced by DPQ (3 μM), a PARP inhibitor that is structurally unrelated to PJ34 (data not shown). Neither of these PARP inhibitors displayed any scavenging activity of oxygen radicals in the isoluminol-dependent chemiluminescence technique (data not shown) (26).

Next, we investigated the intracellular events leading to oxidant-induced cell death. In these experiments, lymphocytes were exposed to mononuclear phagocytes or hydrogen peroxide and assayed for apoptotic features. Three common events in apoptosis are depolarization of the inner mitochondrial membrane (ΔΨm), exposure of phosphatidyl serine on the outside of the plasma membrane, and increased permeability of the plasma membrane. Already after 1 h, H2O2-treated lymphocytes displayed signs of altered mitochondrial membrane potential (Fig. 5A). With time, more cells became apoptotic and eventually lost the integrity of the plasma membrane, as manifested by an increased To-Pro-3 staining. Lymphocytes exposed to phagocytes displayed ΔΨm after 3 h (Fig. 5B) and were fully protected by PJ34; in contrast, Z-VAD.fmK had no significant protective effect against H2O2 or phagocytes (Fig. 5). Externalization of phosphatidyl serine to the outer leaflet of the plasma membrane was a later event than ΔΨm and was evident first after 6 h of incubation (data not shown).

The ability of PARP inhibitors to prevent the early events in radical-induced cell death prompted us to investigate whether the observed caspase activation was PARP dependent. In a series of experiments, lymphocytes treated with PARP inhibitors were subjected to phagocytes or hydrogen peroxide overnight and assayed for caspase activation. As shown in Fig. 4B, PJ34 efficiently prevented caspase activation in radical-exposed lymphocytes, suggesting that caspase activation was a downstream event of PARP activation.

**AIF accumulates in the nucleus in H2O2-exposed lymphocytes**

AIF has been identified as the downstream executioner of PARP-dependent cell-death in an in vitro model of excitotoxic neuronal death (25). Upon extensive PARP activation, AIF is released from mitochondria and translocates to the nucleus (25), where it causes large-scale DNA fragmentation (27). To investigate the potential role of AIF in phagocyte-induced lymphocyte cell death, lymphocytes exposed to H2O2 were harvested at different time points and assayed for nuclear AIF by use of Western blot. As shown in Fig. 6A, nuclear extracts from H2O2-treated lymphocytes displayed elevated levels of AIF compared with untreated control cells.
Translocation of AIF to the nucleus has been associated with large-scale DNA degradation with fragments of \(\sim 50\) kb (27). The observed accumulation of nuclear AIF after challenge with \(\text{H}_2\text{O}_2\) prompted us to investigate whether such large-scale DNA fragmentation accompanied lymphocyte cell death. Lymphocytes were exposed to \(\text{H}_2\text{O}_2\), cast into agarose plugs, and analyzed using pulsed-field gel electrophoresis. As shown in Fig. 6B, a distinct band of \(\sim 50\) kb was seen in cells treated with Z-VAD.fmk and \(\text{H}_2\text{O}_2\). A similar band, although less pronounced, appeared in the lane corresponding to cells treated with \(\text{H}_2\text{O}_2\) alone. This finding suggests that large-scale chromatin fragmentation occurs in lymphocytes after oxygen radical exposure and that activated caspases cause partial secondary internucleosomal DNA fragmentation. However, in the presence of a pancaspase inhibitor, the secondary fragmentation is abolished and large 50-kb fragments accumulate.

**PJ34-protected NK cells remain cytotoxic after exposure to oxygen radicals**

As shown in the above experiments, PARP inhibitors offer complete protection of radical-exposed cytotoxic lymphocytes. Next, we investigated whether NK cell cytotoxicity was maintained in PJ34-protected NK cells after exposure either to autologous mononuclear phagocytes or to exogenously added hydrogen peroxide. As shown in Fig. 7, NK cells pretreated with PJ34 remained cytotoxic to leukemic K562 cells, which are prototypically NK cell sensitive. Similar results were obtained in experiments in which PJ34 was replaced by DPQ (3 \(\mu\)M; data not shown). Caspase inhibitors did not restore NK cell cytolytic activity in the presence of phagocytes or hydrogen peroxide (data not shown).

**Discussion**

In recent years, several studies have shown that lymphocytes in the malignant microenvironment and in the peripheral blood of subjects with solid or hematological cancers display signs of functional impairment, unresponsiveness to activating signals, and apoptosis, suggesting that the immunosuppressive milieu associated with malignant cell expansion counters an appropriate antitumor response (28–31). An immunosuppressive role has been ascribed to oxygen radicals produced and released by mononuclear phagocytes (5, 6). In this study, we present data suggesting that phagocyte-derived reactive oxygen species trigger PARP- and AIF-dependent cell death in human cytotoxic lymphocytes. Inhibitors of PARP activity efficiently maintained lymphocyte viability in the presence of suppressive phagocytes or after exposure to exogenous oxygen radicals, and also upheld the tumor-killing function of lymphocytes exposed either to radicals or to phagocytes.

The induction of cell death apparently occurred independently of caspases, since pancaspase inhibitors did not protect radical-exposed lymphocytes. However, later in the apoptotic process caspase-3 activation was observed, suggesting a role for caspases in the execution rather than in the induction phase of phagocyte-induced lymphocyte apoptosis. In fact, caspases seemed to affect the DNA fragmentation pattern observed in radical-exposed lymphocytes. In the presence of caspase inhibitors, a band corresponding to 50-kbp DNA fragments appeared, suggesting that the caspase activation observed in this study played a role in secondary internucleosomal fragmentation of the 50-kbp fragments into shorter fragments. Collectively, these results suggest the following tentative scheme of events: oxygen radicals trigger PARP activation, which in turn induces a nuclear translocation of mitochondrial...
AIF with subsequent fragmentation of DNA into large (50-kbp) fragments. Finally, caspase activation causes partial internucleosomal DNA fragmentation.

