Biochemical and Biophysical Research Communications **281,** 63–70 (2001)
doi:10.1006/bbrc.2001.4300, available online at http://www.idealibrary.com on IDEAL®

V3 Induces in Human Normal Cell Populations an Accelerated Macrophage-Mediated Proliferation-Apoptosis Phenomenon of Effector T Cells When They Respond to Their Cognate Antigen

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Received December 27, 2000

The semi-conserved domain of V3 of HIV-1 was synthesised in a lipopeptide form to be presented on the surface of liposome particles. Composite liposomes were constructed with entrapped tetanus toxoid as a recall antigen (lipo-V3/TT liposomes) to study the influence of V3 on effector T cells of human normal peripheral lymphocyte populations. We demonstrated that lipo-V3/TT liposomes induce a V3-specific response characterised by an early, enhanced proliferation of effector CD4+ T cells, followed by a sharp apoptosis. The phenomenon required the presence of monocyte-derived macrophages and CD4+ T cells, but it was qualitatively and quantitatively distinct from the normal soluble antigen-mediated antigen presenting cell: T cell interaction. Presence of the β-chemokine RANTES in the culture medium inhibited the phenomenon, suggesting that V3 plays a costimulatory role that involves the chemokine receptor CCR5 pathway during the process of antigen presentation to T cells. This observation may be very important if it occurs also in HIV-1 infection, as it may explain the selective and progressive depletion of non-infected effector CD4+ T cells.

Key Words: **V3; lipopeptides; liposomes; T cells; macrophages; tetanus toxoid; HIV; proliferation; apoptosis.**

The human immunodeficiency virus (HIV) is involved in a number of sophisticated interactions with the human immune system (1). Asymptomatic infection with HIV is characterized by a chronic activation of the immune system (2, 3), although the mechanism of this activation is not precisely known. The major surface glycoprotein gp120 has been reported to induce cytokine production in monocyte-derived macrophages (4) and elevation in intracellular calcium and inositol triphosphate in T lymphocytes (5). It is now generally accepted that this is the result of the interaction between gp120 and one of several chemokine receptors from the G-protein coupled receptor super-family, CCR5 and CXCR4 being the main coreceptors for the macrophage-tropic and T cell line-tropic HIV-1 strains respectively (1).

Macrophages appear to play a crucial role in sustaining HIV-1 *in vivo,* serving at least as reservoirs for the virus, implicated also in pathogenesis (2, 3). The precise involvement of macrophages in HIV-1 infection and how they consequently affect the immune system is far from clear.

The third hypervariable region (V3 loop) of gp120 participates in a number of postattachment processes such as virus-cell fusion, syncytium formation and viral tropisms (6–10). Despite reports of the involvement of V3 in T helper cell dysfunction (11, 12), the mechanism via which it contributes to the pathogenesis is still not clearly understood. By using cells isolated from healthy blood donors, we investigated the effect of the semi-conserved domain of V3 in the interaction of macrophages with T helper cells during antigen presentation. In this study, we present evidence of a V3-specific phenomenon characterised by an early and enhanced proliferation of memory T helper cells (CD4+/CD45RO+) which then undergo an early apoptosis. This phenomenon was inhibited by RANTES suggesting involvement of the CCR5 receptor.

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0006-291X/01 $35.00

MATERIALS AND METHODS

Peptides and liposomes. The HIV-1 gp120 peptides V3 (LAI strain, a.a. 304–318) RKSIRIQRGPGRAFY, C4 (CD4 receptor, binding site) SFINMWQEVGKAMYAPPISG, tetanus toxin peptide (a.a. 830–844) QYIKANSKFIGITEL and the control mucin core antigen MUC-1 PAHGVTSAPDTRPAPGSTAP were synthesized using *F-moc*/tBu chemistry (13). These were converted to lipopeptides according to the manufacturer's instructions by covalent binding of serine-S-[2,3-bis(palmitoyloxy)-(2rs)-propyl]-n-palmitoyl-(r)-cysteine, (Boehringer Mannheim Biochemica, Germany) to the above peptides. Liposomes were constructed by the dehydration-rehydration method (14). Briefly, 0.5 μmol of phosphatidylcholine (100 mg/ml, Sigma), 0.5 μmol of cholesterol (10 mg/ml), and 50 μg of the appropriate lipopeptide were mixed and dried under a flow of nitrogen. The film was resuspended in 0.5 ml distilled water and 0.5 ml PBS and sonicated for 2 min. The sample was centrifuged at 10,000*g* for 30 min and 3 μg of tetanus toxoid was added to the supernatant. The mixture was frozen in liquid nitrogen and lyophilised. The liposomes were reconstituted with 100 μl distilled water for 30 min. Non-entrapped material was removed by washing twice with PBS.

