

# Reconstitution of Immune Responses to Tuberculosis in Patients With HIV Infection Who Receive Antiretroviral Therapy\*

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**Study objectives:** To assess the restoration of immune responses to tuberculosis, as manifested by secretion of T-helper type 1 cytokines (interferon [IFN]- $\gamma$ , interleukin [IL]-12, and IL-2) and T-helper type 2 cytokines (IL-10), in HIV-positive patients who receive antiretroviral therapy (ART).

**Design:** Prospective cohort study.

**Setting:** University hospital.

**Patients:** Ten HIV-positive patients, all naïve to ART and all about to start ART for clinical indications, and 11 healthy, HIV-negative control subjects.

**Interventions:** Assessment of T-cell proliferation and cytokine production after administration of ART to patients with HIV infection.

**Measurements and results:** All patients had a negative tuberculin skin test result at baseline and were anergic. Highly active ART reduced the viral load to very low levels in all patients within a short time after starting therapy. Blood samples were drawn every 2 months after starting therapy, and continued for 1 year while the patients continued to receive ART. There were trends toward increased proliferation of peripheral blood mononuclear cells (PBMCs) in response to *Mycobacterium tuberculosis*-specific stimuli, but these were delayed until several months of ART had elapsed. Similar trends were noted in relation to the secretion of IFN- $\gamma$ . Neither PBMC proliferation nor IFN- $\gamma$  secretion reached levels seen in healthy control subjects. No consistent trends in IL-2, IL-10, or IL-12 production were noted.

**Conclusion:** ART restores immune responses to *M tuberculosis*, although this restoration is delayed and does not reach levels seen in healthy, HIV-negative control subjects. These results may explain in part the phenomenon of paradoxical reactions to antituberculosis therapy in patients with HIV infection. A larger study in which patients are followed up for a longer period of time will allow the magnitude and timing of this reconstitution to be more precisely defined. (CHEST 2002; 122:597-602)

**Key words:** AIDS; immunity; treatment; tuberculosis

**Abbreviations:** ART = antiretroviral therapy; BCG = bacille Calmette-Guérin; IFN = interferon; IL = interleukin; PBMC = peripheral blood mononuclear cell; PHA = phytohemagglutinin; PPD = purified protein derivative

Immune function in patients with HIV infection is restored, at least partially, by antiretroviral therapy (ART). This has been demonstrated in several ways. First, patients receiving ART have a rise in their

CD4+ T-lymphocyte counts, a leading indicator of immune function<sup>1,2</sup> and presumably a marker of at least partially restored cellular immunity. Second,

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studies<sup>3</sup> conducted early after the introduction of ART demonstrated an increased T-cell proliferation in response to antigens from common infectious agents, such as *Mycobacterium tuberculosis*. Third, studies<sup>4-6</sup> in which prophylaxis against certain opportunistic infections such as *Pneumocystis carinii* pneumonia has been discontinued in patients receiving ART show that this can be performed safely

\*From the Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY. Presented in part at the American Thoracic Society International Conference, May 2000, Toronto, Canada. Supported in part by National Institutes of Health grant No. K24 HL004074 (Dr. Schluger). Manuscript received August 16, 2001; revision accepted February 5, 2002.

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within certain parameters. However, questions still remain regarding the extent, time course, and exact nature of the reconstitution of immune function after ART is instituted.

In the absence of ART, patients with HIV infection and latent tuberculosis infection have an extremely high rate of having active tuberculosis develop, perhaps as much as 10%/yr, as opposed to a 10% lifetime risk in otherwise healthy persons with latent tuberculosis infection.<sup>7</sup> This high rate of active tuberculosis developing may be directly related to the loss of interferon (IFN)- $\gamma$ -producing CD4+ T cells, because this cytokine has been demonstrated to be of major importance in the human host response against tuberculosis.<sup>8</sup> It stands to reason then that improvements in immune function will be accompanied by restoration of the ability to secrete IFN- $\gamma$  in response to certain mycobacterial stimuli.

