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Proteomic fingerprinting of human immunodeficiency virus type 1–associated dementia from patient monocyte-derived macrophages: A case study

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The emergence of a subset of circulating monocytes during human immunodeficiency virus type 1 (HIV-1) disease has been shown to correlate with cognitive impairment. Thus, it is hypothesized that diagnostic protein profiles may be obtained from these cells from patients with or at risk for HIV-1–associated dementia (HAD). To address this possibility, we used ProteinChip assays to define a unique monocyte-derived macrophage (MDM) protein fingerprint during HAD and whether it is affected by highly active antiretroviral therapy (HAART). The study included five Hispanic women, one with HAD, two HIV-1–infected without cognitive impairment, and two seronegative controls. All patients were matched by age and immune status. Monocytes were recovered from the peripheral blood leukocytes by Percoll gradient centrifugation and allowed to differentiate in vitro for 7 days. Cell lysates and supernatants were collected from the MDM and analyzed by surface enhanced laser desorption/ionization–time of flight ProteinChip assays. Seven unique protein peaks between 3.0 and 20.0 kDa were found in the HAD MDM sample. Each of these proteins were abrogated after HAART. Additional studies extending this one time point determination would serve to confirm the general utility of MDM protein profiling for the diagnosis and monitoring of HAD. *Journal of NeuroVirology* (2004) **10(suppl. 1),** 74–81.

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Introduction

Mononuclear phagocytes (MPs; perivascular macrophages and microglia) are the principle target cells for persistent viral replication and the source of neurotoxins during human immunodeficiency virus type 1 (HIV-1) disease, which affect neuronal function, leading to neurological impairment (Swindells *et al*, 1999). It is generally agreed that the persistently infected blood-borne MPs are the vehicles for central nervous system (CNS) disease leading to the pathological changes seen in the later stages of HIV-1–associated dementia (HAD) (Zheng and Gendelman, 1997) although the virus persists in the brain at very low levels and is regulated by both the innate and

adaptive immune system (Resnick *et al*, 1988; Garcia *et al*, 1999) in disease, it is the breakdown of these regulatory mechanisms that leads to CNS disease. Indeed, very low levels of both HIV DNA and viral proteins are found in the brains of asymptomatic individuals. Such signs of active viral replication are increased during disease (Bell *et al*, 1993; Kibayashi *et al*, 1996).

Emerging evidence suggests that virus is carried into the brain through infected monocyte-derived macrophages (MDMs) in the absence of critical immune control (Betts *et al*, 1999; Altfeld and Rosenberg, 2000; Rowland-Jones *et al*, 2001). During HIV encephalitis, perivascular $CD14^+/CD16^+$ macrophages accumulate and co-localize with HIV-1 (Fischer-Smith *et al*, 2001). A study has shown that $CD14^+/CD16^+$ monocytes correlate with the development of cognitive impairment and secrete factors that damage neurons (Pulliam *et al*, 1997).

To explore these issues at a phenomic level, we utilized "state-of-the-art" proteomic techniques to look at the functional unit of the genome, also known as the proteome (derived from protein complement of the genome), of human MDMs from a patient with HAD. We utilized surface enhanced laser desorption/ionization (SELDI)-time of flight (TOF) mass spectrometry to uncover protein profiles from monocyte lysates and supernatants recovered from a Hispanic patient with HAD (as defined by an Memorial Sloan Kettering [MSK] cognitive score of 2), two HIV-1–infected patients without cognitive deficits, and two virus-seronegative controls. The goal of these works is to evaluate whether a circulating monocyte subset can affect the disease course.

Results

Neurological and neuropsychological evaluation
On examination, HIV-infected patients 1 and 2 had a normal neurological examination, including measures of cognitive function. At the initial evaluation of the cognitively impaired patient (MSK of 2), the patient complained of diminished concentration, memory difficulty, and needing help to keep lists of tasks and special events. The patient had decreased concentration, memory difficulty, and could not perform everyday household chores. On examination, clumsiness and slow ambulation was shown along with decreased distal vibratory sensation in hands and feet. Cells, cerebrospinal fluid, and sera were obtained at both 3 weeks [samples referred to as HAD (1)] and 6 months after highly active antiretroviral therapy [HAART; consisting of Combivir and Viracept; samples referred to as HAD (2)]. The outcome of the neuropsychological evaluation (eight tests with 18 variables) was statistically analyzed by the analysis of variance (ANOVA) test, which showed significant changes in five tests (eight variables) when compared to control (uninfected) or HIV-1–infected subjects without memory symptoms ($P \leq .05$). After 6 months of treatment, the HAD patient improved in six of the eight variables. These included the Grooved Pegboard (dropped from 86 to 68 for dominant hand; from 93 to 76 for nondominant hand), the Stroop test (improved from 60 to 68 for words; from 45 to 68 for words/color), and the Rey Auditory (improved from 5 to 6 for part A and 1 to 3 for Memory—Retention A).

