Differential down-regulation of CD95 or CD95L in chronically HIV-infected cells of monocytic or lymphocytic origin: cellular studies and molecular analysis by quantitative competitive RT-PCR

Marcello Pinti[a], Jessica Pedrazzi[a], Francesca Benatti[a], Veronica Sorrentino[b], Cira Nuzzo[a], Valeria Cavazzuti[b], Priscilla Biswas[c], Daniela Nicoleta Petrusca[d], Cristina Mussini[a], Bruno De Rienzo[a], Andrea Cossarizza[b,*]

[a] *Department of Internal Medicine, Infectious Disease Clinics, University of Modena and Reggio Emilia School of Medicine, via del Pozzo 71, 41100 Modena, Italy*
[b] *Chair of Immunology, Department of Biomedical Sciences, Section of General Pathology, University of Modena and Reggio Emilia School of Medicine, via Campi 287, 41100 Modena, Italy*
[c] *Laboratory of Clinical Immunology, CSL, Istituto Scientifico San Raffaele, Via Stamira d'Ancona 20, 20127 Milan, Italy*
[d] *Center of Immunology, Department of Clinical Immunology, University of Bucharest, Sos. Mihai Bravu 285, 79650 Bucharest, Romania*

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Abstract **We analysed the expression of CD95/CD95L in two widely used models for studying the cellular effects of chronic infection with human immunodeficiency virus type 1 (HIV-1), i.e. ACH-2 cells, derived from the lymphocytic cell line A301, and U1, derived from monocytic U937 cells. A301 and ACH-2 mounted the same amount of plasma membrane CD95, while U1 had a consistent decrease in CD95 expression. Using different antibodies, we failed to detect the plasma membrane form of its ligand, CD95L, but we could see the intracellular presence of that molecule in A301 cells and, to a lesser extent, in ACH-2 cells, but not in U937 or U1 cells. To confirm the cytofluorimetric data and quantify the expression of CD95L at the RNA level, we developed a quantitative competitive RT-PCR assay. The HUT78 cell line had about 50 000 copies mRNA/1000 cells, three times more after induction with a phorbol ester and ionomycin. ACH-2 expressed about 400- (basal) or 10- (induced) fold less CD95L mRNA than the parental cell line A301; U937 and U1 were below the limit of detection. In cells of lymphoid origin (ACH-2) chronic HIV infection inhibits the expression of CD95L, the phenomenon occurring at the transcriptional level. In cells of monocytic origin (U1) the infection decreases the plasma membrane expression of CD95. This suggests that HIV could trigger different anti-apoptotic strategies which likely depend upon the cell line which is infected. In monocytic cells which act as a viral reservoir, the expression of the molecule whose binding triggers apoptosis decreases, while in lymphoid cells, capable of exerting cytotoxicity, the expression of a molecule which induces apoptosis is reduced.**

Key words: HIV; AIDS; CD95L; FasL; Apoptosis; Quantitative competitive RT-PCR

1. Introduction

Infection with human immunodeficiency virus type 1 (HIV-1) causes a loss of T helper lymphocytes, which is crucial to the development of the immunodeficiency. The mechanism(s) by which HIV-1 provokes death of CD4+ T cells has been under investigation for many years. A fundamental issue is whether HIV-1 primarily induces direct killing of infected cells or indirectly causes death of uninfected bystander cells. Apoptosis of T cells plays an important role in the pathogenesis of HIV infection [1–4], and correlates with the progression of disease, as shown by many investigators who studied different groups of patients, ranging from the earliest moments of primary infection to the so-called long-term non-progressors [5–8].

