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T cell survival/proliferation reconstitution by trifluoperazine in human immunodeficiency virus-1 infection

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Abstract

Recent findings support an indirect relationship between T cell depletion in HIV-1 infection and the rate of virus replication with implications for treatment strategies. We have initiated a new approach to recover immune function through the use of novel chemical agents. A cationic amphiphilic drug that binds to Ca^{2+}-calmodulin at high concentrations, [10-{3-(4-methyl-1-piperazinyl)-propyl}-2-(trifluoromethyl)-^{10}H-phenothiazine dihydrochloride] [denoted trifluroperazine dihydrochloride (Tfp); molecular weight 480.43] TFP was found at low concentrations (10^{-6} to 10^{-10} M) to help T cells from AIDS patients to restore proliferation in vitro. Here we show that the Tfp molecule can restore the cell survival of T lymphocytes from PBMCs derived from HIV-1-infected patients in vitro. Tfp enhances T cell proliferation and Th-cell responses by selectively inhibiting cell mortality and apoptosis. The restored antigen-specific response is associated with the synthesis of IL-2 and γ-interferon. Even though this drug does not possess any detectable antiviral effect, it might be considered as a potential therapeutic agent in HIV-infected patients, to correct immune defects. Besides antiviral compounds, these data may facilitate immune reconstitution in patients with HIV infection and other immunosuppressive diseases.

Keywords: HIV-1; Pathogenesis; Therapy; Immunity; Cationic; Amphiphilic; Trifluoperazine

Restoration of the immune system is a crucial element in the successful clinical management of human immunodeficiency virus (HIV)-1 infection. Current therapy for HIV infection consists of highly active antiretroviral therapy (HAART) and protease inhibitors, which are presumed to exert their positive effects on $CD4^+$ T cell number and immune function by inhibiting viral replication (Autran et al., 1997; Pakker et al., 1998). However, the clinical benefits of HAART still have limitations, namely HIV-1-infected persons receiving HAART have responded with partial restoration of immune cell populations (Bohler et al., 1999; Hengel et al., 1999), immune functions (Autran et al., 1997; Dam-Nielsen et al., 1998; Ledermann et al., 1998), and homeostasis (Dyrhol-Riise et al., 1999). Immune recovery generally appears incomplete and variable. Many additional strategies to reconstitute the immune system during HAART have been attempted (Pantaleo, 1997; Butera, 2000), including the in vivo expansion of the T cell population with the use of interleukin-2 (Chun et al., 1999) and the development of novel chemical agents (Achour et al., 1998; Achour, 2001, 2002; Butera, 2000).

A remarkable chronic immune activation is observed in HIV-1-infected individuals throughout infection leading to lymphadenopathy and dysregulated expression of various cytokines (Clerici et al., 1993; Copeland and Heeney, 1996). Impaired proliferative responses (anergy) and increased apoptosis of activated T lymphocytes are the hallmarks of the pathogenesis characteristic of HIV-1 infection. The difficulties associated with the use of agents that induce proliferation of $CD4^+$ T cells, the major reservoir of HIV-1, which in turn leads to increased viral replication limits the therapeutic usefulness of these approaches involving immune reconstitution. Several hypotheses have been suggested to explain the deple-

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tion of $CD4^+$ T lymphocytes (Zinkernagel and Hengartner, 1994). The model of high lymphocyte turnover has been proposed postulating that HIV-1 infection leads to a rapid turnover of lymphocytes (Ho et al., 1995; Wei et al., 1995). It has been reported that perìpheral T cell proliferation is a consequence of generalized immune activation (Hazenberg et al., 2000). The activation of HIV-1 expression and viral production, in addition to the limited clonogenic potential of the uninfected T cells, represents the two major obstacles for $CD4^+$ T cell proliferation. Moreover, infection with HIV-1 results in a remarkable loss of cell-mediated function. The virus infects CD4-positive cells inducing lysis of infected lymphocytes and releasing virions. However, direct killing of infected cells cannot account by itself for the progressive immune deficiency characteristic of AIDS. It has been recently reported that CD4 cell depletion is directly associated with immune activation and only indirectly to viral load (Broussard et al., 2001; Deeks et al., 2000; Kaur et al., 1998). Infection with HIV is associated with apoptosis, which is partially responsible for the depletion of $CD4^+$ T cells, independent of virus-mediated cytolysis (Finkel et al., 1995). AIDS is also characterized by an impairment of the immunoregulatory network (Fauci, 1993).

In a previous study we have studied cationic amphiphilic drug effect in the form of AY 9944 molecule and provided a new target for manipulation of immune responses. We have shown that AY 9944, a nonspecific calmodulin antagonist, can restore the expression of the IL-2 receptor and Th-cell responses, favoring cytokine and chemokine production (Achour et al., 1998; Achour, 2000). In this study, we report the activity of another cationic amphiphilic drug [trifluoroperazine dihydrochloride (Tfp)] for its anti-HIV-1 activity in peripheral blood mononuclear cells (PBMC) derived from healthy HIV-1- seronegative individuals and chronically infected AIDS patients. This molecule belongs to the series of antipsychotic agents, phenothiazines, possessing a wide range of pharmacological action (Breugnot et al., 1990; Jeding et al., 1995; Slater, 1968). Tfp binds to calmodulin in the presence of calcium and inhibits its effect at high concentrations (Hait and Lee, 1985; Levin and Weiss, 1977; Nel et al., 1986). We found that this compound, at low concentrations (where anticalmodulin effects are not observed), was able to reduce HIV-induced disorders. More importantly, the drug appears to restore the survival of T cells by decreasing cell mortality and apoptosis. The development of Tfp-based treatment that can restore immune functions may offer a complementary approach to AIDS therapy besides anti-HIV compounds.

Results

Effect of the drug on mitogen-induced proliferative responses of PBMC from healthy seronegative individuals

Tfp inhibited the growth of cells, including lymphocytes at high concentration of the drug. Thus, cells grew normally at 10^{-6} M Tfp but showed >90% inhibition at 10^{-5} M. To assess the biological effects of Tfp at lower concentrations, we studied the proliferative responses of PBMC after in vitro activation with phytohemagglutinin (PHA). PBMC derived from four healthy seronegative donors were either infected in vitro with HIV-1 or not infected. Following 10 days in culture, the number of viable HIV-1-infected PBMC (Fig. 1A) was significantly lower in the untreated controls as compared with noninfected cells (Fig. 1B). Moreover, when PBMC were cultured in the presence of Tfp (from 10^{-6} to 10^{-10} M), the proliferative responses of the infected PBMC were significantly higher than the untreated cells ($P < 0.001$). The survival of HIV-1-infected PBMC increased by 2.9- to 4.9-fold (at day 14) with 10^{-6} to 10^{-10} M TFP (peak response at 10^{-8} M). In noninfected PBMC a lower but significant ($P < 0.001$) change in the survival rate was found following culture with Tfp. At 14 days of culture, the survival increased by 1.1- to 2-fold (Fig. 1B). The number of untreated living cells in the HIV-1-infected cultures progressively decreased as a consequence of infection (Fig. 1A, control). In contrast, when these cells were cultured in media containing Tfp, the reduced survival of in vitro HIV-infected PBMC was corrected to the level seen in cultures of uninfected cells in the presence of the drug (Figs. 1A and B). Such a beneficial effect of Tfp on the survival of PBMC (50% of the maximal effect at 5×10^{-10} M) appears to be within the range of pharmacological concentrations (Fig. 1A). The mechanisms by which TFP increased cell survival might be at either the level of T cells or the level of adherent cells (AC). To address this question, nonadherent $CD4^+$ T cells and $CD8^+$ T cells from healthy donors were purified. No increase in cell survival was observed when $CD4^+$, $CD8^+$, or $CD4^+$ and $CD8^+$ cells were cultured in absence of adherent cells (Table 1). Moreover, preincubation of (AC) with an inhibitor of protein synthesis (actinomycin D), completely abolished the Tfp effect (Table 1).