Proteomic analyses
To substantiate the data set shown in this report, we investigated protein profiles from HIV-1–infected MDMs by ProteinChip analyses. Protein profiles were analyzed within the 2 to 50-kDa molecular mass range by using weak cation exchange (WCX2) chips with the alpha-cyano-4-hydroxycinnamic acid (CHCA) energy absorbing molecule (EAM). Previously, the sensitivity and reproducibility of the system had been tested and found adequate (Luo *et al*, 2003). The MDM lysate and supernatant samples were tested and 60 peaks from the cell lysates (Figure 1, representative data) and 20 peaks from the supernatant were found. Comparisons between HIV-1–infected patients without cognitive deficits while on HAART [HIV (P1) and HIV (P2)], HIV-1, -2–seronegative controls [Con (P1) and (P2)], and the HAD patient early after HAART was initiated [HAD (1)] and 6 months later [HAD (2)] are shown in Figure 1. Figure 1A and B demonstrate similar and discordant peaks among samples. Control, HIV, and HAD patient samples (both 3 weeks and 3 months after HAART treatment) share some common peaks in the cell lysate samples between 3.5 and 6.0 kDa; and a small number of peaks are shared by all the samples (Figure 1A, *vertical lines*). The most prominent unique peaks in the cell lysate are from the HAD patient [HAD (1)], which appear between 6.5 and 8.0 kDa (Figure 1B). These peaks do not appear in any of the other samples and they disappear from the same patient after three months of HAART [HAD (2)] (Figure 1B). In order to exclude that this series of unique peaks came from viral protein, both pure viral lysate (HIV-1_{ADA}) and lysate obtained from HIV-1_{ADA}–infected MDMs were studied (Figure 2). The spectra show no similar peaks in the 5.0 to 8.0-kDa range in either the pure HIV-1_{ADA} lysate or HIV-1_{ADA}–infected MDM lysate (Figure 2).

To determine whether the phenomic protein profiles observed in the MDM lysates reflect similar or different proteins secreted by the macrophages, we performed parallel experiments examining supernatant fluids collected from the same patient (Figure 3A–C). The results displayed a unique group of proteins from the supernatant fluids demonstrating discordance. Figure 3A–C shows the comparisons made between the supernatant samples from HIV (P1) and HIV (P2), Con (P1) and Con (P2), HAD (1) and HAD (2). Similar peaks in the supernatant are shared by either some or all the patient samples as shown

