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Distinct CpG DNA and Polyinosinic-Polycytidylic Acid Double-Stranded RNA, Respectively, Stimulate CD11c$^-$ Type 2 Dendritic Cell Precursors and CD11c$^+$ Dendritic Cells to Produce Type I IFN[1]

Norimitsu Kadowaki,[2] Svetlana Antonenko, and Yong-Jun Liu

Two classes of nucleic acids, bacterial DNA containing unmethylated CpG motifs and dsRNA in viruses, induce the production of type I IFN that contributes to the immunostimulatory effects of these microbial molecules. Thus, it is important to determine which cells produce type I IFN in response to CpG DNA and dsRNA. CD4$^+$CD11c$^-$ type 2 dendritic cell precursors (pre-DC2) were identified as the main producers of type I IFN in human blood in response to viruses. Here we asked whether pre-DC2 also produce type I IFN in response to CpG DNA and dsRNA. Oligodeoxynucleotides containing particular palindromic CpG motifs induced pre-DC2, but not CD11c$^+$ blood DC or monocytes, to produce IFN-α. In contrast, a synthetic dsRNA, polyinosinic polycytidylic-acid, induced CD11c$^+$ DC, but not pre-DC2 or monocytes, to produce IFN-$\alpha\beta$. These data indicate that CpG DNA and polyinosinic-polycytidylic acid stimulate different types of cells to produce type I IFN and that it is important to select oligodeoxynucleotides containing particular CpG motifs to induce pre-DC2 to produce type I IFN, which may play a key role in the strong adjuvant effects of CpG DNA. ***The Journal of Immunology,*** **2001, 166: 2291–2295.**

The innate immune system has the capacity to recognize invariant molecular patterns shared by microbial pathogens (1). Recent studies have revealed that this recognition is a crucial step in inducing effective immune responses (1). The main mechanism by which microbial components augment immune responses is to stimulate APC, especially dendritic cells (DC),[3] to produce proinflammatory cytokines and to express high levels of costimulatory molecules for T cells (1, 2). These activated DC subsequently initiate primary T cell responses and dictate the type of T cell-mediated effector function (3).

Two classes of nucleic acids, namely 1) bacterial CpG DNA that contains immunostimulatory unmethylated CpG dinucleotides within specific flanking bases (referred to as CpG motifs) (4) and 2) dsRNA synthesized by various types of viruses (5), represent important members of the microbial components that enhance immune responses. Recent studies have shown that oligodeoxynucleotides (ODNs) containing CpG motifs (6, 7) and synthetic dsRNA, e.g., polyinosinic-polycytidylic acid (poly(I:C)) (8, 9), are capable of inducing DC to produce proinflammatory cytokines and to express high levels of costimulatory molecules.

A series of studies by Tokunaga and Yamamoto et al. has shown that bacterial DNA or synthetic ODNs containing unique palindromic CpG motifs induce human PBMC (10) and mouse spleen cells (11) to produce type I IFN (IFN-$\alpha\beta$) (reviewed in Ref. 12). Poly(I:C) was originally synthesized as a potent inducer of type I IFN (13, 14). Type I IFN plays an essential role in antiviral innate immunity and is widely used to treat viral hepatitis and various types of cancers (15). These effects appear to be due to direct inhibition of viral replication in infected cells and to the pleiotropic immunomodulating activity of type I IFN (15), such as 1) enhancing the cytotoxicity of NK cells and macrophages (15), 2) inducing T cell activation (16), 3) maintaining the survival of activated T cells (17), 4) stimulating human CD4$^+$ T cells to produce a Th1 cytokine IFN-γ (18), and 5) inducing the expression of TNF-related apoptosis-inducing ligand on T cells and thereby enhancing T cell cytotoxicity (19). Thus, CpG DNA and poly(I:C) are believed to be promising adjuvants for vaccination against infections and cancers due to their DC-stimulating and type I IFN-inducing capacity.

