Fas Antigen Stimulation Induces Marked Apoptosis of T Lymphocytes in Human Immunodeficiency Virus–infected Individuals

By Peter D. Katsikis,* Eric S. Wunderlich,* Craig A. Smith,† Leonore A. Herzenberg,* and Leonard A. Herzenberg*

From the *Department of Genetics, Stanford University School of Medicine, Stanford, California 94305; and †Immunex Research and Development Corporation, Seattle, Washington 98101

Summary

Apoptosis (programmed cell death) has been proposed as a mechanism which plays an important role in the pathogenesis of human immunodeficiency virus (HIV) disease. Activation of Fas (CD95) can either result in costimulation of proliferation and cytokine production or in the induction of apoptosis of T lymphocytes. This raises the possibility that Fas is involved in the observed T cell apoptosis during HIV disease. In this report we show that peripheral blood CD4+ and CD8+ T lymphocytes from HIV-infected individuals undergo apoptosis in vitro in response to antibody stimulation (cross-linking) of Fas at a much higher frequency than from uninfected controls. This anti-Fas–induced T cell apoptosis is markedly higher than spontaneous T cell apoptosis in HIV-infected individuals. Antibodies against other members of the tumor necrosis factor (TNF)/nerve growth factor receptor family such as CD27, CD30, CD40, 4-1BB, p55 TNF receptor, p75 TNF receptor, and TNF receptor–related protein did not result in any increase of T cell apoptosis above that spontaneously observed in HIV+ individuals. Anti-Fas–induced apoptosis was much higher in symptomatic HIV-infected individuals; and the magnitude of anti-Fas–induced CD4+ T cell apoptosis correlated inversely with peripheral blood CD4+ T cell absolute counts. Surface expression of Fas on T cells was also found to be higher in HIV-infected individuals. Resting and activated CD4+ and CD8+ T cells both underwent apoptosis in response to anti-Fas antibody. L-Selectin positive memory CD4+ T cells were especially susceptible to anti-Fas–induced apoptosis. These findings show that CD4+ and CD8+ T lymphocytes in HIV-infected individuals are primed in vivo to undergo apoptosis in response to Fas stimulation, suggesting that Fas signaling may be responsible for the T lymphocyte functional defects and depletion observed in HIV disease.

Spontaneous and activation-induced T cell apoptosis (programmed cell death) has been well-documented in peripheral blood mononuclear cell cultures of HIV-infected individuals (1–3) and has been proposed as a mechanism involved in both the functional T cell defects observed even at early stages of the disease and the later depletion of T lymphocytes (4). The mechanisms however involved in this apoptosis have not yet been elucidated. Fas, a member of the TNF/nerve growth factor (NGF)1 receptor superfamily (5), and its ligand (FasL) have recently been cloned (6, 7), and signaling through Fas has been shown to induce apoptosis of transformed cell lines and chronically activated T cell clones and lines (8–10). On peripheral blood T cells of healthy individuals, however, Fas acts as a costimulating receptor enhancing proliferation and cytokine production (10). Cytotoxic T lymphocytes have recently been shown to use both the perforin system and Fas–FasL interactions to kill their target cells, including virally infected cells (11, 12). These observations raise the question whether Fas is involved in T cell apoptosis of HIV disease.

In this study we examined whether Fas stimulation by cross-linking with monoclonal antibodies induces peripheral blood T cell apoptosis in HIV-infected individuals. We found that Fas induces marked T cell apoptosis in HIV-infected individuals. In contrast, antibodies against other members of the TNF/NGF receptor family, such as CD27, CD30, CD40, 4-1BB, p55 TNF receptor, p75 TNF receptor, and TNF receptor–related protein (TNFRrp) all failed to induce apoptosis. Anti-Fas–induced apoptosis involved both CD4+ and CD8+ T cells and correlated with disease progression and severity. These findings indicate that Fas/Fas ligand interactions may play an important role in T cell apoptosis and therefore the pathogenesis of HIV disease.

