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HIV-1-SPECIFIC PRODUCTION OF IFN-γ AND MODULATION BY RECOMBINANT IL-2 DURING EARLY HIV-1 INFECTION[1]

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Specific cellular immune responses to human immunodeficiency virus type 1 (HIV-1) were assessed in mononuclear leukocyte cultures from homosexual men with documented, early phase HIV-1 infection. Cell cultures from men with a mean duration of 1.3 yr (range, 0.3 to 2.2 yr) of HIV-1 infection were treated with UV-inactivated, whole, purified HIV-1 Ag together with various concentrations of rIL-2. Cell supernatants were harvested after 5-day incubation and assayed for IFN activity against encephalomyocarditis virus in human WISH cells. IFN subtypes were characterized by neutralization of antiviral activity with antiserum specific for human IFN-γ and IFN-α. Results showed that cultures from 68% (17 of 25) of the HIV-1-seropositive subjects produced "immune" IFN-γ in response to whole HIV-1 Ag plus rIL-2. IFN-γ was induced in only 20% (5 of 25) of cultures treated with HIV-1 Ag alone. Enhancement of HIV-1-specific IFN-γ production by rIL-2 was synergistic rather than additive in that titers induced by the mixture were consistently higher than the sum of IFN titers induced by HIV-1 or rIL-2 alone. This effect was not demonstrable in cultures from 18 HIV-1-seronegative men. Similarly, HIV-1-immune specific augmentation of IFN-γ production by rIL-2 was noted for PENV9, a recombinant HIV-1 envelope glycoprotein gp41 and gp120 fragment. Production of IFN-γ may be an important, HIV-1-immune specific parameter in the host response to this retrovirus.

Infection with HIV type 1 (HIV-1) (1–4) is known to result in a spectrum of clinical responses ranging from asymptomatic illness to AIDS (5). These clinical conditions can develop in association with relatively efficient humoral antibody responses to HIV-1 (6). This suggests that other antiviral mechanisms, particularly cell-mediated immunity, are significant in host resistance to HIV-1. Although cellular immune responses are known to occur in many other retroviral infections (7–9), they have been difficult to demonstrate and are poorly understood in HIV-1 infection. Treatment of mononuclear leukocytes (MNL)[3] from HIV-1 seropositive subjects with various whole virus and recombinant HIV-1 protein preparations has resulted in relatively low level and inconsistent stimulation of lymphocyte blastogenesis (10–12). This may be related to immunosuppressive properties of the complete virus (13) or viral envelope (14). It is also unclear as to what effect duration of HIV-1 infection has on cellular responses to this retrovirus, as these studies did not differentiate early from later phase infection.

IFN-γ production is known to occur during immune specific reactivity of T lymphocytes to viral Ag (15) and is regulated by IL-2 (16). In the present study, we assessed production of "immune" IFN-γ in blood MNL cultures from HIV-1-seropositive homosexual men as a parameter of HIV-1-specific cellular immunity. We show that inactivated, purified HIV-1, or a recombinant envelope fragment of HIV-1, together with rIL-2 can induce IFN-γ in cultures from the majority of homosexual men with relatively short duration of HIV-1 infection, but not in cultures from HIV-1-seronegative subjects. Production of IFN-γ may be a significant, HIV-1-immune-specific parameter in assessing both the natural history of this human retrovirus infection and the efficacy of intervention strategies.

MATERIALS AND METHODS

Subjects. Homosexual male subjects were part of the Pitt Men's Study, the Pittsburgh portion of the Multicenter AIDS Cohort Study, a prospective investigation of the natural history of HIV-1 infection (17). The volunteers were enrolled in the epidemiologic study from April 1984 through March 1985, and were reexamined at 6-mo intervals for clinical status, risk factors for AIDS and HIV-1 infection, and HIV-1 serostatus. Duration of HIV-1 infection was defined as the time from the midpoint between two semiannual study visits in which a change in HIV-1 ELISA and Western blot antibody status occurred, and the time of this cellular immune testing (18).

HIV-1 serology. Sera were assayed for HIV-1 antibody by enzyme immunoassay using a commercial kit (LAV-EIA, Genetic Systems, Seattle, WA). Positive samples were confirmed by Western blot in a commercial laboratory (Biotech Laboratories, Rockville, MD). Protein bands were scored as negative (zero score) or +1, +2, and +3 based on increasing intensity. Sera with a cumulative score of +3 or more were considered as true positives (18).