Among the large variety of molecules involved in this process, members of the tumour necrosis factor (TNF) family such as CD95 (APO-1/Fas) and its ligand (CD95L) have been extensively studied because of their capacity to trigger apoptosis [9–11]. However, the role of CD95/CD95L interactions in determining the increased tendency to undergo apoptosis in cells from HIV+ subjects is far from being clear. In fact, although it is known that up-regulation of CD95 expression during HIV infection enhances the lymphocytes' propensity to cell death, different and contrasting reports exist on the expression of CD95L in immune cells (reviewed in [12]). Many reasons, mostly due to technical problems, may account for the discrepancies regarding CD95L expression in different cell types or in cells from HIV+ patients. Over the past years, different groups have used anti-CD95L polyclonal rabbit antibodies [13–15], which only recently have been shown to be unable to detect cell surface CD95L when analysed by flow cytometry [16]. Similar problems arise with different monoclonal antibodies (mAbs) directed to the plasma membrane form of CD95L.

On the other hand, molecular biology techniques based upon polymerase chain reaction (PCR) have been employed in studies on cells from HIV+ patients, demonstrating a decrease in CD95L expression at the mRNA level [17]. However, the limitation of these techniques is that they are only semiquantitative, and do not take into consideration the intrinsic variability of the type of analysis. In order to contribute to a better analysis of CD95L, we developed a sensitive system to quantify the amount of CD95L mRNA using a PCR-based competitive approach, comparing it with a classical, cytofluorimetric approach performed using different commercially available anti-CD95L mAbs.

*Corresponding author. Fax: (39) (59) 428 623.
E-mail: cossariz@unimo.it

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2. Materials and methods

2.1. Cell lines

The following cell lines of human origin were studied: A301, ACH-2, U937, U1 and HUT78. The human ACH-2 and U1 chronically HIV-infected cells derived from limiting dilution of cells surviving acute in vitro infection with HIV-1 (HTLV-III/LAV) of the T cell line A301 and the promonocytic cell line U937, respectively [18,19]. HUT78 cells derived from a human cutaneous T cell lymphoma which expresses high levels of plasma membrane CD95L and is largely used in studies on cytotoxicity via CD95 [20,21]). Cells were collected during the log phase of growth, when viability was >95%, and immediately used for the cytofluorimetric analyses described below. In order to increase CD95L expression, 1.5×10^6 cells were then resuspended in 1 ml of RPMI supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin and 10 μg/ml streptomycin and cultured in the presence or in the absence of 3 ng/ml phorbol 12-myristate 13-acetate (TPA) and 2 μM ionomycin (Ion) for different periods (up to 24 h). Each experiment was repeated a minimum of four times, with only negligible variations.

2.2. Flow cytometry

2.2.1. Phenotype analysis and quantitation of CD95 and CD95L expression. Flow cytometric analysis was performed with fluorochrome-labelled mAbs, according to standard methods [22]. Living cells were identified on the basis of their physical characteristics (forward and side scatter, i.e. FSC and SSC, respectively), and their percentage, as assessed in parallel samples, was always >95%. We used anti-CD95 mAbs from Pharmingen (San Diego, CA, USA; clone DX2, mouse IgG1,κ directly conjugated with phycoerythrin, PE); for the detection of CD95L, we used two different mAbs: clone Alf-2.1 (Caltag Lab., Burlingame, CA, USA, mouse IgG2a,κ directly conjugated with PE) and clone NOK-1 (Pharmingen, mouse IgG1,κ whose binding was revealed by FITC-conjugated goat anti-mouse Ig, from Becton Dickinson, San Jose, CA, USA). The anti-CD95L rabbit polyclonal antibody C-20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was also tested. Control samples were treated with control rabbit IgG, and showed an unspecific staining <0.2%. All samples were treated with C-20 or control rabbit serum (from Pigei, Cavezzo, Italy) for 45 min at 4°C, followed by PE-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) as the secondary reagent (40 min at 4°C). For the analysis of CD95 and CD95L on the cell surface, cells were incubated for 30 min at 4°C with adequate amounts of mAbs, washed twice and analysed. Intracellular staining of CD95L was performed after fixation in 2% paraformaldehyde and permeabilisation of cells with 0.5% Triton-X, according to standard methods [23]. A FACScan (Becton Dickinson) flow cytometer was used, as described [24].