1 Abbreviations used in this paper: NGF, nerve growth factor; TNFRrp, TNF receptor–related protein.
Materials and Methods

Samples and Reagents. Heparinized blood samples were obtained after informed consent of HIV+ (n = 79) and HIV- (n = 16) individuals. Patients were characterized as symptomatic (n = 48) when presented with Kaposi's sarcoma or opportunistic infections. Mouse monoclonal antibodies to Fas antigen (M3, M38, and M33, all IgG1 isotypes), anti-CD27 (M48), anti-CD30 (M44 and M67), anti-CD40 (M3), anti-4-1BB (M127), anti-CD57 TNF receptor (M50), anti-775 TNF receptor (M11), and anti-TNF receptor-related protein (M12) were provided by Immuneex Corp., Seattle, WA. Anti-CD3 monoclonal antibody OKT3 and the isotype control antibody DS-1 (anti-mouse IgM of the a allotype) were kindly provided by Drs. M. Feldmann, Kennedy Institute, London, UK, and A. B. Kantor, Dept. of Genetics, Stanford, respectively.

Anti-Fas Stimulation and Apoptosis Measurement. Freshly isolated PBMCs from HIV+ and HIV- individuals were cultured for 24 h at 1 × 10^6 cells/ml in RPMI 1640 + 10% FCS in 24-well plates coated with or without monoclonal anti-Fas antibody M3 or isotype control IgG, monoclonal antibody DS-1. Plates were washed with 500 µl/well RPMI 1640 and then washed twice with 500 µl media alone, solid phase bound to anti-Fas antibody M3 or isotype control IgG1 monoclonal antibody DS-1. Plates were coated with 500 µl/well of 10 µg/ml of antibodies in RPMI 1640 for 2 h at 37°C, plates were then washed twice briefly with 500 µl/well RPMI 1640 10% FCS and then washed once more in RPMI 1640 at 37°C. In six HIV+ individuals cells were incubated in plates coated with antibodies to Fas, CD27, CD40, 4-1BB, 775 TNFR, p55 TNFR, p75 TNFR, TNFRp, and DS-1. Apoptosis was determined by staining cells with 1 µg/ml Hoechst 33342 (HO342; Molecular Probes, Eugene, OR) for 7 min on ice (13). Cells were costained with anti-CD3-PE (Becton Dickinson and Co., Mountain View, CA), anti-CD4-FITC (PharMingen, San Diego, CA) and anti-CD4-CyChrome 5 (Cy5) (PharMingen) for 15 min on ice. After fixing in paraformaldehyde, 30,000 cells were analyzed by flow cytometry using a Facstar™ (Becton Dickinson and Co.) and Desk software (14). Apoptosis was determined by measuring HO342 staining cells gated on forward and side scatter for live lymphocytes. To verify that HO342 was indeed staining apoptotic cells in a number of the above experiments, cells were simultaneously stained for HO342 and Merocyanine 540 (Molecular Probes), another apoptotic stain which stains cells with loosely packed membranes (15). This latter stain showed that all HO342 staining cells also stained positive for Merocyanine 540. In another set of experiments DNA content was also measured by staining cells from the above cultures with hypotonic propidium iodide solution. Percentages of hypodiploid nuclei were close to those obtained for HO342-positive cells.

DNA Fragmentation. For DNA fragmentation analysis PBMCs (2 × 10^6 cells) were treated with media alone, solid phase bound anti-Fas antibody M3, or control antibody DS-1 for 5 h. Cells were centrifuged and DNA was extracted by resuspending cells in 20 µl of 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% sodium lauryl sarcosylate, 0.5 mg/ml Proteinase K, and incubating for 1 h at 50°C. RNase A was then added at 100 µg/ml and the extract was further digested for 1 h at 30°C. DNA was run on a 2% agarose gel in 1× TBE buffer, stained with ethidium bromide, and analyzed under UV light.

Flow Cytometry. T lymphocyte Fas surface expression was determined by monoclonal antibody staining and flow cytometry. Freshly isolated PBMCs from 24 HIV+ and 11 HIV- individuals were stained on ice with biotinylated monoclonal anti-Fas antibody M38 or isotype control monoclonal DS-1 for 15 min, cells were washed twice and incubated with streptavidin-PE (PharMingen) for 15 min and after two washes cells were incubated for 15 min with a biotinylated anti-PE monoclonal antibody (kindly provided by Dr. P. Lansdorp, The Terry Fox Laboratory for Hematology/Oncology, Vancouver, Canada) (17). After two more washes cells were incubated with streptavidin-PE for another 5 min. These last two incubation steps with their washes were performed two more times for 5 min each. Finally cells were incubated with anti-CD3-FITC (Becton Dickinson and Co.) and anti-CD4-CyChrome (PharMingen) or anti-CD3-FITC and anti-CD8-CyChrome (PharMingen) for 15 min. Cells were washed twice and fixed in 0.5% paraformaldehyde and analyzed as above.