In addition to restoring immune function, ART has been associated with so-called paradoxical reactions in patients concurrently being treated for tuberculosis.<sup>9,10</sup> This phenomenon is generally associated with enlarging lymph nodes and high temperatures in patients with AIDS who receive ART at the same time that they are receiving antituberculosis therapy. These syndromes do not usually occur at the outset of tuberculosis treatment, but rather after several weeks. Diligent searching for drug-resistant organisms or other evidence of tuberculosis treatment failure is fruitless, also it appears that the syndrome is somehow related to immunologic phenomena, and it is as yet unexplained.

Understanding the pace and quality of immune reconstitution could have significant implications for the treatment of active and latent tuberculosis. To explore the extent, nature, and timing of immune responses to tuberculosis in HIV-positive patients who were receiving ART, we studied a cohort of such individuals at regular intervals after beginning ART.

## MATERIALS AND METHODS

### Study Design

A cohort of patients naïve to ART was followed up for 12 months after the institution of ART. Peripheral blood was drawn every 2 months after initiation of ART, and peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with purified protein derivative (PPD), bacille Calmette-Guérin (BCG), and *M tuberculosis* strain H37Ra. After stimulation, both T-cell proliferation and production of cytokines IFN- $\gamma$ , interleukin (IL)-10, IL-2, and IL-12 were assessed.

### Patient Recruitment

This study was carried out at the Columbia Presbyterian Medical Center in New York City. We enrolled 10 individuals

who recently received a diagnosis of HIV infection and who were naïve to ART. These individuals were recruited from the infectious disease outpatient clinic and from individuals admitted to the hospital. Screening included a history, physical examination, and a short questionnaire regarding history of opportunistic infections, especially tuberculosis. Individuals enrolled were followed up in the clinic every 2 months after the initiation of ART. Blood samples were obtained during their visit, and a PPD tuberculin skin test with an anergy panel was performed every 4 months. Two patients included in the study received a diagnosis of pulmonary tuberculosis during their initial hospitalization. Responses to ART were monitored with frequent HIV DNA viral loads and CD4 T-cell quantification. A group of healthy, HIV-negative individuals served as control subjects. Six of the control subjects had negative tuberculin skin test results; five control subjects had positive tuberculin skin test results. The study was approved by the Institutional Review Board of Columbia Presbyterian Medical Center.

### Isolation of PBMCs

PBMCs were isolated by density centrifugation with Ficoll-Hypaque.<sup>11</sup> The viability of cells was determined by trypan blue exclusion test. PBMCs were resuspended to a concentration of  $1 \times 10^6$  cells/mL with complete RPMI-10.

### PBMC Proliferative Assays

PBMCs were plated in triplicate ( $2 \times 10^5$  cells per well) in round-bottom microtiter plates (Nunc Surface; NUNC; Roskilde, Denmark). PBMCs were cultured in 5% carbon dioxide at 37°C for 5 days with the following stimuli: PPD, 2.5 U/mL; phytohemagglutinin (PHA), 10  $\mu$ g/mL, *Mycobacterium bovis* BCG strain,  $2 \times 10^7$  organisms per milliliter; and live and heat-killed *M tuberculosis* H37Ra,  $2 \times 10^7$  organisms per milliliter. The PBMCs were pulsed with [<sup>3</sup>H]thymidine for 18 h before being harvested. Incorporation of radioactivity was measured with a scintillation counter. A triplicate of unstimulated cells served as background proliferation.

### Enzyme-Linked Immunosorbent Assays

IFN- $\gamma$ , IL-10, IL-2, and IL-12 were assayed from cell culture supernatants by commercial kits (R&D Systems; Minneapolis, MN). PBMCs ( $1 \times 10^6$  cells/mL) are incubated in complete RPMI-10 in an atmosphere of 5% carbon dioxide at 37°C. Supernatants from these cell cultures were collected at 24 h, 48 h, and 72 h. The supernatants from the cell cultures were stored at -70°C until further use. The same stimuli prepared for the proliferation assays were added to the cultures.

### Statistical Analysis

Levels of cytokine production and T-cell proliferation at various time points were compared with baseline levels by paired *t* tests.

