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Can the peripheral blood monocyte count be used as a marker of CSF resistance to antiretroviral drugs?

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Resistance to antiretroviral drugs in human immunodeficiency virus (HIV) disease may be discordant between blood and cerebrospinal fluid (CSF). However, there is no method by which patients who are likely to harbor resistant HIV in the CSF can be noninvasively identified. Activated monocytes are known to traffic through the brain and perivascular microglia are considered to "turnover" regularly from bone marrow–derived monocytes. Monocytes lack certain kinases necessary to metabolize some antiretroviral drugs, making it possible that monocytes, could deliver antiretroviral drugs to the brain. Low monocyte counts in the peripheral blood, however, would be expected to lead to decreased trafficking and turnover of monocytes, with less drug delivery to the brain. The authors hypothesized that this would increase the likelihood of drug resistant HIV in the central nervous system. To test this, 24 matching CSF and plasma samples that had been prospectively collected and stored from patients treated with nucleoside analogue reverse transcriptase inhibitor drugs were assessed for genotypic resistance. Those CSFs, with evidence of resistance mutations, were compared to those without for peripheral blood monocyte count, hemoglobin, CD4 cell count, and zidovudine (ZDV) use. The same analyses were repeated on the plasma samples. There were 11 CSFs with evidence of resistance mutations. The peripheral blood monocyte count was significantly lower in the CSF resistant group (0.29 ± 0.16) *versus* (0.52 ± 0.21) × 10^9/L ($P < .001$). There was no difference between the groups according to hemoglobin, CD4 cell count, total white cell count, or use of ZDV. There was no difference between resistant and sensitive plasma samples according to peripheral blood monocyte count. To further test the hypothesis, the authors determined the concentrations of ZDV, stavudine, and abacavir in monocytes after each drug had been added to monocyte cultures. There was a significant decline in the concentration of each drug in the supernatant, implying that it had been "taken up" by the monocytes. These preliminary data suggest that peripheral blood monocytes may be important in delivery of antiretroviral drugs to the brain and the development of resistance. *Journal of NeuroVirology* (2004) **10(suppl. 1),** 38–43.

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Introduction

Resistance to antiretroviral drugs used in the treatment of patients with human immunodeficiency virus (HIV) disease is becoming increasingly common. The resistance pattern of HIV that has been derived from a patient's blood may be different to that which is found in HIV derived from the patient's cerebrospinal fluid (CSF) (Cunningham *et al*, 2000; Lanier *et al*, 2001; Venturi *et al*, 2000). Although it is certainly true that CSF-derived HIV may not correspond to brain-derived HIV, it is the only viral isolate from the central nervous system (CNS) that is readily attainable *in vivo*. Moreover, CSF-derived HIV does

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seem to have clinical relevance: the CNS efficacy of antiretroviral drugs used in the treatment of AIDS dementia complex (ADC) appears to be related to their ability to enter the CSF in appropriate concentrations (Brew, 2001), and resistance to antiretroviral drugs in the CSF most commonly occurs in those patients with neurological disease (Cunningham *et al*, 2000; Lanier *et al*, 2001; Venturi *et al*, 2000), suggesting a causal relationship. At present there is no way of knowing *a priori* whether it is likely that CSF-derived virus will in fact have a different resistance profile from the blood in a particular patient. A noninvasive method of targeting patients who are likely to harbor such a different resistance pattern in the CSF is needed.

Activated monocytes are known to traffic through the CNS. A subset of monocytes expressing the marker CD14/16 can support productive HIV infection and can "deliver" HIV to the brain, the so-called Trojan horse hypothesis. Perivascular microglia are known to "turn over" from bone marrow-derived monocytes, though the extent and frequency are unknown (Gartner and Liu, 2002). Meningeal and choroid plexus macrophages also "turn over" from bone marrow–derived monocytes. Again, however, the extent and frequency with which these happen are not known. Monocytes can take up antiretroviral drugs but the precise details of this are not understood. Some drugs are poorly phosphorylated once in the cell. Stavudine is an example of this where the dominant intracellular moiety is the unphosphorylated form (Gao *et al*, 1994; Hoggard *et al*, 1997), whereas other drugs are variably phosphorylated (Stein *et al*, 2001). Such a turnover of cells with variable metabolism for some antiretroviral drugs (Aquaro *et al*, 2002; O'Brien *et al*, 1994) raises the possibility that monocytes or a subset thereof may be able to act as "delivery vehicles," allowing access to the brain, for antiretroviral drugs. If monocyte trafficking were reduced, then this might not only diminish delivery of HIV to the brain, but it might lessen the amount of antiretroviral drugs in the CNS. In the setting of existing HIV brain infection and reduced concentration of antiretroviral drugs, resistance would be more likely to occur.