All HO342-positive cells (apoptotic) double stained for TNFR, p75 TNFR, p55 TNFR, p75 TNFRp, and DS-1. Apoptosis was determined by staining cells with 1 µg/ml Hoechst 33342 (HO342; Molecular Probes, Eugene, OR) for 7 min on ice (13). Cells were costained with anti-CD3-PE (Becton Dickinson and Co., Mountain View, CA), anti-CD4-FITC (PharMingen, San Diego, CA) and anti-CD4-CyChrome 5 (Cy5) (PharMingen) for 15 min on ice. After fixing in paraformaldehyde, 30,000 cells were analyzed by flow cytometry using a Facstar™ (Becton Dickinson and Co.) and Desk software (14). Apoptosis was determined by measuring HO342 staining cells gated on forward and side scatter for live lymphocytes. To verify that HO342 was indeed staining apoptotic cells in a number of the above experiments, cells were simultaneously stained for HO342 and Merocyanine 540 (Molecular Probes), another apoptotic stain which stains cells with loosely packed membranes (15). This latter stain showed that all HO342 staining cells also stained positive for Merocyanine 540. In another set of experiments DNA content was also measured by staining cells from the above cultures with hypotonic propidium iodide solution. Percentages of hypodiploid nuclei were close to those obtained for HO342-positive cells.

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Flow Cytometry. T lymphocyte Fas surface expression was determined by monoclonal antibody staining and flow cytometry. Freshly isolated PBMCs from 24 HIV+ and 11 HIV- individuals were stained on ice with biotinylated monoclonal anti-Fas antibody M38 or isotype control monoclonal DS-1 for 15 min, cells were washed twice and incubated with streptavidin-PE (PharMingen) for 15 min and after two washes cells were incubated for 15 min with a biotinylated anti-PE monoclonal antibody (kindly provided by Dr. P. Lansdorp, The Terry Fox Laboratory for Hematology/Oncology, Vancouver, Canada) (17). After two more washes cells were incubated with streptavidin-PE for another 5 min. These last two incubation steps with their washes were performed two more times for 5 min each. Finally cells were incubated with anti-CD3-FITC (Becton Dickinson and Co.) and anti-CD4-CyChrome (PharMingen) or anti-CD3-FITC and anti-CD8-CyChrome (PharMingen) for 15 min. Cells were washed twice and fixed in 0.5% paraformaldehyde and analyzed as above.

Analysis of T cell subsets for memory and activation markers was performed by multicolor staining of cells and flow cytometry. PBMCs were stained for 15 min on ice with the following combinations of monoclonal antibodies: anti-CD3-FITC (PharMingen)/anti-CD4-CyChrome/anti-HLA-DR-PE (Becton Dickinson and Co.), anti-CD3-FITC/anti-CD4-CyChrome/anti-CD38-PE (PharMingen), anti-CD3-FITC/anti-CD8-CyChrome (PharMingen)/anti-HLA-DR-PE and anti-CD3-FITC/anti-CD8-CyChrome/anti-CD38-PE, anti-CD4-CyChrome/anti-CD45RA-PE/anti-CD62L-FITC (PharMingen) or anti-CD8-CyChrome/anti-CD45RA-PE/anti-CD62L-FITC (PharMingen). Cells were fixed in paraformaldehyde and analyzed as above.

Statistical Analysis. Data were analyzed by Mann-Whitney U test, Wilcoxon's signed-rank test for paired data, Student's t test, and Spearman's rho correlation using JMP Statistics Guide (SAS Institute Inc., Cary, NC).