Cells. Buffy coats were obtained from healthy, HIV-1 seronegative donors from Venizelio Hospital Blood Transfusion Service, Heraklion, Crete. The isolated peripheral blood mononuclear cells were treated with L-leucyl-L-leucine methyl ester hydrobromide (LLOMe) (Bachem Feinchemikalien AG, Budendorf, Switzerland), as previously described (15). Briefly, the cells were incubated in RPMI 1640, containing 2% foetal calf serum (CIBCO), together with a freshly prepared 0.25 mM LLOMe solution, for 15 min at room temperature. The cells were washed three times in culture medium, containing 2% human serum. After resuspension in RPMI containing 10% human serum the cells were incubated for 2 h at 37°C. During the last 15 min of the incubation, 10 μg DNAse was added to the culture (16). Where appropriate, separation of the CD4+, CD8+, and CD19+ cell populations was carried out with the MACS system (Mylteniil Biotech, Germany) according to the manufacturer's protocol.

Isolation of human macrophages. Monocytes were isolated from peripheral blood mononuclear cells by selective adherance to the solid phase and maintained in RPMI containing 5% human serum and 50 mM 2-mercaptoethanol.

Antigen-specific proliferation and ELISA assays. For proliferation assays, 1×10^6 LLOMe treated cells per ml were cultured in 96-well plates (COSTAR) in RPMI, containing 5% human serum, 50 mM 2-mercaptoethanol and antigen together with 1×10^6/ml irradiated (40 Gy) autologous macrophages. At an appropriate time period cells were harvested after a pulse period of 18 h with ^{3}H-thymidine (Amersham, UK). Counts per minute of each sample were measured in a LS1701 beta counter (Beckman, USA). In inhibition experiments with the regulated-upon-activation normal T expressed and secreted (RANTES) β-chemokine (R&D Systems) were used. Various concentrations of the above chemokine and proliferation were also measured at appropriate time periods. Negative controls used the same cells without any kind of stimulation. IL-2, IL-4, and IFN-γ were measured with ELISA according to the manufacturer's instructions (Quantikine, R&D Systems Europe, UK).

Measurement of cell apoptosis. In order to measure cell apoptosis of certain cell sub-populations, 1×10^6 LLO Me treated cells/ml cocultured with irradiated macrophages and various liposome or soluble TT combinations at appropriate time periods were stained first for CD4/CD45RO surface expression. The cells were collected in tubes, resuspended in 150 μl cold PBS supplemented with 3% inactivated FCS (GIBCO), pH 7.4–7.6, and incubated for 25 min on ice with 5 μl of each of the following fluorochrome labelled antibodies: CD4-RPE-Cy5 (DACO) and CD45RO-RPE (Becton Dickinson). After washing with PBS the cell pellet was resuspended in 100 μl annexin V-fluos labelling solution, containing a certain amount of annexin V-fluos (Boehringer Mannheim Biochemica, Germany) in a buffer of 10 mM Hepes/NaOH, 140 mM NaCl, 5 mM $CaCl_2$ in pH 7.4 and incubated in R.T for 10–15 min. Finally 400 μl of incubation buffer was added to each tube and apoptosis was analysed immediately on a FACS Callibur (Becton Dickinson) using the CELLQuest programme. FLUO has the spectral characteristics of fluorescein (FITC) both excited at 488 nm with emission intensity at the rate of 515 nm.

