Inflammatory and Immune Mediators in Crevicular Fluid From HIV-Infected Injecting Drug Users*

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Gingival crevicular fluid (GCF) levels of the polymorphonuclear leukocyte (PMN) lysosomal enzyme beta-glucuronidase (βG), the pro-inflammatory cytokine interleukin 1β (IL-1β), and immunoglobulins (IgA, IgG, and IgM) were examined in 16 HIV seropositive (HIV+) and 10 HIV seronegative (HIV−) injecting drug users (IDU). Each subject received a periodontal examination including assessment of probing depth, attachment level, bleeding on probing, and plaque and calculus accumulation. GCF was collected from the mesial surfaces of premolar and molar teeth using filter paper strips. Although HIV+ subjects had a significantly lower number of peripheral blood CD4+ T cells/mm³ compared to HIV− subjects, there were no significant differences in mean probing depth, percentage of sites exhibiting bleeding on probing, or plaque and calculus accumulation between HIV− and HIV+ subjects. When the GCF components were analyzed, we found no significant differences between HIV− and HIV+ subjects in GCF levels of βG, IL-1β, IgA or IgM, but GCF levels of IgG were significantly increased in HIV+ subjects. When sites were categorized by probing depth, no differences in the levels of βG, IgA, IgG, and IgM existed between sites with probing depth ≤ 3 mm compared to sites with probing depth ≥ 4 mm in both HIV− and HIV+ IDU. However, levels of IL-1β in GCF were increased in the deeper sites (≥ 4 mm) in HIV+ IDU when compared to sites with PD ≤ 3 mm. Analyzing GCF constituents in relation to the CD4 cell number, no differences were found between subjects with ≤ 400 or > 400 CD4 cells/mm³ with respect to the levels of IL-1β, IgG, and IgM. However, the level βG was significantly decreased in the HIV+ IDU with ≤ 400 CD4 cells when compared to those with > 400 CD4 cells/mm³, while levels of IgA were significantly higher in HIV+ subjects with ≤ 400 CD4 cells/mm³. Our results suggest that levels of IgG, and in immunodeficient subjects IgA were increased in GCF of HIV+ IDU while decreased levels of βG were found in immunodeficient HIV+ IDU. These findings may be local manifestations of systemic alterations and suggest that analysis of GCF may provide insight into the immune and inflammatory responses of HIV-infected individuals to periodontal microorganisms. J Periodontol 1997;68:249–255.

Key Words: Glucuronidase; gingival crevicular fluid/analysis; HIV seronegativity; HIV seropositivity; interleukin-1β; IgA; IgG; IgM; substance use disorders.

Infection with the human immunodeficiency virus (HIV) leads to progressive immunosuppression caused primarily by the loss of CD4+ (T helper) cells, although other cell types may also be affected (i.e., follicular dendritic cells). These immunologic changes may alter the ability of the patient to respond appropriately to infection, including the presence of subgingival bacterial. Alteration in the host response to the plaque challenge has been implicated as a cause for increased periodontal attachment loss observed in HIV+ individuals.

In this study, we examined the relationship of periodontal clinical parameters to the presence of inflammatory and immunologic mediators in gingival crevicular fluid (GCF). The levels of the polymorphonuclear leukocyte (PMN)-derived lysosomal enzyme beta-glucuronidase (βG) were used to assess PMN activity in GCF. Levels of βG have been shown to be increased in patients...
at risk for periodontal breakdown.\textsuperscript{8,9} Also, altered PMN function has been implicated in the pathogenesis of periodontal disease associated with HIV infection.\textsuperscript{10} In addition, levels of the pro-inflammatory cytokine interleukin 1\(\beta\) (IL-1\(\beta\)) were studied, as well as levels of immunoglobulins (IgA, IgG, and IgM) in GCF. Alterations in the inflammatory and/or humoral immune responses in GCF in patients infected with HIV could help define the role of the local host response in periodontal disease.