## RESULTS

Ten patients (mean age  $\pm$  SE,  $46.8 \pm 3.1$  years), of whom two were women, were studied. The clinical characteristics and drug regimens of the patients are shown in the Table 1. Patients had low CD4+ T-cell counts before institution of ART (mean,

**Table 1—Clinical Characteristics\***

Patient No.	Age, yr/Sex	BCG	Baseline CD4 Count, $\mu$ L	Antiviral Regimen	Baseline PPD/Anergy	Post-ART PPD/Anergy
1	45/Female	No	79	L, Z, E	-/-	-/-
2	35/Male	Yes	8	L, Z, E	-/-	-/+
3	44/Female	Yes	443	L, Z, N	-/+	-/+
4	29/Male	Yes	63	S, L, N	-/-	-/-
5	63/Male	Yes	28	L, Z, N	-/-	-/+
6	48/Male	Yes	80	L, Z, N	-/-	-/-
7	64/Male	Yes	36	L, Z, N	-/-	-/-
8	41/Male	Yes	69	S, L, E	-/-	-/+
9	53/Male	Unknown	8	L, Z, E	-/-	-/+
10	35/Female	No	344	S, L, E	-/+	-/-

\*All patients were Hispanic, except for patients 1 and 10, who were African American. Patients 1, 5, and 9 had *P carinii* pneumonia prior to starting ART, and patient 4 had cryptococcosis. Only one patient (patient 2) had an opportunistic infection (CNS toxoplasmosis) after starting ART. L = lamivudine, Z = zidovudine, E = efavirenz, N = nelfinavir, S = stavudine.

115.8  $\pm$  47.7/ $\mu$ L). Seven of the patients were born outside the United States, all of whom had received BCG vaccination.

*Viral Load, CD4+ T-Cell Number, and Tuberculin Skin Testing*

At initiation of ART, mean viral load in the patients were 158,657  $\pm$  101,089 copies (range, 47,285 to 337,473 copies). HIV viral loads dropped promptly (within 4 weeks) to generally undetectable levels (< 400 copies) in all patients receiving ART. Similarly, rises in CD4+ T-cell numbers occurred soon after initiation of ART. Tuberculin skin testing was performed using 5 U of intermediate-strength tuberculin at baseline and every 4 months thereafter.

Five millimeters of induration was considered to be a positive test finding. Anergy testing using *Candida* and mumps antigens was also performed. The full results are shown in Table 1. Only two patients were not anergic at baseline, and all had negative tuberculin skin test results. No patient had a positive tuberculin skin test finding (*ie*, > 5 mm induration after placement of PPD) during ART, although 6 of 10 patients had restoration of delayed-type hypersensitivity responses to either *Candida* or mumps during the study.

*Proliferation of PBMCs*

Figure 1 shows proliferative responses of PBMCs over time after initiation of ART. Proliferative re-

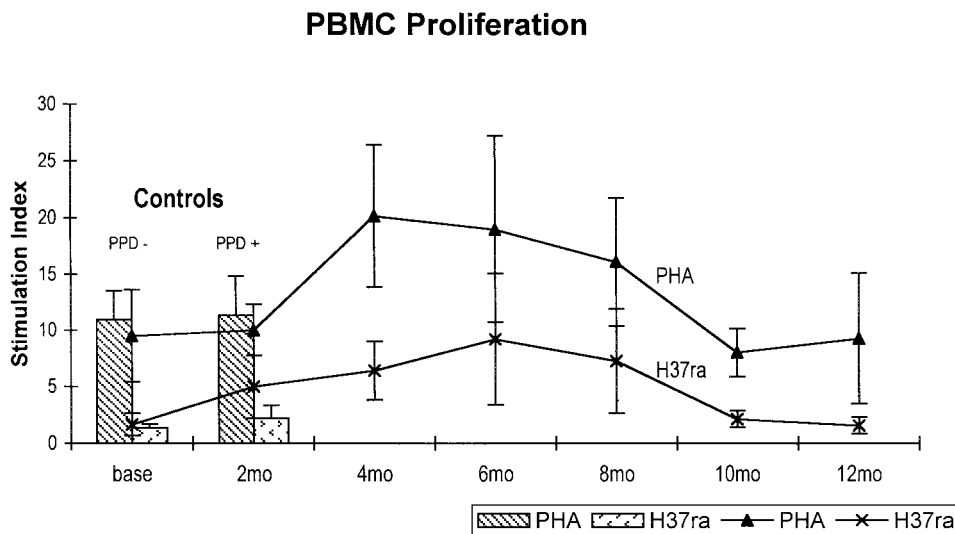


FIGURE 1. PBMC proliferation assay. PBMCs were stimulated with PHA and *M tuberculosis* H37Ra before and during 12 months of ART. Stimulation indices were determined by comparison with paired unstimulated cells from each patient (n = 10). Normal values in this and all figures refer to data from the healthy control subjects.