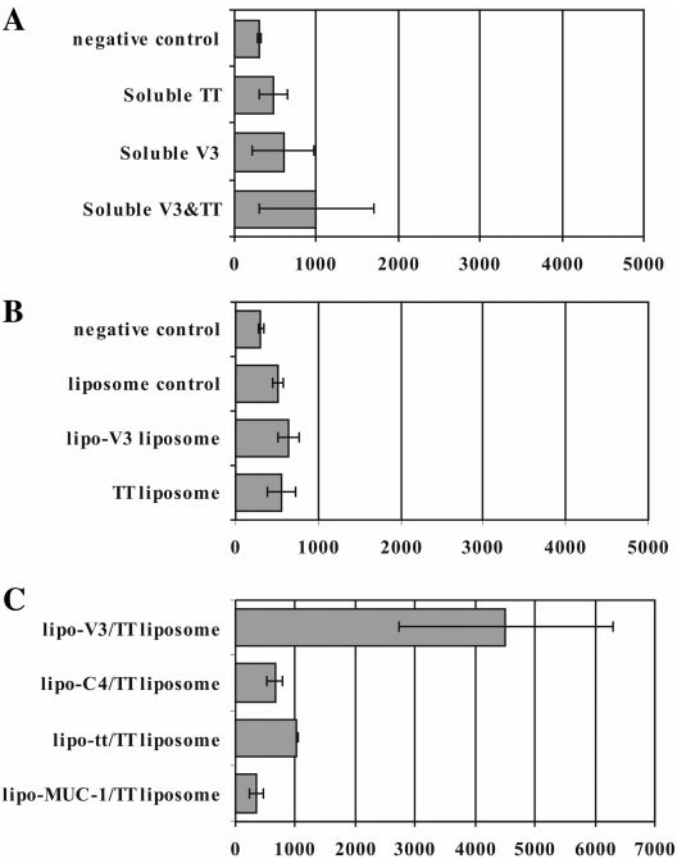

FIG. 1. Activation of human T cells after a 3-day exposure to antigen. (A) Stimulation with 1 μg/ml soluble antigens; TT tetanus toxoid; V3 15 a.a. synthetic peptide from the V3 region (LAI strain). (B) Stimulation with one dose of liposome preparation (see Materials and Methods); liposome control, plain liposomes; lipo-V3 liposome, liposomes with incorporated V3 lipopeptide; TT liposome, liposomes with entrapped tetanus toxoid. (C) Stimulation with composite liposomes with different incorporated lipopeptides and with entrapped tetanus toxoid; lipo-V3, V3 lipopeptide; lipo-C4, lipopeptide from the CD4+ binding site of the gp 120 C4 domain; lipo-tt, lipopeptide from a Th2 epitope of the tetanus toxoid (a.a. 830–844); lipo-MUC-1, lipopeptide from the core mucin MUC-1 tandem repeat unit. Amino acid sequences of the above peptides are stated under Materials and Methods. The cells used were 2×10^5 irradiated APCs and 2×10^5 LLOMe-treated lymphocytes per well; each value represents a mean of tests in triplicate.

RESULTS AND DISCUSSION

LipoV3/TT Liposomes Induce an Antigen-Specific Proliferation of the Corresponding Effector T Cell Population

The human *in vitro* cell system we applied to study the influence of V3 on the host T helper cell-mediated