Cell death has traditionally been divided into two forms: active programmed cell death, apoptosis, mediated by the caspase cascade which orchestrates cell degradation without release of toxic substances into the surrounding tissue, and passive accidental cell death, necrosis, in which cells rapidly lose plasma membrane integrity and are degraded uncontrollably. Recently, it has become evident that apoptosis and necrosis are not always distinguishable, because dying cells may fulfill criteria for apoptosis and necrosis at the same time. Furthermore, it was recently reported that there are forms of programmed cell death in which caspases are of minor or even no importance (32, 33). Cell death in neural tissue commonly follows caspase-independent routes (34, 35), and several studies have suggested caspase-independent cell death in lymphocytes (36–38).

Overactivation of PARP has been identified as an alternative route to the triggering of cell death. PARP is a nick sensor enzyme, which becomes activated by DNA single-strand breaks. Upon activation, PARP transfers poly-ADP polymers to various nuclear proteins, including PARP itself, leading to cell death.

FIGURE 4. PARP inhibitors prevent caspase activation and lymphocyte cell death. Lymphocytes pretreated with PJ34 (500 nM), Z-VAD.fmk (100 μM), or medium were subjected to mononuclear phagocytes (ratio 1:1) or H₂O₂ (250 μM). PJ34 protected lymphocytes from cell death induced by phagocytes (p < 0.05; n = 5) and hydrogen peroxide (p < 0.001; n = 5), while Z-VAD.fmk failed to protect lymphocytes against radical-induced cell death (A). B. After overnight incubation with mononuclear phagocytes (Ph, ratio 1:1) or H₂O₂ (250 μM) in the presence or absence of PJ34 (500 nM), lymphocytes were stained with a FITC-labeled caspase inhibitor (FAM-VAD.fmk) and assayed for caspase activation using flow cytometry. Phagocytes and H₂O₂ triggered caspase activation in overnight-incubated lymphocytes, and this event was reversed by pretreatment of lymphocytes with PJ34. Similar results were obtained using the PARP inhibitor DPQ (3 μM; data not shown). Data are from one representative experiment of three similar ones. The level of caspase activation in lymphocytes not exposed to H₂O₂ or phagocytes was consistently <3% (data not shown).

AIF with subsequent fragmentation of DNA into large (50-kbp) fragments. Finally, caspase activation causes partial internucleosomal DNA fragmentation.

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FIGURE 5. Depolarization of the mitochondrial membrane potential after oxygen radical exposure. Lymphocytes exposed to H₂O₂ (250 μM) or mononuclear phagocytes (Ph, ratio 1:1) were assayed for altered ϕₘ and plasma membrane integrity at various time points. Depolarization of the ϕₘ is seen as an increase in green fluorescence (mitosensor monomers). Viable cells with normal light scatter are shown in red, apoptotic cells with altered ϕₘ are shown in yellow, and end-stage apoptotic cells are shown in blue. Lymphocytes treated with H₂O₂ displayed altered ϕₘ already after 1 h (A). With time, more cells obtained depolarized mitochondrial membranes, acquired modified scattering properties, and became increasingly stained by the live cell-impermeant stain To-Pro-3. Similarly, after 3 h, lymphocytes incubated with mononuclear phagocytes started displaying signs of altered ϕₘ (B). PJ34 (500 nM) protected lymphocytes from oxidant-induced alterations of ϕₘ, while Z-VAD.fmk failed to display any protective effect (A and B). Ctrl, Control.
proteins, thereby initiating recruitment of DNA repair systems to the site of damage. However, extensive PARP activation instead transmits a death signal to mitochondria. The nature of this signal is not known in detail, but as a result, depolarization of the mitochondrial transmembrane potential occurs, leading to opening of high-conductance permeability pores and release of the mitochondrial protein AIF into the cytoplasm. AIF is translocated to the nucleus, where it induces DNA fragmentation (25). PARP activity is instrumental in various models of neural cell death, and, accordingly, genetic knockout of the gene encoding PARP or pharmacological inhibition of PARP results in neuroprotection in animal models (39–41).

This study is, to our knowledge, the first to show a role for PARP activation in oxygen radical-induced cell death in human cytotoxic lymphocytes. In murine thymocytes, peroxynitrite was shown to trigger PARP-dependent cell death (42). Peroxynitrite can be formed in vivo through a reaction between superoxide anion (O$_2^-$) and NO (43); however, the oxidant-induced cell death observed in this study is not likely to be mediated by peroxynitrite or other NO-derived radicals because phagocyte-exposed lymphocytes were not protected by the NO synthase inhibitor L-nitroarginine methyl ester, used at final concentrations of 1–100 μM (44) (data not shown).

PARP plays profound roles in diverse cellular processes including cell death, DNA repair, and gene expression, and has therefore been an interesting target for pharmacological intervention in several diseases, such as ischemia, cancer, and inflammatory pathologies (45). In cancer, the role of PARP in DNA repair has been exploited to increase the efficacy of chemotherapy and radiotherapy, based on the notion that inhibition of PARP could incapacitate the DNA repair systems in tumor cells, and thus render them sensitive to the DNA-damaging effect of chemotherapy and radiotherapy (46). The finding in this study, that PARP inhibitors protect lymphocytes from phagocyte-induced cell death, suggests an additional role for PARP inhibitors in malignant diseases in that the
PARP inhibitors could protect pivotal cytotoxic lymphocytes to alleviate oxygen-radical-induced immunosuppression.

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