For the quantitation of CD95 and CD95L expression, samples were immediately analysed after staining, and data acquired using a logarithmic amplification of the flow cytometer photomultiplier devoted to collect the fluorescence from PE or FITC. Then, data were transformed into a linear scale, as described elsewhere [25]. As CD95 or CD95L was supposed to be expressed by almost all cells, to calculate the changes in antigen expression, we took the linearised median value from each sample and compared it with its adequate negative control (i.e. the signal from the same cells stained with a PE- or FITC-labelled irrelevant mAb of the same isotype). The difference between these two values, i.e. the relative fluorescence intensity (expressed in Fig. 1 by the Greek letter Δ) indicates the number of linear channels representing the net fluorescence due to the binding of the mAb to its antigen. Autofluorescence from the cell populations we analysed was always negligible, and of the same order (not shown).

2.3. RNA extraction and purification

Total RNA was purified from different cell lines with High Pure RNA purification kit (Boehringer, Mannheim, Germany) according to the procedure recommended by the supplier. RNA was resuspended in 100 μl of elution buffer. The total amount of RNA extracted from HIV+ and HIV− cells was always of the same order, i.e. 10–20 μg/10^6 cells.

2.4. Construction of an RNA competitor for FasL

An RNA competitor for FasL mRNA was constructed carrying out an internal deletion in a 259 bp region of DNA encoding the human FasL gene. In brief, a 259 bp region, spanning the first exon, was amplified with primers 60D (5′-GGGAATTCTGAGAAGAAG-TAAAACCGTTTGCTG-3′) and 276R (5′-GGGGATCCGGTGG-CAGCGGTAGTGGAG-3′), which bring a tail with *Eco*RI and *Bam*HI restriction sites, respectively. This fragment was then cloned in pGEM-3Z; the recombinant plasmid obtained, named pFM3259, was digested with *Apa*I and *Bgl*II, blunt-ended with Klenow fragment and self-ligated; a deletion of 33 bp was obtained. One microgram of this plasmid, named pFasL, was purified from transformed *Escherichia coli* DH5α cells and digested with *Xba*I. Linearised pFasL was in vitro transcribed using 40 U of T7 polymerase, in the presence of rNTPs 1 mM, 40 mM Tris-HCl, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine and 0.5% Tween 20. The reaction was stopped by heating at 70°C for 15 min. The integrity of RNA was verified on 2% denaturing agarose gel. DNA used for transcription was eliminated by digestion with 40 U Dnase-Rnase-free (Boehringer). A PCR without previous reverse transcription was performed on serial dilutions of transcribed RNA, to test for the presence of DNA contamination. A ratio of about 300 000:1 was found between RNA and DNA. DNA can thus be considered not capable of influencing the subsequent competitive RT-PCR. The concentration of purified RNA was estimated by spectrophotometer evaluation and confirmed by end-point dilution. The competitor was immediately divided into several aliquots and stored at −80°C.

2.5. Competitive RT-PCR

Before the reverse transcription reaction, 5 μl of RNA from cells (i.e. 500 ng of RNA), 5 μl of competitor at various concentrations (from a minimum of 1.75×10^3 to a maximum of 1.75×10^6 molecules per tube), and primer 276R (5 pmol) were heated at 70° for 5 min and immediately cooled on ice, to prevent formation of secondary structures. Then reverse transcription was performed at 42°C for 45 min, in the presence of dNTPs 400 μM, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 200 U MuMLV RT (Promega, Madison, WI, USA), 40 U RNasin (Promega), 276R primer 200 nM, 5 μl of RNA from cells and 5 μl of competitor RNA at various concentrations. The final volume of the reaction was 25 μl. After reverse transcription, MuMLV RT was inactivated by heating at 95°C for 5 min. The product of reverse transcription was used directly for PCR, after addition of 25 μl of 1×PCR buffer, containing 2.5 U of *Taq* polymerase (Promega), primers 60D and 276R 200 nM. Amplification was carried out for 50 cycles in a Perkin Elmer Thermal Cycler 9700. Each cycle consisted of 30 s denaturation at 94°C, 35 s annealing at 56°C and 45 s extension at 72°C. The first cycle was preceded by 5 min denaturation at 94°C and the last one was followed by 7 min extension at 72°C.

