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CUTTING EDGE

Cutting Edge: Antioxidative Properties of Myeloid Dendritic Cells: Protection of T Cells and NK Cells from Oxygen Radical-Induced Inactivation and Apoptosis[1]

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Dendritic cells (DCs) communicate with nonadaptive and adaptive lymphocytes on multiple levels. Efficient DC-lymphocyte interactions require that lymphocytes remain viable and functional also under conditions of oxidative stress, such as in microbial infection or in the malignant microenvironment. For this study, we exposed human T and NK cells to oxidants delivered either by autologous phagocytes or in the form of exogenous hydrogen peroxide. In accordance with earlier studies, these lymphocytes became dysfunctional and subsequently apoptotic. The presence of myeloid DCs efficiently rescued T cells ($CD4^+$ and $CD8^+$) and NK cells from oxidant-induced inactivation and apoptosis. The mechanism of the myeloid DC-mediated lymphocyte protection was, at least in part, explained by the capacity of the myeloid DCs to neutralize extracellular oxygen radicals, which, in turn, was reversible upon coincubation with a catalase inhibitor. Our results are suggestive of a novel aspect of DC-lymphocyte interaction that may have implications for lymphocyte function in inflamed tissue. The Journal of Immunology,* 2007, 179: 21–25.

Dendritic cells (DCs)[3] form a pivotal part of adaptive immunity by virtue of their uniquely efficient presentation of Ag to T cells (1). However, DCs also communicate with NK cells (2–4), which are innate effector cells that are important in the early phase of infection (5, 6). The reciprocal interaction between DCs and NK cells has been identified as a putative link between innate and adaptive immunity. For example, DCs are stimulated by pathogen-derived components, which results in the production of NK cell-activating cytokines (7, 8). In parallel, interactions between NK cells and target cells can activate NK cells to secrete cytokines leading to DC activation (9–11), and NK cell-mediated lysis of foreign cells provides DCs with Ags (12). In addition, NK cells have been shown to selectively eliminate DCs that fail to differentiate into mature APCs (3, 9).

In infected tissue, viral and bacterial components activate the NADPH oxidase of neutrophils and mononuclear phagocytes (MPs), resulting in the production of large amounts of reactive oxygen species ("oxygen radicals") (13). These toxic compounds are important for killing phagocytosed microorganisms or infected cells and have been implicated in DC activation (14, 15). However, the release of radicals may be detrimental to surrounding cells and tissues ("oxidative stress"), including immune cells. We and others have previously reported that phagocyte-derived radicals compromise NK and T cell function and induce programmed cell death in these cells (16–21). Myeloid DCs (mDCs) are equipped with a functional NADPH oxidase (22, 23) and might thus share the ability of other myeloid cells to inactivate human NK cells and T cells. In this study, we investigated whether human mDCs affected the function and viability of lymphocytes under conditions of oxidative stress in vitro. Our results are suggestive of a novel aspect of DC-lymphocyte interaction that may have implications for lymphocyte function in inflamed tissue.

Materials and Methods

Cells

Peripheral venous blood was obtained as freshly prepared leukopacks from healthy blood donors at the Blood Center, Sahlgren's University Hospital, Göteborg, Sweden. The blood (65 ml/donor) was mixed with 92.5 ml of Iscove's modified DMEM, 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; Nycomed) density gradient. After centrifugation at 380 × *g* for 15 min, mononuclear cells were collected at the interface. The mononuclear cells were further separated into lymphocytes and MPs using a countercurrent centrifugal elutriation technique as described elsewhere (18). In brief, cells were fed into an ultracentrifuge at 2100 rpm, where the sedimentation rate of cells is balanced by a counter-directed flow through the chamber. By slowly increasing the flow rate, fractions of cells of well-defined sizes were collected. From this separation procedure, a fraction with >90% MPs was recovered. Lymphocyte fractions enriched for NK cells and T cells contained <3% phagocytes. These lymphocyte fractions were pooled and subjected to further separation. Purified preparations of NK cells were obtained from elutriated lymphocytes using the BD IMag NK cell isolation kit (BD Biosciences) according to the instructions provided by the manufacturer.