**MATERIALS AND METHODS**

**Patient Selection**

In this study, 16 HIV seropositive (HIV+) and 10 HIV seronegative (HIV-) injecting drug users (IDU) were examined. These subjects were all recruited from a longitudinal natural history study of HIV infection at Harlem Hospital Center in New York City. All participants had a history of parenteral drug use. Subjects were selected based on a willingness to participate in the trial and the presence of at least 8 posterior teeth. All subjects examined in this study received a through medical and immunologic evaluation including the determination of CD4+ cell numbers and HIV serostatus testing using both an enzyme-linked immunosorbent assay (ELISA) and western blots. This investigation received the approval of the Harlem Hospital Center and Columbia-Presbyterian Medical Center Institutional Review Boards.

**Clinical Examination**

After meeting entry criteria, GCF samples were collected and clinical parameters were recorded. All clinical data were collected by a single, calibrated examiner. All teeth exclusive of third molars were studied, and clinical data including probing depth, attachment level, and bleeding on probing were collected at 6 sites per tooth (mesio-buccal, direct buccal, disto-buccal, mesio-lingual, direct lingual, and disto-lingual). Probing depth was defined as the distance in mm from the coronal-most margin of the gingiva to the most apical penetration of the Michigan-O probe. Attachment level was defined as the distance from the cemento-enamel junction to the most apical penetration of the Michigan-O probe. Bleeding on probing was recorded dichotomously following probing. Plaque and calculus accumulation was assessed by a modification of the Green and Vermillion simplified oral hygiene index (OHI).\textsuperscript{11} The OHI has two components: 1) the debris index (DI) which is based on the coronal extension of the plaque and was recorded on a 0 to 3 scale; and 2) the calculus index (CI) which notes the coronal extension of supragingival calculus and, was recorded on a 0 to 3 scale. Buccal and lingual surfaces were scored separately for each tooth and averaged. The OHI for each of the teeth examined was calculated as \(\frac{DI + CI}{2}\). The OHI for each patient was then determined as the mean OHI for 6 teeth: the maxillary and mandibular right and left first molars, the maxillary right central incisor, and the mandibular left central incisor. In the absence of first molar teeth, the most distal tooth in the quadrant was examined. In the absence of the maxillary right or mandibular left central incisor, the contralateral tooth was examined.

**Collection and Analysis of Gingival Crevicular Fluid**

GCF was collected prior to the clinical examination from the mesial surface of all posterior teeth (premolars and molars) present in the mouth, exclusive of third molars. Areas of collection were isolated with cotton rolls and gently dried with air. Supragingival plaque was carefully removed. Precut methylcellulose filter paper strips\textsuperscript{4} were inserted in the mesio-buccal and mesio-lingual aspects of the crevice. The strips were moved apically until mild resistance was felt and left in place for 30 seconds. The mesiobuccal strips were analyzed for lysosomal \(\beta\)G using a fluorometric assay.\textsuperscript{8} The mesiolingual strips were eluted and the GCF/diluent solution were analyzed for IgA, IgG, and IgM using an ELISA technique described previously.\textsuperscript{12} In addition, the GCF/diluent solution obtained from the mesiolingual strips were used to determine levels of IL-1\(\beta\) using a commercially available ELISA.\textsuperscript{3} For all mediators, the data were reported as total amount per 30-second sample.

**Data Analysis**

Data analysis was accomplished using a software program\textsuperscript{4} for an Apple Quadra 950 computer. Comparison between the means of the two groups (HIV-, HIV+) was by unpaired Student \(t\) test. Correlations were determined by the Spearman rank correlation method using the patient as the unit of analysis. For some evaluations, analysis of covariance (ANCOVA) was also performed to control for the effect of probing depth.

**RESULTS**

**Relationship of Clinical and Immunologic Findings to HIV Serostatus**

The clinical and immunologic findings for the HIV- and HIV+ IDU are shown in Table 1. No significant differences were present between the two populations with respect to mean probing depth, mean percentage of sites exhibiting bleeding on probing, and mean OHI. HIV+ subjects did exhibit a trend for greater mean attachment loss, but the difference was not statistically significant \((P = 0.19)\). The groups did not differ in age. It is important to note that both groups displayed significantly more tissue inflammation when compared to an "unknown" sero-

\textsuperscript{1}IDE, Amityville, NY.