Since HIV-1 replicates in activated cells and Tfp increases cell survival, primary concern with the use of Tfp in cultures of HIV-infected cells might be that such cultures would result in an increase in target cells and thus increased viral burden. As shown in Fig. 1C, no consistent changes in viral production in the PBMC, as evaluated by serial measurement of P24 antigen, were detected during the culture. At 18 days of the culture cells treated with the drug exhibited a decline in P24 antigen levels. However, from 8 to 20 days, P24 antigen levels in the supernatant was not significantly different from those in untreated cells (Fig. 1C). A quantitative PCR was also performed to determine amounts of HIV proviral DNA. Whereas HIV-1 Gag proviral DNA became detectable in all cultures at early time points, by day 14 and 17 days PBMC treated with Tfp molecule showed a slight decrease in HIV gag DNA (more than twofold). However, expression of HIV gag DNA was comparable in all the samples at 20 days of culture (Fig. 1D).

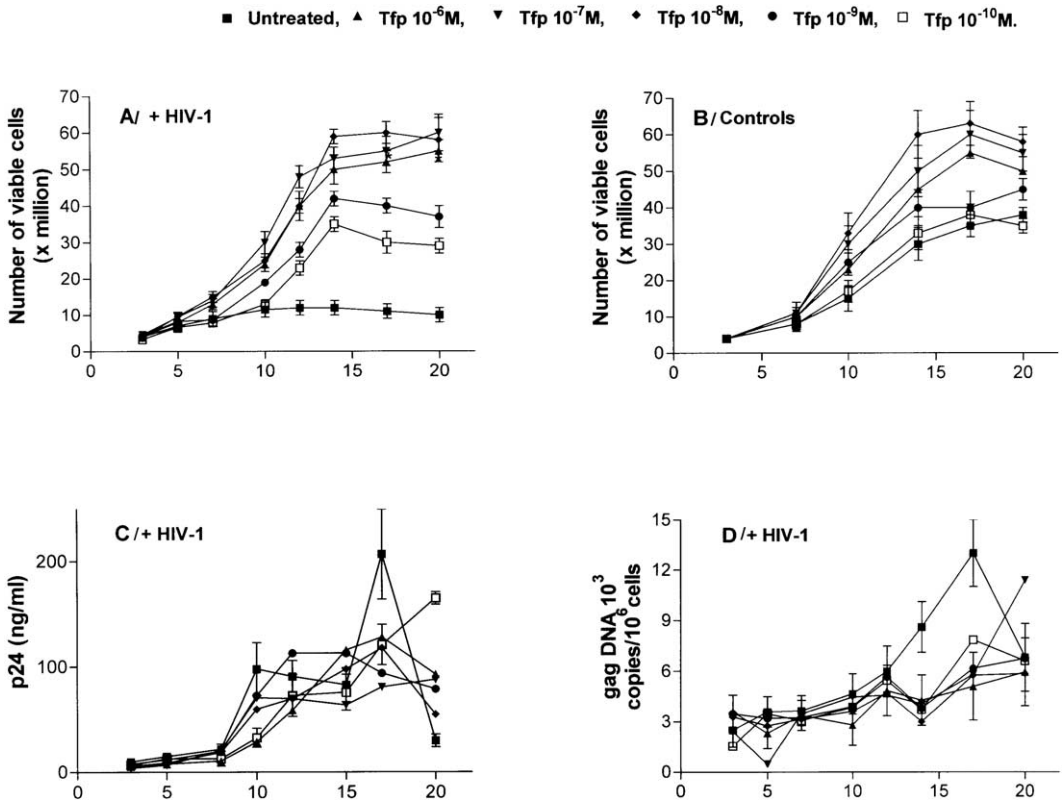

Fig. 1. Cells derived from four healthy HIV-1-seronegative individuals were either infected with HIV-1 (A, C, D) or not infected (B). Data represent the cell-proliferation cultures from 3 to 20 days poststimulation of cells alone or in the presence of Tfp molecule (10^{-6} to 10^{-10} M). Results are expressed as the number of viable cells (means ± SD) (A,B). Cell-free culture supernatants were analyzed for p24 ELISA (C) and quantitative proviral DNA was done by PCR with 1×10^6 cells (D).

Effect of the drug on PBMC from naturally infected patients

To compare the results obtained with cells obtained from healthy individuals, lymphocytes were cultured from four patients (P1, P2, P3, P4) (CD4 counts from 196 to 664 cells/mm^3, Table 2) with PHA and IL-2 to evaluate the effect of the drug on naturally infected cells for 4 weeks. As illustrated in Fig. 2, when T cells were cultivated with Tfp (10^{-7} M), the cell survival increased by 2.5- to 6-fold more than the control cells after 2 weeks of culture ($P < 0.001$). Compared to controls, PBMCs cultured in the presence of Tfp demonstrated substantially decreased cell mortality revealed by trypan blue exclusion (Figs. 2A′–D′). This reduction of mortality seems polyclonal and Tfp increased cell proliferation of $CD4^+$ in the same level as $CD8^+$ T cells (data not shown). In the PHA-stimulated culture, the data revealed an initial expansion of treated cells at week 2, resulting in termination on weeks 3 and 4 of the culture.

T cell anergy is a characteristic of HIV-1 infection. To

Table 1
Effects of adherent cells (AC) on the cell survival of T cells stimulated with PHA and cultured in the presence of 10^{-7} TFP[a]

	Day 0 of culture		Day 5 of culture		Day 10 of culture	
	Medium	TFP	Medium	TFP	Medium	TFP
Total PBMCs (+AC)[b]	2	2	4	5	8	12
$CD4^+$ T cells (−AC)[c]	2	2	3	3.5	4.9	5.2
$CD8^+$ T cells (−AC)[c]	2	2	3.3	4	5	5.6
$CD4^+$ + $CD8^+$ T cells (−AC)[c]	2	2	4	4.5	5.4	6
$CD4^+$ + $CD8^+$ T cells (+ treated AC)[d]	2	2	3.9	4	5	5.6

[a] PPMCs were derived from healthy HIV-1 seronegative donors and cultured as described in Fig. 1.
[b] Data are expressed as the total number of viable cells.
[c] Nonadherent T cells were purified using the magnetic beads (Materials and methods).
[d] Inhibitor of protein synthesis Actinomycin D was added to AC prior to coculture.