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[3] Abbreviations used in this paper: DC, dendritic cell; adMP, adherent mononuclear phagocyte; ALM-633, Alexa Fluor 633-coupled maleimide; 3-AT, 3-amino-1,2,4-triazole; mDC, myeloid dendritic cell; MFI, median fluorescence intensity; MP, mononuclear phagocyte; PHPA, *p*-hydroxyphenyl acetic acid; TP3, TO-PRO-3.

Differentiation of mDCs

MPs were differentiated to DCs using a 7-day standard protocol with GM-CSF and IL-4 (24). After 7 days, the majority of cells were immature $CD14^-CD11c^+CD83^-$ DCs as assessed by flow cytometry. Seven-day cultures of MPs ($CD14^+CD11c^+$, henceforth referred to as adherent MPs (adMP)) in the absence of cytokines were used as controls. In some experiments, pure preparations of $CD14^-CD11c^+CD83^-$ DCs were sorted out using a BD FACSAria cytometer equipped with three laser lines (405, 488, and 633 nm) and FACSDiva version 5 software (BD Biosciences). In other experiments, mDCs were maturated by the addition of IL-1β (4 ng/ml), IL-6 (100 U/ml), PGE_2 (0.5 μg/ml), and TNF-α (10 U/ml) to the DC cultures on day 5.

Lymphocyte cell death

One hundred thousand lymphocytes were incubated in a total volume of 200 μl with or without 50,000 mDCs or adMPs in the presence of oxidants, either derived from freshly isolated MPs (50,000 or 100,000 per well) or as exogenously added hydrogen peroxide (H_2O_2). After overnight incubation, lymphocytes were assayed for end-stage, oxidant-induced cell death by 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 (16). Apoptosis was confirmed using annexin V staining as described elsewhere (18). In some experiments, lymphocytes were exposed to MPs that were stimulated with the formylated bacterial peptide fMLF (0.1 μM) or yeast cells (MP/yeast ratio of 1:4).

Cytotoxicity

NK cell cytotoxicity was assayed using PKH-26-labeled K562 cells as described elsewhere (18). Labeled cells were incubated for 4 h with NK cells at an E:T ratio of 2.5:1. Before flow cytometry analysis, 0.5 μM TO-PRO-3 (TP3) was added. Lysed target cells were identified as double-positive $PKH\text{-}26^+TP3^+$ cells. NK cytotoxicity was also assayed using a traditional ^{51}Cr-release assay as described elsewhere (25).

Staining for cell surface thiols

The relative number of cell surface thiols on cells was determined using Alexa Fluor 633-coupled maleimide (ALM-633; Invitrogen Life Technologies) (26, 27). In brief, cells were incubated with 5 μM ALM-633 for 15 min on ice. After extensive washing, cells were stained with Abs directed against CD3, CD8, CD56, and CD16. Data were acquired and analyzed using a BD FACSCanto II with FACSDiva version 5 software, and results are presented as the median fluorescence intensity (MFI) values of ALM-633 staining.

H_2O_2 consumption assay

DCs or adherent mononuclear cells were resuspended in Krebs-Ringer glucose buffer (200,000 cells/ml) and incubated with 30 μM H_2O_2 for 15 min. The remaining H_2O_2 was determined fluorometrically by measuring the oxidation of *p*-hydroxyphenyl acetic acid (PHPA) (0.5 mg/ml) catalyzed by HRP (4 U/ml) using a PerkinElmer fluorescence spectrophotometer (LC50) (excitation at 320 nm; emission at 400 nm) (28).

Abs and reagents

The following antihuman mAbs were purchased from BD Biosciences: anti-CD3 (PerCP and allophycocyanin), anti-CD8 (AmCyan, PerCP, and allophycocyanin), anti-CD11c (PE-Cy5 and allophycocyanin), anti-CD14 (FITC), anti-CD16 (FITC and allophycocyanin-Cy7), anti-CD56 (PE, PE-Cy7, and allophycocyanin), anti-CD83 (PE). BD CompBeads and FITC- and PE-conjugated annexin V were from BD Biosciences. The following compounds were used: PHPA, PJ34, and PKH-26 (Sigma-Aldrich); dextran (Kabi Pharmacia); acid citrate dextrose (Baxter); BSA (ICN Biomedicals); EDTA and hydrogen peroxide (VWR International); Ficoll-Hypaque, Lymphoprep (Nycomed); TP3 and ALM-633 (Invitrogen Life Technologies); and HRP (Boehringer-Mannheim).