To understand the mechanisms underlying the induction of type I IFN by CpG DNA and poly(I:C) and to increase their efficacy as immunological adjuvants, it is important to determine which cells produce type I IFN in response to CpG DNA and poly(I:C). Two groups have recently shown that the main producers of type I IFN in human blood, designated natural IFN-$\alpha\beta$-producing cells (IPC), are identical with CD4$^+$IL-3Rα^{high}CD3$^-$CD11c$^-$ type 2 dendritic cell precursors (pre-DC2) (20, 21), which differentiate into DC in response to IL-3 (22) or viruses (23). Pre-DC2 IPC produce 1000 times more type I IFN than do CD11c$^+$ blood immature DC, monocytes, and monocyte-derived DC in response to viral stimulation (20). In this study we asked whether pre-DC2 will produce type I IFN in response to CpG DNA and poly(I:C). We show that 1) pre-DC2, but not CD11c$^+$ DC or monocytes, produce type I IFN in response to CpG ODNs containing particular palindromic

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[3] Abbreviations used in this paper: DC, dendritic cells; ODN, oligodeoxynucleotide; poly(I:C), polyinosinic-polycytidylic acid; IPC, natural IFN-$\alpha\beta$-producing cells; pre-DC2, type 2 dendritic cell precursors.

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sequences, and that 2) CD11c$^+$ DC, but not pre-DC2 or monocytes, produce type I IFN in response to poly(I:C).

Materials and Methods

Oligodeoxynucleotides

The following ODNs were purchased from Research Genetics (Huntsville, AL): 1668, TCCATGACGTTCCTGATGCT (6); 2117, T*CGT*CGTTTTGT*CGTTTTGT*CGTT (*C, methylated cytosine) (24); 2006, TCGTCGTTTTGTCGTTTTGTCGT (24); ACC-30, ACCGATACCGGTGCCGGTGACGGCACCACG (10); AAC-30, ACCGATAACGTTGCCGGTGACGGCACCACG (10); and GAC-30, ACCGATGACGTCGCCGGTGACGGCACCACG (10) (underlines indicate palindromic sequences). ODNs 1668, 2117, and 2006 are phosphorothioate forms, and ACC-30, AAC-30, and GAC-30 are phosphodiester forms. The phosphorothioate ODNs were added at 0 h at 0.1 μM (0.6 μg/ml) or 1 μM (6 μg/ml), and the phosphodiester ODNs were added at 0, 4, and 16 h, 5 μM (46 μg/ml) at each time point, to compensate for their degradation by DNase activity in medium.

Isolation and culture of cells

Monocytes, CD11c$^+$ DC, and pre-DC2 were isolated from human peripheral blood as previously described (22, 23). The cells were cultured for 24 h in RPMI 1640 containing 10% FCS at 2×10^4/200 μl in round-bottom 96-well culture plates in the presence of ODNs or 50 μg/ml poly(I:C) (Sigma, St. Louis, MO). Adult dermal fibroblasts (Clonetics, Walkersville, MD) were maintained as recommended by the company and were stimulated with 50 μg/ml poly(I:C) for 24 h at 7.5×10^4/ml in 12-well culture plates.

Analysis of viability of pre-DC2 stimulated with ODNs or poly(I:C)

Pre-DC2 cultured for 24 h without stimulation or with ODNs or poly(I:C) were stained with propidium iodide and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). After cell debris was excluded by an appropriate forward scatter threshold, the percentages of propidium iodide-negative cells were calculated.

Flow cytometric analysis of the expression of CD80 and CD86

Freshly isolated pre-DC2 and pre-DC2 stimulated with ODNs for 24 h were stained with PE-conjugated anti-CD80 (L307.4; Becton Dickinson), PE-conjugated anti-CD86 (2331; PharMingen, San Diego, CA), or an isotype control Ab. CD11c$^+$ DC cultured for 24 h without stimulation or with poly(I:C) were stained with FITC-conjugated anti-CD80 (L307.4), FITC-conjugated anti-CD86 (2331), or an isotype control Ab. The cells were analyzed with a FACScan flow cytometer. Dead cells were excluded by staining with propidium iodide.

Quantitation of cytokines by ELISA

An IFN-α ELISA kit, an IL-12 ELISA kit (BioSource International, Camarillo, CA), and an IFN-β ELISA kit (FUJIREBIO, Tokyo, Japan) were used to analyze cytokine production.

