ELSEVIER

Antiviral Research 39 (1998) 47–53

Antiviral Research

The sequential occurrence of *pol* 215 and *pol* 41 zidovudine resistance mutations is associated in an additive fashion with low CD4 cell counts and high plasma and cellular HIV viral load

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Received 6 November 1997; accepted 17 April 1998

Abstract

We report on a cross-sectional study of virological and immunological surrogate markers of HIV infection in 115 patients for whom a determination of the *pol* 215 and *pol* 41 zidovudine (ZDV) resistance mutations had been described between January 1995 and February 1996. The patients received ZDV alone or a combination of ZDV and zalcitabine or didanosine. A total of 55, 15 and 45 patients exhibited a wild (W), a mixed (MIX) or a mutant (M) genotype at codon *pol* 215, respectively; 85, 10 and 20 patients exhibited a W, a MIX or a M genotype at codon *pol* 41, respectively. Patients exhibiting the *pol* 215 M genotype had lower CD4 cells, higher plasma viral load and higher proviral burden than patients exhibiting the *pol* 215 W genotype. Patients who had variants exhibiting both *pol* 215 M and *pol* 41 M or MIX genotypes had significantly worsened surrogate marker values than patients having variants only carrying the *pol* 215 M genotype. These observations demonstrate that the two mutations additively associate with pejorative surrogate markers.

Keywords: HIV; Zidovudine; Genotype

1. Introduction

Zidovudine (ZDV) has extensively been used as an antiretroviral agent in HIV disease for the last 10 years. Phenotypic resistance to ZDV occurs

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0166-3542/98/$19.00
PII S0166-3542(98)00026-6

within 9 months of ZDV monotherapy in almost all of the patients (Larder and Kemp, 1989). The resistance is associated with the sequential selection of mutations at codons 70, 215, 41, 67 and 219 in the reverse transcriptase *pol* gene (Boucher et al., 1992). Phenotypic resistance in vitro is directly related to the number of selected ZDV resistance mutations (Kellam et al., 1992). The association of ZDV resistance with a poor clinical outcome of patients receiving ZDV monotherapy has been documented in the retrospective virological analysis of specimens of patients included in ACTG 116B-117 (D'Aquila et al., 1995; Japour et al., 1995).

In the present study, we investigated immunological and virological surrogate markers of HIV infection in an unselected group of patients treated with ZDV alone or in combination with another nucleoside analogue, according to the presence or absence of ZDV resistance mutations at codons *pol* 215 and *pol* 41. The data indicate that both mutations are associated in an additive fashion with low CD4 cell counts and increased plasma and cellular viral load.

2. Patients and methods

2.1. Patients

All HIV type 1 seropositive patients for whom at least one determination of ZDV resistance mutation has been prescribed by the physician in charge at Hôpital Broussais, Paris, between January 1995 and February 1996, were included in this cross-sectionnal study. All patients had been treated for over 1 year with ZVD, alone or in combination with either dideoxyinosine (ddI) or zalcitabine (ddC); none of the patients had received a protease inhibitor.

2.2. Samples

Plasma was separated from EDTA-anticoagulated blood and stored in aliquots at -80°C until use. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of blood on Ficoll Hypaque and kept frozen at -80°C until processing.

2.3. Virological studies

Plasma HIV-1 RNA was quantitated using the bDNA signal amplification assay (Quantiplex HIV-RNA 1.0, Chiron, Emeryville, CA). Values below the threshold of detection of 10^4 copies/ml were arbitrarily recorded as 5000 copies/ml for the purpose of statistical analysis.

Analysis of the *pol* 215 and *pol* 41 codons in proviral DNA was performed by primer mismatch selective PCR on 1.0 μg of DNA extracted from cryopreserved PBMC (2×10^6), as described (Larder and Boucher, 1993), with slight modifications. The first round of PCR used the primers $A_{(35)}$ and NE-$1_{(35)}$. In order to analyze the codon *pol* 215, 5 μl of the first PCR product were subjected to serial 10-fold dilutions in distilled water (up to 10^{-9}), and further subjected in parallel to two amplifications, using the primer set B and 3 W, specific for the wild type codon (215 W), and the primer set B and 3 M specific for the mutant Thr_{215}Phe and Thr_{215}Tyr condons (215 M). The 3 M primer hybridizes strictly with the three bases of the TTC (Phe) codon and partially with two bases of the TAC (Tyr) codon. The cross-hybridization is sufficient to recognize the Tyr (TAC) pol 215 mutant in addition to the Phe (TTC) mutation (Larder and Boucher, 1993; Shafer et al., 1996). To analyze the *pol* 41 codon, the same procedure was followed, except that the diluted products of the first PCR product were subjected to three amplifications: one used the primer set BR and 5 WR specific for the wild type codon (41 W); one used the primer set (BR and 5 MR) specific for the mutant Met_{41}Leu/TTG codon (41 MR); the third used the primer set BR and 5 MCR specific for the mutant Met_{41}Leu/CTG codon (41 MCR). All primers were used in the same molar amounts (25 pM). The amplified products were separated on a 2.0% agarose gel electophoresis, and the appropriately sized products (210 bp for *pol* 215, and 370 bp for *pol* 41) were visualized under UV illumination following staining with ethidium bromide. Gels were read by comparison with patterns obtained with mutated strains used as positive controls, kindly provided by F. Brun-Vézinet, Paris, and by H. Fleury, Bordeaux. The predominantly wild (W) or