Statistics

Paired samples *t* tests were used throughout the study. MFI values were transformed logarithmically before statistical calculation. All reported *p* values are two-sided.

Results and Discussion

Oxygen radicals, which are synthesized by the NADPH oxidase-dependent reduction of oxygen, are toxic to a variety of cells and tissues. Within the population of lymphocytes, NK cells appear to be unusually sensitive to radical-induced toxicity. NK cells exposed to oxygen radicals, either delivered by adjacent phagocytes or in the form of exogenous H_2O_2, are thus rapidly inactivated and highly prone to apoptosis (16, 18). Although the mechanisms of radical-induced toxicity in NK cells have only been partially explored, it seems clear that the inactivation and ensuing apoptosis are critically dependent on the integrity of the poly(ADP-ribose) polymerase-1/apoptosis-inducing factor axis, with a minor contribution by traditional pathways of apoptosis such as the caspase cascade (18). T cells, in particular $CD8^+$ cells, are sensitive to oxygen radical-inflicted toxicity by similar intracellular pathways of apoptosis induction (18).

Several myeloid cell types have been shown to inactivate NK cells and T cells by this mechanism. Thus, freshly recovered MPs and neutrophils as well as malignant granulocytic cells from subjects with chronic myeloid leukemia all efficiently trigger dysfunction and apoptosis in lymphocytes by synthesizing and releasing oxygen radicals (16, 29, 30). mDCs are of myeloid origin, and we therefore chose to explore whether these cells share the apoptosis-inducing properties of other myeloid cells. In these experiments, enriched MPs were differentiated to DCs by using a standard protocol with GM-CSF and IL-4. After 7 days, immature DCs with the $CD14^-CD11c^+CD83^-$ phenotype were recovered from the culture and incubated with enriched NK or T cells overnight. Neither the mDCs nor the population of adherent mononuclear cells significantly affected lymphocyte viability in these experiments (not shown). These data imply the following: 1) that mDCs do not induce apoptosis in lymphocytes; and 2) that MPs lose their capacity to trigger apoptosis in lymphocytes after adherence; the latter finding is in agreement with a previous report (31).

In the next series of experiments we assessed the function and viability of NK and T cells under conditions of oxidative stress that were inflicted either by the addition of freshly recovered MPs or by exogenous H_2O_2. In agreement with earlier studies, a large proportion of lymphocytes acquired apoptotic features and became dysfunctional when exposed to MPs as well as after treatment with H_2O_2 (16, 18). The presence of mDCs, however, efficiently rescued NK and T cells from inactivation and apoptosis (Figs. 1 and 2). Thus, NK cells remained viable and functional in the presence of radical-producing phagocytes or H_2O_2 when coincubated with mDCs, and a similar mDC-mediated protection was observed for $CD8^+$ and $CD4^+$ T cells (Fig. 1). No apparent difference was observed between the protection exerted by immature mDCs and that by mature mDCs (data not shown). The capacity to rescue lymphocytes was significantly lower for adherent MPs, i.e., MPs cultured for the same period of time in the absence of GM-CSF and IL-4, than for corresponding mDCs (Fig. 1). To ensure that mDCs rather than contaminating cells in the GM-CSF/IL-4-stimulated cell preparations mediated the protection, mDCs were sorted using a FACS on the basis of $CD11c^+CD14^-$ phenotype and added to NK/T cell preparations. The sorted mDCs were as efficient as the crude preparation of mDCs in conveying NK and T cell protection against oxidant stress. Notably, mDCs were almost completely resistant to H_2O_2 used at concentrations sufficient to induce significant apoptosis in lymphocytes (60–250 μM; data not shown).