\textsuperscript{2}Cistron Biotechnology, Pine Brook, NJ.

\textsuperscript{3}Systat 4.0, Systat, Inc., Evanston, IL.
status group of age-matched non-IDU, controlled for probing depth (data not shown). For these groups of subjects, HIV infection did not appear to modify the severity and extent of gingival inflammation as assessed by bleeding on probing. Rather, gingival inflammation in this population was pronounced and observed in all individuals. As a result, correlation analysis between percent bleeding on probing and other variables was not performed.

As would be expected, the HIV+ subjects had significantly lower numbers of CD4+ cells with 9 of the 16 subjects having less than 400 CD4+ cells/mm³ and 5 of the 16 having less than 200 CD4+ cells/mm³. None of the HIV− subjects had less than 600 CD4+ cells/mm³.

Our data demonstrated a significant correlation between median probing depth and the mean total βG in the HIV− group (r = 0.760; P = 0.02). This association is similar to what has been reported in previous studies from our laboratory.13-15 In contrast, for HIV+ subjects, mean total βG activity was not associated with mean probing depth (r = 0.203; NS). The association of mean probing depth and levels of IL-1β was similar in both the HIV− (r = 0.236) and the HIV+ (r = 0.386) groups.

Correlations between mean probing depth and total immunoglobulin in GCF differed based on serostatus: IgA (HIV−: r = 0.341; HIV+: r = 0.911), IgG (HIV−: r = 0.043; HIV+: r = 0.419) and IgM (HIV−: r = 0.797; HIV+: r = 0.570). Since there was a relationship between mean probing depth and the levels of some inflammatory mediators, an ANCOVA analysis was performed to control for the covariate of probing depth.

### Relationship of the Levels of GCF Mediators to HIV Serostatus

The mean levels of the PMN enzyme βG were found to be nearly identical in HIV− and HIV+ IDU (Table 2). An analysis of IL-1β levels in GCF revealed that HIV+ IDU demonstrated higher levels of IL-1β than HIV− IDU, but the difference did not reach statistical significance (P = 0.07).

When we compared the levels of IgA and IgM in GCF collected from HIV− and HIV+ subjects, we did not find differences between groups, although a trend for higher levels of IgA in GCF from HIV+ subjects was noted.

Significantly (P < 0.05) higher total IgG was found in the GCF from HIV+ IDU.

To determine whether inflammatory and immune mediators in GCF differ with different probing depths, sampled sites were categorized as 1 to 3 mm and ≥ 4 mm. HIV− and HIV+ subjects were examined separately. Levels of βG in GCF were similar regardless of probing depth and HIV serostatus (Fig. 1). Levels of IL-1β were similar for HIV− and HIV+ IDU in the shallow sites (Fig. 2). At deeper sites the HIV+ IDU demonstrated significantly greater levels of IL-1β in GCF than HIV− IDU. Levels of IgA were increased in HIV+ IDU (Fig. 3); however, the differences between the HIV+ and HIV− subjects were not significant. Levels of IgG were significantly (P < 0.05) increased in the HIV+ subjects in both shallow and deeper sites (Fig. 4), however, no differences between shallow and deep sites were observed within groups. Figure 5 demonstrates that total IgM did not differ between the HIV− or HIV+ subjects at either the shallow or deeper sites. Furthermore, no significant differences between shallow and deeper sites were observed for either the HIV− or HIV+ groups.

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**Table 1. Clinical and Immunologic Parameters in HIV+ and HIV− Injecting Drug Users (mean ± standard error of the mean)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HIV−</th>
<th>HIV+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean attachment level (mm)</td>
<td>3.39 ± 0.20</td>
<td>4.01 ± 0.32</td>
</tr>
<tr>
<td>Mean probing depth (mm)</td>
<td>3.21 ± 0.11</td>
<td>3.40 ± 0.14</td>
</tr>
<tr>
<td>Percent of sites exhibiting BOP</td>
<td>86.1 ± 5.0</td>
<td>86.9 ± 4.3</td>
</tr>
<tr>
<td>Oral hygiene index</td>
<td>1.80 ± 0.15</td>
<td>1.86 ± 0.14</td>
</tr>
<tr>
<td>Age</td>
<td>41.7 ± 2.2</td>
<td>43.6 ± 2.0</td>
</tr>
<tr>
<td>CD4+ T cells (per mm³)</td>
<td>990 ± 140</td>
<td>500 ± 113*</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>

*P < 0.05.