Results

Anti-Fas-induced Apoptosis. We found that cross-linking of Fas with solid phase (plastic bound) anti-Fas antibody (M3) for 24 h induces marked in vitro apoptosis of PBMC T cells from 79 HIV-infected individuals. Apoptosis was measured by a modified HO342 stain and flow cytometry (Fig. 1 A). All HO342-positive cells (apoptotic) double stained for Merocyanine 540, which also stains apoptotic cells. Apoptosis induced by immobilized anti-Fas antibody was also confirmed by measurement of hypodiploid nuclei stained with hypotonic propidium iodide solution. Stimulation of PBMCs from HIV-infected individuals with the same anti-Fas antibody on plastic for 5 h resulted in enhanced DNA fragmentation, a hallmark of apoptosis (Fig. 1 B). In PBMCs from HIV-negative individuals little or no DNA fragmentation could be shown (Fig. 1 B). The levels of anti-Fas-induced apoptosis in HIV-infected individuals, as assessed by HO342 staining, were markedly higher than those spontaneously observed (Fig. 1 C). This anti-Fas-induced T cell apoptosis in HIV+ individuals was much higher than in HIV-negative controls (Fig. 1 A and C; Table 1). This increase in apoptosis observed with anti-Fas was also accompanied by a reduction in the number of T cells recovered in culture (data not shown). Apoptosis could be induced by two different mouse monoclonal anti-Fas antibodies (M3 and M38) both of which also kill the Fas-sensitive Jurkat cell line. In PBMCs from some HIV+ individuals which showed very high M3-induced apoptosis, a third anti-Fas monoclonal antibody (M33) that is a weak killer of the Jurkat cell line also induced significant killing (data not shown). This indicates that the threshold for triggering Fas induced T cell apoptosis in cells from some HIV+ individuals is lower than that of the Jurkat cell line.
4-1BB, p55 TNF receptor, and p75 TNF receptor on PBMCs from 6 HIV+ individuals failed to induce T cell apoptosis above background or control antibody induced levels (Fig. 2). Antibodies to TNFRrp also did not induce apoptosis (data not shown).

Anti-Fas-induced apoptosis, as measured by flow cytometry, occurred in both the CD4+ and CD8+ T lymphocytes subpopulations (Fig. 3A). Preliminary results suggest that CD8+ T cells may have earlier kinetics of apoptosis induction compared to the CD4+ T cells (data not shown).

| HIV+ Individuals (n = 79): | CD3+ lymphocytes | CD4+ lymphocytes | CD8+ lymphocytes |
|---------------------------|------------------|------------------|------------------|
| Asymptomatic (n = 48):    | 31 ± 2 (28)*     | 45 ± 3 (44)      | 17 ± 2 (9)       |
| Symptomatic (n = 31):     | 27 ± 3 (25)      | 38 ± 3 (35)      | 17 ± 3 (8)       |
| HIV- Individuals (n = 16):| 36 ± 3 (38)      | 56 ± 4 (58)      | 17 ± 2 (10)      |

* Mean ± SE (median).

Table 1. Anti-Fas Antibody-induced Apoptosis of T Lymphocytes
Figure 2. Representative experiment showing T cell apoptosis induced by cross-linking with immobilized antibodies members of the TNF/NGF receptor family on PBMCs from asymptomatic HIV+ individuals. T cell apoptosis could be induced only by Fas antigen stimulation. Experiment was repeated with six HIV+ individuals yielding similar results.

Spontaneous and anti-Fas-induced apoptosis of CD4+ T cells from HIV+ individuals was higher in symptomatic (n = 31) compared to asymptomatic individuals (n = 48), whereas no such difference could be shown for CD8+ T cells (Fig. 3 B). Spontaneous and anti-Fas antibody induced CD4+ T cell apoptosis correlated inversely with peripheral blood CD4+ T cell counts in both asymptomatic (P <0.001 in both, Spearman's rho) and the total of HIV+ individuals (P <0.001, Spearman's rho) (data not shown and Fig. 4).

Fas Surface Expression. The percentage of Fas+ CD4+ T cells in freshly isolated PBMC was significantly higher in 24 HIV+ compared with 11 HIV- individuals (Fig. 5 A). However the percentage of Fas+ CD4+ T cell in HIV+ individuals did not correlate with anti-Fas-induced apoptosis of these cells. The numbers of Fas+ CD4+ T cells also did not differ between symptomatic and asymptomatic HIV+ individuals and did not correlate with peripheral blood CD4+ cell counts (data not shown).