We therefore tested the hypothesis that in patients with evidence of resistance in CSF-derived virus, there would be a lower peripheral blood monocyte count as opposed to those in whom there was no evidence of resistance. This hypothesis is based on the premise that the "turnover" of perivascular macrophages and microglia is dependent on the peripheral blood monocyte count. Although this seems to be a reasonable assumption, there is no proof as yet. The hypothesis was tested in two ways. *In vivo*, we analyzed the resistance patterns to antiretroviral drugs in the CSF and blood according to the peripheral blood monocyte count. We assessed, *in vitro*, the antiretroviral drug uptake into monocytes from a buffered medium after various concentrations of monocytes had been added.

Results

In vivo *experiments*

Fifty-three same-day samples of paired blood and CSF had been collected from 49 patients (4 had repeat samples for different clinical reasons and were considered independent observations). The details of this data set have already been published (Cunningham *et al*, 2000). The patients' diagnoses were ADC ($n = 15$), cryptococcal meningitis ($n = 11$), cytomegalovirus (CMV) encephalitis ($n = 5$), progressive multifocal leukoencephalopathy (PML) ($n = 4$), and HIV neuropathy ($n = 6$). The remainder had assessment for nonspecific neurological symptoms and where not found to have any significant disease. The mean CD4 count was 30 cells/μl, whereas the mean HIV viral load in the CSF was 4.92 log (median 3.67; range 2.6–5.9) and the mean plasma HIV viral load was 5.35 log (median 4.95; range 2.6–5.9). The HIV reverse transcriptase (RT) sequences could only be determined when the viral load was in excess of 1000 copies/ml. Consequently, only 31 of the 53 samples had RT sequences available for analysis.

Of these 31, peripheral blood monocyte counts were available in 24 (Table 1): 9 had ADC, 6 had cryptococcal meningitis, 3 had CMV radiculopathy, and 1 had PML. The remainder had assessment for nonspecific neurological symptoms and were not found to have any significant disease.

CSF samples were dichotomized into those containing no resistance mutations (CSF sen) and those containing resistance mutations (CSF res). Blood samples were similarly dichotomized (b sen and b res). In the CSF res group there were 11, whereas in the b res group there were 12. In the CSF sens group, there were 13, whereas the b sen group had 12.

Peripheral blood monocyte counts were significantly lower in the CSF res group compared to the CSF sen group ($P < .001$) (see Table 1). Indeed, all the samples in the CSF res group had monocyte counts $\leq 0.6 \times 10^9$/L. In contrast, there was no difference between the b res ($[0.34 \pm 0.17] \times 10^9$/L) and b sen ($[0.51 \pm 0.25] \times 10^9$/L) groups, though there was perhaps a trend ($P = .06$). The results were unchanged when the monocyte counts that had been taken 2 weeks either side of the CSF acquisition were analyzed (data not shown).

Other parameters were not different between the CSF res and sen groups: CD4 count ($P = .57$), hemoglobin ($P = .8$), total white cell count ($P = .12$). Moreover, there was no difference between the groups in zidovudine (ZDV) use (presence or duration of use in months) ($P = .65$) or albumin index as a marker of blood brain barrier integrity ($P = .8$). Of the 10 patients with opportunistic infections, the 3 with CMV infection were all in the CSF res group, as was 1 of the 6 with cryptococcal meningitis; the remaining patients were in the CSF sens group.

Table 1 Patients with HIV-1 reverse transcriptase mutations associated with resistance in paired blood and cerebrospinal fluid (CSF) specimens