Ten μl of PCR product was loaded on 0.6% LE/2.4% NuSieve agarose gel (FMC) and run at 90 V for 40 min, stained with ethidium bromide (EtBr) and analysed with a GelDoc 2,000 (Bio-Rad) video-densitometer. The competitor band was observed as a lower 223 bp band with respect to the 259 bp band of the wild type. The relative intensity of the competitor band was corrected by a factor of 1.16, to compensate for minor EtBr incorporation. Multiple reactions were performed for the same sample, using increasing amounts of the competitor molecules. The ratio between the lower and the upper bands was linearly correlated with the input number of competitor molecules (r^2 always >0.9), and the number of copies of wild type RNA was calculated as described elsewhere [26].

A PCR without previous reverse transcription was always performed to test for DNA contamination, and gave negative results.

2.6. Statistical analysis

To analyse the differences in the fluorescence intensity of cells stained with anti-CD95 mAbs we used unpaired, two-tailed Student's *t*-test. The same test was used to analyse differences in the expression of CD95L mRNA between control and infected lines; the paired *t*-test was used to analyse the effects of the treatment with TPA/Ion.

3. Results and discussion

Interactions between CD95 and CD95L have a crucial importance in apoptotic phenomena, and, consequently, in several pathologies including the infection by the virus that causes AIDS [27]. In the past years, the expression and func-

