Detection of resistant mutations in the reverse transcriptase of HIV-1-infected children

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Development of viral resistance to the antiretroviral drugs used for treating human immunodeficiency virus (HIV) infections is an important cause of treatment failure and limits the options for alternative antiretroviral regimens [1–4]. This resistance develops gradually in a stepwise series of mutations. The number and the pattern of mutations influence the level of phenotypic resistance, which in some cases results in cross-resistance to two or more drugs [5–7]. Aggressive multidrug regimens are currently being introduced into HIV-positive pediatric populations, both in clinical practice and in controlled trials [8,9]. It is not yet clear whether the experience and guidelines developed for adults will be applicable to infants and children [10]. Detection of genotypic resistance, which would be faster and cheaper than phenotypic studies, may help in choosing an initial regimen or in deciding to change the antiretroviral therapy for a patient. Our objectives were to detect genotypic resistance to the antiretroviral nucleoside analogs among the perinatally HIV-1-infected children treated at our hospital and to determine if the changes implemented in treatment regimens would influence plasma RNA, $CD4^+$ lymphocyte count and disease progression.

Eleven perinatally infected children treated at the Pediatric Infectious Diseases Section of the University Hospital 'Virgen Macarena' (Seville, Spain) were included in the study and were subsequently followed and evaluated quarterly by analyzing their plasma RNA, $CD4^+$ lymphocyte counts, genotypic resistance to nucleoside analogs and clinical state. During the study, the patients were treated with different antiretroviral regimens after an individualized analysis of their previous parameters, intolerance to drugs and disease progression.

Viremia levels were determined in batches using EDTA-treated plasma samples, stored in aliquots at −70 °C, using the Amplicor HIV-1 Monitor test (Roche Molecular System, Basel, Switzerland) according to the manufacturer's instructions, with a detection limit of approximately 200 HIV-1 RNA copies/mL. Those samples with a viremia level of < 200 copies/mL were retested using a modification of the Amplicor HIV Monitor test (Ultra Direct assay) with a detection limit of 20 HIV-1 RNA copies/mL [11].

Total fresh blood was used for $CD4^+$ lymphocyte counts. $CD4^+$ cell percentage and absolute counts were measured at the same points in time as HIV-1 RNA. Flow cytometry was performed according to standard techniques.

RNA extraction was performed with the High Pure Viral RNA kit (Boehringer Mannheim GmbH, Mannheim, Germany). cDNA synthesis and PCR amplification were performed with the Titan System RT-PCR (Boehringer Mannheim GmbH), following the instructions of the manufacturer and using nested primers (Murex Innogenetics, Madrid, Spain) to amplify the HIV-1 reverse transcriptase. Detection of nucleoside mutations was performed with the LIPA hybridization assay LIPA HIV-1 RT (Murex Innogenetics). The system is based on amplification of a fragment of the HIV RT gene by PCR and hybridization with specific probes, fixed to a plastic strip, which recognize and discriminate between mutant and wild-type genotypes at positions 41, 69, 70, 74, 184 and 215 [12].

The characteristics of the 11 children included in the analysis are shown in Table 1. The median age at entry in the study group was 2 years (range 0.1–11 years). The majority of the children (73%) were classified as symptomatic (stages B or C) according to the revised 1994 Centers for Disease Control and Prevention [13] classification of HIV infection in children. Three were classified as stage N (two N2 and one N1). The $CD4^+$ lymphocyte count at baseline showed an age-dependent downward trend that was independent of the clinical stage and corroborated the results obtained by other authors [14–16]. This decline is possibly due to the combined effect of disease progression and the natural fall with age. All 11 children had detectable HIV-1 RNA at baseline. The median and geometric mean were 5.12 and 4.89 $\log_{10}$ copies/mL, respectively, with a range from 4.11 to 5.81, similar to that of children in other reports [17–19]. The mean value of the viral load in patients with resistance before a treatment change was 5.02 $\log_{10}$ copies/mL, and 4.43 $\log_{10}$ copies/mL following treatment change after resistance had been detected. In those patients who did not develop resistance, the mean after treatment was 4.2 $\log_{10}$ copies/mL.