MNL cultures and induction of IFN. MNL were separated from heparinized blood on Ficoll-Hypaque gradients, washed, and cultured at 10^6 cells/ml in medium 199 supplemented with 10% human AB^+ serum (heat inactivated; negative for antibodies to HIV-1 and

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[3] Abbreviations used in this paper: MNL, mononuclear leukocytes; PENV9, HIV-1 envelope glycoprotein fragments of gp41 and gp120.

CMV (19). HIV-1 Ag was sucrose gradient-purified virus (HTLV-IIIb/H9) containing HIV-1 proteins p24, gp41, p53/55, p64, and gp120 as detected by Western blot (obtained from Dr. Steve Alexander, Biotech). The virus was UV-inactivated (10 min at 4000 ergs/cm^2/s). In the first series of experiments, inactivated HIV-1 was added to the cultures at a final dilution of 1/10,000 of purified virus, or a final concentration of 4 ng protein/ml. The rIL-2 (Cetus, Emeryville, CA) was added to replicate cultures at final concentration of 1 commercial U equivalent to 1.9 Biologic Response Modifier Program (BRMP) U/ml as determined by bioassay with the IL-2-dependent CTLL-2 cell line in comparison with a BRMP IL-2 standard (lot ISDP-841) (assayed by Dr. M. Lyte, University of Pittsburgh).

In the second series of experiments, cells were treated in the same manner with final dilutions of 1/1,000, 1/10,000, and 1/100,000 of HIV-1 whole viral Ag (40, 4, and 0.4 ng protein/ml) with and without 0.1, 1.0, and 10 commercial U of rIL-2. Controls included untreated cell cultures and cells treated with these concentrations of rIL-2 alone. Similar experiments were done using PENV9, a recombinant envelope gp120-gp41 fragment of HIV-1 (20) at final concentrations of 1 μg, 100 ng, and 10 ng. Treated and untreated MNL were cultured for 5 days at 37C in flat-bottom microwells (0.2 ml cells/well). Replicate cell cultures were also treated with PHA-P (Difco, Chicago, IL) (25 μg/ml) for 4 days and CMV Ag (Davis strain; 1/200 final dilution of UV-inactivated preparation) for 5 days (19) together with 1 U of rIL-2. Preliminary experiments had shown that these time periods were optimal for detection of IFN-γ and blastogenic responses. All subjects were seropositive for CMV as determined by immunofluorescence assay (FIAX, M.A. Bioproducts, Silver Spring, MD).

IFN assay and characterization. Cell supernatants were assayed for IFN by 50% reduction of cytopathic effect using a modification of the method of Armstrong (21), which utilizes WISH cells and encephalomyocarditis virus as the challenge virus. IFN titers were adjusted with an international reference human IFN-α standard (MRC Research Standard B, preparation 69/19) and expressed as U per milliliter, and as geometric means ($\log_{10}$) of the Δ U of IFN (differences between IFN titers in stimulated cultures and controls).

The antiviral activity in the culture supernatants was characterized for the type of IFN by neutralization with antiserum to human IFN-α or IFN-γ (National Institutes of Health GO 26-502-568 and GO 34-501-565, respectively). Each sample to be tested was diluted to 100 U and mixed with a dilution of anti-IFN serum previously determined to neutralize 100 U of IFN-α (Flow Laboratories, McLean, VA) or IFN-γ (Biogen Research Corp., Cambridge, MA) standards. A subset of specimens was also confirmed by an RIA using mAb to IFN-γ (Centocor, Malvern, PA).

T cell enumeration. T lymphocytes were identified by direct immunofluorescence with mAb anti-Leu-4 (pan T), anti-Leu-3a (helper T), and anti-Leu-2a (suppressor-cytotoxic T) (Becton Dickinson, Mountain View, CA) using a flow cytometer (EPICS C, Coulter, Hialeah, FL). Data for T cell counts are arithmetic means.