Results

CpG ODNs, but not poly(I:C), maintain the survival of pre-DC2 and induce them to differentiate into DC

Without any stimuli, the majority of pre-DC2 rapidly die within 24 h (22, 23) (Fig. 1*A*). We first examined whether different CpG ODNs and poly(I:C) prevent pre-DC2 from dying. We used three types of CpG ODNs: 1) 2006, which up-regulates the expression of costimulatory molecules on human blood DC (7) and B cells (24); 2) AAC-30 and GAC-30, which have palindromic CpG motifs and stimulate human PBMC (10) and mouse spleen cells (11) to produce type I IFN; and 3) 1668, which stimulates mouse DC to express high levels of costimulatory molecules and to produce proinflammatory cytokines TNF-α, IL-6, and IL-12 (6).

As shown in Fig. 1*A*, 1 μM CpG ODN 2006 efficiently blocked the death of pre-DC2. The same concentration of control CpG ODN 2117 having the same sequence except for methylated cytosines blocked the death of pre-DC2 to a lesser extent than 2006 (Fig. 1*A*). In the presence of a lower concentration (0.1 μM) of

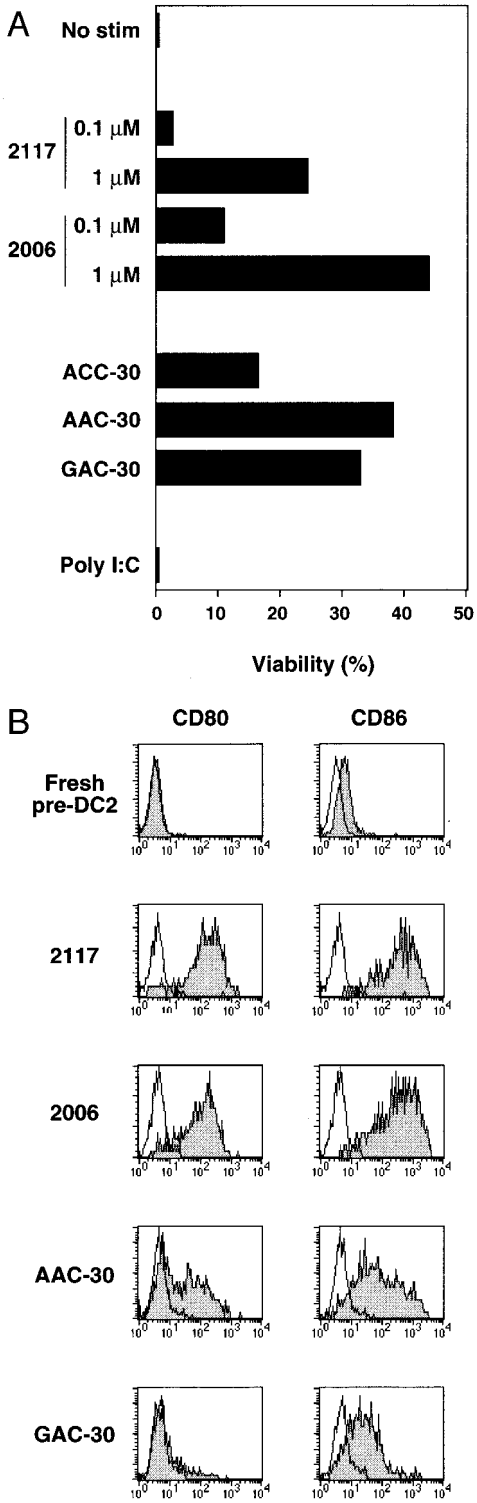

FIGURE 1. CpG ODNs, but not poly(I:C), maintain the survival of pre-DC2 and up-regulate the expression of CD80 and CD86. Pre-DC2 were cultured for 24 h without stimulation, with the indicated ODNs, or with 50 μg/ml poly(I:C). ODNs ACC-30, AAC-30, and GAC-30 were added at 5 μM (46 μg/ml). *A*, Viability. ODNs 2117 and 2006 were added at 0.1 μM (0.6 μg/ml) or 1 μM (6 μg/ml). After cell debris was excluded by an appropriate forward scatter threshold, the percentages of propidium iodide-negative cells were measured by flow cytometry. Data are representative of three experiments. B, The expression of CD80 and CD86. Freshly isolated pre-DC2 and pre-DC2 cultured with the indicated CpG ODNs for 24 h were stained with PE-conjugated anti-CD80 or anti-CD86 mAbs. ODNs 2117 and 2006 were added at 1 μM (6 μg/ml). Data are representative of three experiments.