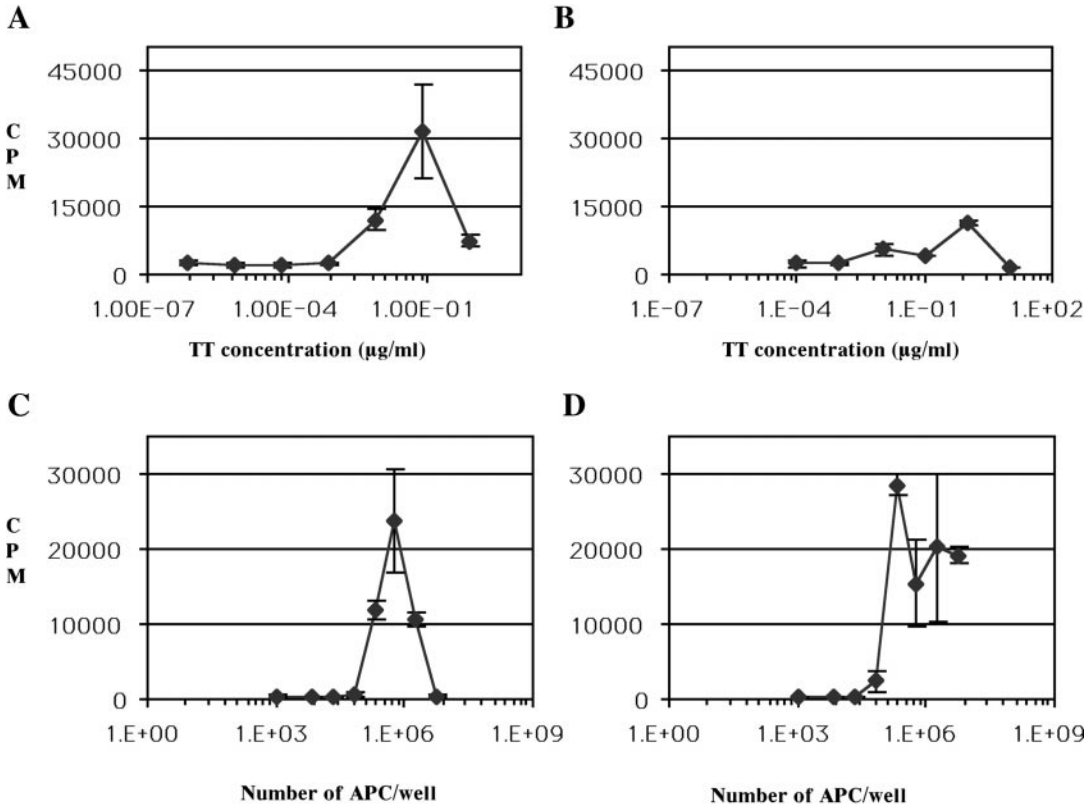

FIG. 2. Comparison of T cell stimulation by composite lipo-V3/TT liposomes and soluble tetanus toxoid: Tetanus toxoid dose-response with lipo-V3/TT liposomes (A) and soluble TT (B); APC requirements for optimal T cell stimulation with lipo-V3/TT liposomes (C) and soluble TT (D). Unless shown otherwise, the cells used were 2×10^5 APCs and 2×10^5 lymphocytes per well; each value represents a mean of tests in triplicate.

immune mechanism (15) consisted of polymorph- and monocyte-depleted peripheral blood lymphocytes (PBL). The average cell composition was 70% CD3+/CD4+, 15% CD3+/CD8+, and 10% CD19+, as estimated by flow cytometry (FACScan, Beckton and Dickinson, USA), and irradiated autologous monocyte-derived macrophages as antigen presenting cells (APCs), all freshly isolated from HIV seronegative normal blood donors. As V3 antigen we used a synthetic 15 amino acid peptide from the semi-conserved domain of the V3 loop containing the GPGRAF motif. Activation of lymphocytes was assessed with proliferation assays using tetanus toxoid (TT) as the recall antigen, hence all donors in the study were selected to be responsive to TT (15). The V3 peptide was presented to the cell cultures in two forms: (i) as soluble peptide antigen, and (ii) as a lipopeptide (lipoV3) immobilised onto the surface of liposomes. Exposure of the cells for 3 days to soluble V3 or TT had no proliferative effect on T cells, although a slight increase was observed when using a mixture of soluble V3 and TT (Fig. 1A). Similar results were obtained when using liposomes with either lipoV3 or entrapped TT (Fig. 1B). Cells stimulated with composite liposomes containing lipo-V3 on the surface and entrapped TT (lipoV3/TT liposomes) exhibited a marked increase in proliferation of T cells (Fig. 1C). This effect was not observed when we used the control lipopeptides lipo-C4 (CD4 binding domain of HIV), lipo-tetanus toxin peptide (a.a. 830–844) or lipo-MUC-1 (core mucin monomer) in composite liposomes with entrapped TT. The above phenomenon of enhanced proliferation was observed in 28 out of 30 donors tested, indicating that it is a highly reproducible event and clearly associated with the immobilised V3, as none of the other formulations had such an impact on T cell activation. Our results concur with similar observations that multi-branched GPGRAF peptides appeared to mimic part of the V3 loop and interact with host cells, in contrast to soluble peptides which had no such effect (17). The observations of both Yahi *et al.* (17) together with our own strongly suggest a multiple interaction of V3 with the cell target.

The Lipov3/TT Liposome-Induced Proliferation Was Distinctly Different from That of Antigen Presentation

To determine whether the lipoV3/TT liposome-induced T cell activation represented a normal antigen-mediated APC − T cell interaction or a distinct