RESULTS

Peripheral blood MNL from HIV-1 serum antibody-positive homosexual men were cultured for 5 days with a 1/10,000 final dilution of purified, UV-inactivated HIV-1 (4 ng protein/ml) and 1 U of rIL-2, and the culture medium was assayed for IFN. Cultures were treated with both HIV-1 Ag and rIL-2 because preliminary experiments showed that HIV-1 Ag alone induced IFN in MNL from only a minority of HIV-1-seropositive subjects. Furthermore, mitogen and Ag-induced IL-2 production, which is required for production of IFN-γ (16), is defective in certain HIV-1-infected subjects (22, 23), and this defect can be overcome by addition of IL-2 (24, 25). Results in Figure 1 show that HIV-1 Ag plus rIL-2-induced IFN-γ in 83% (5/6) of MNL cultures from six different seropositive men with early documented duration of HIV-1 infection (mean, 1.2 yr; range, 0.3 to 2.0 yr). IFN titers averaged 38-fold higher in cultures treated with HIV-1 Ag plus rIL-2 than in cultures treated with HIV-1 Ag or rIL-2 alone. The IFN activity was completely neutralized by anti-IFN-γ but not by anti-IFN-α serum in all of the samples (data not shown).

Addition of rIL-2 alone induced IFN-γ in MNL from some of the HIV-1-seropositive subjects (Fig. 1). A similar effect of IL-2 in the absence of Ag or mitogens has been described in MNL cultures from healthy donors (26, 27). However, in our study, MNL from 67% (4/6) of the subjects produced more IFN in response to HIV-1 Ag plus rIL-2 than the sum of the titers of IFN induced separately by HIV-1 Ag or rIL-2 (Fig. 1). Therefore, rIL-2 enhanced HIV-1-specific IFN-γ production in a synergistic rather than an additive fashion.

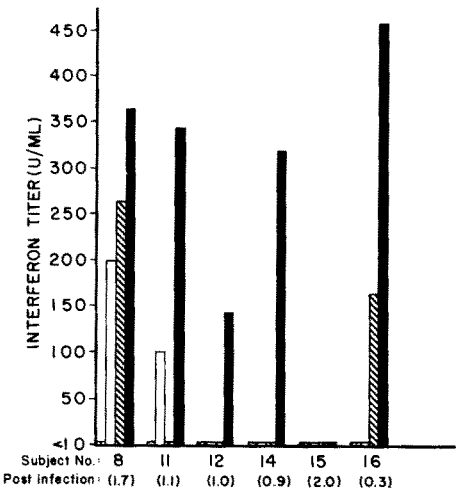

Figure 1. Enhancement of HIV-1 Ag-induced IFN-γ production by rIL-2. MNL from six homosexual men with documented mean duration of HIV-1 infection of 1.2 yr (range, 0.3 to 2.0 yr) were treated for 5 days at 37°C without Ag or rIL-2 (▨), or with rIL-2 alone (□), HIV-1 Ag alone (▧), or rIL-2 plus HIV-1 Ag (■).

The enhancement of HIV-1 induced IFN-γ production by rIL-2 was consistently HIV-1 immune specific. Evidence for this is that HIV-1 Ag alone did not induce IFN in MNL cultures from 10 HIV-1-seronegative men. Addition of rIL-2 was done in four of these cultures. In two of these MNL cultures, IFN was not produced in response to either rIL-2 alone or rIL-2 plus HIV-1 Ag; in the other two cultures, rIL-2 alone induced more IFN-γ (500 and 230 U) than did rIL-2 plus HIV-1 Ag (80 and 165 U, respectively).

To define more fully the IFN response to HIV-1 Ag, MNL from 19 men with similar duration of HIV-1 infection as above (average, 1.4 yr; range, 0.5 to 2.2 yr) were treated with various concentrations of HIV-1 Ag and rIL-2. MNL from 16% (3/19) of the subjects produced IFN-γ in response to HIV-1 Ag alone. Addition of rIL-2 resulted in enhanced production of HIV-1 Ag-induced IFN in 63% (12/19) of the MNL cultures (representative example, Fig. 2*A*). Antiviral activity in the samples was neutralized by antiserum to IFN-γ but not IFN-α. Additional testing of samples from two of these subjects for IFN-γ by RIA confirmed this pattern of augmented IFN-γ production induced by various concentrations of HIV-1 Ag and rIL-2. The effect of rIL-2 was dose dependent, with more IFN being induced by higher concentrations of rIL-2. The results also indicated that the lower concentrations of HIV-1 Ag (1/10,000 and 1/100,000 dilutions, 4.0 and 0.4 ng protein/ml cells) combined with 1 or 10 U of rIL-2 were the most consistent inducers of IFN-γ. Furthermore, we noted that the highest concentration (1/1,000 dilution) of HIV-1 Ag in combination with rIL-2 consistently induced less IFN (example, Fig. 2). This effect was not related to loss of cell viability, as no evidence of cellular