2117, most pre-DC2 died, whereas a significant number of pre-DC2 survived in the presence of 0.1 μM 2006 (Fig. 1*A*). These are consistent with the finding that methylation of cytosine residues significantly diminishes immunostimulatory activity of CpG DNA (7). Another group of CpG ODNs, AAC-30 and GAC-30, also maintained the survival of pre-DC2, while ACC-30, which has been shown to lack the ability to induce human PBMC to produce type I IFN (10), was less efficient (Fig. 1*A*). Although 1 μM CpG ODN 1668 has been shown to activate mouse DC (6), the same concentration of 1668 did not maintain the survival of pre-DC2 (data not shown). This is consistent with the finding that CpG DNAs with strong stimulatory activity in the mouse system does not necessarily activate human immune cells (24, 25). In contrast to the CpG ODNs immunostimulatory for human cells, poly(I:C) did not maintain the survival of pre-DC2 (Fig. 1*A*).

The up-regulation of CD80 and CD86 expression on pre-DC2 is a hallmark of their differentiation into DC (22, 23). Unmethylated CpG ODN 2006 and methylated CpG ODN 2117 (1 μM) strongly up-regulated the expression of CD80 and CD86 (Fig. 1*B*). AAC-30 and GAC-30 also up-regulated CD80 and CD86, albeit to a lesser extent than 2006 and 2117 (Fig. 1*B*).

Taken together, these data indicate that CpG ODNs containing appropriate sequences efficiently maintain the survival of pre-DC2 and induce them to differentiate into DC. In marked contrast, poly(I:C) does not stimulate pre-DC2 to survive and differentiate into DC.

Distinct CpG ODNs, but not poly(I:C), induce pre-DC2 to produce IFN-α

Next we examined whether CpG ODNs and poly(I:C) induce pre-DC2 to produce IFN-α. Although 2006 strikingly up-regulated CD80 and CD86 (Fig. 1*B*), this CpG ODN as well as 2117 and 1668 did not induce pre-DC2 to produce detectable levels of IFN-α (Fig. 2). In contrast, AAC-30 and GAC-30 induced pre-DC2 to produce large amounts of IFN-α (AAC-30 508–1806 pg/ml; GAC-30 931–1362 pg/ml; $n = 3$; Fig. 2). ACC-30 induced pre-DC2 to produce much smaller amounts of IFN-α (18–161 pg/ml; $n = 3$; Fig. 2). None of the CpG ODNs used here induced CD11c$^+$ DC and monocytes to produce detectable levels of IFN-α (data not shown). In line with the finding that poly(I:C) did not maintain the survival of pre-DC2 (Fig. 1*A*), this reagent did not induce pre-DC2 to produce a detectable level of IFN-α (Table I). These data indicate that ODNs containing particular CpG motifs, but not poly(I:C), induce pre-DC2 to produce IFN-α.

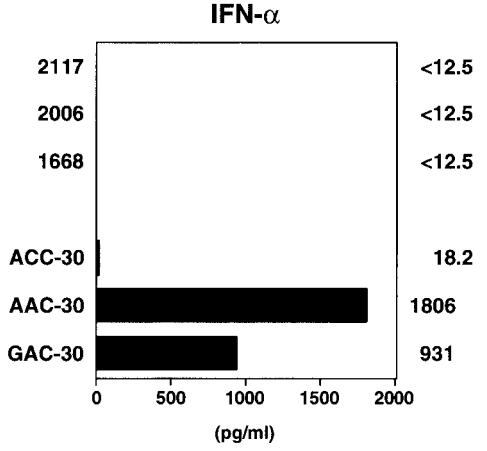

FIGURE 2. ODNs containing particular palindromic CpG motifs induce pre-DC2 to produce IFN-α. Cells were stimulated with the indicated CpG ODNs (2117, 2006, and 1668, 1 μM (6 μg/ml); ACC-30, AAC-30, and GAC-30, 5 μM (46 μg/ml)) for 24 h, and the concentrations of IFN-α in the supernatants were measured by ELISA. Data are representative of three experiments.