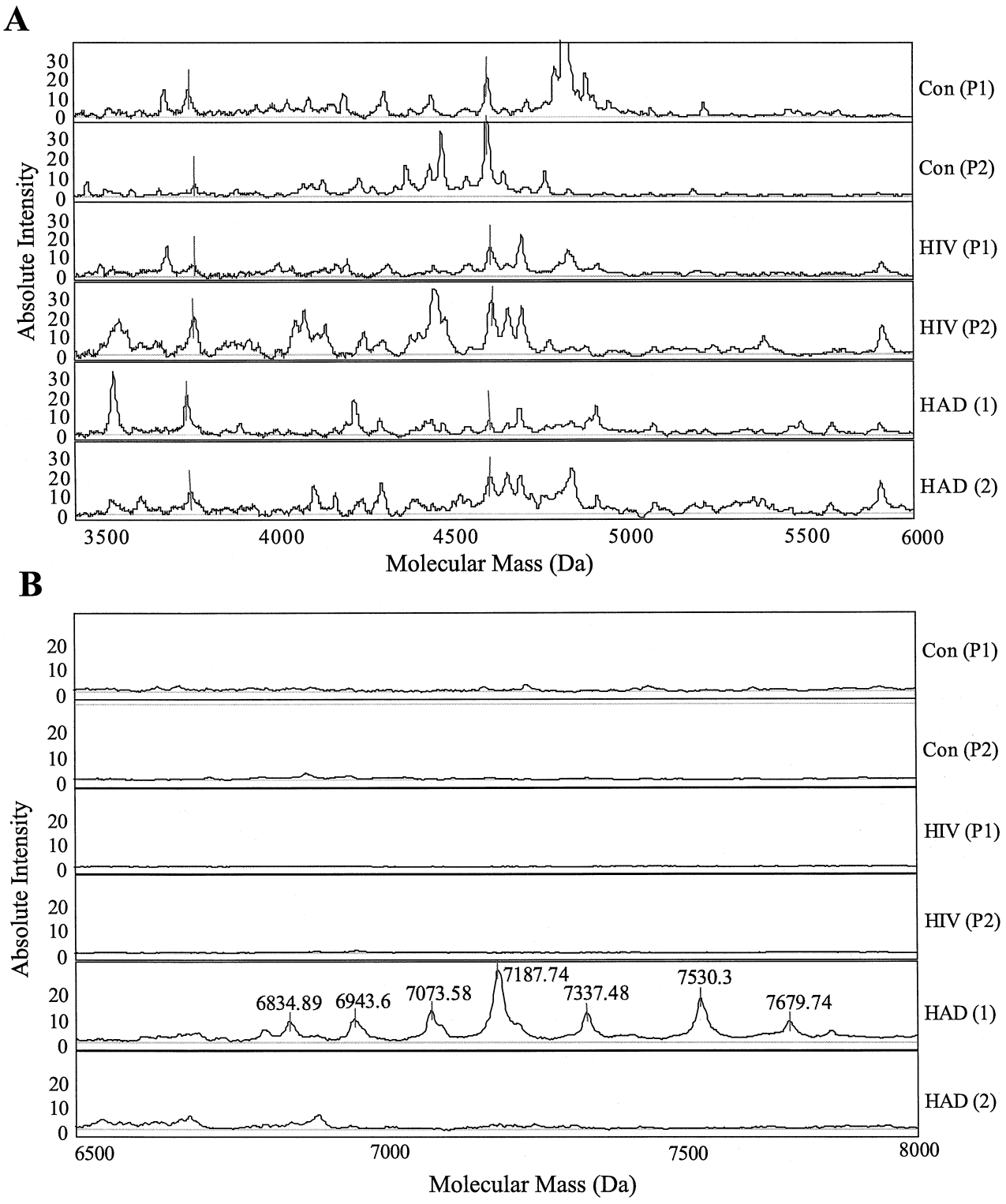

Figure 1 Protein peak analysis in MDM lysates taken from control (uninfected) and HIV-1–infected subjects with or without neurocognitive deficits. MDMs from Con (P1) and Con (P2); HIV (P1) and HIV (P2); and HAD (1) and HAD (2) were evaluated. **A** shows that similar protein peaks are found among samples within 3.5 to 6.0 kDa. Two of the peaks (*vertical lines*) are common to all samples, and most of the remaining proteins are shared among the samples. **B** shows unique peaks (labeled by mass) within 6.5 to 8.0 kDa from HAD (1). These proteins were absent in Con (P1), Con (P2), HIV (P1), HIV (P2), and HAD (2). All of the spectra in **B** are normalized against the absolute intensity of the HAD (1) spectra. Non-normalized spectra show the appearance of very-low-intensity peaks in the identical mass range, but neither the mass nor the absolute intensity was comparable to the HAD (1) sample.