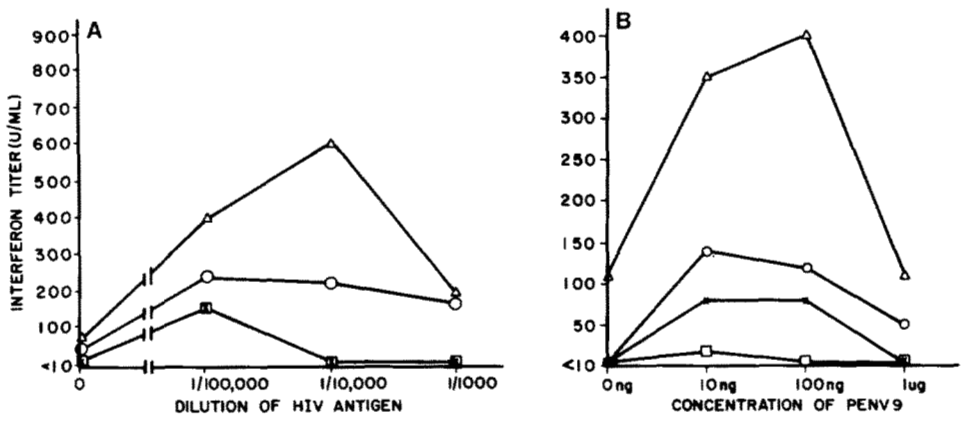

Figure 2. *A*, induction of IFN-γ in MNL cultures from an HIV-1-seropositive man by different combinations of HIV-1 Ag and rIL-2 concentrations. The cells were treated with various dilutions of HIV-1 Ag (see *Materials and Methods*). The same samples were also treated without rIL-2 (□), or with rIL-2 at final concentrations of 0.1 (×), 1.0 (○), or 10 (△) commercial U/ml. Data shown are from subject 32 (1.4 yr postinfection), one of the 12 subjects tested in these experiments whose MNL produced IFN in response to one or more combinations of HIV-1 Ag and rIL-2. *B*, induction of IFN-γ in MNL cultures from an HIV-1-seropositive man by different concentrations of PENV9 Ag and rIL-2. Data shown are from subject 6 (1.6 yr postinfection).

toxicity was observed with any of the various Ag-rIL-2 combinations. A similar pattern of IFN-γ production was shown in MNL cultures treated with the recombinant form of HIV-1 envelope Ag, PENV9 (Fig. 2*B*).

Additional analysis was done to determine why MNL from some of these HIV-1-seropositive men failed to produce IFN-γ in response to HIV-1 Ag plus rIL-2. A strong correlation was observed between immune-specific induction of IFN-γ by HIV-1 Ag plus rIL-2 and that induced by another viral Ag (CMV) and with numbers of circulating $CD4^+$ lymphocytes. Of the 19 HIV-1-seropositive men tested, 12 had both an HIV-1-specific and CMV-specific IFN response (group 1, Table I; mean duration of infection 1.4 yr, range 0.6 to 2.2 yr). In contrast, MNL from only two of the other seven men, who did not have an IFN response to HIV-1, showed such a response to CMV Ag (group 2, Table I; mean duration of infection 1.3 yr, range 0.5 to 2.1 yr) ($p = 0.005$, Fisher's exact test). Compared to group 1, men in group 2 also had a lower, although not significantly different, IFN-γ response to the mitogen PHA plus rIL-2. PHA induction of IFN-γ in group 2 men, however, was significantly lower than in HIV-seronegative subjects. Moreover, HIV-1-specific IFN-γ production was found to be significantly associated with numbers of Th ($CD4^+$) cells (Table I). Mean $CD4^+$ cell counts were higher in the 12 group 1 men who had an HIV-1-specific IFN response as compared with the seven group 2 subjects with a similar duration of infection whose MNL did not respond.

