Replication of HIV-1 Deleted Nef Mutants in Chronically Immune Activated Human T Cells

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Lymphocytes (PBMC) obtained from blood of HIV-sera negative Ethiopian immigrants (ETH) were highly susceptible to HIV-1 infection in vitro with no need for stimulation by mitogens. As the HIV nef gene product has been shown to enhance viral replication in stimulated primary lymphocytes, we investigated in this work the role of Nef in viral replication in the ETH cells. Lymphocytes obtained from ETH individuals supported high replication of wild-type HIV-1 and low but significant replication level of the two deleted Nef mutants (encode truncated Nef proteins consisting only of either the first 35 or the first 86 amino acids of Nef). In contrast, no replication was observed in nonactivated cells obtained from non-ETH individuals. After activation of the PBMC from ETH individuals with PHA, replication of both wild-type strains and the two deleted Nef mutant viruses further increased. The CD4+ T cells of ETH individuals exhibited elevated levels of the surface activation markers CD45RO and HLA-DR, compared with T cells derived from non-ETH group. Likewise, expression of the chemokine receptors CCR5 and CXCR4 on these cells was higher in the ETH group than in the non-ETH group. Replication of HIV-1 wild-type and the isogenic-deleted Nef mutants was significantly correlated with the proportion of ETH cells expressing CD45RO and the chemokine receptors. This study suggests that HIV-1 may respond differently to several activation states characteristic of T cells. One activation state, defined by chronically activated lymphocytes from ETH individuals, is permissive to the wild-type HIV-1 and, to a lesser degree, to the Nef mutants. Further activation of these cells by exogenous stimuli enhances replication of the virus. Our results support the notion that Nef enhances the basal level of T cell activation and consequently, viral replication. © 2002 Elsevier Science (USA)

Key Words: HIV-1 replication; nef gene mutants; T cell activation.

INTRODUCTION

The issue of HIV-1 disease progression in Africa is controversial; earlier reports from Africa suggested a faster course of HIV infection with shorter period of survival after the onset of AIDS (Anzala et al., 1995; Mbagga et al., 1990). However, more recent reports, based on a large incidental cohort in rural Uganda, did not find significant differences in HIV disease progression and mortality than similar cohorts in the West (Morgan and Whitworth, 2001; Morgan et al., 2002a,b). Recent studies have implicated immune cell activation, blood level of secreted inflammatory cytokines, and chemokines with frequencies of HIV-1 infection and subsequently with the rate of disease progression (Mellors et al., 1995; Pantaleo et al., 1994; Safrit and Koup, 1995; Rizzarde et al., 1996, 1998; Bentwich et al., 1995; Messele et al., 2001).

We have previously reported that fresh lymphocytes isolated from healthy new immigrants of Ethiopian (ETH) origin were highly susceptible to HIV-1 infection in culture, with no need for stimulation by immune cell activators, such as phytohemagglutinin (PHA). In contrast, lymphocytes obtained in parallel from non-Ethiopian (non-ETH) individuals could be productively infected only following activation by standard T cell activators such as PHA (Shapira-Nahor et al., 1998). Analysis of T cell surface markers in fresh peripheral blood mononuclear cells (PBMC) of the two populations has indicated a striking increase of activated CD4+ cells (HLA-DR+) in the ETH group as compared with the non-ETH group (Shapira-Nahor et al., 1998; Messele et al., 1999). This chronic activation state of the T-lymphocytes could explain the ETH susceptibility to HIV-1 infection.