T Cell Surface Phenotype and Apoptosis. The representation of live (HO342-negative) activated (HLA-DR+ or CD38+) or resting (HLA-DR- or CD38-) CD4+ and CD8+ T cells remaining in culture after 24 h did not differ between control antibody and anti-Fas antibody-treated cultures of PBMCs from eight asymptomatic HIV+ individuals, indicating that activated and resting T cells populations both underwent apoptosis in response to anti-Fas stimulation to a similar extent (data not shown). In 13 asymptomatic HIV+ individuals tested, L-selectin expressing memory CD4+ T cells (CD62L+ CD45RA-) were found to be particularly depleted in response to anti-Fas stimulation (Fig. 5 B). In these 24-h cultures CD62L- CD45RA- and CD62L- CD45RA+ memory CD4+ populations were spontaneously depleted (data not shown).

Discussion

Spontaneous and activation-induced apoptosis of T cells has been proposed as a mechanism involved in both the T cell functional defects which are observed early on in HIV infection and in the depletion of CD4+ T cells which occurs later (4). Members of the TNF/NGF receptor family have been shown to act as costimulants of normal peripheral blood T cells inducing proliferation and cytokine production (5). Fas antigen in particular has also been shown to induce T cell apoptosis in transformed cell lines and chronically activated T cell lines and clones. In this study, we found that stimulation of Fas antigen induces marked apoptosis of freshly
isolated PBMC CD4+ and CD8+ T cells in HIV-infected individuals. Although it has been reported that other members of the TNF/NGF receptor family, such as p55 TNF receptor, CD30, and 4-1BB, can induce apoptosis under certain conditions (18–20), this apoptosis of T cells in HIV-infected individuals appears to be specific for Fas antigen. We found that antibodies against other TNF/NGF receptor family members such as CD27, CD30, CD40, 4-1BB, p55 TNF receptor, p75 TNF receptor, and TNFRp55 all failed to induce such apoptosis. Spontaneous and Fas-induced apoptosis correlated inversely with peripheral blood CD4+ T cell counts in the overall HIV+ population and the asymptomatic subset examined, indicating that apoptosis may be involved in disease progression. In addition Fas-induced apoptosis was higher in symptomatic versus asymptomatic HIV+ individuals indicating an association with disease severity.

Fas-expressing CD4+ T cells were significantly increased in HIV+ individuals when compared to the HIV− age-matched controls. However the percentage of Fas+ CD4+ T cells did not correlate with anti-Fas antigen induced CD4+ T cell apoptosis. This is not surprising as no correlation between the amount of anti-Fas-induced apoptosis and the levels of Fas cell surface expression has been found (21).

Since memory CD4+ T cells have been reported to be preferentially infected in HIV-infected individuals and this has been proposed as an important contributor to the impaired immune responses resulting from HIV infection (22, 23), we analyzed by multicolor flow cytometry T cell surface expression of memory and activation markers (CD45RA, CD62L, HLA-DR, and CD38) on PBMCs treated with antibody to Fas to determine which T cell subsets were depleted. As has been previously reported for spontaneous apoptosis (24), activated and resting CD4+ and CD8+ T cells were equally susceptible to anti-Fas treatment. Memory T cells were more susceptible than naive cells to Fas-induced apoptosis. Interestingly L-selectin (CD62L+) expressing CD45RA− memory CD4+ T cells, a subpopulation which responds potently to recall antigens and provides help for immunoglobulin production and isotype switching for B cells (25, 26), were found to be especially depleted in response to Fas stimulation. During an in vivo immune response, memory T cells responding to antigen may be selectively eliminated, as Fas ligand would be upregulated on these cells (7, 27) signaling apoptosis. Such Fas-mediated apoptosis in vivo could result in the described hyporesponsiveness to recall antigens of HIV disease (23).

Fas-induced apoptosis may also be involved in the described skewing of the immune system in favor of a Th2 like response which has also been proposed as a mechanism for the T cell defects of HIV infection (28). Differential expression of Fas on Th1 and Th2 type cells leads to the resistance of Th2 T cell clones to undergo activation-induced cell death (29). In HIV infection, a selective Th1 depletion that involves Fas/Fas ligand interactions may be accountable for the described skewing in favor of Th2 responses.