Table 2
Patient characteristics

Patient no.	CD4 count[a]	Plasma viral load[b]	Antiviral[c]
P1	196	620	3TC, D4T, PI
P2	561	6990	(—)
P3	664	692	3TC, D4T
P4	362	1842	(—)
P5	389	<50	3TC, D4T, PI
P6	554	<50	DDC, D4T, PI
P7	450	19,978	3TC, D4T
P8	41	6080	3TC, D4T, PI
P9	800	<50	(—)
P10	329	123	AZT, 3TC, PI
P11	713	983	3TC, D4T, PI
P12	600	146	3TC, D4T
P13	259	879	(—)
P14	811	<50	3TC, D4T, PI
P15	935	<50	3TC, D4T, PI
P16	353	49,178	3TC, D4T, PI
P17	30	263,935	(—)

[a] $CD4^+$ T cells per mm^3 of blood.
[b] Plasma HIV-1 RNA, copies per ml of plasma (commercial kit).
[c] Abbreviations: AZT, zidovudine; 3TC, lamivudine; D4T, stavudine; PI, protease inhibitors; (—), without treatment.

investigate this issue, we tested the ability of lymphocytes to proliferate in vitro in response to CD3 stimulation in the absence and presence of CD28 costimulation. First, lymphocytes from two patients (P5, CD4: 389/mm^3 and P6, CD4%: 554/mm^3) were cultured with immobilized monoclonal antibody to CD3 and IL-2. Cell survival, the percentage of cell mortality, and the percentage of apoptotic cells were compared during the first 2 weeks of culture in the presence or absence of Tfp. The results shown in Fig. 3 revealed an initial expansion of cells from day 7 to day 9. This pattern was coincident with decreased cell mortality compared to untreated cells. However, by day 11 the proportion of apoptotic cells diminished in TFP-treated cells compared to untreated cells (30% variation, $P < 0.005$).

In a second step, we tested the T cell receptor (TCR) and the CD28 costimulatory signal engagement to amplify T cell activation. We cultured lymphocytes from three patients with HIV-1 infection with CD3 mAb and CD28 mAb. P9 is a recent HIV-1 seroconverter (CD4: 800/mm^3, plasma viral load: <50 copies/ml), whereas individuals P7 (CD4: 450/mm^3, plasma viral load: 19778 copies/ml) and P8 (CD4: 41/mm^3, plasma viral load: 6080 copies/ml) were patients under treatment (Table 2). As shown in Fig. 4, at 10–20 days of culture, cell survival from two naturally infected donors with clinical symptoms (P7 and P8) (Figs. 4A and B) were significantly lower than the response of PBMC from asymptomatic individual P9 (Fig. 4C) ($P < 0.001$). Moreover, when cells were cultured in the presence of Tfp molecule (10^{-6}–10^{-10} M) proliferative responses from all individuals were significantly higher than in control untreated cells. Under Tfp (10^{-7} M), cell survival of PBMC from individuals P7 and P8 up regulated in the same range as the cells from patient P9. After 2 weeks of culture, the number of viable cells conditioned by Tfp molecule for the donor P7 the donor P8 and the asymptomatic individual P9 were 55×10^6, 63×10^6, and 65×10^6, compared to untreated cells (9×10^6, 8×10^6, and 30×10^6), respectively (Figs. 4A–C). As seen in figs. 4A′, B′, and C′, the number of untreated living cells provided from patients (P7 and P8) progressively decreased. In contrast, when these cells were supplemented with complete medium containing Tfp molecule, the cell mortality remained low. Such a beneficial action of Tfp on the survival or the mortality of PBMC provided from HIV-1-infected donors was dose dependent and fitted within the range of pharmacological conditions as indicated in Fig. 4 (peak response at 10^{-8}, 50% of the maximal effect at 10^{-10} M). We further determined whether Tfp could also prevent naturally infected PBMC from viral production. Since it is difficult to detect p24 antigen in the cultures of lymphocytes from AIDS patients, a quantitative PCR was used to determine amounts of HIV-1 RNA. By day 7–16 of the culture, no specific diminution in virus production was observed in cells from patients P7 and P8 and no virus production was detected in PBMCs originated from recent seroconverter individual P9 (data not shown).

Effect of the drug on the expression of Ki-67 antigen

To answer the question of whether increased T cell proliferation could be involved in T lymphocyte depletion, we measured expression of Ki-67 nuclear antigen, which is expressed in late GI, SI, G2, and M phase of the cell division circle in our cultures. After stimulation with mAbs anti-CD3 and -CD28, PBMCs provided from two patients (P10, CD4: 329/mm^3; P11, CD4: 713/mm^3) were cultured in complete medium in the presence or absence of Tfp (10^{-7} M). As shown, from day 14 of the culture, we observed a decline in the percentage of Ki-67 lymphocytes (Figs. 5A′ and B′). In parallel, higher increases in the percentage of cell survival and diminution of apoptotic cells were observed. When cells derived from 10 patients were studied, the expression of the cell-cycle marker Ki-67 and the fraction of apoptotic decreased sigificantly after 14 days of the culture in the presence of TFP (Table 3). Analysis by flow cytometry indicated that the naive ($CD45RA^+$/$CD62L^+$) and memory ($CD45RO^+$) subsets of both CD4 and CD8 T lymphocytes were equally expanded by Tfp (data not shown).