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**Table 2. Inflammatory and Immune Mediators in the Gingival Crevicular Fluid of HIV+ and HIV− Injecting Drug Users (mean ± standard error of the mean)**

<table>
<thead>
<tr>
<th>Mediator</th>
<th>HIV−</th>
<th>HIV+</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-glucuronidase (units)</td>
<td>173 ± 33</td>
<td>175 ± 20</td>
</tr>
<tr>
<td>Interleukin-1 β (pgs)</td>
<td>986 ± 249</td>
<td>1714 ± 377</td>
</tr>
<tr>
<td>IgA (ngs)</td>
<td>189 ± 35</td>
<td>240 ± 35</td>
</tr>
<tr>
<td>IgG (ngs)</td>
<td>2130 ± 432</td>
<td>3198 ± 335*</td>
</tr>
<tr>
<td>IgM (ngs)</td>
<td>243 ± 64</td>
<td>278 ± 42</td>
</tr>
</tbody>
</table>

ANCOVA controlling for the covariate of mean probing depth; *P < 0.05.

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**Figure 1. Levels of β glucuronidase (βG) in GCF. Stratification by probing depth of the collection site and serostatus. For the 1 to 3 mm and ≥ 4 mm sites, there were no differences between the HIV− and HIV+ subjects. There were no significant differences between probing depth categories within each patient category.**

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The Relationship of GCF Mediators to CD4 Lymphocyte Count

To analyze the effect of immunodeficiency on the levels of GCF mediators, we categorized HIV+ subjects into 2 groups based on their CD4 cell numbers (≤ 400 CD4 cells/mm³, N = 9 and > 400 CD4 cells/mm³, N = 7).

Levels of IL-1β and IgM also did not demonstrate a significant difference between groups as stratified by the number of CD4 cells, though there was a trend towards lower total IL-1β in GCF with immunosuppression (Table 3). The elevation in total IgG was observed for both groups, suggesting that HIV infection was more important than the CD4 cell count in determining the levels of IgG in GCF.

In contrast, levels of βG in GCF were significantly decreased in the subjects with ≤ 400 CD4 cells/mm³ when compared to those with greater than 400 CD4/mm³ cells. Total βG in the HIV− subjects was intermediate between
Table 3. Inflammatory and Immune Mediators in the Gingival Crevicular Fluid of HIV+ Injecting Drug Users With ≤ 400 and > 400 CD4+ Cells/mm³ (mean ± standard error of the mean)

<table>
<thead>
<tr>
<th>Mediator</th>
<th>&lt;400 CD4+</th>
<th>&gt;400 CD4+</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-glucuronidase</td>
<td>136 ± 23</td>
<td>226 ± 58*</td>
</tr>
<tr>
<td>Interleukin-1 β</td>
<td>1336 ± 376</td>
<td>2200 ± 745</td>
</tr>
<tr>
<td>IgA (ngs)</td>
<td>278 ± 68</td>
<td>204 ± 32*</td>
</tr>
<tr>
<td>IgG (ngs)</td>
<td>3142 ± 629</td>
<td>3275 ± 334</td>
</tr>
<tr>
<td>IgM (ngs)</td>
<td>265 ± 56</td>
<td>291 ± 78</td>
</tr>
</tbody>
</table>

ANCOVA controlling for the covariate of mean probing depth; *P < 0.05.

those HIV+ individuals with CD4 cells ≤ 400 and those with CD4 cells > 400 (see Table 2). These results suggest that HIV+ subjects with > 400 CD4 cells/mm³ have a hyperactive local PMN response, while the HIV+ subjects with ≤ 400 CD4 cells/mm³ have a reduced PMN response.