mutant (M) genotype was determined by comparing the intensity of the PCR bands at each dilution tested. When the intensities of the W and M bands were similar at all dilutions tested, the genotype was considered as being mixed (MIX).

HIV proviral load was calculated from the last dilution of the first PCR product that gave a signal after having been subjected to a second round of amplification for the analysis of the *pol* 215 codon. The results were expressed as log of the last positive dilution obtained from 1.0 μg of DNA (corresponding to approximately 10^5 PBMC). The interassay variation of the assay is of 2.9%.

The presence of p24 antigen and of anti-p24 IgG antibodies in plasma was determined by means of ELISA (VIDAS, BioMerieux, Marcy l'Etoile). Positive results for p24 antigen were confirmed by a neutralization assay.

2.4. CD4 cell counts

Enumeration of CD4 T cells was performed by flow cytometry using the FACScan® cytometer and the Cellquest® software (Becton Dickinson, Montain View, CA).

2.5. Statistical analysis

Results are expressed as mean $\pm$ S.E.M. Comparison of the viral load and CD4 cell counts between groups of patients was achieved by analyzing the data by means of the non-parametric Kruskal-Wallis ANOVA test. Comparison between two groups was further performed using the Mann-Whitney test. The χ^2 test and the Fisher exact test were used to compare the prevalence of positive samples for p24 antigen and anti-p24 antibodies in the study groups.

3. Results

3.1. Patients

The study included 115 patients, 92 males and 23 females of mean age 37 years (range, 28–40). A total of 39 of the individuals were homosexual/bisexual men; 30 patients were intravenous drug users; 14 were infected heterosexually. The mode of contamination was unknown in 32 patients. A total of 44 patients fulfilled the criteria of category A according to the 1993 revised CDC classification; 42 belonged to category B, and 29 to category C. At the time of blood sampling, 83 of the patients were receiving monotherapy with ZDV; 17 were receiving ZDV in association with ddI; 15 were receiving ZDV in association with ddC.

3.2. Prevalence of ZDV resistance mutations at codons pol 215 and pol 41

Fifty five (47.8%) patients exhibited a predominant *pol* 215 W genotype, 15 (13%) exhibited a MIX genotype, and 45 (39.2%) exhibited a predominant M genotype. For the *pol* 41 codon, 85 patients (73.9%) had a 41 predominant W genotype, 10 (8.7%) exhibited a MIX genotype and 20 (17.4%) had a predominantly *pol* 41 M genotype. Among the patients with the *pol* 41 genotype, 12 exhibited the *pol* 41 MR genotype and eight exhibited the *pol* 41 MCR genotype. A total of 18 of the 20 patients with the *pol* 41 M genotype exhibited a predominant *pol* 215 M genotype; two of the patients exhibited a *pol* 215 MIX genotype. Nine of the 10 patients with a *pol* 41 MIX genotype exhibited a predominant *pol* 215 M genotype; one patient exhibited a *pol* 215 W genotype.