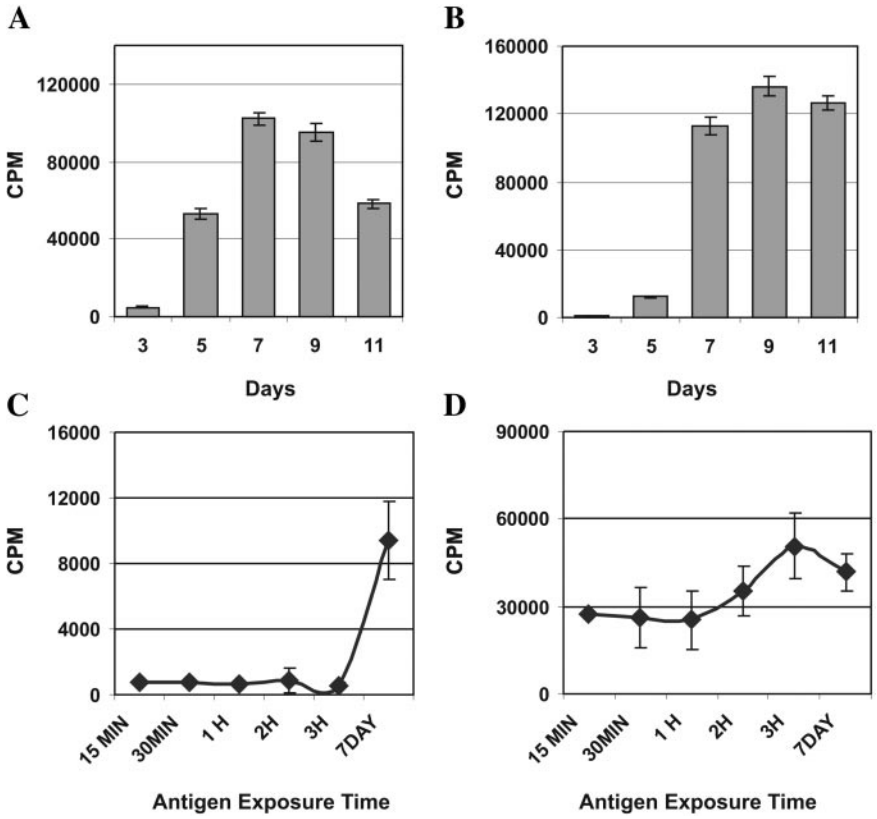

FIG. 3. Comparison of the time course activation by lipoV3/TT liposomes and soluble tetanus toxoid: Proliferation as measured at 2-day intervals with lipo-V3/TT liposomes (A) and soluble TT (B); T cell activation after exposure to antigen lipo-V3/TT liposomes (C) and soluble TT (D) for different lengths of time. Unless shown otherwise, the cells used were 2×10^5 APCs and 2×10^5 lymphocytes per well; each value represents a mean of tests in triplicate.

phenomenon, we compared the requirements for the T cell activation as well as the proliferation profiles in relation to those induced by soluble TT (Fig. 2) and observed noticeable differences:

In dose-response studies, lipoV3/TT liposomes were effective in a narrow window of concentrations with an optimum at 75 ng/ml TT, whereas soluble TT was effective on a much broader range with an optimum at 1 μg/ml (Figs. 2A–2B). The phenomenon was macrophage-dependent, as no proliferation was obtained in total absence of these cells. Indeed, the number of macrophages required for the T cell stimulation by the lipoV3/TT liposomes was restricted to a narrow range with an optimum response at 6×10^5 APCs per well, whereas soluble TT seemed to be effective from 2×10^5 to over 1×10^7 APCs per well (Figs. 2C–2D).

In a time course analysis of T cell proliferation from five donors, we noticed in all cases that in addition to enhanced proliferation, a much earlier response to lipoV3/TT liposomes peaking on days 4–7 followed always by a sharp decline, whereas the response to soluble TT was slower with a peak 2–3 days later (days 7–9) and a less defined decline. A representative response is shown in Figs. 3A–3B.

These results suggest that although the phenomenon was affecting the antigen presentation process, it was distinct from the normal antigen-mediated APC − T cell interaction for the following reasons: it was V3-specific, and the differences in proliferation could not be attributed (a) to excess TT since the liposomes contained 13 times less TT than that used in the soluble TT experiments and (b) to the more efficient phagocytosis of the liposomes by the APCs, as in pulse experiments (Figs. 3C–3D) the uptake of soluble TT was much faster with a maximum T cell activation after less than 3 h exposure to soluble TT. In contrast, no proliferation was induced by the lipoV3/TT liposomes incubated over the same time period.

Preliminary results from a kinetics study of cytokine secretion in three donors (data not shown) indicated that cytokines did not appear to play a major role in the V3-induced phenomenon but were probably produced as a result of the activation.