The levels of IgA were found to be significantly higher in the more severely immunodeficient group after adjusting for the covariate (probing depth). Further analysis of the IgA data suggested that increased levels of IgA were manifested in HIV+ subjects with less than 200 CD4 cells/mm³ (data not shown). Two of the 5 subjects with CD4 cell counts less than 200 cells/mm³ had IgA levels exceeding 500 ng/s per sample. Therefore, in some severely immunodeficient patients, very high levels of IgA in GCF were present.

DISCUSSION

Unusual and severe forms of periodontal pathology have been described in patients with HIV infection, including HIV-associated gingivitis (HIV-G; later termed linear marginal erythema) and HIV-associated periodontitis (HIV-P; later termed necrotizing ulcerative periodontitis).15-18 Recent investigations have determined that the prevalence of these lesions is less than originally believed.7,19-20 Although the exact pathologic mechanisms responsible for these acute periodontal lesions in HIV disease are unknown, they may be unrelated to the progression of chronic periodontal disease. Furthermore, the progression of chronic adult periodontitis has been examined in individuals infected with HIV, and a number of studies suggest that the rate of progression is increased in seropositive patients.6,7,21

In a longitudinal study designed to determine the rate of attachment loss, Barr and co-workers7 found that HIV+ patients who were over the age of 35 and had less than 200 CD4 cells/mm³ demonstrated a significantly higher rate of periodontal attachment loss than a control group. An inappropriate response to plaque was proposed as a contributory mechanism.

In this study, we attempted to determine whether immunologic changes that accompany HIV infection were associated with changes in the levels of inflammatory and immunologic mediators in GCF. Regardless of serostatus, the IDU subjects examined here displayed more periodontal disease as measured by mean attachment level, mean probing depth and percentage of sites exhibiting bleeding on probing compared to age-matched HIV− and HIV+ homosexual men.20 In addition, compared to a group of age-matched subjects of unknown serostatus but strongly believed to be seronegative with comparable periodontal disease severity, the IDU had considerably higher levels of plaque and bleeding on probing (data not shown). Consequently, it is important to note that the results presented here may be unique to this specific population. Clearly, the intense bacterial challenge these patients experience plays a significant role in the type of inflammatory and immunologic responses observed, but this intense challenge may also allow differences between groups to be more easily observed.

Although the subjects in our study exhibited considerable existing periodontal disease, there were no significant differences between groups in terms of the severity of periodontal disease as assessed by the mean probing depth or mean percentage of sites which exhibited bleeding on probing. A trend was observed for greater existing attachment loss in the HIV+ subjects, but the difference between groups did not reach significance. This finding supports our previous study,20 which found no significant differences between HIV− and HIV+ homosexual men with respect to parameters of clinical periodontal disease.

Analysis of immunoglobulin levels revealed elevated total IgG in HIV+ subjects. Since GCF levels of an immunoglobulin are influenced by the serum levels of that immunoglobulin,22 the higher levels of IgG in the HIV+ IDUs may be a manifestation of the hypergammaglobulinemia reported in HIV-infected patients.23,24 In our study, this finding does not appear to be related to the level of immunodeficiency as measured by the number of CD4+ cells.

We observed increased IgA with pronounced immunodeficiency (< 200 CD4 cells/mm³). Levels of IgA did not differ between shallow and deeper sites, indicating that levels of this isotype were independent of the site, and appears to be dependent upon the contribution of serum to the GCF pool.13 Similar to IgG, increasing levels of IgA have been found to be inversely correlated with the number of CD4+ cells and shown to be associated with the progression of disease.25 Increased serum levels of IgA and IgG may be related to the antigenemia these patients experience.23 This antigenemia may function to stimulate production of the pro-inflammatory cytokine, interleukin-6, which has been found in increased levels in the serum of seropositive patients, and correlated with increased serum levels of IgG and IgA.26 However, unlike IgG, the increase in GCF IgA is primarily seen in severely immunodeficient patients.

Our laboratory has previously demonstrated that de-
creased levels of IgA in the GCF were found in patients who experienced periodontal attachment loss at multiple sites. In addition, we demonstrated increased IgA levels in patients with gingivitis in comparison to patients with periodontitis. These data suggest that patients with increased IgA in GCF may be protected against periodontal breakdown. Therefore, the increased levels of IgA seen in GCF from HIV-infected, immunodeficient IDU may be compensating for the effects of a deficient cellular immune response in the periodontium of seropositive individuals.