In acute viral infections such as EBV infection there is high transient T cells apoptosis which results in a transient im-
infection can induce T cell apoptosis, even though B cells infected by the virus in HIV+ individuals (35, 36). Acute EBV by PCR only 1% of peripheral blood CD4+ T cells are infected cells are prone to apoptosis in response to stimulation of Fas (34), this however cannot account for the levels of anti-Fas-induced apoptosis which we observe, since by PCR only 1% of peripheral blood CD4+ T cells are infected by the virus in HIV+ individuals (35, 36). Acute EBV infection can induce T cell apoptosis, even though B cells are primarily infected, and this is accompanied by increased Fas expression on T cells (30). Similarly, priming of T cells during HIV infection for apoptosis is more likely to be the result of extracellular stimulation or viral proteins entering the cells and altering the Fas signal pathway. In vivo cross-linking of CD4 on T cells in mice induces apoptosis which is mediated by Fas (37). Treatment with gp120 and antibodies to gp120 has also been shown to prime CD4+ T cells for activation induced apoptosis although the role of Fas has not been examined in this system (38, 39). This mechanism would directly account for anti-Fas-induced apoptosis of CD4+ but not CD8+ T cells, different mechanisms of apoptosis however may be operative in these two subsets (40). The possibility of other viral protein-T cell surface molecule interactions leading to Fas-mediated apoptosis such as gp120 or Tat and CD26 (41, 42) also cannot be excluded. Recently intracellular expression of gp160 was shown to enhance susceptibility to Fas-induced apoptosis, indicating that intracellular interactions with viral proteins may also be involved in this apoptosis priming (43). Finally, HIV-induced alterations in the cytokine network in the form of over- or underproduction of cytokines may also be playing a role in Fas-related apoptosis.

Our findings show that the Fas signal transduction pathway of T cells is primed to induce apoptosis in HIV-infected individuals. The mechanism by which HIV infection primes T cells to Fas-related apoptosis is not at the moment clear. HIV-infected cells are prone to apoptosis in response to stimulation of Fas (34), this however cannot account for the levels of anti-Fas-induced T cell apoptosis which we observe, since by PCR only 1% of peripheral blood CD4+ T cells are infected by the virus in HIV+ individuals (35, 36). Acute EBV infection can induce T cell apoptosis, even though B cells are primarily infected, and this is accompanied by increased Fas expression on T cells (30). Similarly, priming of T cells during HIV infection for apoptosis is more likely to be the result of extracellular stimulation or viral proteins entering the cells and altering the Fas signal pathway. In vivo cross-linking of CD4 on T cells in mice induces apoptosis which is mediated by Fas (37). Treatment with gp120 and antibodies to gp120 has also been shown to prime CD4+ T cells for activation induced apoptosis although the role of Fas has not been examined in this system (38, 39). This mechanism would directly account for anti-Fas-induced apoptosis of CD4+ but not CD8+ T cells, different mechanisms of apoptosis however may be operative in these two subsets (40). The possibility of other viral protein-T cell surface molecule interactions leading to Fas-mediated apoptosis such as gp120 or Tat and CD26 (41, 42) also cannot be excluded. Recently intracellular expression of gp160 was shown to enhance susceptibility to Fas-induced apoptosis, indicating that intracellular interactions with viral proteins may also be involved in this apoptosis priming (43). Finally, HIV-induced alterations in the cytokine network in the form of over- or underproduction of cytokines may also be playing a role in Fas-related apoptosis.

In this study we show that CD4+ and CD8+ peripheral blood T cells from HIV-infected individuals undergo apoptosis in response to stimulation through Fas antigen. The level of CD4+ T cell apoptosis correlates inversely with peripheral blood CD4+ T cell counts as well as the presence of symptoms thus implying an association of anti-Fas-induced apoptosis with disease progression. These findings provide evidence of Fas involvement in T cell apoptosis of HIV disease, a process which may play an important role in the pathogenesis of the disease. Understanding the mechanisms of T lymphocyte apoptosis in HIV disease may prove of great importance not only in the design of new therapeutic approaches but also in vaccine development.

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Address correspondence to Dr. P. D. Katsikis, Dept. of Genetics, Beckman Center B007, Stanford University School of Medicine, Stanford, CA 94305.

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