Lymphocyte Populations Induced by Lipov3/TT Liposomes

To identify the cell populations involved in the lipoV3/TT liposome-induced activation, we measured pro-

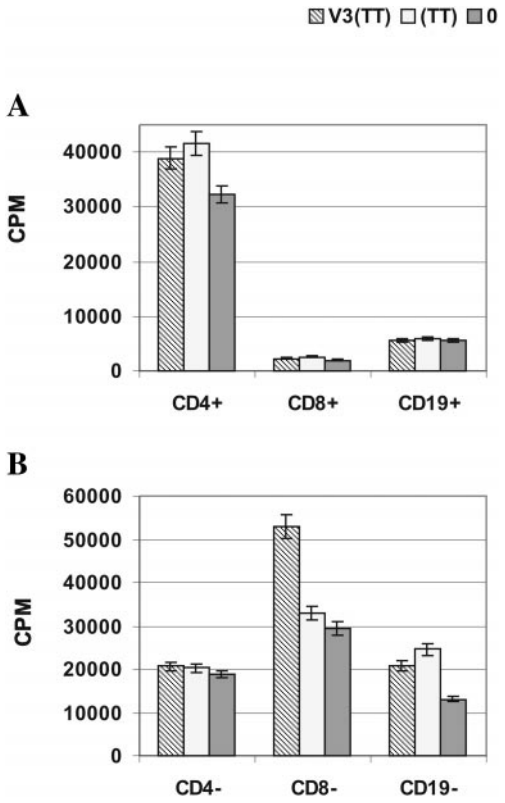

FIG. 4. Lymphocyte subset populations induced by the lipoV3/TT liposomes. (A) Positive selection of CD4+, CD8+, and CD19+ lymphocytes; (B) Removal of CD4+, CD8+, and CD19+ lymphocytes from the LLoMe-treated PBL. V3(TT), LipoV3/TT liposomes; (TT), TT liposomes; 0, negative control. The cells used were 2×10^5 selected lymphocytes and 2×10^5 irradiated APCs per well; each value represents a mean of tests in triplicate.

liferation activity after a positive and negative cell selection in the presence of monocyte-derived macrophages (Fig. 4). In the case of positive selection, CD3+/CD4+ cells responded equally to both lipo-V3/TT liposomes and TT liposomes (Fig. 4A). This suggests that effector CD4+ T cells were activated only by their cognate antigenic stimulus. The relatively high nonspecific proliferation observed may be attributed to cell stimulation by the anti-CD4 antibody used to isolate the cells, while CD3+/CD8+ and CD19+ cells did not respond to liposomes. Depletion of the CD3+/CD4+ cells from the cell mixture (Fig. 4B) rendered proliferation ineffective, which was to be expected since the CD4+ T cells were the only cell population responding in the positive selection experiments. Depletion of CD3+/CD8+ did not affect the lipo-V3/TT liposome-specific proliferation. Interestingly, depletion of CD19+ cells reduced the proliferation. Although this observation may indicate a potentially key involvement of CD19+ cells, we surmise that it is probably due to the severe reduction in availability of CD40 and MHC class II molecules which have been reported to influence the function (18) and survival (19) of the CD4+ T cells.

Occurrence of Apoptosis in Lipov3/TT Liposome-Activated Effector CD4+ Cells

To determine whether the observed sharp decline in lipoV3/TT liposome–induced proliferation was associated with cell death, we performed a parallel time course analysis of T cell activation using proliferation assays and apoptosis measurements using annexin-V staining, a calcium ion-dependent phospholipid-binding protein with high affinity for phosphatidylserine, an exposed surface molecule of lymphocytes during the early stages of apoptosis (20, 21). In preliminary experiments with flow cytometry it was noted that specific apoptosis occurred in $CD4^+/CD45RO^+$ T cells. Similar observations have been reported with T cell subsets expressing CD45RO from HIV-infected patients (22). In all subsequent experiments, apoptosis was monitored in this T cell sub-population. The results indicated that (A) minimal apoptosis was detected at the time the lipoV3/TT liposomes were introduced into the cultures, and (B) the sharp decline of proliferation (Fig. 5A) during exposure of cell cultures to lipoV3/TT liposomes was accompanied by a parallel sharp rise in apoptosis of at least the $CD4^+/CD45RO^+$ cells (Fig. 5C). In contrast, proliferation induced by soluble TT (Fig. 5B) was always followed by a less distinct apoptotic rate of the same sub-population (Fig. 5D). The above results suggest that lipoV3/TT liposomes induce an early and enhanced activation of $CD4^+/CD45RO^+$ cells after which they go into a state of apoptosis. These events are qualitatively and quantitatively distinct from the equivalent events of antigen presentation with soluble reporter antigen (TT). Interestingly, there have been reports that HIV induces indirect apoptosis in uninfected T cells mediated by antigen-presenting cells (23, 24).