3.3. Plasma and cellular HIV viral load

We first compared the mean plasma levels of HIV RNA of patients classified into three groups, according to the *pol* 215 genotype. The ANOVA analysis indicated that the mean plasma viral load differed significantly between patients with the *pol* 215 W (48795 ± 11625 eq-copies/ml), *pol* 215 MIX (158740 ± 10650 eq-copies/ml) and *pol* 215 M (232265 ± 10440 eq-copies/ml) genotypes ($P < 0.003$) (Fig. 1). The mean plasma viral load was 4.7-fold lower in patients with the predominant *pol* 215 W genotype as compared with patients with the *pol* 215 M genotype ($P < 0.001$), irrespective of the presence or absence of a resistance mutation at codon *pol* 41. The mean plasma viral

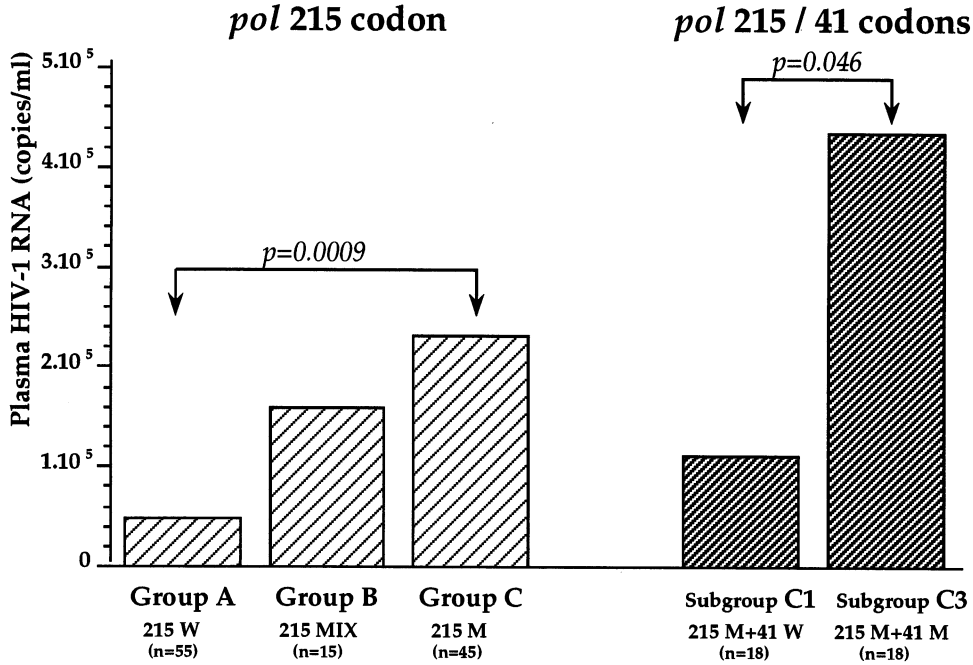

Fig. 1. Plasma levels of HIV-1 RNA, according to *pol* 215 and *pol* 41 genotypes. Left: mean plasma viral load of patients harboring *pol* 215 W, *pol* 215 MIX and *pol* 215 M variants. Right: mean plasma viral load of patients harboring *pol* 215 M and *pol* 41 W, and *pol* 215 M and *pol* 41 M genotypes.

load of patients with a *pol* 215 MIX genotype did not differ significantly from that of patients with *pol* 215 M or *pol* 215 W genotypes. In patients exhibiting a *pol* 215 M genotype, the mean plasma viral load was 3.9-fold higher in patients exhibiting a *pol* 41 M genotype (433900 ± 25520 eq-copies/ml) than in patients with a *pol* 41 W genotype (110890 ± 35120 eq-copies/ml) ($P < 0.05$). We have also analyzed the relationship between the presence of a mutation at codon *pol* 41, CD4 cell counts and plasma HIV RNA, independently of the pol 215 genotype. The mean plasma viral load in patients harboring *pol* 41 MIX or M variants was significantly higher (284469 ± 155293 eq-copies/ml) than that of patients harboring *pol* 41 W variants (97312 ± 27399 eq-copies/ml) ($P = 0.005$).

Similarly, the mean cellular proviral load differed significantly between patients with the *pol* 215 W, *pol* 215 MIX and *pol* 215 M genotypes ($P < 0.01$ by ANOVA). The mean proviral load in patients harboring *pol* 215 M variants ($7.43 \pm 0.24/10^5$ PBMC) was significantly higher than that of patients harboring *pol* 215 W variants ($6.60 \pm 0.19/10^5$ PBMC) ($P < 0.05$). Patients with both *pol* 215 M and *pol* 41 M genotypes exhibited a higher mean proviral load ($8.30 \pm 0.45/10^5$ PBMC) than patients with, the *pol* 215 M or *pol* 41 W genotype ($7.21 \pm 0.50/10^5$ PBMC) ($P < 0.01$).

3.4. p24 antigenemia and circulating antibodies to p24

The prevalence of positive p24 antigenemia was higher in patients with *pol* 215 M variants than in patients harboring *pol* 215 W variants ($P < 0.04$). The prevalence of detectable circulating anti-p24 antibodies was higher in the latter group of patients as compared with that of patients with *pol* 215 M variants ($P < 0.02$).