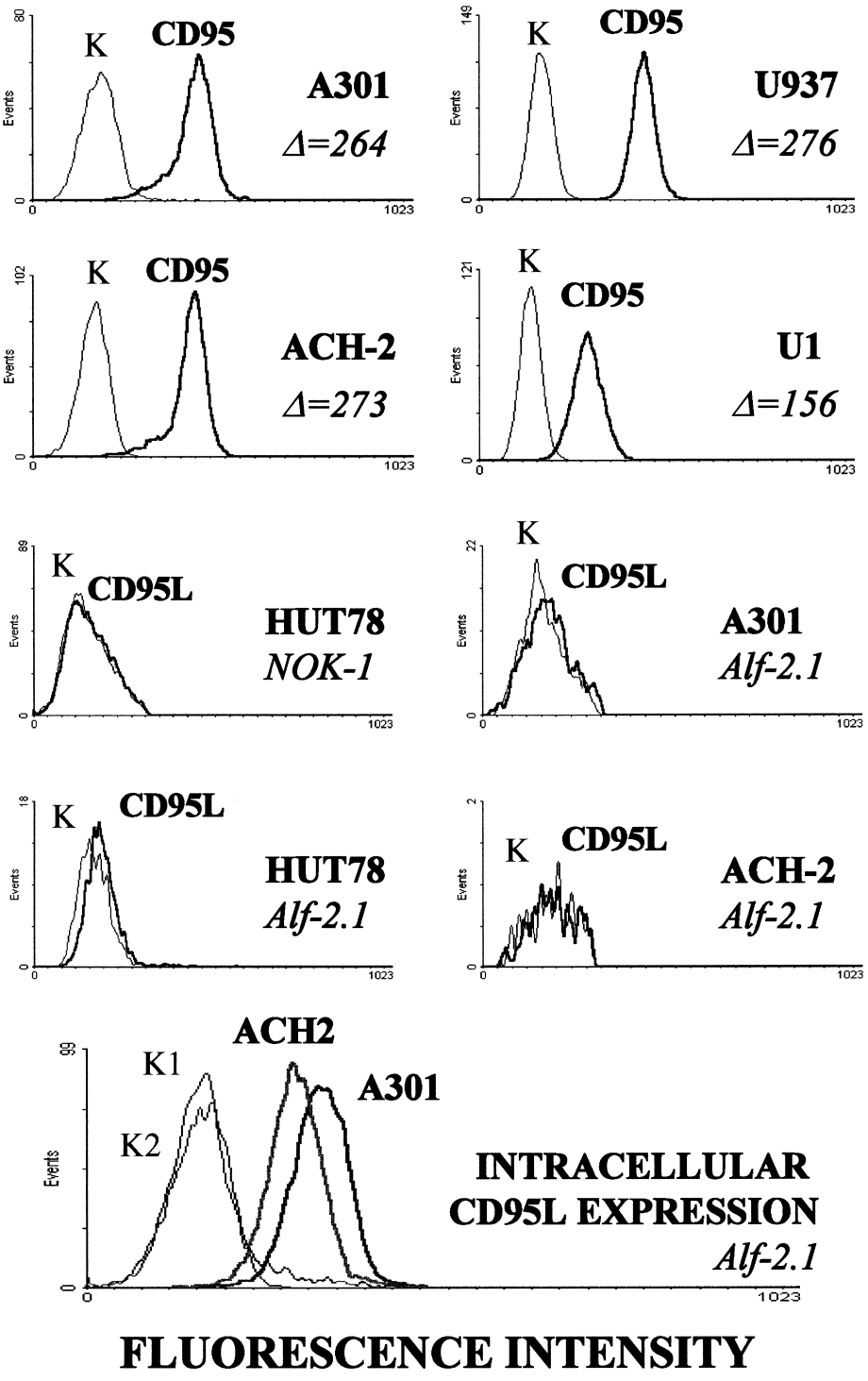

Fig. 1. Cytofluorimetric analysis of: (i) the plasma membrane expression of CD95 in parental (A301 and U937) and HIV-infected (ACH-2 and U1) cell lines. K indicates negative controls. The analysis of the relative fluorescence intensity (Δ, related to the difference in fluorescence channels between controls and the stained cells) shows that U1, but not ACH-2, cells have a consistent decrease in the expression of CD95; (ii) the plasma membrane expression of CD95L in different cell lines, including HUT78, after induction with TPA/Ion. Two mAbs, NOK-1 and Alf-2.1, were used. K indicates negative controls. In no case could we detect the plasma membrane form of CD95L; (iii) the intracellular presence of CD95L in A301 and ACH-2 cells. K1 and K2 indicate the relative negative controls. Note that A301 cells have a higher fluorescence intensity than ACH-2. One representative experiment out of four is shown.

tion of CD95 have been widely studied. It is well known that this molecule is expressed, and can increase its presence, in a variety of conditions, including cell activation, cell proliferation, different viral infection, among others. We have studied the plasma membrane expression of CD95 in HIV-infected cell lines of lymphocytic (ACH-2) and monocytic (U1) origin, and found that there is a consistent decrease of this molecule in U1 ($P < 0.01$) but not in ACH-2 cells (Fig. 1). Functional analyses indicate that, using a variety of stimuli, U1 have a lesser tendency to undergo apoptosis than the parental line

U937 (J. Pedrazzi et al., manuscript submitted). This is fully consistent with a previous report, which showed that the viability of U1 cells was not affected by treatment with anti-CD95 mAb, and they did not die of apoptosis [28]. Moreover, although these cells expressed CD95 on the surface, TNF-α was unable to up-regulate the expression of this molecule.

Interestingly, we could not extend this observation to ACH-2 vs A301 cells. In fact, the HIV-infected cell line of lymphocytic origin had the same amount of CD95 as the uninfected, parental line, but an increased tendency to undergo apoptosis after different stimuli (submitted). Thus, HIV-infected cells of different origin regulate, and likely use in a different manner, a molecule that has a crucial importance in the induction of apoptotic phenomena.