There was no relationship discernible between HIV-1 IFN responses and clinical symptoms, because only one of the 19 men tested had severe symptoms (AIDS-related complex) at the time of this study. IFN was not detected in HIV-1 Ag-treated cultures from this individual (member of group 2, Table I). Additional studies have shown that MNL from three AIDS patients (after the first episode of *Pneumocystis carinii* pneumonia) failed to produce IFN-γ in response to HIV-1 Ag and rIL-2.

MNL from eight HIV-1-seronegative men neither produced IFN in response to various concentrations of HIV-1 Ag alone, nor had enhanced levels of IFN-γ in response to rIL-2 plus HIV-1 Ag (Table I). Cultures from five of the eight seronegative men produced IFN-γ in response to rIL-2 alone (e.g., mean, 62 U IFN in response to 10 U rIL-2). Similar to our initial studies, addition of HIV-1 Ag together with rIL-2 resulted in reduced titers of IFN in all

TABLE I

Relationship of HIV-1-specific production of IFN-γ with other T cell immune parameters in homosexual men[a]

	HIV-1 Seronegative Subjects $n = 8$		HIV-1 Seropositive Subjects			
			Group 1 $n = 12$		Group 2 $n = 7$	
IFN Titers	Mean	n responders[b]/n tested	Mean	n responders/n tested	Mean	n responders/n tested
HIV-1 Ag + rIL-2	<10	0/8	198[c]	12/12	<10	0/7
CMV Ag + rIL-2	294	8/8	287	12/12	7[d]	2/7
PHA + rIL-2	4027	8/8	2783	11/11	1579[e]	6/6
T Cell Nos.	Mean ± (SE)		Mean ± (SE)		Mean ± (SE)	
CD^{3+}	1415 (144)		1730 (133)		1461 (172)	
CD^{4+}	879 (85)		836 (89)		533 (85)[f]	
CD^{8+}	512 (65)[g]		846 (96)		855 (91)	

[a] Results are given as the geometric mean of the Δ U of IFN-γ. For HIV-1 Ag, this is the difference between the peak IFN titer induced by either a 1/10,000 or 1/100,000 dilution of HIV-1 Ag (4.0 or 0.4 ng protein/ml) together with 1 U or 10 U of rIL-2 (Cetus), and the combined titer induced by that concentration of HIV-1 Ag alone and 1 U or 10 U of rIL-2 alone. Results for CMV Ag and PHA are also presented as geometric means of the Δ units of IFN-γ, or the difference between IFN titers induced by CMV or PHA plus 1 U of rIL-2, and the combined titer induced by CMV or PHA alone and 1 U of rIL-2 alone. IFN-γ produced in response to CMV Ag alone was 151, 155, and 4 U, and to PHA alone was 2692, 1622, and 1023 U for HIV-seronegative and HIV-seropositive group 1 and group 2 men, respectively. Data for T cell numbers are arithmetic means of the absolute count per cubic millimeter. Duncan's New Multiple Range Test was used to compare all group means.

[b] "Responders" refers to those cultures showing positive Δ units of IFN in response to mitogen or Ag plus rIL2.

[c] $p < 0.01$ as compared with group 2 and HIV-seronegative men.

[d] $p < 0.01$ as compared with group 1 and HIV-seronegative men.

[e] $p < 0.05$ as compared with HIV-seronegative subjects.

[f] $p < 0.05$ compared with group 1 and HIV-seronegative subjects.

[g] $p < 0.05$ as compared with group 1 and group 2 subjects.

five of these cultures (mean, 19 U). This suppressive effect was greater with increasing concentrations of HIV-1 Ag, as shown in Figure 3 with representative data from cultures of two HIV-1-seronegative men.