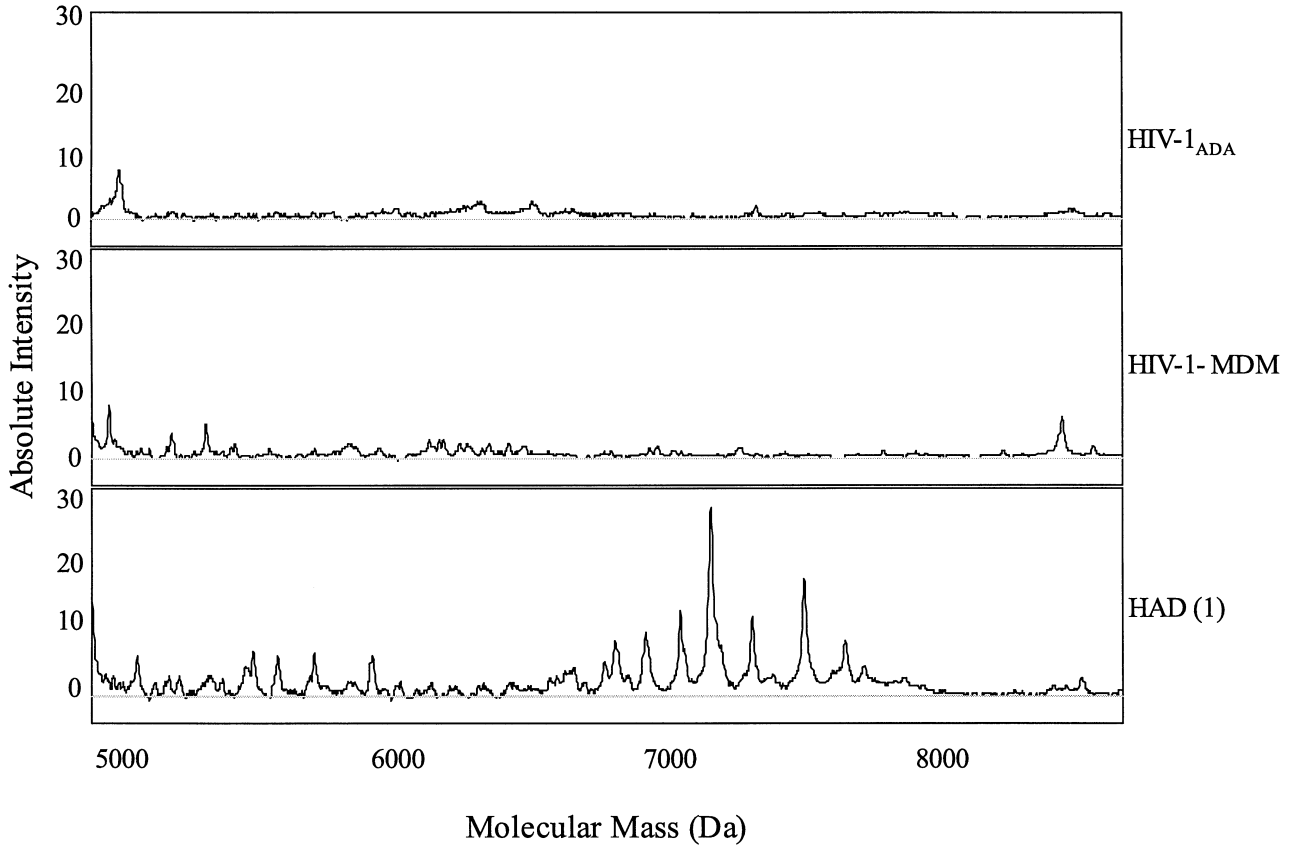

Figure 2 The unique protein peaks found in the HAD (1) patient MDM profile are cellular proteins. In order to exclude the possibility that the unique peaks were HIV-1 proteins, lysates were prepared from purified virions (HIV-1_{ADA}) and from HIV-1_{ADA}–infected MDMs. In either virus-containing lysate, no similar peaks were found within the 6.5- to 8.0-kDa range as demonstrated in the HAD (1) patient sample.

by vertical lines (Figure 3A), whereas in the HAD (1) sample, a small number of unique peaks appeared between 4.5 and 4.9 kDa (Figure 3B) and 13.0 and 17.0 kDa (Figure 3C). These HAD (1) unique peaks disappeared after HAART treatment [HAD (2)] (Figure 3B–C). Conversely, there were many peaks detected in HIV-infected subjects [HIV (P1), HIV (P2), and HAD (1)] that were not present in the uninfected controls [Con (P1) and Con (P2)] and that were abrogated in the HAD patient after 3 months of HAART (Figure 3A–C).