To further study the state of activation of T cells derived from ETH individuals and the correlation with susceptibility to HIV-1 infection, we investigated, in the present study, infection of PBMC from ETH individuals by HIV-1 mutants, carrying frameshift mutations in the nef gene. The nef gene encodes for an N-terminal modified myristilated protein that is found associated with raft structures in the inner surface of the plasma membrane of the infected cells (Wang et al., 2000; Kaminchik et al., 1994). Several groups have studied the role of Nef in lentiviral replication, using both quiescent and activated
T cells. The nef gene, which is dispensable for HIV-1 replication in T cell lines, enhances viral replication in primary stimulated lymphocytes (De Ronde et al., 1982; Spina et al., 1994; Miller et al., 1994). This property of Nef is especially evident when stimulation by PHA is carried out subsequent to the time of infection, rather than by the standard activation protocols, i.e., cell stimulation prior to infection (Siekevitz et al., 1987; Tong-Starksen et al., 1987; Ott et al., 1997). Expression of the nef gene in primary lymphocytes has also been shown to play a positive role in T cell activation, by lowering the threshold of activation through the T cell receptor (Schrager and Marsh, 1999; Rhee and Marsh, 1994; Alexander et al., 1997). Moreover, recent studies have demonstrated that expression of Nef mediated superinduction of IL-2 and subsequent activation of NFκB transcription factor (Wang et al., 2000; Schrager and March, 1999). Since HIV, through the nef gene, modulates the activation state of the infected cells and thus may regulate its own replication, it was of interest to analyze specifically the phenotype of Nef in T cells displaying different activation states. With this aim in mind, we investigated the replication of two strains of HIV-1 and their isogenic-deleted Nef mutants in PBMC of ETH individuals. We demonstrate in this study that there is a direct correlation between the activation state of T cells and extent of HIV-1 replication.

RESULTS

Replication of HIV-deleted Nef mutants

Our previous studies with lymphocytes obtained from ETH individuals indicated that productive HIV-1 replication in vitro occurred without a need of mitogenic stimulation. The present study was designed to examine whether mutations introduced in the nef gene of two X4-tropic HIV-1 strains of different clades, LAI (clade B) and ELI (clade D), would affect infection of the ETH lymphocytes. The mutations in nef were frameshift mutations leading to the expression of truncated proteins consisting of either the first 35 amino acids of Nef plus 11 missense residues (Nef1) or the first 86 amino acids of Nef with two missense residues (Nef2) (Melanie et al., 1995). PBMC were obtained from 42 ETH individuals (during the first week of their arrival to Israel) and from 6 non-ETH individuals. As can be seen in Fig. 1, at 3, 6, and 9 days postinfection, the deleted Nef mutant viruses replicated in the PBMC of ETH donors to some extent, albeit lower compared with their wild-type (wt) counterparts. The two different deleted Nef mutants manifested similar phenotypes, i.e., an 8- to 10-fold reduction in virus production compared with the parental (wt) strains. Since the extent of T cell activation may play a critical role in HIV-1 replication, we next analyzed infection of the HIV-1-deleted Nef mutants following PHA treatment of PBMC obtained from ETH individuals. Interestingly, we found that chronically activated cells of ETH could be further enhanced by PHA treatment to support three- to fourfold higher replication of the wt HIV, as compared to non-treated ETH cells (Fig. 2A). This phenomenon is even more pronounced after infection of ETH lymphocytes with the deleted Nef mutants (Fig. 2B). While low-level p24 production was observed without PHA treatment, some 40- to 200-fold enhancement is observed following PHA pretreatment (Fig. 2B). A completely different pattern of infection is observed with PBMC of the non-ETH individuals. As observed previously, the replication of wt virus is dependent upon cell activation (Fig. 2C). The HIV-deleted Nef mutants could replicate in the non-ETH cells only after treatment with PHA, although to a lower extent than the wt virus (Fig. 2D).

Analysis of the T cell activation profile in correlation with HIV-1 replication

The activation markers of T cells in chronically activated PBMC from the ETH and non-ETH donors participating in this study were measured by flow cytometry. Results of flow cytometry analysis of 11 ETH of 42 and 6 non-ETH individuals are presented in Table 1. Also included is the level of replication of LAI, LAInef1, and LAInef2 in untreated PBMC of the same individuals. There is a significant correlation between the extent of replication of the two deleted Nef mutants and the proportion of CD45RO cells in the PBMC from ETH donors. For example, donors 3, 4, and 5 have a range of CD45RO CD4 cells of between 84 and 88% and HIV-1 p24 levels of 5.6–6.7 (ng/mL) for LAInef1 and 6.9–7.2 (ng/mL) for LAInef2 (see Table 1, noted in asterisks).

Statistical analysis of lymphocyte surface markers indicated that the proportion of CD4+ lymphocytes that expressed the CD45RO+ marker was higher in the ETH group (42 donors) than in the non-ETH group (6 donors) (63.4 ± 2.17% vs 41.5 ± 1.9%, P < 0.0001), respectively.
Regression analysis of the correlation between the proportion of CD45RO/CD4 cells and the replication of the wt HIV-1 LAI showed a significant correlation (Fig. 3A, $r^2 = 0.4637$, $P = 0.021$). Similarly, the proportion of CD45RO/CD8 cells also showed a significant correlation with replication of the wt HIV-1 LAI (Fig. 3B, $r^2 = 0.4100$, $P = 0.0338$).