Inhibition of the Lipov3/TT Liposome-Induced T Cell Activation

The evidence provided by the lipoV3/TT liposome experiments indicates that V3 interferes with a mechanism that may well be associated with the virus attachment in HIV infection. To identify this potential mechanism, we attempted to inhibit the lipoV3/TT liposome-induced activation using ligands to receptors of relevant biological interest.

Maleyated-bovine serum albumin which binds to macrophage scavenger receptors (25) did not appear to greatly affect the lipoV3/TT liposome-induced proliferation. Similarly, anti-CD26 (kindly provided by Dr. E. Bosmans, Eurogenetics, Belgium) did not appear to affect the T cell activation process (data not shown).

Due to strong evidence that gp120 of HIV-1 interacts with chemokine receptors during the attachment of the

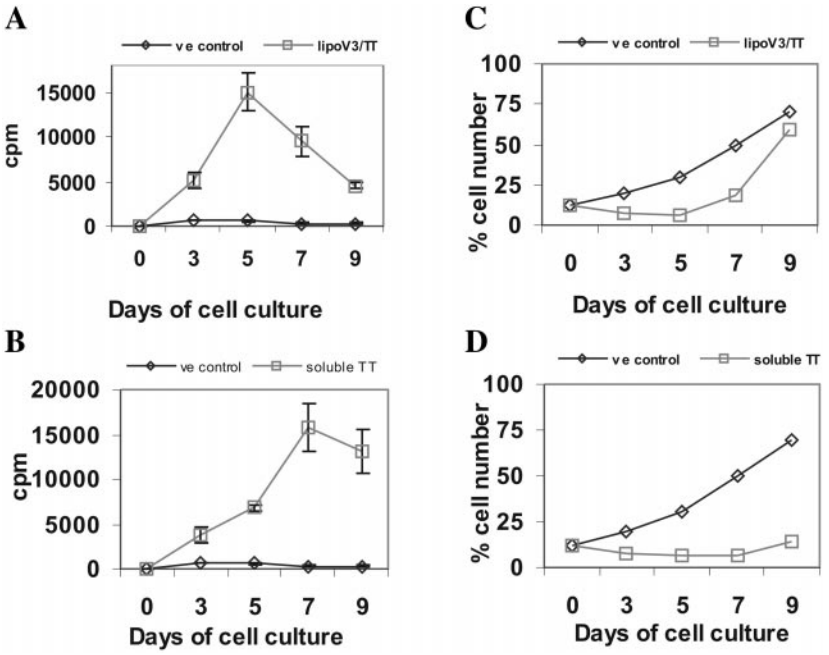

FIG. 5. Comparison of the time course of activation and apoptosis of the subset population $CD4^+/CD45RO^+$ during exposure to lipoV3/TT liposomes and soluble tetanus toxoid: (A) T-cell proliferation after exposure to lipo-V3/TT liposome; (B) with tetanus toxoid; (C and D) percentage of $CD4^+/CD45RO^+$ apoptotic lymphocytes stimulated with the above antigens respectively, as analysed for annexin-V specific binding using FACScan. The cells used in both procedures were 2×10^5 LLoMe-treated lymphocytes cocultured with 2×10^5 irradiated adherent APCs per well, whereas as negative control cells without any antigen stimulation were used. The results represent data from five experiments.

virus to the host cell (see recent review, (26)), we performed competitive inhibition experiments of lipoV3/TT liposome-induced proliferation with regulated-upon-activation normal T expressed and secreted (RANTES) β-chemokine (27), which is the ligand of one of the centrally involved receptors, CCR5. The presence of increasing amounts of RANTES in the culture medium led to a distinct reduction of the T cell proliferation (Fig. 6D). In contrast, RANTES did not affect the soluble TT-mediated activation of T cells (Figs. 6C and 6F). The corresponding control TT liposomes and control cultures containing RANTES did not give any proliferation (Figs. 6B and 6E). The experiment was repeated twice with the same pattern of results. The inhibition of the lipoV3/TT liposome-induced proliferation suggests that V3 may be involved in the CCR5 pathway. If such is the case, either RANTES interferes with the direct interaction of V3 with CCR5, or the interaction of RANTES with CCR5 interferes with the lipoV3/TT liposome-induced proliferation. Further experiments addressing this issue are underway using CCR5 antagonists.