Discussion

The increased transmigration of circulating peripheral monocytes into the brain is a central feature of the neuropathogenesis of HIV-1 infection (Kaul *et al*, 2001). A promising hypothesis is that a specific subset of circulating monocytes contributes to blood-brain barrier (BBB) dysfunction and neuronal demise is emerging (Pulliam *et al*, 1997; Fischer-Smith *et al*, 2001). Our data show the presence of unique peaks in both the cell lysate and supernatant samples of the monocytes from the HAD patient, which strongly supports the hypothesis that a subset of monocytes appear with a unique protein profile in HAD, as compared to HIV-infected and -uninfected controls. The significance of the presence of multiple peaks in the HIV-infected subjects (as compared to uninfected) that disappeared in the HAD patient following HAART is not as clear. Because we did not study pre-HAART samples from the HIV infected patients without cognitive deficits [HIV (P1) and HIV (P2)], we do not know if a similar response would have been seen in these patients, which might suggest that these peaks are not specific to HAD but to HIV infection. The subsequent disappearance of the unique peaks following 3 months of HAART therapy corresponded with improvement of the neuropsychological tests in this patient, suggesting that alterations in monocyte function may correlate with the progression or improvement of dementia. The disappearance of the unique protein profiles after 3 months of HAART is consistent with other studies in which neurotoxicity is abrogated in monocyte supernatants from HAD patients after HAART therapy (Kusdra *et al*, 2002) and also consistent with the fact that the neuropsychological tests in the HAD patient, although remaining overall dysfunctional, showed improvement after 3 months of HAART. HAART is designed to inhibit