DISCUSSION

This study provides evidence of HIV-1-specific in vitro induction of IFN-γ during HIV-1 infection. Addition of rIL-2 along with HIV-1 Ag was necessary to detect this cellular immune reactivity consistently, possibly to compensate for the direct inhibition of IL-2 gene expression by HIV-1 (28). In contrast, HIV-1 does not inhibit expression of genes for the IL-2R or IFN-γ that are involved in the induction of IFN-γ. A second mechanism of interaction of HIV-1 with IL-2 is suggested by our data showing that HIV-1 Ag suppressed rIL-2-induced IFN-γ production in MNL from HIV-1-seronegative subjects. HIV-1 may induce suppressor factors thereby reducing lymphocyte activation, as suggested by other studies (29). Alternatively, whole, inactivated HIV-1 and PENV9 may have direct, competitive effects on IL-2 activation of lymphocytes. Recent reports indicate that HIV-1 envelope protein included in both of our HIV-1 Ag preparations contains a region homologous with a portion of IL-2 (30, 31). This region of IL-2 may be important in binding to the IL-2R and subsequent activation of T lymphocytes (32). Such a mechanism could be significant in immunosuppression caused by HIV-1 and requires further study.

Assessment of HIV-1-specific production of IFN-γ may be important to our understanding of how HIV-1 infection results in AIDS. IFN-γ has been shown to inhibit HIV-1 infection in vitro (33), although conflicting reports have been published (34). A soluble mediator such as IFN-γ has been postulated to be the mechanism by which $CD8^+$ lymphocytes inhibit HIV-1 replication in $CD4^+$ cells (35). IFN-γ is also known to be an important modulator of various immunologic functions (36). Thus, decreasing ability to produce IFN-γ in response to HIV-1 may be related to development of more severe HIV-1 infection and AIDS, as has been shown for IFN responses to other Ag (37, 38).

Our data demonstrate the MNL from most but not all of the men infected for relatively short time periods were capable of producing IFN-γ in response to HIV-1 Ag plus rIL-2. These results show for the first time that defects in T cell immunity specific for HIV-1 can occur relatively early in the course of infection. The dysfunction is associated with lower $CD4^+$ cell counts, but occurs before the onset of clinically significant manifestations of HIV-1 infection. Prospective studies of this T cell-specific response to HIV-1 may allow delineation of which subjects are at greatest risk for developing AIDS. Of further interest is that HIV-1-specific production of IFN-γ could be useful in monitoring efficacy of antiviral and immunomodulating drug intervention in HIV-infected subjects.

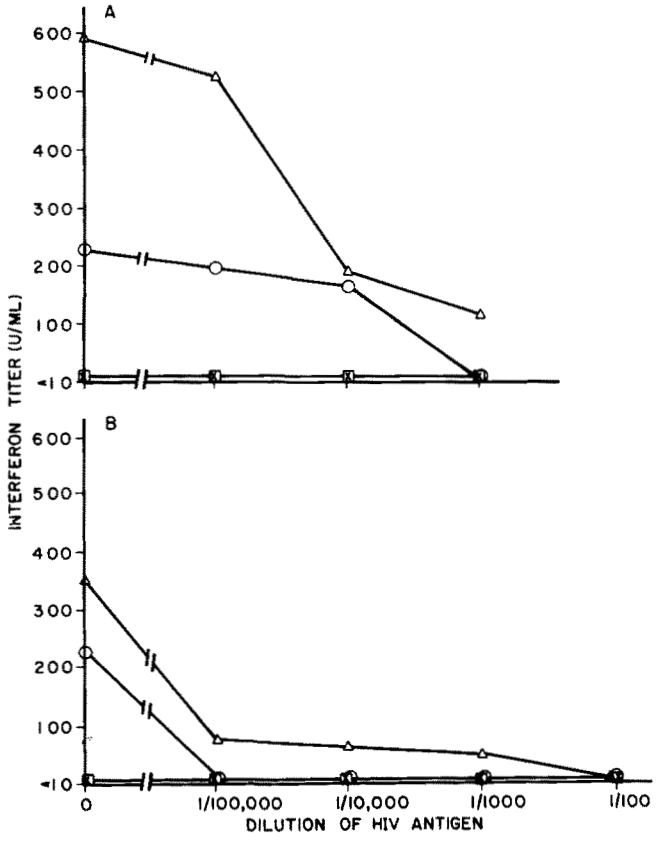

Figure 3. Suppression of rIL-2-induced IFN-γ production by HIV-1 Ag in MNL cultures from two HIV-1-seronegative homosexual men (*A* and *B*). Symbols are described in Figure 2.

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