Levels of IgM in GCF were found to be similar for HIV− and HIV+ subjects in both shallow and deep sites. In contrast to IgG and IgA, levels of IgM in GCF may be influenced primarily by the ability of the large IgM molecule to diffuse into the sulcus. In this study as well as previous studies, total IgM in GCF was shown to be related to both the intensity of tissue inflammation and probing depth. The high levels of IgM in GCF seen in both the HIV− and HIV+ relative to our previous studies were probably associated with the intensity of the tissue inflammation.

The cytokine IL-1β has been implicated as an important mediator involved in periodontal disease. IL-1β has been found to be increased in the tissues of patients with periodontitis and is presumed to be produced by macrophages resident in the periodontal tissues. In addition, IL-1β has been shown to stimulate osteoclastic activity in vitro. HIV infection has been shown to enhance macrophage activation and this could also account for the relative increase in IL-1β levels found in the deeper (≥ 4 mm) sites in HIV+ subjects.

The data also suggested that PMN function in the crevicular environment, as measured by the levels of the PMN lysosomal enzyme βG in GCF, may be altered in HIV infection. Evidence for this is based upon two findings from this study. First, the mean levels of βG in GCF were not correlated to the mean probing depth in HIV+ subjects, but these parameters were highly correlated in HIV− subjects. Other studies from our laboratory have found a good correlation of mean βG levels and mean probing depth. These correlations have typically been in the r = 0.7 range. In this study, we did not observe an association between these parameters in seropositive patients. The significance of this finding is unclear. Nevertheless, it does suggest altered regulation of PMN influx into the gingival crevice in HIV+ subjects.

Second, we observed that HIV+ subjects with a CD4 count of less than 400 cells/mm³ had lower levels of βG in GCF than HIV+ subjects with a CD4 cell count above 400 cells/mm³. These data suggest an inappropriate PMN response in immunodeficient, HIV-infected subjects. Ryder et al. studied the function of blood and crevicular PMN in HIV-infected patients and found that phagocytosis and killing by crevicular PMNs was depressed. They suggested that an abnormal PMN response could account for the periodontal pathology seen in HIV+ patients. Other investigators have found that patients infected with HIV have impaired neutrophil function including decreased intracellular enzyme activity. Impaired neutrophil function in HIV patients has been associated with increased bacterial infections and decreased survival. The decrease in crevicular PMN activity may be a manifestation of systemic neutrophil dysfunction and therefore assessment of βG in GCF may provide a quick and relatively inexpensive method of assessing neutrophil function in HIV disease.

The apparent dysregulation of PMN activity observed in our HIV+ patients may help explain the findings of Barr et al. Although HIV infection has been shown to directly affect the cellular arm of the immune system, a multitude of secondary effects on other aspects of the immune and inflammatory responses have been identified. PMN function in the gingival sulcus in HIV-infected patients could be affected by a number of different factors. Specifically, 1) the bacteremia and viremia seen in HIV+ subjects could cause activation of PMN either directly or indirectly; 2) activation of PMN in the peripheral blood may impair local PMN activity; 3) other pro-inflammatory mediators (i.e., IL-1β, IL-8, leukotriene β4, tumor necrosis factor-α) could also modulate PMN function. Therefore our findings suggest that further investigation of the role of PMN in the periodontium of HIV-infected patients is warranted.

In conclusion, rampant periodontal breakdown does not normally occur in HIV-infected patients. Our study suggests that several of the immunologic and inflammatory changes seen in GCF may serve to protect the host and compensate for the deficiency in other aspects of the host immune response. For example, the elevated levels of IgG and IgA in GCF from these patients serve a protective function. Our data suggest that changes in inflammatory and immune mediators in GCF do occur in association with HIV infection and the resultant reduction in the number of CD4+ lymphocytes. A number of these changes can be suggested to be local manifestations of systemic alterations. Our findings have emphasized the multifaceted nature of the host response in periodontal disease, and suggest that GCF analysis may be a useful approach for studying periodontal disease in HIV infection.

Acknowledgments

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