Poly(I:C) stimulates CD11c$^+$ myeloid DC to produce IFN-αβ and IL-12 and to undergo maturation

Although poly(I:C) did not induce pre-DC2 to survive (Fig. 1*A*) or to produce IFN-α (Table I), this reagent has been shown to induce human monocyte-derived DC to mature and to produce IL-12 (8, 9). Thus, we asked whether poly(I:C) stimulates CD11c$^+$ myeloid DC (26) to produce type I IFN and IL-12 and to undergo maturation. As shown in Table I, poly(I:C) induced CD11c$^+$ DC, but not pre-DC2 or monocytes, to produce small, but significant, amounts of IFN-α (24.1–97.9 pg/ml; $n = 3$), IFN-β (33.6–166.5 pg/ml; $n = 3$), and IL-12 (17.7–137.7 pg/ml; $n = 4$). Poly(I:C)-stimulated fibroblasts produced similar amounts of IFN-β (28.6–48.5 pg/ml; $n = 2$; Table I). Poly(I:C) strongly up-regulated the expression of CD80 and CD86 on CD11c$^+$ DC during 24-h culture (Fig. 3). Thus, poly(I:C) stimulates CD11c$^+$ myeloid DC, but not pre-DC2, to produce type I IFN and IL-12 and to become mature DC.

Discussion

One of the main effects of CpG DNA (12) and poly(I:C) (13, 14) is the induction of type I IFNs. In addition to the essential role of type I IFNs in antiviral innate immunity (15), they appear to be key cytokines to induce effective adaptive immunity due to their pleiotropic effects on various types of immune cells (15–19). Therefore, it is important to determine which cells produce type I IFN in response to CpG DNA or poly(I:C) to understand the mechanisms by which these nucleic acids augment immune responses and to exploit their ability as immunological adjuvants. The question we asked in this study was whether pre-DC2/IPC, the most potent producers of type I IFN in response to viruses (20, 21), are the target of CpG DNA and poly(I:C) for type I IFN production. We found that 1) CpG ODNs containing certain palindromic sequences induce pre-DC2, but not CD11c$^+$ DC, to produce type I IFN; and that 2) poly(I:C) stimulates CD11c$^+$ DC, but not pre-DC2, to produce type I IFN.

Hartmann et al. screened an extensive series of CpG ODNs to find those with the highest immunostimulatory activity for human cells (24, 27). They and others found that CpG ODN 2006 most potently activates human B cells (24), monocytes (25), and DC (7). In line with these findings, 2006 induced marked up-regulation of CD80 and CD86 on pre-DC2. However, this CpG ODN did not induce pre-DC2 to produce detectable levels of IFN-α. In contrast, another class of CpG ODNs, AAC-30 and GAC-30, which have been shown to induce human PBMC (10) and mouse spleen cells (11) to produce type I IFN, induced pre-DC2 to produce IFN-α. On the other hand, monocytes or CD11c$^+$ DC did not produce

Table I. *Poly(I:C) induces CD11c$^+$ DC, but not pre-DC2, to produce IFN-αβ and IL-12*[a]

Cells	IFN-α (pg/ml)	IFN-β (pg/ml)	IL-12 (pg/ml)
PBMC	<12.5	<8.3	<7.7
Monocytes	<12.5	<8.3	<7.7
CD11c$^+$ DC	24.1	56.6	28.5
Pre-DC2	<12.5	<8.3	<7.7
Fibroblasts	<12.5	28.6	ND

[a] PBMC, monocytes, CD11c$^+$ DC, pre-DC2 from the same donor(2×10^4/200 μl), or adult dermal fibroblasts (7.5×10^4/ml) were stimulated with 50 μg/ml poly(I:C) for 24 h, and cytokine concentrations in the supernatants were measured by ELISA. Data are representative of three experiments.