Proposed Model of Action by LipoV3/TT Liposomes

The evidence from the present study indicates that the semi-conserved domain of V3 presented in a composite lipopeptide form can act in a manner not reported before. It appears to deliver a signal that results in an early, enhanced proliferation of effector CD4+ T cells when responding to their cognate antigen, and which is followed by a sharp apoptosis. This pronounced T cell activation could be inhibited by RANTES, an agonist to CCR5, a chemokine receptor that has been implicated in HIV-1 infection. The characteristic differences in cell activation and apoptosis from those when using a soluble form of the same antigen renders this phenomenon unique, with features that resemble certain immune dysfunctions during HIV-1 infection.

We speculate on a potential model of action for the lipoV3/TT liposomes: when antigen-specific memory T helper cells recognise the presented TT epitope via their T cell receptor, they become activated by the classical pathway. These T cells, however, receive an additional signal from a multiple interaction of macrophage membrane-bound V3 molecules and T cell CCR5 molecules. This signal leads to an early and enhanced activation of the interacting T cell and a triggering of the apoptotic pathway. A recent study of HIV-1 and T cell interaction reports that the V3 region of M-tropic HIV-1 strains physically interacts with the sulfated aminoterminal of CCR5 (28), although the effect of such interaction to the T cell was not described.

It has been reported that interaction of HIV-1 gp120 and CCR5 of host T cells leads to calcium influx and

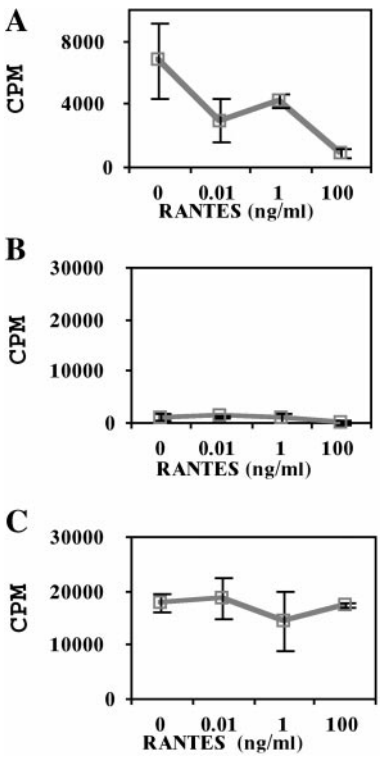

FIG. 6. T lymphocyte proliferation in the presence of a range of concentrations of RANTES β-chemokine and (A) lipoV3/TT liposomes, (B) TT liposomes, or (C) soluble tetanus toxoid. The cells used were 2×10^5 T lymphocytes and 2×10^5 irradiated APCs per well. Cells not exposed to antigen and RANTES were used as negative controls. Each value represents a mean of tests in triplicate after subtraction of the negative control counts.

signalling (29, 30). Normal signal transduction through CCR5 alone has not been shown to have either proliferative or apoptotic effect on T cells, in agreement with our observations. Somma *et al.* (2000) have recently reported that synchronous stimulation of cloned human T cells by normal antigen presentation and gp120 could induce Fas-independent apoptosis implying an apoptotic function within the gp120 molecule (31). This suggests that exposure of HIV-infected macrophages to other pathogens could induce a gp120-dependent apoptosis in the respective effector CD4+ T cells, as the rescue signals would be perturbed. HIV also induces Fas ligand expression in infected macrophages causing apoptosis of uninfected T cells (23, 32). The net result of such events could be selective activation followed by progressive depletion of those effector CD4+ T cells which are most frequently and persistently activated by exogenous stimuli as in the case of opportunistic and chronic pathogens. This hypothesis would explain the frequent occurrence of such infections among HIV patients (33). It also offers a reasonable explanation for the observation that cellular activation is a prerequisite to HIV-induced apoptosis of CD4+ T cells (34), and that most CD4+ T cells which die in HIV-infected patients are not infected (35, 36). In our hypothesis, the macrophages not only act as a reservoir for the virus, but also play a vital role in the depletion of non-infected memory CD4+ T cells. Verification of the contribution of V3 to this intrinsic immunological dysfunction will contribute to a better understanding of the mechanisms that lead to immune deficiency by the HIV-1 virus thus allowing a rational design of novel therapeutic and prophylactic interventions.

ACKNOWLEDGMENTS

We thank Hara Vlata and Stefania Androulaki, Institute of Molecular Biology and Biotechnology, for their technical support. This study was supported by the Hellenic Center for Infectious Disease Control (HCIDC) Grant 3065/95 and the Regional Secretariat General of Crete Grant 40/94.

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