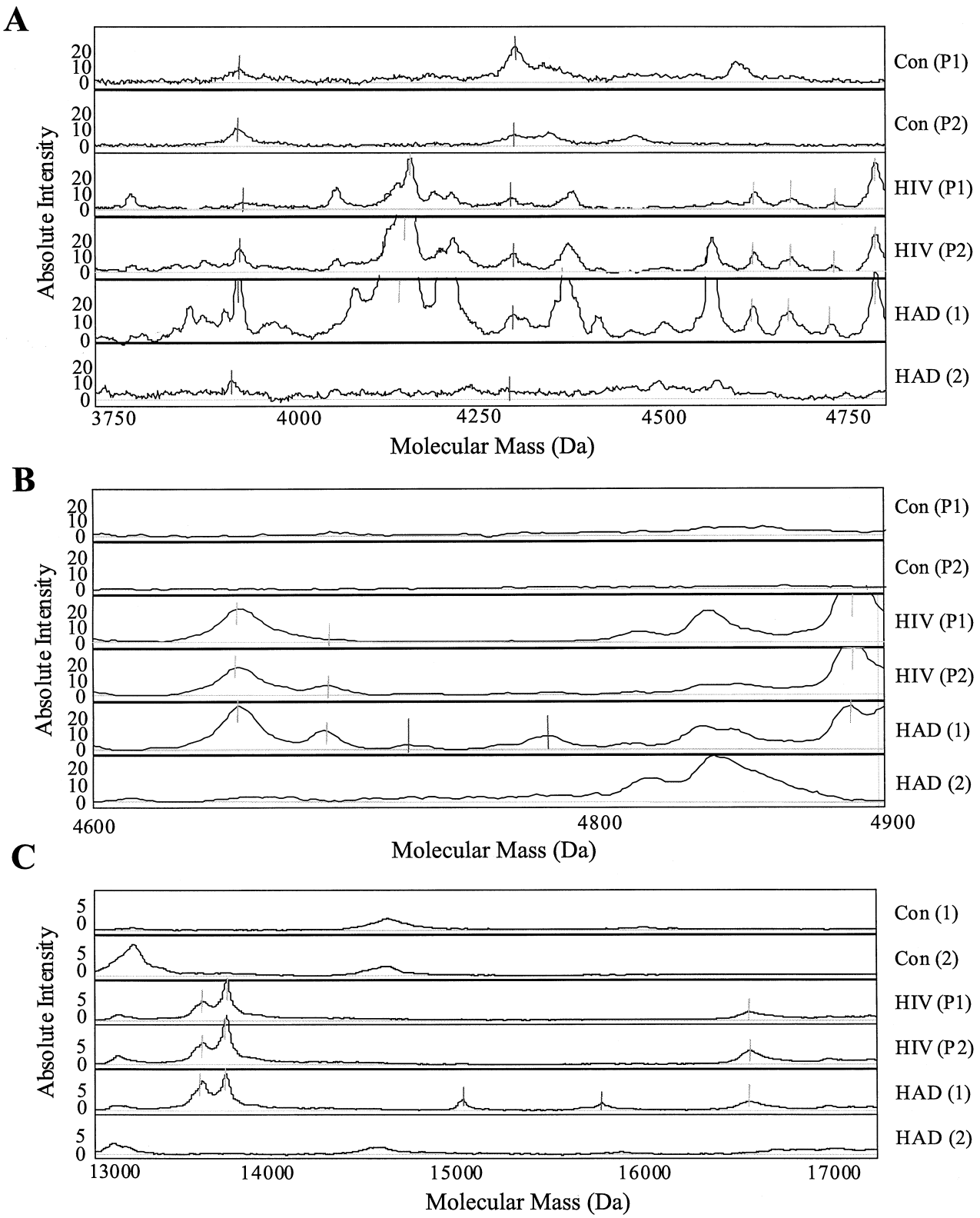

Figure 3 Protein peak analysis in MDM supernatant fluids taken from control (uninfected) and HIV-1–infected subjects with or without neurocognitive deficits. MDMs from Con (P1) and Con (P2); HIV (P1) and HIV (P2); and HAD (1) and HAD (2) were evaluated. **A** shows the similar peaks among samples. Two of these (*vertical lines*) were common among all samples. Five of these (*vertical lines*) are common to all the HIV-infected subjects but not found in the uninfected controls or the HAD patient following 3 months of HAART. **B** shows the spectra from 4.6–4.9 kDa and **C** shows the spectra from 13 to 17 kDa. The unique peaks (*red lines*) in the HAD patient can be found at 3 weeks of HAART [HAD (1)]. These proteins were absent in all other patients, whereas several peaks (*green lines*) are again found to be common to all the HIV-infected subjects but not detected in the uninfected controls or the HAD (2) subject after HAART. All of the spectra are normalized against the absolute intensity of the HAD (1) data.

viral replication, which can reduce the plasma viral load by 2 logs in 2 weeks (Siliciano, 1998), but whether it has direct or indirect effects on monocyte function, and whether it can be restored to normal, is unclear.

In order to exclude the possibility that the unique peaks in the cell lysate and supernatant samples originated from virus particles, we tested *in vitro* infected MDMs as well as pure viral lysate. When both profiles were compared to the profile from the HAD patient, there were no peaks similar to the unique peaks in the dementia patient in the area of 6.5 to 8.0 kDa. It is unlikely that the unique peaks came from another cell type, such as lymphocyte contamination during monocyte extraction and culture, because growth factors essential for lymphocyte growth are absent in the culture medium. Also, lymphocytes that may be contaminating the cell sample usually detach from the plastic surface of the culture dish and are washed off by changing the medium before the cell lysates and supernatants are collected. Hence, the numbers of any contaminating lymphocytes would be so low that they would unlikely contribute to the protein profile in a quantity sufficient for detection. Because only one dementia case was studied, the possibility that the genetic background of this patient may account for the phenomic protein profile cannot be ruled out. However, the disappearance of the unique peaks after HARRT in both the cell lysate and supernatant samples indicates these peaks are more related to the disease process, rather than to the patient genetic background. Nonetheless, such differences remain a possibility, at least, for some of the peaks (Figure 3A–C, marked with vertical lines).

The TagIdent website (www.expasy.ch/tools/tagident.html) with known molecular weight and relative isoelectric point of these peaks was searched to determine the possible identity of the unique peaks. In the MDM supernatants, the most relevant proteins for the two unique peaks between 4.0 and 5.0 kDa are vasoactive intestinal peptide precursor and vitronectin precursor. Vasoactive intestinal peptide precursor can cause vascular dilation, which could be related to increased permeability of the BBB and the vitronectin precursor is a cell adhesion and spreading factor, which could aid in macrophage transmigration. For the cell lysate, the most relevant proteins are mainly related to the highly active intracellular activity, for example, ATP synthase lipid binding protein, Na^+/K^+-ATPase gamma subunit, and copper transport protein, which may reflect the intracellular protein preparation for trafficking or secretion.

In conclusion, our data demonstrate that a unique subset of circulating peripheral monocytes exists for HAD and that its emergence in blood is associated with a unique phenomic protein signature that is abrogated after treatment. These findings may prove to be an effective marker for the diagnosis of dementia in HIV-infected individuals, as well as for measurements of therapeutic efficacy.