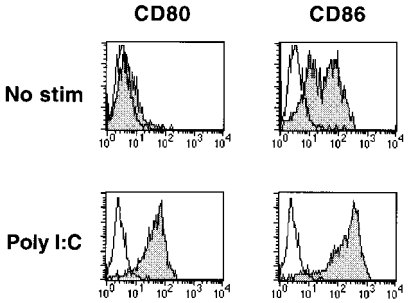

FIGURE 3. Poly(I:C) strongly up-regulates the expression of CD80 and CD86 on $CD11c^+$ DC. $CD11c^+$ DC cultured for 24 h without stimulation or with 50 μg/ml poly(I:C) were stained with FITC-conjugated anti-CD80 or anti-CD86 mAbs. Data are representative of three experiments.

detectable levels of IFN-α in response to 2006, AAC-30, or GAC-30. These data suggest that 1) pre-DC2 is the cell type that produces IFN-α in response to CpG DNAs; and that 2) it is important to select CpG DNAs containing particular sequences to induce pre-DC2 to produce IFN-α. In addition, the finding that 2006 induced pre-DC2 to up-regulate CD80 and CD86, but not to produce IFN-α, suggests that CpG DNA may induce pre-DC2 to differentiate into DC and to produce IFN-α through distinct signaling pathways.

Previous studies showed that methylation of cytosines in the CpG motifs abrogates the activity of CpG DNA to induce B cell proliferation (28) and NK cell activation (29). In this study, however, ODN 2117 containing methylated cytosines up-regulated CD80 and CD86 as strongly as ODN 2006 containing unmethylated CpG motifs, although 2117 did not support the survival of pre-DC2 as well as 2006 did. These data indicate that the methylation of cytosines abrogates the activity of CpG ODN 2006 only partially. It has been shown that ODNs with methylated CpG have a diminished, but significant, level of IFN-inducing capacity when encapsulated in liposomes (30). In addition, inverting CpG to GpC reduces, but does not totally abrogate, the activity of ODNs (including 1668 and 2006) to induce B cell proliferation and the up-regulation of costimulatory molecules (25). These findings suggest that although the presence of unmethylated CpG is most important for the optimal activity of immunostimulatory ODNs, those having appropriate flanking sequences can retain residual activity even without unmethylated CpG.

Our data suggest that $CD11c^+$ DC is the only blood cell type that produces a significant amount of type I IFN in response to poly(I:C). $CD11c^+$ DC and fibroblasts produced similar levels of IFN-β per cell. Since the number of fibroblasts in tissues is probably greater than that of $CD11c^+$ DC, the main producers of type I IFN in response to poly(I:C) and dsRNA may be fibroblasts, not blood cells, as has been shown (13, 14). It has recently been shown that poly(I:C) induces the maturation of monocyte-derived immature DC (8, 9). Whereas $CD11c^+$ DC appear to be myeloid-derived DC because they express myeloid markers (26), pre-DC2-derived DC may be lymphoid-derived DC because pre-DC2 lack myeloid markers (26) and express mRNA of pre-T receptor α-chain (31). Thus, poly(I:C) may stimulate myeloid-derived, but not lymphoid-derived, DC.

CpG DNA has pleiotropic effects on the immune system through activating APC, i.e., B cells, macrophages, and DC (4). In particular, a strong Th1-inducing effect of CpG DNA makes it a promising immunological adjuvant to treat infectious diseases (32), cancers (33), and allergic diseases (34). It is conceivable that type I IFN induced by CpG DNA contributes to the immunostimulatory effects of CpG DNA through various mechanisms, such as enhancing NK cell activity (11), inducing T cell activation (16), and enhancing IFN-γ production by T cells (18). Therefore, CpG ODNs that induce pre-DC2 to produce type I IFN may be suitable reagents for clinical application. DNase-resistant phosphorothioate forms of AAC-30 and GAC-30 do not have a type I IFN-inducing effect on pre-DC2 (N. Kadowaki, unpublished observations). Designing phosphorothioate forms of CpG ODNs having such an effect may be an important future direction for CpG immunology.

The molecular mechanisms by which the innate immune system recognizes microbial components are a major topic of current immunology (35). Although both CpG DNA (36, 37) and dsRNA (38) activate NF-κB and Jun NH_2-terminal kinase, upstream signaling pathways were shown to be different; CpG DNA, but not dsRNA, triggers a myeloid differentiation marker-88- and TNF receptor-associated factor-6-dependent pathway (39). The system we have developed here provides an opportunity to identify the different receptors involved in the recognition of CpG DNA vs dsRNA, which are possibly members of the Toll-like receptor family (40).

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