To shed light on the mechanisms of mDC-mediated lymphocyte protection, we first investigated whether supernatants from 7-day cultures of mDC could reconstitute the protective

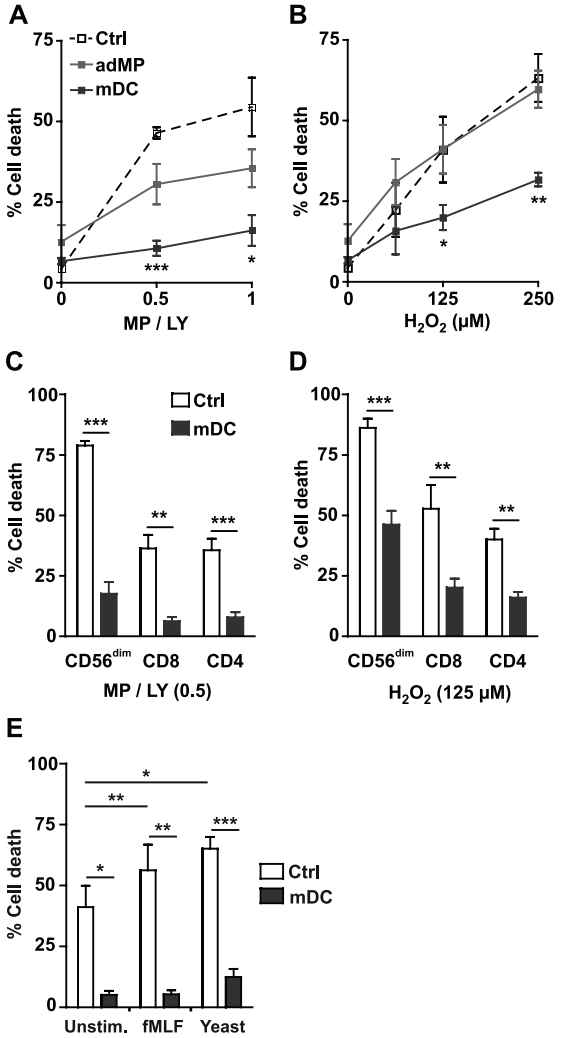

FIGURE 1. mDCs protect human lymphocytes from oxidant-induced cell death. *A* and *B*, Human lymphocytes (Ly) were incubated overnight with freshly isolated autologous MPs or H_2O_2 in the presence or absence of DCs or corresponding adMPs (mDC/lymphocyte and adMP/lymphocyte ratios at 1:2). Lymphocytes were significantly protected by mDCs against phagocytes (ratio 0.5, $p < 0.001$; ratio of 1, $p < 0.05$) (*A*) and H_2O_2 (125 μM, $p < 0.05$; 250 μM, $p < 0.001$) (*B*), but not by the adherent MPs. *C* and *D*, Oxidant-exposed lymphocytes were stained for lymphocyte surface Ags, and the percentages of apoptotic NK cells (with $CD3^-CD56^{dim}$ phenotype) and $CD8^+$/$CD4^+$ T cells were determined. All lymphocyte subsets were significantly protected by mDCs ($CD56^{dim}$, $p = 0.0002$ and $p < 0.0001$; $CD8^+$, $p = 0.003$ and $p = 0.004$; $CD4^+$, $p = 0.0003$ and $p = 0.003$ for MPs and H_2O_2, respectively; $n = 6$). *E*, Activation of MPs by addition of the bacterial peptide fMLF or by triggering phagocytosis of yeast cells enhanced the production of oxidants (data not shown) and the ensuing lymphocyte cell death (fMLF, $p = 0.004$; yeast, $p = 0.016$; $n = 5$). The addition of mDCs significantly protected lymphocytes from oxygen radicals released from fMLF- or yeast-activated MPs (fMLF, $p = 0.008$; yeast, $p = 0.001$,; $n = 5$). Ctrl, Control; Unstim., unstimulated.