sponses of PBMCs to the nonspecific-stimulant PHA rose at 4 months of ART, peaked at 6 months of ART, and remained higher than baseline values (and those of HIV-negative control subjects) after 12 months of treatment. In contrast, proliferation of PBMCs after stimulation with *M tuberculosis* strain H37Ra was not nearly as pronounced, but there was also a marked increase over baseline (and compared with control subjects) after 6 months of treatment.

### Cytokine Release by PBMCs

Release of the cytokines IFN- $\gamma$ , IL-2, and IL-12 is shown in Figures 2–4. Most notably, secretion of IFN- $\gamma$ , initially much lower than for HIV-negative control subjects, rose somewhat slowly after initiation of ART, and peaked (at near normal levels) only after 8 months of treatment. Production of IFN- $\gamma$  continued at near-normal levels after that time point. A similar trend was seen with IL-12, although the increase in secretion was even more delayed and the magnitude of the increase was not as great. After 10 months of ART, there were small increases in IL-2, although these did not reach levels achieved in HIV-negative individuals, nor were they as high as those induced by the nonspecific stimulus, PHA. No consistent trends were noted in secretion of the T-helper type 2 cytokine IL-10 (data not shown).

## DISCUSSION

We have demonstrated that after initiation of ART, immune responses to mycobacteria are re-

stored to a certain degree, but this reconstitution is both delayed (in comparison with suppression of HIV viral load and increase in peripheral CD4+ T-cell number) and often submaximal, as compared with responses seen in PBMCs obtained from healthy volunteers. This was most clearly demonstrated with regard to the proinflammatory cytokine IFN- $\gamma$ , which plays a major role in host defense against *M tuberculosis*. Levels of this cytokine produced by PBMCs in response to stimulation with mycobacteria reached levels seen in HIV-negative volunteers only after several months of ART. These results provide a possible explanation for the phenomenon of so-called paradoxical reactions in patients with AIDS who are being treated for tuberculosis. Although it might have been preferable to include only patients with documented tuberculosis infection in the study, most if not all patients with AIDS about to begin ART have an absence of cutaneous delayed-type hypersensitivity responses, making actual assessment of their tuberculin status difficult. The patients in our study were either born outside the United States in countries with a high prevalence of tuberculosis or had risk factors for tuberculosis infection such as homelessness and injection drug use, and we judged them as likely to have latent infection.

Our study is limited by the relatively small number of patients and possibly also by the different ART regimens used by the patients in the cohort. Although all regimens reduced viral loads significantly, it is possible that different regimens had different