	Treatment history (months)					*HIV reverse transcriptase codon*						
Specimen	*ZDV*	*DdI*	*ddC*	*3TC*	*Diagnosis*	*41*	*69*	*70*	*74*	*184*	*215*	*Monocyte count*
Patient 1	9	—	—	—	ADC							
Blood						wt/M	M	M	wt	wt	M	0.3
CSF						wt	wt	wt	wt	wt	M	
Patient 2	60	—	—	—	HIV neuropathy							
Blood						wt/M	M	M	wt	wt	wt/M	0.2
CSF						M	wt	wt	wt	wt	M	
Patient 3	1	—	—	—	ADC							
Blood						wt	wt	wt	wt	wt	wt	0.2
CSF						wt	wt	wt	wt	wt	M	
Patient 4	30	30	—	—	ADC							
Blood						wt/M	wt	wt/M	wt	wt	NS	0.4
CSF						wt	wt	wt	wt	wt	wt/M	
Patient 5	27	1	—	—	CMV polyradiculopathy							
Blood						wt	NS	NS	NS	wt	M	0.4
CSF						M	wt	wt	NS	wt	M	
Patient 6	1	—	—	—	ADC							
Blood						M	wt	wt	wt/M	NS	M	0.5
CSF						wt	wt	wt	wt	wt	M	
Patient 7	15	—	—	15	ADC							
Blood						M	wt	M	wt	NS	M	0.6
CSF						M	wt	M	wt	NS	M	
Patient 8	18	4	5		CMV polyradiculopathy							
Blood						M	M	wt	wt	wt	M	0.0
CSF						M	M	wt	wt	wt	M	
Patient 9	18	4	5		CMV polyradiculopathy							
Blood						wt	M	wt	wt	wt/M	M	0.1
CSF						wt	M	wt	wt	wt/M	M	
Patient 10	—	—	6	6	ADC							
Blood						wt	wt	wt	wt	M	M	0.5
CSF						wt	wt	wt	wt	M	M	
Patient 11	5	—	5	—	Cryptococcal meningitis							
Blood						wt	wt	wt	wt	M	wt	0.3
CSF						wt	wt	wt	wt	M	wt	
Patient 12	50	30	—	—	ADC							
Blood						M	M	wt	wt	wt	M	0.4
CSF						wt	wt	wt	wt	wt	wt	
Patient 13	34	8	34		Cryptococcal meningitis							
Blood						M	M	M	wt	wt	wt	0.4
CSF						NS	wt	wt	wt	wt	NS	
Patient 14	—	—	26		ADC							
Blood						wt	M	M	wt	M	wt	0.4
CSF						wt	wt	wt	wt	NS	wt	
Patient 15	20	6	8		Bacterial pneumonia							
Blood						wt	wt	wt	wt	wt	NS	0.8
CSF						wt	wt	wt	wt	wt	wt	
Patient 16	—	—	—		Demyelinating Polyneuropathy							
Blood						wt	wt	wt	wt	wt	wt	0.9
CSF						wt	wt	wt	wt	wt	wt	
Patient 17	—	—	—	—	Cryptococcal meningitis							
Blood						wt	wt	wt	wt	wt	wt	0.5
CSF						wt	wt	wt	wt	wt	wt	
Patient 18	36	—	—	—	Mycobacterium avium							
Blood						wt	wt	wt	wt	wt	wt	0.4
CSF						wt	wt	wt	wt	wt	wt	
Patient 19	9	3	2	—	No significant finding							
Blood						wt	wt	wt	wt	wt	wt	0.4
CSF						wt	wt	wt	wt	wt	wt	
Patient 20	2	—	—	2	PML							
Blood						wt	wt	wt	wt	wt	wt	0.5
CSF						wt	wt	wt	wt	wt	wt	
Patient 21	0.5	—	—	0.5	Cryptococcal meningitis							
Blood						wt	wt	wt	wt	wt	wt	0.3
CSF						wt	wt	wt	wt	wt	wt	
Patient 22	—	25	—	—	ADC							
Blood						wt	wt	wt	wt	wt	wt	0.4
CSF						wt	wt	wt	wt	wt	wt	
Patient 23	—	49	—	—	Cryptococcal meningitis							
Blood						NS	NS	NS	NS	wt	NS	0.9
CSF						NS	NS	NS	NS	wt	NS	
Patient 24	—	—	—	24	Cryptococcal meningitis							
Blood						wt	wt	wt	wt	wt	wt	0.2
CSF						wt	wt	wt	wt	wt	wt	

Note. Patients 1 to 11 are in the CSF-resistant group.
ZDV, zidovudine; ddI, didanosine; ddC, zalcitabine; 3TC, lamivudine; wt, wild-type; M, mutant; wt/M, mixed population; NS, no signal; ADC, AIDS dementia complex; PML, progressive multifocal leukoencephalopathy; CMV, cytomegalovirus.

Table 2 The effect of different monocyte numbers on nucleoside reverse transcriptase inhibitor drug concentrations in the supernatant

	Stavudine	*Abacavir*	*Zidovudine*
Mean % decrease in concentration in experiment 1 (5×10^6 monocytes after 18 h)	7.3	2.8	0.5
Mean % decrease in concentration in experiment 2 (40×10^6 monocytes after 1 h)	7.5	2.8	0
Mean % decrease in concentration in experiment 3 (100×10^6 monocytes after 1 h)	14.4	8.3	1.4

In vitro *experiments*
Although the experimental numbers are small, the data suggest that drug does enter monocytes, the drug entry appears to be low and varies for different drugs (Table 2). Stavudine appears to enter monocytes more easily than abacavir or zidovudine; however, zidovudine entry into cells was low in all experiments even when the monocyte numbers were increased substantially.