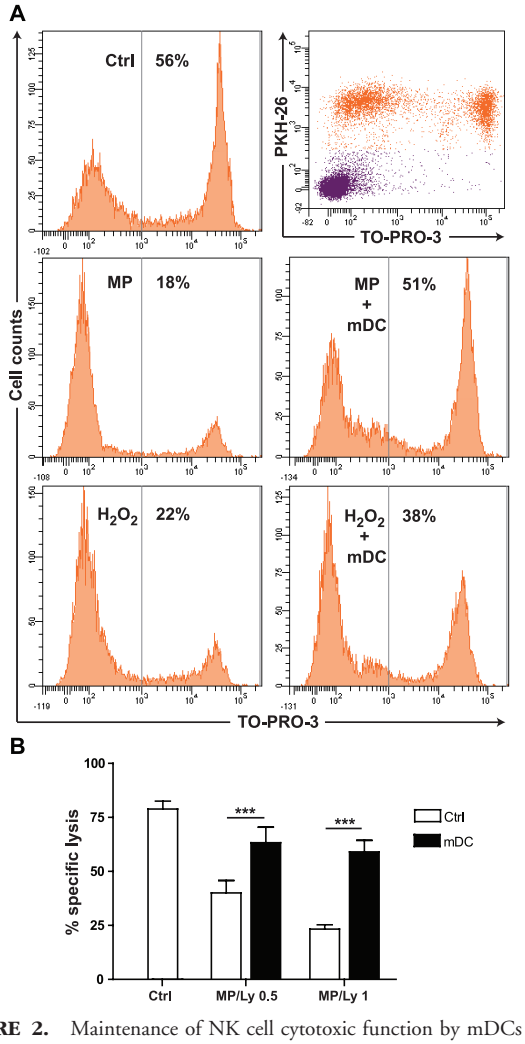

FIGURE 2. Maintenance of NK cell cytotoxic function by mDCs during oxidative stress. *A*, *Upper right dot plot*, NK cells are negative for the membrane dye PKH-26 and are displayed as purple dots. The orange PKH-26^+ K562 target cells form two populations; the one to the *right* is lysed and thus stained by TP3 whereas intact cells are TP3 negative and found to the *left*. In the histograms, PKH-26^+ K562 cells were assayed for TP3 staining. Numbers are the percentage of lysed target cells in a representative experiment. NK cell cytotoxic function was maintained by mDCs despite oxidative challenge by MPs (MP/NK ratio of 0.5, $p = 0.02$; $n = 4$) or H_2O_2 (60 μM, $p < 0.05$; $n = 4$). *B*, Similar results were obtained in a chromium-release microcytotoxicity assay. Lymphocytes (Ly) were incubated with or without MPs at designated ratios, and NK cell-mediated cytotoxicity against ^{51}Cr-labeled K562 cells was assessed. mDCs preserved the cytotoxic function of NK cells in the presence of oxygen radical-producing phagocytes ($p < 0.001$ for both MP/Ly ratios; $n = 7$). Ctrl, Control.

properties of these cells. mDC-derived supernatants, however, did not rescue lymphocytes from oxygen radical-induced toxicity (data not shown). Next, we investigated the capacity of mDCs to neutralize exogenous oxygen radicals. mDCs or 7-day cultures of adherent MPs were incubated with 30 μM H_2O_2 for 15 min and the remaining H_2O_2 was determined by measuring PHPA oxidation. As shown in Fig. 3*A*, mDCs were significantly more efficient at degrading H_2O_2 than the adherent MPs.

Catalase is an essential antioxidative enzyme that degrades H_2O_2 and is expressed by several types of myeloid cells (32). A catalase inhibitor, 3-amino-1,2,4-triazole (3-AT), was used in experiments aimed at clarifying whether the catalase expressed by mDCs contributed to the antioxidative properties of these cells. The presence of 3-AT significantly reversed the mDC-induced protection of lymphocytes against H_2O_2-induced apoptosis (Fig. 3*B*). In addition, 3-AT significantly reduced the capacity of mDCs to consume exogenous H_2O_2 (Fig. 3*C*).

Cells use thiols such as glutathione and thioredoxin for protection against the detrimental effects of oxidants. To assess the surface thiol expression by mDCs and adherent MPs, we stained these cells for thiols using fluorochrome-conjugated