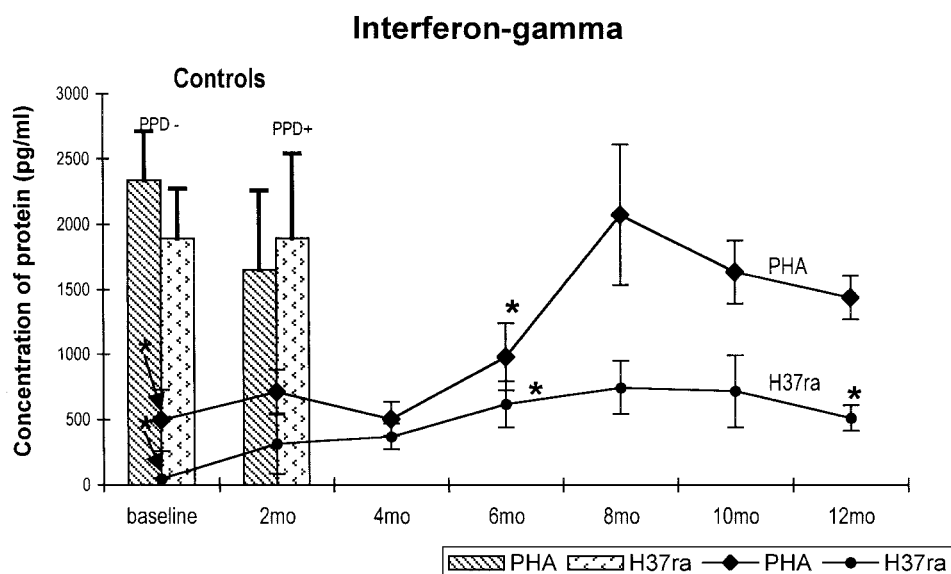


FIGURE 2. Mean levels of IFN- $\gamma$ . Cell culture supernatants from PBMCs stimulated with PHA and H37Ra were collected from patients before and during 12 months of ART. \* $p < 0.05$  compared with baseline.

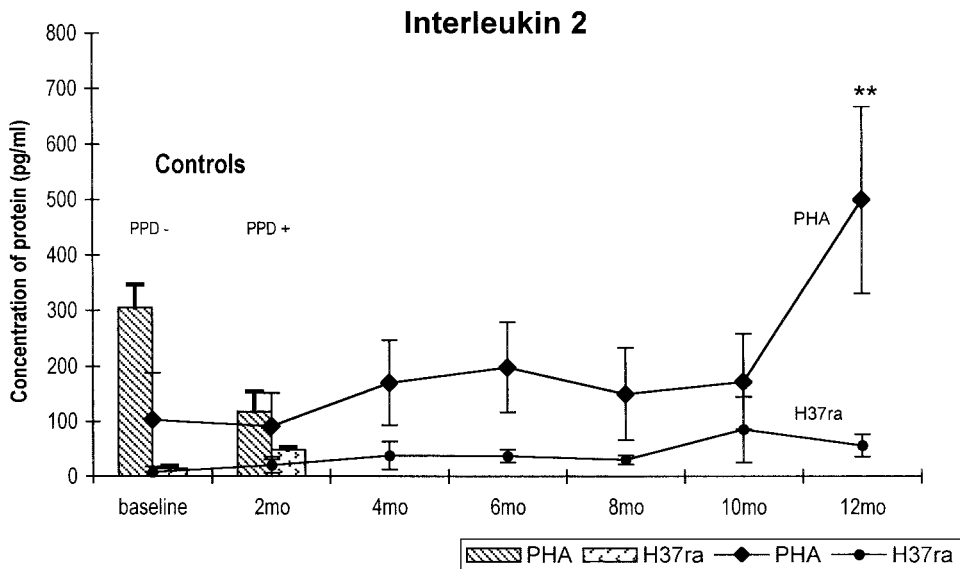


FIGURE 3. Mean levels for IL-2. The methods used were the same as used for INF- $\gamma$ .

effects on reconstitution of immune function that we could not detect because of the numbers of subjects involved.

IFN- $\gamma$  is a major effector cytokine in human host responses to tuberculosis.<sup>8</sup> It is produced mainly by T lymphocytes of both CD4+ and CD8+ types, and it stimulates macrophage function in a variety of ways, including increasing production of both reactive oxygen and reactive nitrogen species (both implicated in intracellular killing or growth inhibition of *M tuberculosis*), increased expression of major histocompatibility class II molecules, and increased

tumor necrosis factor production. Prior studies<sup>12</sup> have shown that patients with AIDS with depressed T-cell counts (and function) have low levels of IFN- $\gamma$  production, and this may be directly related to their increased likelihood of getting active tuberculosis after becoming infected.

Our results are consistent with and extend the observations of Aufran and colleagues,<sup>1</sup> and Li et al,<sup>3</sup> who studied T-cell proliferative responses but not cytokine release in patients receiving ART.<sup>1,3</sup> Recently, Hsieh and colleagues<sup>13</sup> reported that CD69 (a member of the lectin superfamily of transmembrane