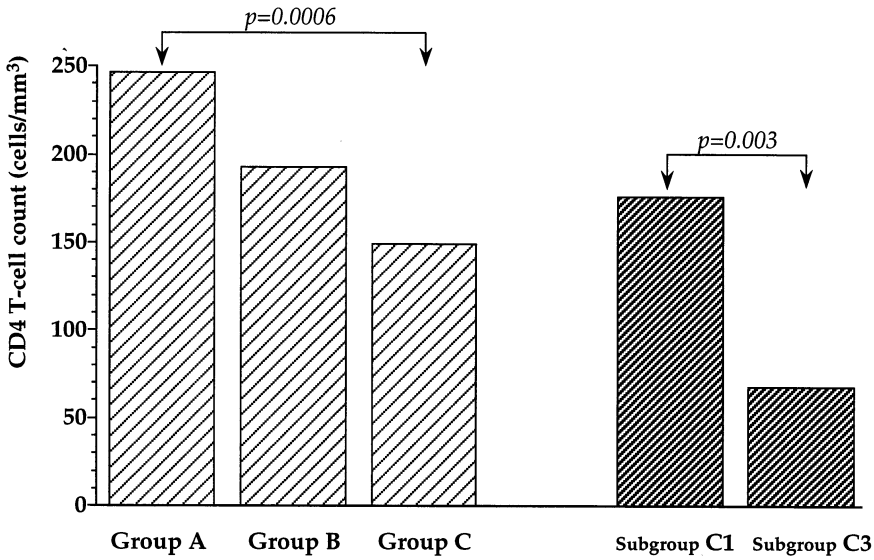

Fig. 2. CD4 cell counts, according to *pol* 215 and *pol* 41 genotypes. Left: mean peripheral CD4 cell counts of patients harboring *pol* 215 W, *pol* 215 MIX and *pol* 215 M variants. Right: mean peripheral CD4 cell counts of patients harboring *pol* 215 M and *pol* 41 W, and *pol* 215 M and *pol* 41 M genotypes.

3.5. CD4 T-cell counts

The mean level of CD4 T-cell counts differed between patients with the *pol* 215 W, *pol* 215 MIX and *pol* 215 M genotypes ($P < 0.003$). It was lower in patients with *pol* 215 M variants ($149 \pm 23/mm^3$) than in patients with *pol* 215 W variants ($246 \pm 22/mm^3$) ($P < 0.001$) (Fig. 2). Patients exhibiting both *pol* 215 M and *pol* 41 M genotypes had a 3-fold lower mean CD4 T-cell count than patients with *pol* 215 M and *pol* 41 W genotypes ($P < 0.005$). When analyzing the data independently of the *pol* 215 genotype the mean CD4 T-cell count in patients harboring *pol* 41 MIX or M variants ($127 \pm 31/mm^3$ was found to be lower than that of patients harboring *pol* 41 W variants ($221 \pm 21/mm^3$ ($P < 0.001$).

3.6. ZDV resistance genotype according to treatment duration

Patients exhibiting a predominant *pol* 215 M viral genotype had been receiving ZDV for a longer period of time (25.9 ± 2.5 months) than patients exhibiting *pol* 215 W variants (17.3 ± 2.3 months) ($P < 0.02$). Patients exhibiting both *pol* 215 M and *pol* 41 M genotypes had received longer treatment with ZDV (27.7 ± 4.9 months) than patients exhibiting a *pol* 215 M or *pol* 41 W genotype (22.8 ± 4.6 months), although the difference did not reach statistical significance. The relative proportion of patients receiving ZDV monotherapy as compared with combined ZDV therapy with another nucleoside analogue did not differ between groups of patients according to *pol* 215 and *pol* 41 genotypes. Thus, 64, 60 and 78% of patients with *pol* 215 W, MIX and M genotypes, respectively, were under ZDV monotherapy. Among patients with *pol* 215 M variants, 67, 78 and 83% of the patients with *pol* 41 W, MIX and M genotypes, respectively, were receiving ZDV as monotherapy.

4. Discussion

The present cross-sectional study demonstrates that the sequential selection of mutations at codons 215 and 41 of the HIV-1 *pol* gene is associated with decreasing CD4 cell counts and increasing plasma and cellular viral load, in an additive fashion. Although ZDV is not recom-

mended anymore for use as monotherapy, the data appear relevant in that the prevalence of selected variants harboring the *pol* 215 or *pol* 41 ZDV resistance mutations in the study population, was similar in patients receiving ZDV monotherapy and in those receiving ZDV in combination with another nucleoside analogue.

