IDENTIFICATION OF AN AMINO ACID SUBSTITUTION INVOLVED IN THE REDUCTION OF SENSITIVITY OF HIV-1 TO AN INHIBITOR OF VIRAL PROTEINASE

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Summary. – Clones derived from HIV variants previously characterized as resistant to Ro31-8959, an inhibitor of viral proteinase (PR), were sequenced. Substitution of glycine by valine at position 48 of the PR protein was found. None of the 20 clones derived from wild type HTLV-IIIB contain this mutation. Since such a position is located in a conserved region of PR, it is possible that the substitution can affect the interaction of the enzyme with the inhibitor.

Key words: HIV-1; Ro31-8959; drug resistance; viral proteinase

The identification of chemotherapeutic agents active in inhibiting the replication of HIV has resulted in improved survival of AIDS patients. Nevertheless, the use of these antiviral agents is thought to be limited by the *in vivo* appearance of HIV drug-resistant variants (Hirsch and D'Aquila, 1993; Richman, 1993). Indeed, HIV-1 strains resistant to most of the nucleoside and non-nucleoside inhibitors of reverse transcriptase have been isolated *in vivo* and *in vitro* (Hirsch and D'Aquila, 1993; Richman, 1993).

The identification of mutations responsible for acquisition of drug-resistance has allowed to set up methods e.g. selective polymerase chain reaction (PCR) aimed to rapidly identify the amino acid substitutions associated with the resistant phenotype (Boucher *et al.*, 1991; Richman *et al.*, 1991; Zhegxian *et al.*, 1992).

Recently it has been shown, by us and others (Dianzani *et al.*, 1993; Craig *et al.*, 1993), that it is also possible to obtain HIV-1 variants with reduced sensitivity to inhibitors of viral PR. These agents have been designed to block the action of viral proteinase which cleaves the gag-pol polyproteins in the course of synthesis of infectious virions (Martin, 1992).

Here, we report on the cloning and sequencing of Ro31-8959-resistant variants of HIV-1 generated *in vitro* and describe the mutation associated with this phenotype.

As previously described (Dianzani *et al.*, 1993), the selection of resistant variants has been achieved by growing HIV-1 (HTLV-IIIB strain prepared from supernatant of chronically infected H9-cells) in the presence of inhibitory concentrations of Ro31-8959, an inhibitor of viral PR (kindly provided by Roche Products Ltd. U.K.). C8166 cells, a $CD4^+$ lymphoblastoid cell line containing the HTLV-I genome and expressing only the *tax* gene (Clapham *et al.*, 1987), were used to propagate resistant variants of HIV-1 referred above as HIV-F6 with an ID_{50} (inhibitory dose at 50%) value of 50 nmol/l and HIV-G8 with ID_{50} value of 35 nmol/l. After infection of C8166 cells with HTLV-IIIB (wild type) and HIV-F6 and HIV-G8 (resistant strains) total cellular DNA was extracted and subjected to PCR aimed to amplify the coding region of PR sequence. The chosen forward and reverse primers (Genset), were based on sequences flanking the PR gene of strain HXB2. Specifically the forward primer contained the sequence 5'-GTCGACTAGTCAGAGCCAACAGCCCCACCAGAAGAGA-3' (including Spe I site) and reverse primer contained the sequence 5' CAGCTGCGGCCGCTCTTCTGTCAATGGCCATT GTTTAAC-3' (including Not I site). The PCR was performed using a thermal cycler (Perkin Elmer) programmed for 30 cycles. PCR conditions for each cycle included (a) denaturation at 94 °C for 40 secs; (b) annealing at 55 °C for 1 min and (c) extension at 72 °C for 1 min. PCR products were digested with Spe I and Not I (Boehringer

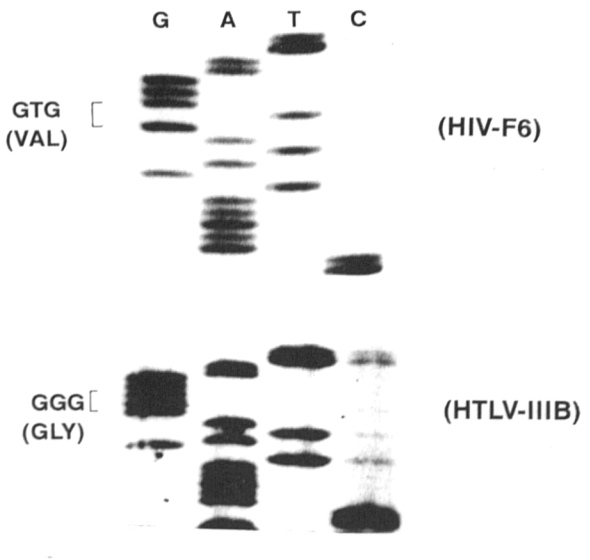

Fig. 1
Portions of polyacrylamide sequencing gels showing substitution of G in HTLV-IIIB by T in HIV-F6
GGG and GTG codons coding for gly and val, respectively.

Mannheim), purified from agarose gel and ligated into the pBluescript SK(+/-) plasmid (Stratagene). The ligation mixture was used to transform *E. coli* strain HB101. DNA obtained from the clones was used as substrate for DNA sequencing by the dideoxynucleotide chain termination procedure, using reagents provided in the Sequenase version 2.0 T7 DNA polymerase kit (USB).

The results of the sequence analysis of the PR open reading frame of HIV-1-resistant genomes show that most of the clones derived from HIV-F6 (18 out 20) and HIV-G8 (19 out 20) contain a mutation when compared with parental drug sensitive HTLV-IIIB (20 out 20). Fig. 1 shows a portion of the polyacrylamide sequencing gel including the observed mutation. This mutation (G to T) is located at the codon for amino acid at position 48 of the protein and leads to substitution of gly by val. This mutation plus an additional one at position 90 leu to met has been reported previously to occur in another Ro31-8959-resistant strain (Jacobsen *et al.*, 1993).

The existence of only two clones of HIV-F6 and one clone of HIV-G8 containing nonmutated nucleotide sequence indirectly indicates that the aforementioned strains do not represent absolutely homogeneous population. The variability in the clones could reflect the fact that (a) these strains have not been molecularly cloned (Dianzani *et al.*, 1993) or (b) the parental HTLV-IIIB does not itself represent a homogeneous population, although all HTLV-IIIB derived clones, tested before propagation in the presence of the drug, displayed the same degree of sensitivity to Ro31-8959 (data not shown).

Thus, from these results one cannot draw definite conclusions. Just the following consideration can be made: (a) site-directed mutagenesis study should be performed to directly and definitely correlate the presence of the described mutation to the drug-resistant phenotype; (b) a possibility cannot be ruled out that multiple mutations are involved in such a phenomenon e.g. in virus having a higher or lower degree of resistance; (c) finally, these results must be complemented by studies *in vivo* as soon as viral isolates from Ro31-8959-treated patients become available.

Similarly to the problem of azidothymidine resistance (Boucher *et al.*, 1991; Richman *et al.*, 1991; Zhegxian *et al.*, 1992), this study can tentatively offer the opportunity to set up methods to detect Ro31-8959-resistant virus *in vivo*.

We believe that addressing the problem of drug-resistance with a new drug is particularly important since these studies can provide information useful to overcome or prevent the selection of resistant strains *in vivo*.

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