Less is clear concerning the role of the CD95/CD95L pathway in HIV infection, and especially about the quantitative expression of CD95L in different cells and models (reviewed in [12]). Reports exist in the literature claiming that the CD95/CD95L system is hyper-activated or hyper-expressed, and highly responsible for the induction of apoptosis in cells

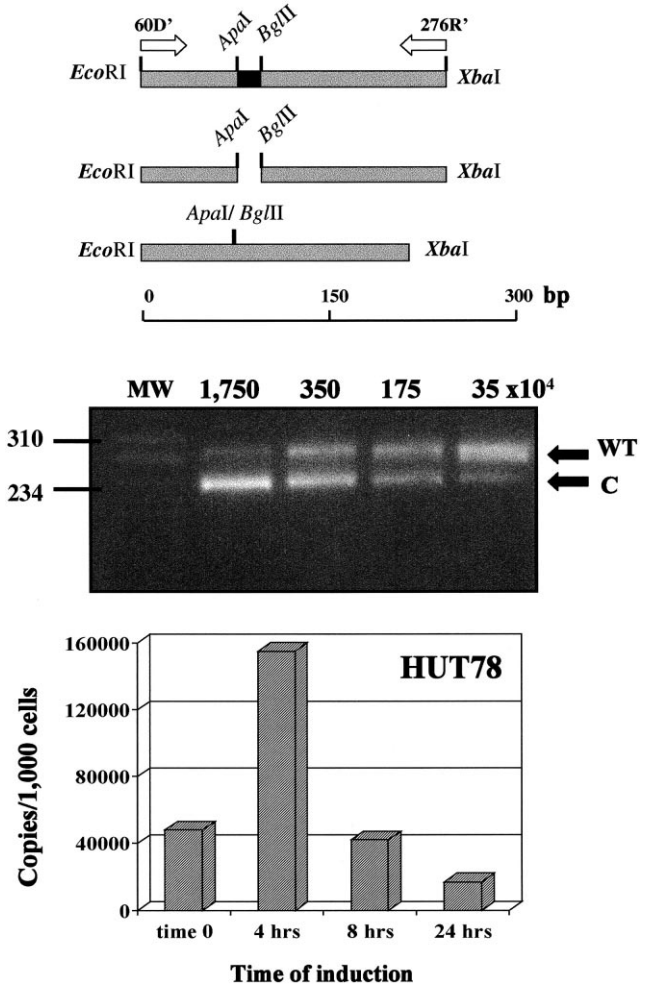

Fig. 2. Upper part: Strategy for the construction of a mRNA competitor for CD95L. See text for details. Middle part: Typical example of quantitative competitive RT-PCR with scalar amounts of competitor (C), i.e. $35–1750\times10^4$ copies/tube, and a fixed dose of wild type RNA (WT). MW: molecular weight marker (ϕX174 digested with *Hae*III); two values are indicated in the left part (310 and 234 Da). Lower part: Kinetics of the induction of CD95L mRNA in HUT78 cells stimulated with TPA/Ion.