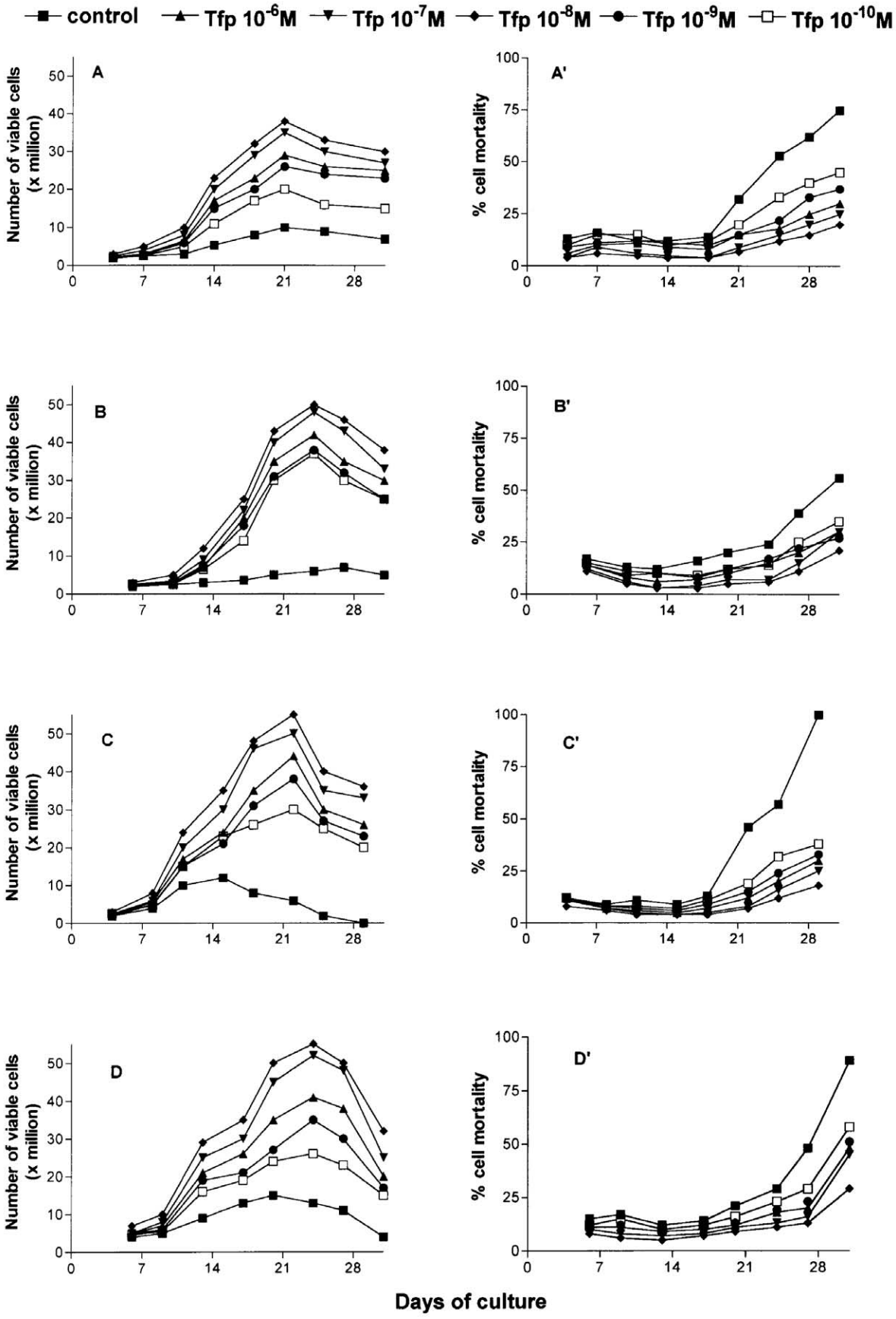

Fig. 2. Effect of Tfp molecule on the survival (total number of viable cells) (A, B, C, D) and the percentage of dead cells assessed by Trypan blue exclusion (A′, B′, C′, D′) of PBMCs from four AIDS patients (P1, P2, P3, P4). After stimulation with PHA, PBMCs were cultured with (10^{-6} to 10^{-10} M) or without Tfp. Data are expressed as the means for triplicate cultures. The standard deviation was less than 10% of the mean.

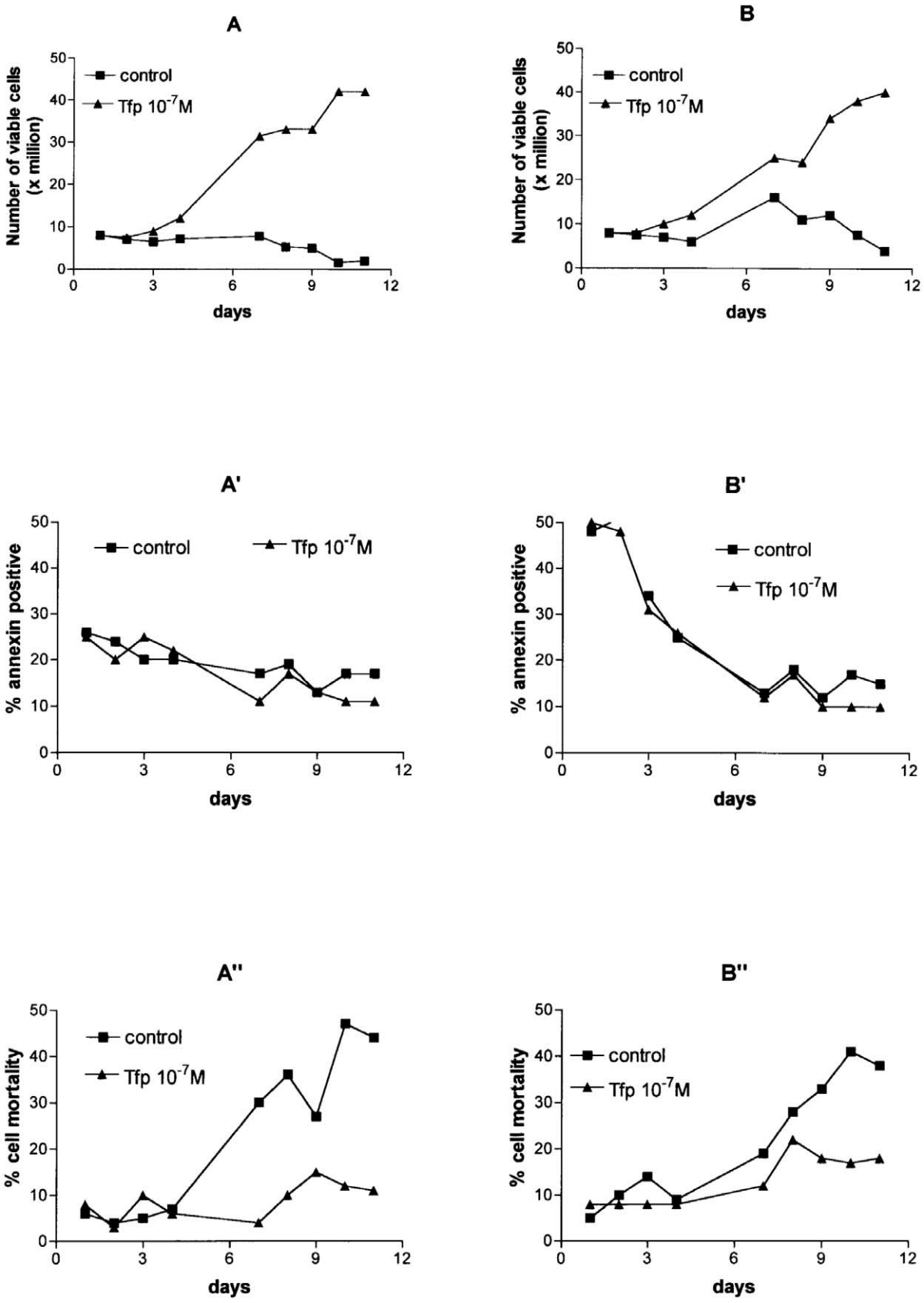

Fig. 3. Effect of Tfp on the cell survival, the percentage of cell morality, and the percentage of apoptotic cells in cultured PBMCs stimulated with immobilyzed mAb anti-CD3. Cells were derived from two patients [A, P5, $CD4^+$: 389/mm^3 (A) and P6, B $CD4^+$: 554/mm^3] and cultured with or without Tfp. For apoptosis the results are expressed as the percentage of annexin-positive cells evaluated by the FITC-labeled annexin V assay. Data are expressed as the means for triplicate cultures. The standard deviation was less than 10% of the mean.