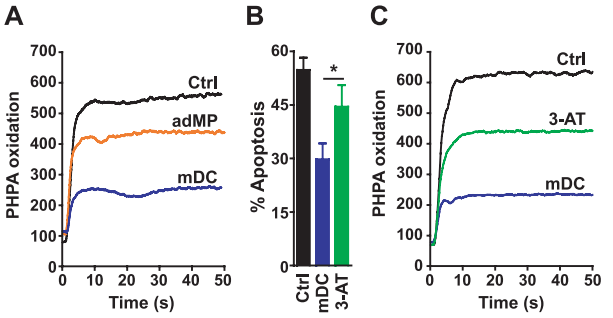

FIGURE 3. Consumption of hydrogen peroxide by mDCs. mDCs or corresponding adherent MPs were incubated with 30 μM H_2O_2 for 15 min. The remaining H_2O_2 was determined by measuring PHPA oxidation. *A*, mDCs neutralized significantly more H_2O_2 than adherent MPs ($p = 0.002$, $n = 5$). *B*, The mean ± SEM of four experiments in which the catalase inhibitor 3-AT (30 mM) reversed the mDC-mediated protection of lymphocytes against H_2O_2 ($p = 0.02$). *C*, 30 mM 3-AT blocked the capacity of mDCs to neutralize H_2O_2 ($p = 0.007$; $n = 4$). Ctrl, Control.

maleimide. As shown in Fig. 4, the thiol expression was significantly higher in mDCs than in the adherent MPs. In addition, lymphocytes incubated with mDCs, but not with adherent MPs, rapidly up-regulated the levels of surface thiols, thus suggesting that mDCs may convey resistance to oxidants by elevating thiol expression in lymphocytes (Fig. 4*B*).

It was recently shown that DCs release thioredoxin upon interaction with Ag-specific T cells, a process that is inhibited by glutamate (33). To investigate whether thioredoxin release contributed to the protection of lymphocytes, we added glutamate, at concentrations reportedly sufficient to inhibit thioredoxin release, to the mDC-lymphocyte cocultures and subsequently exposed them to oxidants. The addition of glutamate did not affect the ability of mDCs to protect lymphocytes from oxidants in these experiments (not shown), thus suggesting that thioredoxin release was not the principal mechanism of mDC-induced protection against oxidants.

We conclude that human mDCs are endowed with an efficient antioxidative defense system that conveys protection of NK and T cells from oxidant-induced inactivation. mDCs thus may have the capacity to uphold lymphocyte viability and function under conditions of oxidative stress such as in acute and chronic infections or in the malignant microenvironment. Our data suggest that a significant part of the protection against oxidants exerted by mDCs is related to catalase activity in these cells. In addition, our data reveal that mDCs convey protection against oxidants by increasing thiol expression in lymphocytes by an as yet undefined mechanism. It should be pointed out, however, that recent proteomic-based analyses have revealed that several antioxidative proteins are up-regulated as MPs differentiate into mDCs (34), and multiple mechanisms are therefore likely to contribute to the observed mDC-mediated lymphocyte protection.

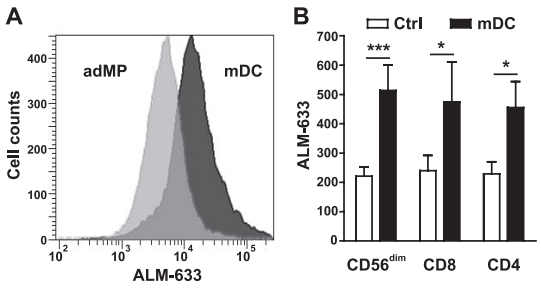

FIGURE 4. mDCs express high levels of surface thiols and induce thiol up-regulation on lymphocytes. *A*, mDCs displayed higher binding of the thiol-reactive reagent ALM-633 than adherent MPs ($p = 0.019$; $n = 5$). *B*, Experiments in which lymphocytes were incubated in the presence or absence of mDCs before ALM-633 staining. mDCs approximately doubled the expression of cell surface thiols on NK cells and $CD8^+$ and $CD4^+$ T cells after overnight incubation ($CD56^{dim}$ NK cells, $p = 0.0008$; $CD8^+$, $p = 0.028$; $CD4^+$, $p = 0.012$; $n = 5$). The effect was evident already after 1 h of lymphocyte-mDC incubation ($CD56^{dim}$ NK cells, $p = 0.036$; $CD8^+$, $p = 0.003$; $CD4^+$, $p = 0.007$; $n = 5$; not shown). Ctrl, Control.

Disclosures

The authors have no financial conflict of interest.

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