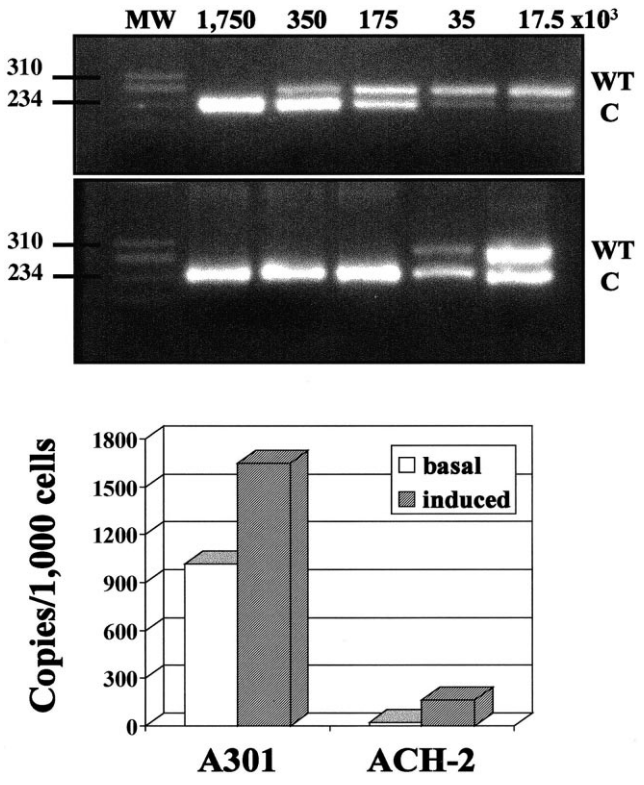

Fig. 3. Upper part: Representative examples of quantitative competitive RT-PCR in A301 (upper picture) and ACH-2 cells (lower picture). Numbers indicate the scalar amounts of competitor (C), i.e. $17.5–1750\times10^3$ copies/tube, co-amplified with the wild type RNA (WT). Molecular weight marker as in Fig. 2. Lower panel: The graphics show that HIV-infected ACH-2 cells have a decreased expression of CD95L in comparison with the parental line A301 either in basal conditions or after stimulation with TPA/Ion.

from HIV patients or in HIV-infected cell lines [15,27,29–34]. Other reports go in the opposite direction, casting some doubts on the role of that pathway, and in particular on the hyper-expression of CD95L in HIV-infected cells [35,36]. Many discrepancies concerning the expression of CD95L could be explained by the existence of problems related to the antibodies used. Indeed, not only polyclonal [16] but also mAbs exist that have a low capacity to detect the surface expression of CD95L. In fact, we searched for the presence of plasma membrane CD95L in a cell line which is known to produce high amounts of CD95L and efficiently kills CD95+ targets (i.e. HUT78) [21], as well as in HIV-infected cells (ACH-2 and U1) and in their parental lines (A301 and U937), but we failed to detect it using either two different mAbs (NOK-1 or Alf-2.1, see Fig. 1) or polyclonal antibodies (not shown). Recently, it was shown that, before degranulation, CD95L can be stored in specialised secretory lysosomes in both CD4+ and CD8+ T lymphocytes and natural killer cells [37]. We thus analysed the amount of intracellular molecule by a flow cytometric approach, and indeed could detect the presence of CD95L either in A301 or in its HIV+ clone, ACH-2. The latter cells, however, had a slightly lower amount of molecule, as assessed in several repeated experiments (Fig. 1). To confirm the cytofluorimetric data, we decided to quantify the expression of CD95L at the RNA level using a PCR-based approach.

PCR analyses performed by others, coupled with cytofluorimetric analyses, have shown a decreased CD95L expression in cells from HIV+ individuals [17]. However, PCR assays provide results which are only semiquantitative, and do not take into account the intrinsic variability of the type of analysis. A commonly experienced problem with PCR is indeed its poor reproducibility, even under the most controlled experimental conditions. Minor differences in the efficiency of amplification result in large differences in the product yield, especially when the amount of initial template is low. Assays based on competitive PCR are much more precise for the quantitation of a given gene or for its expression. In principle, by this method two DNA species (wild type and competitor) compete for PCR amplification. Since results are calculated from the final wild type/competitor ratio, any variable affecting the rate of PCR amplification has no effect on the accuracy of the ratio measurement. The same concept can be applied for measurements at the mRNA level, when an RNA competitor is used and is present in the reaction tube from the beginning of the reaction, i.e. the retrotranscription phase. In all cases, even large variations in the experimental conditions (number of PCR cycles, sample volumes and extracted DNA or RNA quality) do not interfere with the precision of the measurement of the wild type/competitor ratio [38]. For all these reasons, we thought it worth while to develop an approach to rapidly and precisely analyse CD95L expression, by using quantitative competitive RT-PCR, and constructed an RNA competitor using the strategy described above (see Fig. 2, upper part). The minimum level of detection, considering that the efficiency of extraction of RNA from cells was satisfactory and constant (i.e. in the order of 10–20 μg/10^6 cells, for all lines), was about 2000 copies CD95L mRNA/10^6 cells. We first analysed the expression of CD95L in a classical cell line widely used as effector for exerting CD95L-mediated cytotoxicity, i.e. HUT78. Fig. 2, middle part, shows a typical example of competitive PCR analysis. It has to be underlined that with this approach the calculation of the copies of wild type RNA uses the ratio between the two bands in each single lane, and not their relative intensity in different lanes. This allows a precise analysis even in the case that the reactions were not optimal in a given PCR tube, as retrotranscription and amplification are identical for either the wild type or the competitor, both present in the same tube. Fig. 2, lower part, reports the kinetics of induction of CD95L mRNA, showing that the expression of mRNA was maximal after 4 h of stimulation with TPA/Ion, as HUT78 could express about 150 000 copies/1000 cells. This amount decreased after a few hours to basal levels (about 40 000 copies), and decreased again in 24 h.