Patients having received ZDV as monotherapy or as combination therapy with nucleoside analogues for more than 1 year, are at high risk of selecting variants with the ZDV resistance mutations at codon *pol* 215 or at codons *pol* 215 and *pol* 41 (Larder and Kemp, 1989; Richman, 1993; Richman et al., 1994; Shafer et al., 1994; Brun-Vézinet et al., 1997; Rey et al., 1998). In our study population, the selection of ZDV resistant variants occurred at a similar frequency in patients under monotherapy as in those taking a combination of ZDV with ddI or ddC, suggesting that these two reverse transcriptase inhibitors do not protect against the selection of ZDV resistant variants. The selection of ZDV resistance was directly related to the duration of ZDV intake, as previously reported (Richman, 1993; Brun-Vézinet et al., 1997). Except for one patient, the *pol* 41 mutation was associated with the *pol* 215 mutation, further documenting that these resistance mutations arise sequentially (Boucher et al., 1992; Richman, 1993; Brun-Vézinet et al., 1997).

Our data indicate that the selection of ZDV resistance mutations at codons *pol* 215 and *pol* 41 is associated with increased plasma and cellular viral load and with decreased CD4 cell counts, which were even more pronounced when the two mutations were present. The selection of ZDV-resistant HIV variants in our patients is likely to have derived from a high rate of mutations occurring when the viral load is elevated, as a function of replicative events in the population of wild type viral strains (Nowak, 1995). Viral strains with ZDV resistance mutations may have been sequentially selected at high level to become predominant viral populations, because of a selective advantage for replication in the presence of the drug. Thus, patients whose viruses carried the *pol* 215 and/or the *pol* 41 ZDV resistance mutations, exhibited high plasma and cellular viral loads, indicating that mutant viruses replicate at high level in vivo. These observations are consistent with previous reports demonstrating that viruses exhibiting the *pol* 215 mutation replicate at a similar rate to wild-type variants from which they originated, in vitro (Gingeras et al., 1991; Larder et al., 1995). In addition, the selective replicative disadvantage of a virus carrying a single ZDV resistance mutation at codon *pol* 215 was shown to be very low in vivo, in the absence of continuing ZDV-related pressure (Kozal et al., 1993; Goudsmit et al., 1996). Taken together, our observations may suggest that the mutations at codon *pol* 215 alone and at both *pol* 215 and *po1* 41 codons do not provide a selective disadvantage for HIV replication in vivo, at least in patients still receiving zidovudine.

In the present cross-sectional study, the *pol* 215 and *pol* 41 ZDV resistance mutations were associated with the deterioration in all surrogate markers that we tested, including plasma and cellular viral load, peripheral blood CD4 T-cell counts, p24 antigenemia and the absence of circulating anti-p24 antibodies. The previously published virological results of ACTG 116B/117 indicated that isolates showing high-levels of ZDV phenotypic resistance (50% inhibitory concentration $\geq$ 1.0 μM) were associated with a 1.74-fold increase in the risk of progression to an AIDS-defining event or death, independently of the treatment arm and of any other known risk factor (D'Aquila et al., 1995). In addition, the presence of ZDV resistance mutations at both the codons *pol* 215 and *pol* 41 also conferred an increased risk of progression (relative hazard, 1.82) and death (relative hazard, 5.42) in ACTG 116B/117 (Japour et al., 1995). A strong association between the *pol* 215 mutation and a decline in CD4 cell counts has been reported in patients receiving ZDV monotherapy (Kozal et al., 1993). In a recent study, the mutation at codon *pol* 215 has been shown to be an independent predictor of progression in ZDV-experienced asymptomatic subjects (Merigan et al., 1996). Finally, the data of the ACTG 175 study indicated that immunologic and virologic failure of ZDV monotherapy occurred after the selection of viruses bearing the *pol* 215 ZDV resistance mutation (Rey et al., 1998). Taken together, these reports and our observa-

tions demonstrate that high-level resistance to ZDV is associated with lack of therapeutic efficacy and therefore disease progression.

Acknowledgements

We are grateful to Drs C. Beroud and P. Castiel for their help in gathering the patients' data. Grant support: Institut National de la Sante et de la Recherche Medicale (INSEAM), Agence Nationale de Recherches sur le SIDA (ANRS) and SIDACTION, France.

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