Three children (patients 1, 4 and 6) had not received antiretroviral treatment before entry into the study. The other eight

Table 1 Characteristics of the patients at study entry

Patient no.	Age[a]	Sex	CD4$^+$ (cells/μL)	Log_{10} RNA copies/mL	CDC clinical stage	Treatment before study[b]	Primary resistance	Resistance developed
1	1.1	F	1494	5.12	N2	None	None	ZDV (K70R)
2	5	M	390	4.56	N2	ZDV + ddI	None	ZDV (T215Y)
3	5	F	223	5.16	C3	ZDV + ddI	ZDV (T215Y)	ZDV (T69D + M41L)
4	0.2	F	3071	5.29	B1	None	None	None
5	0.9	M	2000	4.08	B1	ZDV + ddI	None	None
6	4	M	646	4.47	B1	ZDV + ddI + RTV	None	None
7	0.2	F	3841	5.81	N1	None	None	None
8	0.8	F	1110	4.56	C3	ZDV + ddI	None	ZDV (T215Y)
9	2	M	814	4.11	B2	ZDV	None	None
10	7	F	150	5.42	B1	ZDV + ddI	None	None
11	11	F	28	5.66	C3	ZDV + ddI	None	None

[a] Age in years. [b] ZDV, zidovudine; ddI, didanosine; RTV, ritonavir.

children had undergone different treatment regimens: one child had been treated with zidovudine (ZDV) alone; six children with ZDV plus didanosine (ddI); and one child with ZDV, ddI and ritonavir (RTV). During the study, all the children received antiretroviral therapy: six were treated with triple therapy; four with double therapy, and only one with ddI alone, because of intolerance to most of the available drugs.

Of the children without previous treatment, none showed any mutation conferring resistance to the antiretroviral drugs investigated at study entry. Among the eight pretreated children, one—patient 3—showed evidence of ZDV resistance (T215Y mutation) at study entry—after only 15 days of ZDV treatment—and developed two new mutations, T69D and M41L, after 4 months of treatment with ZDV and ddI. Another three children developed ZDV resistance during treatment: in one child—patient 1—the viral population showed a K70R mutation, but this mutation disappeared after a triple therapy regimen was established; the other two children showing resistance—patients 2 and 8—presented a T215Y mutation.

This study provides information on HIV-1 resistance in vertically infected children of different ages and at different clinical stages. When a genotypic mutation was detected, the treatment regimen was changed, eliminating ZDV and adding another nucleoside analog and/or a protease inhibitor. These changes always produced a decrease in the copies/mL present in the patients in later samples. In the present study, the patients whose treatment regimens were changed after detection of a resistant mutation had a mean 1.5 log decrease (range 1–2) in HIV-1 RNA levels within 2 months of the change of therapy. The results of the test to detect genotypic resistance were always available before the viral load increased, demonstrating that detection of resistance mutants could be more relevant in monitoring disease progression, although more data are needed to confirm this observation. Moreover, since it has been demonstrated [18] that rises in the HIV-1 RNA load can predict rapid progression to AIDS and death, it would be important to detect resistance quickly in order to avoid this increase by an appropriate treatment change.

Only resistance to ZDV developed in this study, probably because ZDV was the only antiretroviral agent used alone before resistance detection, and the most common mutation in the RT gene was T215Y. This mutation was present in three patients showing resistance. One patient alone showed two other resistant mutations (M41L and T69D) to ZDV as well. No relationship was found between development of resistance and clinical stage.

In conclusion, in this study of vertically HIV-infected children, very high viral loads were seen in children but with no correlation with the clinical stage. Genotypic mutations to resistance were detected before a change in the disease progression markers was noted (an increase of the viral load or a decrease in CD4$^+$ counts) and those children on a triple therapy regimen did not develop resistance. In the absence of more data to provide guidance on the detection of resistance to antiretroviral drugs, pediatricians should bear in mind that the sooner the treatment is modified to suit the individual case, the less the possibility of viral loads rising to high levels and of the development of resistant mutations.

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Pharmacodynamics of doxycycline

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Doxycycline is a long-acting/second generation tetracycline antibiotic, and currently is one of the most commonly prescribed antibiotics in the world, used to treat a wide variety of infectious agents including susceptible intracellular/zoonotic pathogens [1–6].

Because doxycycline was introduced prior to an appreciation of pharmacodynamic concepts, the optimal dosing regimen has not been determined. This in vitro study was conducted to determine the optimal dosing regimen for doxycycline based upon pharmacodynamic data.

Doxycycline was tested against selected Gram-positive pathogens, e.g. *Staphylococcus aureus* and *Streptococcus pneumoniae* and Gram-negative pathogens, e.g. *P. multocida* and *E. coli*. Time-kill studies were performed with each of these organisms at various serum concentrations representing two, four, eight, and 16 times the MIC of each test organism. The growth of the organisms was assessed by colony counts at various time points during a 24 h period to determine if doxycycline kills susceptible organisms by concentration or time-dependent kinetics. Studies were also carried to determine the PAE of doxycycline.