We then studied the expression of CD95L in HIV+ cell lines. U937 and its derived clone, U1, had undetectable levels of CD95L (not shown). In contrast, in agreement with the cytofluorimetric observations, ACH-2 expressed significantly lower levels of CD95L mRNA than the parental line A301, either in basal conditions or after stimulation (Fig. 3). The increase of mRNA after treatment with TPA/Ion was always significant ($P < 0.01$). Experiments were repeated a minimum of four times, indicating that although very few copies of CD95L per cell were present in comparison with the HUT78 cell line, their presence could be quantified either in basal conditions or after stimulation with TPA/Ion. The difference between HIV+ and uninfected cells was statistically significant ($P < 0.01$). It has to be underlined that RNA was extracted from all cells, and thus the low number of copies we found per cell represents the mean from the entire population, whose single components have likely different amounts of CD95L mRNA, for example because of differences in the cell cycle. Further studies on synchronised cells are required to clarify this point.

In conclusion, the main results of this study can be simply summarised as follows: (i) in a cell line of lymphoid origin such as ACH-2 chronic infection with HIV inhibits the expression of CD95L, the phenomenon occurring at the transcriptional level; (ii) in cells of monocytic origin such as U1 the infection decreases the plasma membrane expression of CD95. Studies at the protein and mRNA levels with new RNA competitors are under way to ascertain whether the reduced expression of CD95 on the plasma membrane is associated with changes in the production of the CD95 soluble form.

The data we present here explore the delicate balance between mechanisms triggered by the virus to favour its replication and survival, and those triggered by the immune system to cope with the infection. HIV regulates apoptosis in a complex way, requiring live, functional cells for its replication. Some of its products can either protect from or induce apoptosis [39,40]. The chronically infected cell lines we used are in a sort of steady state, in which a dynamic equilibrium likely exists between the production of pro- and anti-apoptotic factors of either cellular and viral origin. As the rate of spontaneous apoptosis in HIV+ cell lines was extremely low (not shown), and cells proliferate quite well, it is likely that the balance leans towards the side of anti-apoptotic mechanisms. Therefore, on the one hand, it could be hypothesised that HIV is capable of triggering several, co-ordinated strategies which likely depend upon the cell line which is infected, with the ultimate aim of reducing apoptosis and favouring viral production. Indeed, in monocytic cells which act as a reservoir, HIV down-regulates the expression of a molecule whose activation induces apoptosis, i.e. CD95. In cells of lymphocytic origin, capable of exerting cytotoxic responses, it could be convenient to down-regulate the expression of a molecule which induces apoptosis, i.e. CD95L. On the other hand, assuming that the model we studied has some similarities with the situation present in vivo during chronic infection, paradoxically the down-regulation of CD95L could be favourable for the host because activated, cytotoxic T cells, expressing CD95, can be killed by CD95L+ CD4+ lymphocytes [41]. Even if further studies are needed to clarify the role of CD95/CD95L interactions in vivo, this report shows that in the in vitro models of chronic HIV infection a down-regulation of the CD95/CD95L pathway exists which may favour viral production.

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