Methods

Patient cohort

The HIV-1–infected women at risk for cognitive dysfunction were recruited from HIV clinics at the Puerto Rico Medical Center, Latin American Center for Sexually Transmitted Diseases and the Center of Maternal–Infant HIV infections. The selection criteria included women 21 to 45 years, CD4 <500, at least a 9th grade education, and no evidence of either active systemic infection or other neurodegenerative disorders. This study had the approval of the Institutional Review Board of the University of Puerto Rico Medical Sciences Campus (UPR-MSC), and was carried out with informed consent. The ages, $CD4^+$ T-cell count, MSK scores, and viral load of the patients included in this study are shown in Table 1. All patients studied were on HAART.

Neurological and neuropsychological evaluation

HIV-1–infected and uninfected women had a complete neurological and neuropsychological evaluation. The neurological evaluation included a mini-mental status examination, evaluation of cognition and sensorium (including response slowing, speed of thought, and language), behavior/mood, affect, cranial nerves, coordination, motor system, reflexes, and sensory function. In addition, the neurologist staged each patient for dementia status using the MSK scale developed by Dr. Richard Price (Price and Brew, 1988). The neuropsychological evaluation consisted of the following tests: Wechsler Adult Intelligence Test (Vocabulary Subtest), Woodcook-Muñoz

Table 1 Patient demographic and immune profiles of HIV-1–infected women

					CD4		*CD8*			*Lymphocytes*	
Patient	*Age*	*Plasma viral load*	*CSF viral load*	*MSK*	*Total*	*%*	*Total*	*%*	*Monocytes*	*Total*	*%*
HIV (P1)	37	1683	ND	0	296	9	296	68	1.00	3.3	46.4
HIV (P2)	37	33,274	ND	0	78	8.2	413	49	0.20	0.9	19.6
HAD	41	32,057	214	2	388	12.5	477	63.4	0.50	0.8	24.3

Note. The demographics and viral and immune profiles are listed for each of the HIV-1–infected Hispanic women included in this study. ND = nondetectable copies of HIV RNA/ml.

(Reading Subtest Modalities 23 + 31; this test was used as a Spanish substitution for the Wide Range Achievement Test), Symbol Digit Modalities Test, Stroop Color and Word Test, Rey Auditory Verbal Learning Test, Grooved Pegboard, Trail Making A & B Test, Simple Reaction Time, and Beck Depression Inventory. A neuropsychologist performed all the neuropsychological tests. Because the Puerto Rican population is Spanish speaking, all tests were performed in Spanish. Both the neurologist and the neuropsychologist were blinded to each other's findings.

Viral load
Plasma was obtained after centrifugation and frozen at −80°C until testing. RNA copy numbers from plasma were determined by the Amplicor HIV monitor test (F. Hoffmann–La Roche, Basel, Switzerland).

Isolation and cultivation of monocytes from patient blood mononuclear cells
Monocytes were obtained from peripheral blood of patients or healthy donors by Percoll density gradient (Feige *et al*, 1982). Cells were purified by preferential adherence after cultivation in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated pooled human sera, 50 μg/ml gentamicin (Sigma), 10 μg/ml ciprofloxacin (Sigma) and 1000 units/ml macrophage colony-stimulating factor (MCSF; a generous gift from Genetics Institute, Cambridge, MA). After 7 days in culture, patients' MDMs were 95% to 98% pure by Wright staining and histological examination. Supernatant fluids were removed and replaced with serum-free medium for 24 h for subsequent protein evaluations. Serum-free MDMs supernatants were collected. Adherent MDMs were incubated for 15 min at room temperature with lysis buffer (50 mM Tris-HCl, 0.3 M KCl, 2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA). For cell lysates, the protein concentrations were assessed using the Pierce BCA Kit (Pierce, Rockford, IL) following the manufacturer's instructions. For supernatant samples, cell numbers per milliliter of medium were used to normalize the protein load.

Proteomic analyses
SELDI-TOF ProteinChip analyses were performed using WCX2 chips (Ciphergen Biosystems, Palo Alto, CA) to analyze molecules with a positive charge on the active surface of the chip. The spot surfaces were pretreated with 250 μl 10 mM HCl, equilibrated with wash buffer (100 mM ammonium acetate, pH 4 with 0.1% Triton X-100) and equal amounts (2.5 μg) of total protein applied. Unbound proteins were removed by two washes with wash buffer followed by a high-performance liquid chromatography (HPLC)-grade water rinse. Initial experiments were performed to optimize the chip type and washing conditions for all the patient samples (Luo *et al*, 2003).

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