Discussion

Lower peripheral blood monocyte counts appear to be related to the presence of CSF resistance mutations, at least those related to nucleosides. Moreover, *in vitro*, monocytes do appear to be able to "take up" nucleoside analogue drugs even when they are not infected. Indeed as shown in Table 2, the greater the number of monocytes, the greater the fall in drug concentration.

Currently, there are no published data examining the possibility that monocytes could deliver drugs to the CNS. However, there are aspects of the biology of monocytes that makes this possible. Activated monocytes traffic through the CNS as part of their normal immune surveillance function. Second, there are increasing data pointing to the turnover of microglia and meningeal macrophages from monocytes (Gartner and Liu, 2002).

The cause of the monocytopenia is most likely HIV infection *per se*. Up to 75% of patients with advanced HIV disease may demonstrate some degree of monocytopenia (Mir *et al*, 1989; Treacy *et al*, 1987). Other causes such as *Mycobacterium avium intracellulare* and CMV infection as well as toxicity from zidovudine are also possible. In this study, however, only four patients had such infections and zidovudine use was not different between the groups. The precise means by which HIV infection alone leads to monocytopenia is not clear, but there is evidence that cytokine excess, in particular tumor necrosis factor alpha, can lead to monocytopenia at least acutely (Aulitzsky *et al*, 1991).

There are clear limitations in our data. The *in vitro* analyses were *post hoc*—the original study only aimed to determine the resistance patterns in the CSF and blood of HIV-infected patients. Secondly, the numbers in our study are relatively small. However, they are very comparable with other published studies. Thirdly, not all nucleoside analogue reverse transcriptase drugs were studied either *in vivo* or *in vitro*. Fourthly, it is unknown as to whether the fall in concentration of the antiretroviral drugs with lower monocyte counts is clinically relevant. Nonetheless, given that there was a significant fall in drug concentration even when only 0.1×10^9 monocytes were used, it seems likely. Moreover, it is known that the CNS penetration of antiretroviral drugs is relatively poor even for drugs such as zidovudine. It is conceivable that a further fall in CNS drug concentration could facilitate the development of drug resistance. Fifthly, we did not directly measure the passage of the drugs into the cells and we did not measure their exit from the cells. Although other explanations for the drop in drug concentration are possible, such as binding of the drug to plastic or to the cell surface, we consider these very unlikely. Binding of the drugs to the plastic tubes used in the experiments would have been reflected in the control tube. Furthermore, soft plastic containers with plasticisers are usually implicated in the binding of drugs, but not the more rigid plastic that was used in these experiments. Finally, a strong indicator of drug binding to glass or plastic occurs in the analytical procedure during the evaporation step when erratic and low recoveries are seen. The recoveries were high and consistent (>80%) when this assay was validated, strongly suggesting that binding to glass or plastic did not occur. Binding of the drugs to the cell surface is also unlikely. There is no evidence in the literature to suggest that this type of binding can occur and the available evidence (albeit in different cell types, for example, liver microsomes) suggests that nonspecific binding of zidovudine is negligible (Boase and Miners, 2002). Furthermore, other studies investigating the phosphorylation of the drugs in cell cultures have considered and excluded cell surface binding. The issue of whether the drugs can exit the cell is unknown, but one might argue that phosphorylated drugs might be trapped in the cell because it would be more difficult to cross the membrane in a more polarized state. There are two rejoinders to this criticism. As previously mentioned, the extent of phosphorylation is variable (Stein and Moore, 2001)—indeed stavudine is dominantly present intracellularly in the unphosphorylated form. Additionally, Moore *et al* (1999) have shown that the phosphorylated form of lamivudine is cleared from the cell with a half life of 15 h. However, the precise details as to whether this represents anabolism to the parent compound are unknown.