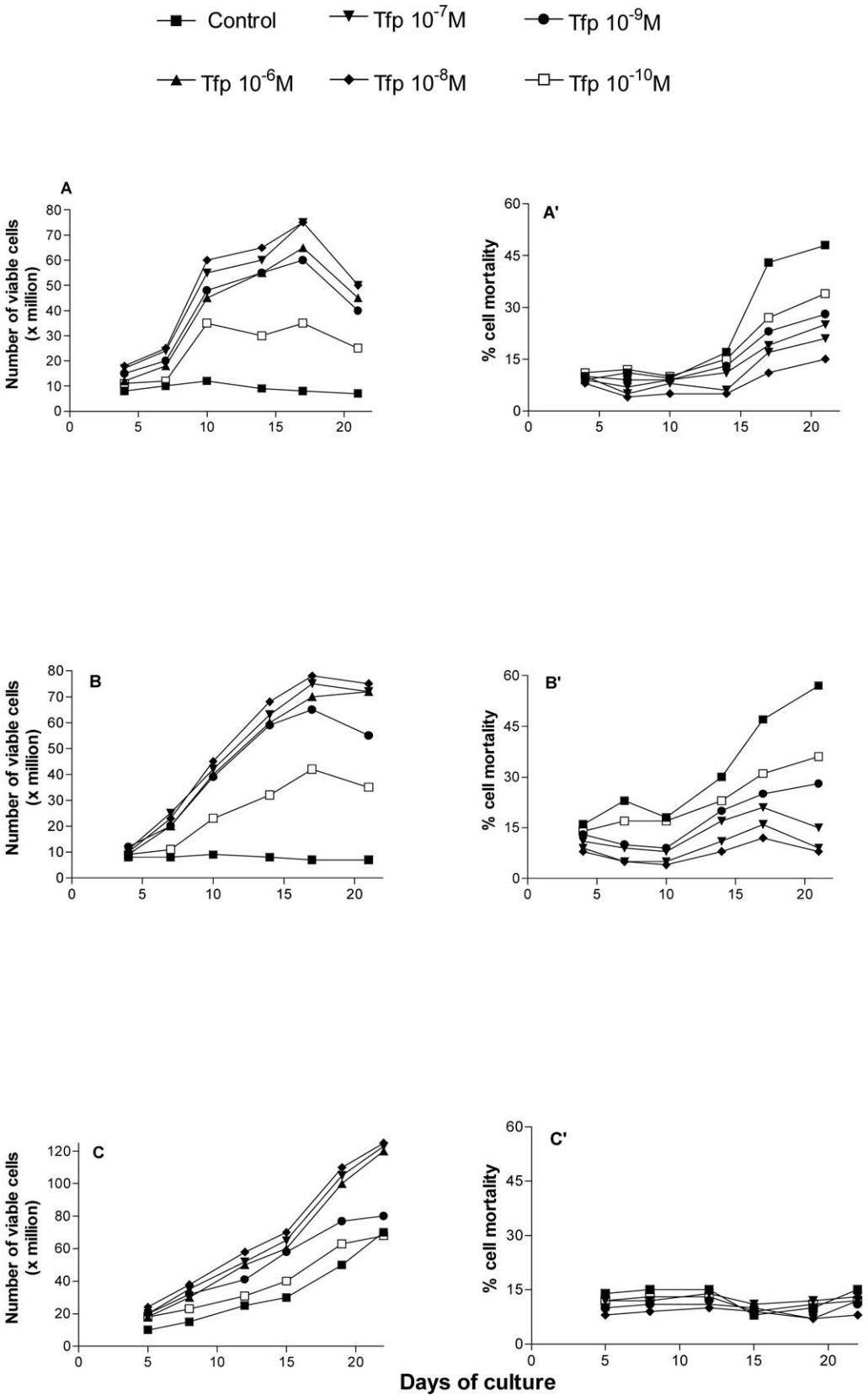

Fig. 4. Effect of Tfp on the cell survival and the percentage of cell mortality (10^{-6} to 10^{-10} M) in cultured PBMCs stimulated with immobilized mAbs anti-CD3 and -CD28. Cells were derived from patients P7 (A, A′), P8 (B, B′), and P9 (C, C′) (Table 2) and cultured with or without Tfp. Each point represents the mean of three experimental values. There was less than 7% variation among them.