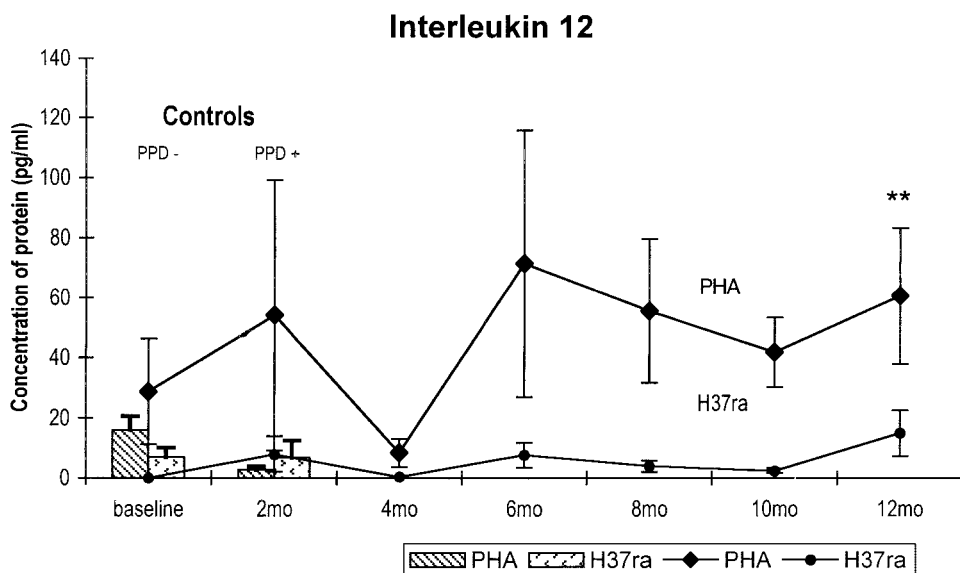


FIGURE 4. Mean levels for IL-12. The methods used were the same as used for INF- $\gamma$ .

signal-transducing receptors and a very early activation surface marker of lymphocytes) expression was increased after initiation of ART (although this increase was not observed in patients who began ART with  $< 50$  CD4<sup>+</sup> T lymphocytes/ $\mu$ L). The percentage of CD69<sup>+</sup> T cells was strongly correlated with the amount of INF- $\gamma$  secretion by CD4<sup>+</sup> cells after stimulation with PPD. Our results are consistent with this observation.

In addition to suppression of viral replication, treatment with highly active ART also restores immune function, as demonstrated by a number of studies that report that the need for primary or secondary prophylaxis against opportunistic infections such as *P carinii* pneumonia may be discontinued if CD4<sup>+</sup> T-cell counts have improved significantly after institution of ART. Currently, recommendations for treatment of latent tuberculosis infection in patients with HIV infection call for treating all such patients with tuberculin skin test reactions of  $\geq 5$  mm of induration.<sup>14</sup> Although in our study, IFN levels and T-cell proliferation responses improved, the restoration of these immune functions was delayed, often incomplete, and occurred to a varying degree in different individuals. In addition, *M tuberculosis* may reside in the host for many years and still be capable of reactivating. Because treatment of latent tuberculosis infection involves provision of medication for a discrete period (rather than continuing lifelong therapy, such as is the case with prophylaxis for other opportunists), the prudent course of action is to continue to provide treatment for latent tuberculosis infection.

Our results also provide insight into the phenomenon of paradoxical reactions to antituberculosis treatment in patients with AIDS. These reactions, typically manifest by recurrence of high temperature and rapidly enlarging lymph nodes, worsening pulmonary infiltrates, development of new pleural effusions, or appearance of a miliary pattern on chest radiography, are often temporally related to the institution of ART.<sup>9,10</sup> Our results suggest that these reactions may be caused by the restored proliferation of T cells and elaboration of proinflammatory cytokines in response to tuberculosis antigens. These observations should be confirmed in a larger cohort in which patients are followed up for  $> 12$  months of ART. The efficacy of treatment of paradoxical reactions with steroids likely is derived chiefly from the ability of prednisone treatment to diminish INF- $\gamma$  production and secretion. The applicability of these findings in other opportunistic infections is not known, although paradoxical reactions are generally not appreciated in conditions other than tuberculosis. In sum, reconstitution of immune responses to tuberculosis in AIDS patients receiving ART is

delayed beyond suppression of viral replication, although near-normal levels of T-cell proliferation and INF- $\gamma$  production may eventually be reached.

ACKNOWLEDGMENT: The authors thank Tiffany Jung, RN, MPH, for her assistance with data collection.

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