There are several interesting implications of our data. First, perhaps there is more to the debate over

the need for CNS-penetrating drugs in the treatment of ADC. Resolution of this controversy has been hampered by lack of consideration of the potential confounding influence of a disrupted blood-brain barrier and the presence of inhibitors of efflux transporter systems (for example, the effect of ritonavir on P-glycoprotein as well as the multidrug resistance protein systems [Huisman *et al*, 2002]). Both these factors may facilitate access to the brain by antiretroviral drugs that would normally have poor penetration. Perhaps in addition to these two factors there is a third: the peripheral blood monocyte count where low monocyte counts may diminish the delivery of antiretroviral drugs to the CNS. This may explain some of the conflicting data on the need for CNS-penetrating drugs thus far published (Antinori *et al*, 2002; Sacktor *et al*, 2001). Such drugs may be important initially as shown by Antinori *et al* (2002), but after several months, the importance appears to be lost (Sacktor *et al*, 2001), possibly because the drugs by this time have been "delivered" to the brain by monocytes and the slow turnover of microglia from monocytes. Second, perhaps those ADC patients who show no evidence of any response to HAART in the absence of any resistance initially fail to respond because of low drug delivery to the brain, with resistance developing only as a later consequence.

Thus our findings lend support for the hypothesis that monocytes may be important in the delivery of antiretroviral drugs to the brain and provide the necessary initial data. More detailed *in vitro* studies of drug influx and efflux from monocytes will need to be conducted.

Materials and methods

An archived data set of samples that had been previously characterized by the LiPA assay (Murex Biotechnology) for the presence of resistance mutations to nucleoside antiretroviral drugs in both blood and CSF was accessed. The assay incorporates nested polymerase chain reaction (PCR) for the wild-type, mutation, and 3rd letter base polymorphisms at codons 41, 69, 70, 74, 184, 214, and 215.

All patients had been assessed neurologically, and other investigations, such as cryptococcal antigen and PCR for JC virus and CMV, had been performed as appropriate. Full blood count and CD4 count had been performed. CSF and plasma samples had been assayed for HIV RNA using the Amplicor HIV-1 Monitor (Roche Diagnostics). The albumin index had been measured as a marker of blood-brain barrier integrity.

In vitro *experiments*

Human peripheral blood mononuclear cells (PBMCs) were isolated from an HIV-1–seronegative buffy coat (Sydney Blood Bank) using a standard Ficoll-Paque (Pharmacia, Uppsala, Sweden) density separation method (Kerr *et al*, 1997). Cells at 100×10^6 were allowed to adhere to plastic 6-well plates (Falcon; Becton Dickinson Labware, NJ, USA) for 1 h. The cells were then washed vigorously five times with phosphate-buffered saline (PBS) to remove any remaining lymphocytes. Pure substance stavudine, abacavir, and zidovudine was dissolved in methanol (Mallinckrodt Baker, KY, USA), and diluted with PBS (JRH Biosciences, Lenexa, Kansas, USA) to produce a final concentration of drug in 2 ml of PBS cell suspension of 200 ng/ml. A final concentration of 200 ng/ml for each drug was chosen as this concentration represented the average steady-state concentration seen in human plasma following standard dosing regimens of the agents. Three experiments were performed. Experiment 1: 5×10^6 monocytes were incubated, in duplicate, with either stavudine, abacavir, or zidovudine for 18 h at 37°C. Experiment 2: 40×10^6 monocytes were incubated, in duplicate, in either stavudine, abacavir, or zidovudine for 1 h at 37°C. Experiment 3: 100×10^6 monocytes were incubated, in duplicate, in either stavudine, abacavir, or zidovudine for 1 h at 37°C. After incubation, the cell suspensions were spun at 1000 rpm for 10 min to remove any remaining cells and a 1.0-ml aliquot of the supernatant was used to quantitate each drug. AIM V medium with diluted drug alone was used as a control to compare uptake of drug into monocytes.

Stavudine, abacavir, and zidovudine were quantitated in control and cell-treated supernatant using high performance liquid chromatography, with ultraviolet detection at 266 nm. Controls and cell-treated supernatant (1.0 ml) were extracted twice with 5.0 ml ethyl acetate after the addition of internal standard (hydroxyethyl theophylline). After mixing and centrifugation, the supernatants were combined and evaporated to dryness under air at 50°C. The residue was resuspended in 200 μl of 50% methanol/water and after sonication and centrifugation, 50 μl was injected onto the liquid chromatograph. Separation was achieved on a Phenomenex Aqua C18 column (250×4.6 mm, 5-μm particles; Phenomenex, CA, USA) using a mobile phase of acetonitrile: 50 mM potassium phosphate buffer (pH 1.5, 10:90). The method was developed and validated to good laboratory practice standards (Shah *et al*, 2000). The concentration of drug in the supernatant was recorded. The difference between the concentration in the supernatant containing monocytes and the supernatant alone was recorded as an average deviation (%).

Statistical comparisons between the groups was assessed by Student's t test with an alpha significance level set at .05.

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