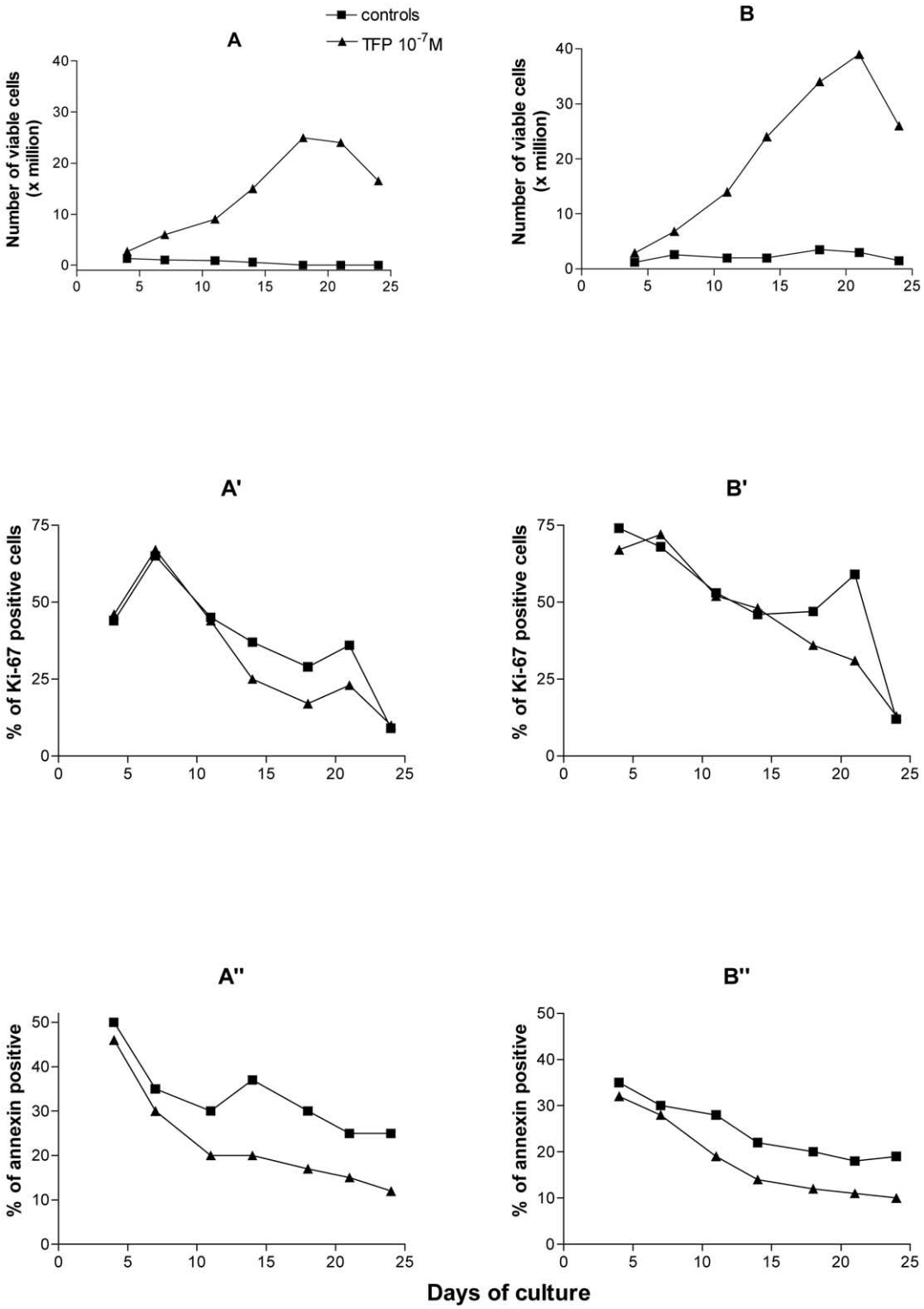

Fig. 5. Effect of Tfp on the cell survival (A,B), the percentage of Ki-67 T cells (A′, B′), and the percentage of apoptotic cells in cultured PBMCs stimulated with immobilized mAbs anti-CD3 and -CD28. PBMC were originated from patients P10 (A, A′, A″) and P11 (B, B′, B″). Data are expressed as the means for triplicate cultures. The standard deviation was less than 5% of the mean.

Table 3
Effects of TFP (10^{-7} M) on the apoptosis and membrane Ki-67 expression of HIV- infected patients[a] (P8–P17, $n = 10$) T cells stimulated with anti-CD3/CD28

	Day 7 of culture		Day 15 of culture	
	Control	TFP	Control	TFP
% apoptotic cells	31 ± 6	28 ± 7	35 ± 9	17 ± 7*
% Ki-67$^+$ T cells	43 ± 8	39 ± 9	35 ± 8	19 ± 5*

[a] Data are expressed as the mean ± SD of the measurements.
* $P < 0.01$ as compared with untreated control cells.

Effect of the drug on recall antigen-, SEB-, and HIV-peptide 18-induced proliferative responses of PBMC

It has been reported that HIV-1$^+$ individuals can be divided into distinct groups, based on in vitro production of IL-2 and proliferation in response to recall antigens and PHA. Negative response to recall antigens is an early event in HIV immune dysfunction compared to PHA. To determine whether Tfp would also increase T helper cell functions to recall antigens as PPD, TT, and superantigen SEB in individuals infected with HIV-1, we stimulated the PBMC of six HIV-1$^+$ individuals (P12–P17) with these antigens. The data of Fig. 6 illustrates that the proliferative responses to SEB of PBMC from HIV-1$^+$ patients was increased approximately 2- to 4-fold; their PPD responses were increased 2- to 3-fold and TT responses were increased 0.5- to 2-fold ($P < 0.005$). Proliferation of unstimulated cultures was not increased by Tfp.

Then, we also investigated the effect of Tfp on HIV antigen similar to P18MN V3 loop envelope peptide. The data of Fig. 7 indicate that the proliferative responses to P18MN in PBMC of six HIV+ individuals was significantly increased in the presence of Tfp. Compared to PBMC activated with PHA or anti-CD3 and CD-28 and cultured with recombinant IL-2, cells activated with antigens could be a more physiological way to determine cytokine production. We tested IL-2 and IFNγ production in response to env P18MN peptide in fresh PBMCs derived from the same HIV+ individuals. In these experimental conditions, when fresh PBMCs were cultured with HIV-1 V3 loopP18MN peptide in medium without recombinant Il-2, we observed an increased expression of interleukin 2 at 3 days of culture as determined by intracellular staining (increasing of 60 to 160%, $P < 0.005$). The T helper effect of Tfp molecule appeared to be associated with its selective capacity to restore the IFNγ-dependent cell activation denoted by significant augmentation of IFN-γ production. As indicated in Fig. 7 P18MN-activated PBMCs from HIV-1-infected indi-

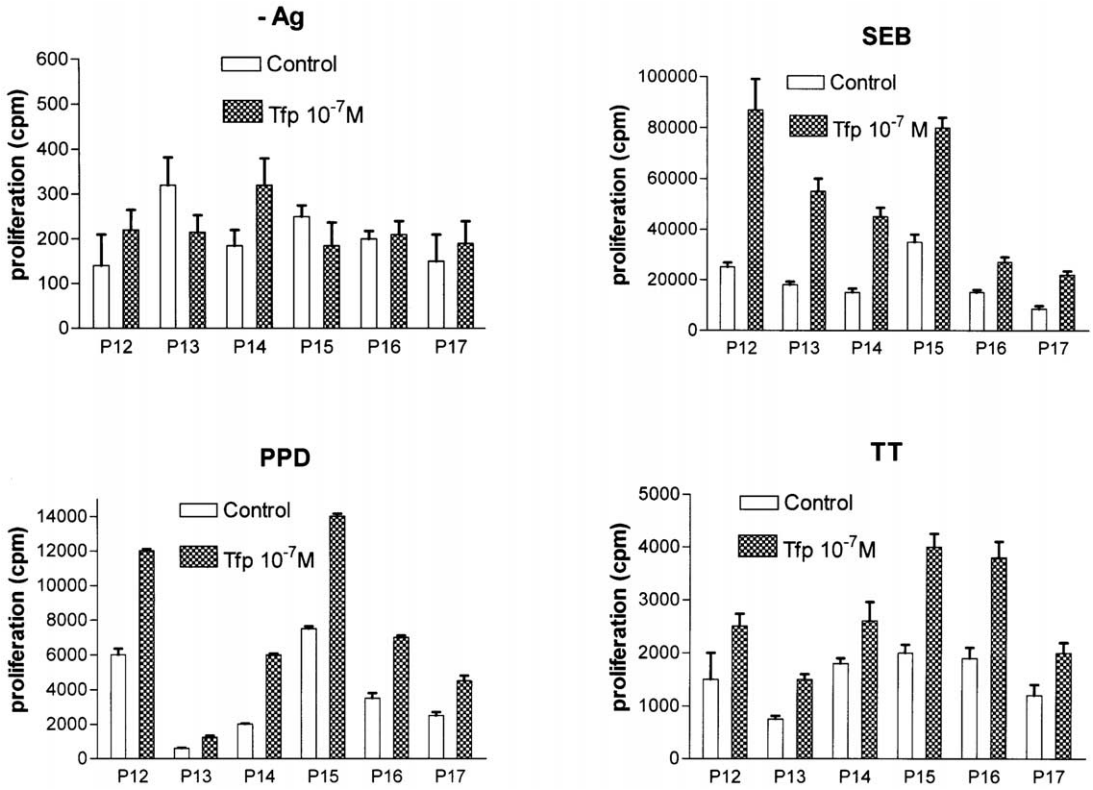

Fig. 6. Human peripheral blood Th-cell responses to recall antigens (PPD, TT) and to superantigen (SEB) in the absence or presence of Tfp (10^{-7} M). Proliferation of PBMC from six HIV+ individuals (P12 through P17) in response to recall antigens (PPD, TT) and to superantigen (SEB), in presence of Tfp (solid bars) or without Tfp molecule (clear bars). (−Ag) indicates unstimulated cultures. Results represent mean ± of triplicate cultures of [^{3}H] uptake at 6 days poststimulation.

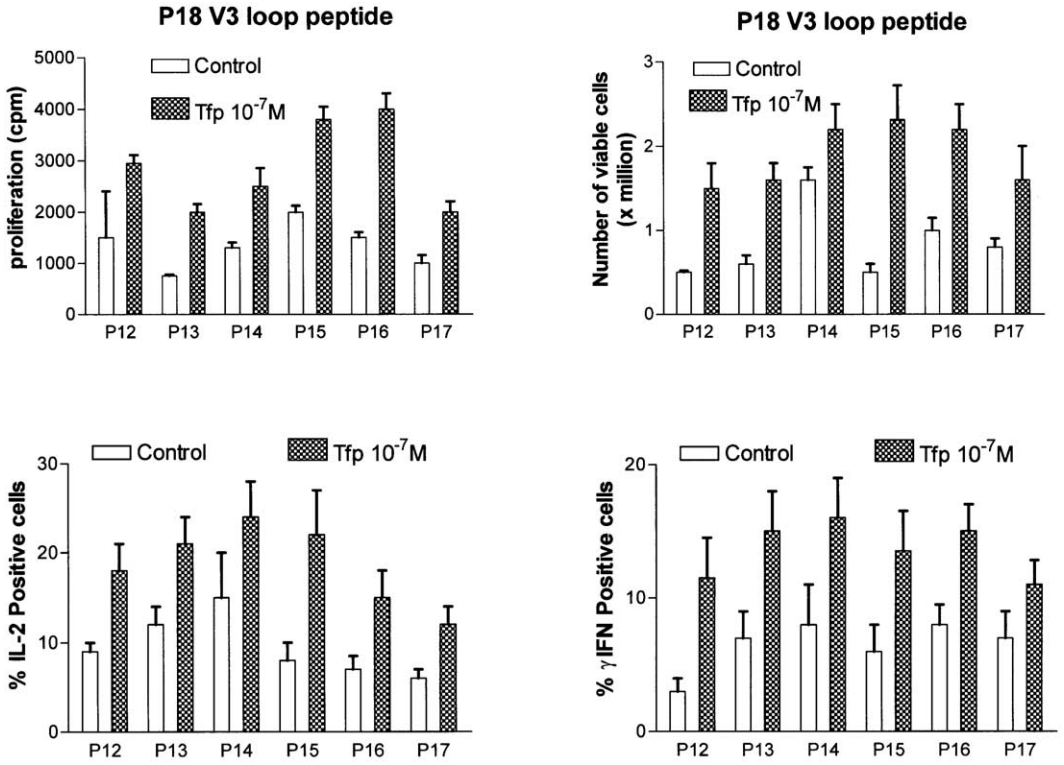

Fig. 7. Effect of Tfp on T cell proliferative response (A), cell survival (B), intracellular production of IL-2 (C), and INF-γ (D) in cultured PBMCs (provided from patients described in Fig. 6) stimulated with HIV-1 envelope peptide (P18 MN). Results represent mean ± of triplicate cultures at 6 days poststimulation.

viduals treated in vitro with Tfp molecule showed a significant increase of IFN-γ-producing cells from 100 to 300%, compared to control cells cultured without the molecule ($P < 0.001$). This proliferative response evaluated through ^{3}H-thymidine incorporation is also associated with cell survival as reflected by the significant increase of the number of viable cells in presence of Tfp compared to control cells after 6 days of culture (Fig. 7B).

Discussion

As calmodulin antagonist, Tfp inhibits cell proliferation at high concentrations. This inhibition was observed within a very narrow range of drug concentrations: cells grew normally in 1 μM Tfp and >90% inhibition occurred at 10 μM and >95% at 50 μM (Hait and Lee, 1985). Previous studies have indicated that calmodulin antagonists such as trifluoperazine and chlorpromazine inhibit the replication of several RNA viruses including measles virus and influenza viruses (Bohr et al., 1983; Krizanova et al., 1982; Ochiai et al., 1991). It has been reported that Tfp inhibited AIDS virus production from lymphocytes at concentrations that were inhibitory for cell proliferation (Srinivas et al., 1994). Inhibition of apoptosis in cells from AIDS patients and ceramide-mediated apoptosis in Jurkat cells by Tfp at high doses have also been reported (Micoli et al., 2000; Pan et al., 1998). In the present study no specific inhibition of viral production was observed at lower concentrations.

The clinical evolution in HIV-1 infection results from immunosuppression characterized by T cell anergy, apoptosis, and chronic activation of T cells. T helper hyporesponsiveness occurs in the context of generalized state of activation. This generalized immune activation is characterized by an increased expression of Ki-67 expression. It has been proposed that apoptosis could be a mechanism whereby $CD4^{+}$ and even $CD8^{+}$ T cells can be killed in HIV-infected individuals without invoking direct infection of the cells by HIV-1 (Ameissen and Capron, 1991). The causal relationship among CD4 cell depletion, HIV replication, and activation are not well understood. Recent reports indicate that CD4 depletion during HIV/SIV infection is more directly related to the activation than to the free virus (Broussard et al., 2001; Deeks et al., 2000; Kaur et al., 1998). Therefore, it has been suggested that elevated immune activation induces T cell depletion but only indirectly to virus load (Sousa et al., 2002). Depression of antigen-specific T cell responses are also an important feature of HIV infection. It involves a qualitative dysfunction of T cells (Fauci, 1993; Clerici et al., 1993).

Here we show that cationic amphiphilic molecule Tfp, at low concentrations (10^{-6} to 10^{-10} M), can restore the proliferation of the T lymphocytes. This restoration is observed in T cells derived from patients receiving or not

receiving HAART with variable CD4 counts and viral load values. Tfp increases normal and HIV-1-infected T cell survival after mitogen stimulation. This activity appears indirect due to the synthesis of molecules by AC. An inhibition of anergy was also observed after CD3/CD28 ligation. As the consequence of restored T cell proliferation an inhibition of apoptosis by Tfp is observed only in the second week of culture. This inhibition of apoptosis is associated with a decreased expression of the cell-cycle marker Ki-67. Since increased expression of Ki-67 expression was caused by generalized immune activation, TFP may facilitate the reentry of the majority of T cells into the Go cell-cycle phase. Tfp does not confer a specific protection against HIV-1 in either levels of viral production and or proviral DNA load. Ongoing work is aimed to study PBMC derived from a large panel of patients with very low CD4 counts the effect of Tfp in relation with the stage of HIV-1 infection.

It clearly appears in our experimental system that a compound which is believed to be classical, "calmodulin antagonist," is efficient in preventing cell mortality and restoring T cell proliferation in HIV-1 infection. The cationic amphiphilic structure of Tfp is characterized by a bulky hydrophobic core associated with a nitrogen-linked cation. In previous studies, we reported the ability of cationic amphiphilic molecules as being potential immunostimulants for HIV-1 infection (Achour, 2001, 2002). More recently, we have reported the immunomodulatory effects of aminoperazine, a phenothiazine derivative able to increase the antigen-specific dendritic cell proliferation (Lu et al., 2001). Since γ-interferon and IL-2 production are induced with Tfp under viral antigen stimulation, recovery of proliferative response may be explained by the production of other unknown growth factors regulating vital biological processes such as regulation of immune responses and cellular proliferation. Whether this activity and its associated responses are due to the activation of T help 1 cells or reflect the known activities of IL-12, IL-15, IL-18 on $CD4^+$, $CD8^+$, NK, and antigen presenting cells remains to be determined. The complete mechanism whereby Tfp modulates these activities has to be elucidated. However Tfp might independently of its action on calmodulin act on cell metabolism by modifying the physicochemical characteristics and the conformation of membrane structure (Maziere et al., 1988).

Regarding clinical practice, the combination of these molecules with HAART might represent a pharmacologic mean for HIV-1 eradication. While our approach does not target latently infected cells, it could enhance immune restoration to more efficiently eliminate latent infected cells upon HIV-1 reactivation as suggested (Butera, 2000). Our findings, therefore, suggest that Tfp molecule and related molecules may represent an important candidate for therapeutic aimed the T cell functions in HIV-infected individuals as well as in other immunosupressive diseases.

Materials and methods

Patients

Blood cells from 17 patients were experimented in this study. Informed consent was obtained from all subjects as reported previously (Lu and Andrieu, 2000). The characteristics of patients were summarized in Table 2. Cells from healthy HIV-1 seronegative individuals were obtained from Laënec Hospital (Paris, France). The study was performed in accordance with local ethical committee standards.

Chemical

The Tfp molecule was provided by Sigma–Aldrich (France).

Isolation of lymphoid cells

Lymphocytes were isolated from peripheral blood over a Ficoll–Hypaque density gradient (Eurobio, France). In this study our experiments were performed progressively with freshly isolated PBMCs.

Cells and culture conditions

Heparinized venous peripheral blood was obtained from HIV-1-seropositive adults with $CD4^+$ T cell counts ranging from 100 to 950/mm^3 and from HIV-1-seronegative healthy controls. PBMC were isolated from heparinized venous blood by Ficoll–Hypaque density gradient centrifugation and were cultured in RPMI 1640 (Life Technologies, France) supplemented with 10% heat- inactivated FCS (Gibco, France), 2 mM L-glutamine (Sigma, Irvine, UK), 1 mM sodium pyruvate, and antibiotics (Sigma). PBMCs were activated by phytohemagglutinin (Murex Biotech Bradford, 1 μg/ml) for 3 days. Thereafter the cells were washed and cultured in the presence or absence of the drug in complete medium supplemented with recombinant IL-2 (20 U/ml, Boehringer Gmbh, Germany). Cell seeding consisted of 1×10^6 of viable cells per milliliter for 3 days of culture. For stimulation with CD3 mAb or CD3 and CD28 mAbs (Pharmingen, Los Angeles CA), plates were coated with antibodies (100 ng/ml) before adding PBMCs. Cells were grown at 37°C in vented upright Costar 3065 flasks containing 5 ml of culture medium. Cells were cultured for 3 days in the presence or absence of the drug and then harvested for counting viable cell number by trypan blue exclusion dye testing. To obtain nonadherent cells, PBMCs were subjected to plate adherence for 12 h. Nonadherent PBMC were enriched for lymphocytes and contained less than 1% $CD14^+$ monocytes. Thereafter $CD4^+$ and $CD8^+$ T cells were purified with the use of magnetic beads/DETACH beads (Dynal, Great Neck, NY). To study mechanisms, inhibitor of protein synthesis actinomycin D (0.1 μg/ml) (Sigma, UK) was added to adherent cells for 12 h.

Thereafter the cells were washed in RPMI and cocultured with T lymphocytes.

In vitro infection of PBMC derived from healthy individuals

PBMCs (5×10^6) were incubated with PHA for 48 h. After washing, the lymphocytes were infected with 100 $TCID_{50}$ of clinical HIV-1 isolate (S5) corresponding to a non-syncytium-inducing (NSI) phenotype. After 2 h of incubation, PBMCs were washed and cultured in complete medium as described (Achour et al., 1998).

Lymphocyte proliferation

PBMCs collected from the patients blood were suspended in culture medium [RPMI 1640 supplemented with 10% heat inactivated normal human (serum AB)] at 2.5×10^6 cells per milliliters. One hundred microliters of the cell suspension was added to 100 μl of culture medium containing SEB superantigen (0.1 μg/ml: Sigma, St. Louis, MO), purified protein derivative (PPD, 3000 U/ml), tetanus toxoid (TT, 1300 U/ml) or V3 loop P18 peptide (1 μg/ml, Genosys, London, UK) in the presence or absence of the drug. Plates were incubated for 6 days, and during the last 18 h 0.5 μCi ^{3}H-thymidine incorporation into DNA was measured. All determinations were done in quadruplicate (Achour et al., 1998).

Intracellular cytokine staining

After 3 days of culture, the cells were fixed in a final concentration of 1% formaldehyde at 4°C overnight. Subsequently they were washed in PBS and then in PBS containing 2% FCS and 0.1% saponin (Sigma, St. Louis, MO). PE-conjugated anti-IL-2 or anti-INF-γ mAb (Becton–Dickinson Immunocytometry Systems, San Jose, CA) in 30 μl of PBS/saponin was used to stain each sample for 60 min (30 min at 4°C followed by 30 min at room temperature). The Ki-67 nuclear antigen, which is expressed in late G1, S1, G2, and M phase of the cell division circle, is evaluated using anti-Ki-67 PE (Pharmingen).

Apoptosis assays

Apoptotic cells were measured by FITC-labeled annexin V, a phospholipid-binding protein that preferentially binds to phosphatidylserine exposed at the cell surface in the early phase of apoptosis using a commercially available kit (Immunotech, Luminy, France).

Immunofluorescence staining and flow cytometry

T lymphocyte phenotyping was carried out by flow cytometry. Washed cells were analyzed on a flow cytometer (FACS, Becton–Dickinson Immunocytometry Systems). The variation in cell staining was calculated as [(% of positive cells in treated cells) - (% of positive cells in control cells) / (% of positive cells in control cells)] $\times$ 100%.

p24 enzyme-linked immunosorbent assay

The production of p24 antigen was measured by enzyme immunoassay. Tissue culture supernatants were harvested and stored at -70°C until analysis. The commercial kit used was Abbott (Abbott Laboratories, North Chicago, IL, USA).

Viral quantification by PCR and reverse transcription (RT)-PCR

Total DNA was isolated from 1×16^6 cultured cells by using a DNA purification kit (Pre-A-Gene DNA Purification Systems, Bio-Rad, Hercules, CA). RNA was extracted from 100 μl of culture supernatants by using a commercial RNA isolation solution (RNAzol; WAK-Chemie Medical, Bad Hombourg, Germany). DNA and RNA were then amplified by RT-PCR using adequate gag primers (Lu et al., 1999). Amplified products were detected by using a solid-phase technique. The absorbance of the samples was determined at 405/450 nm using a microplate Reader (Dynatech MRX, Dynal, Great Neck, NY) and directly matched against the standard curve.

Statistics

All results are expressed as means $\pm$ standard deviations (SD). Comparisons were done by Student's *t* test. A *P* value of <0.05 was considering significant.

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