Expression of CD45RO antigen on CD4 cells was significantly correlated not only with the replication of wt HIV-1 virus but also with the two deleted Nef mutants (Fig. 3C) and LAInef2 ($r^2 = 0.9116$, $P = 0.0001$) (Fig. 3D) and with that of LAInef2 ($r^2 = 0.9464$, $P = 0.0001$). Similarly, the proportion of CD8 cells that expressed the activation marker CD38 was higher among the ETH group (27.9% vs 12.7% $P = 0.029$). No significant correlation was found, however, between HIV-1 replication and the proportion of CD38+CD8 cells.

**Analysis of CCR5 and CXCR4 expression**

We found that CD4+ T cells of both ETH and non-ETH individuals expressed both the CCR5 and the CXCR4 coreceptors (Fig. 4). However, the proportion of CD4+ cells expressing CXCR4 was significantly higher in the ETH group (42 donors) than in the non-ETH group (6 donors): 19.54% ± 1.46% vs 9.75% ± 2.39% ($P = 0.002$), respectively (Fig. 4). We also found a significant correlation between the proportion of CD4+ cells expressing CD45RO and/or the activation marker HLA-DR and the chemokine receptors CXCR4 and/or CCR5 in the ETH group ($P < 0.001$).

**DISCUSSION**

The present study confirms and extends previous observations, indicating that without addition of a mitogenic stimulus, wild-type HIV-1 replicated well in PBMC from ETH individuals. In parallel, T cells of the same ETH individuals exhibited elevated levels of the activation marker HLA-DR and had a higher proportion of memory T (CD45RO+) cells than cells of non-ETH donors. The chronic immune-activation state of the lymphocytes from the ETH population was strongly associated with intestinal helminthic infections (Shapira-Nahor et al., 1998). Furthermore, expression of the chemokine receptors CXCR4 and CCR5, the two major HIV-1 coreceptors, were higher in the T cells from ETH donors than those from non-ETH donors. Expression of the chemokine receptors CCR5 and CXCR4 and replication of wt HIV-1, significantly correlated with the proportions of cells expressing CD45RO, both among the CD4 and the CD8 cells.

Multiple steps during HIV-1 productive infection of T cells may require an immune activation state. First, cell-surface recognition, as well as penetration of the virus, is dependent on CD4 as well as the coreceptors CXCR4 or CCR5 (Alkhatib et al., 1997; Zheng et al., 1996; Picard et al., 1997). Results of this study, as well as a previous study (Kalinkovich et al., 2001), clearly indicated that T
cells of ETH individuals overexpress the two coreceptors and this may account, at least in part, for the permissiveness of their cells to HIV-1 infection, in the absence of an exogenous stimulus. Second, early steps of viral replication, reverse transcription, and nuclear transport of the proviral DNA are suboptimal following infection of resting lymphocytes (Folks et al., 1986; Bukrinsky et al., 1991) and may also be enhanced in the chronically activated ETH lymphocytes. Finally, and perhaps most important for HIV RNA transcription, is the activation of the cellular transcription factor NFκB in the stimulated lymphocyte (Nabel and Baltimore, 1987; Wang et al., 2000). While the optimal level of immune activation required for each of these phases of the viral life cycle is not known at this stage, our data indicate that viral replication is higher in the chronically activated T cells from ETH donors, expressing the highest level of activation markers (Table 1).

Mitogenic stimuli elicit T cell activation by setting into motion a cascade of intracellular events. Nuclear proteins that regulate early T cell activation genes include activator protein-1 (AP-1), octamer factor-1 (Oct-1), and particularly NFκB, which binds to DNA sequences within the promoters of interleukin-2 (IL-2) and IL-2 receptor genes (Emmel et al., 1989; Grillsi et al., 1993). Activation of NFκB, a critical element in T cell stimulation (Bours et al., 1992; Grillsi et al., 1993), is in turn regulated by the ZAP-70/SLP-76 pathways (Herndon et al., 2001). The regulation of NFκB activation by ZAP-70 is controlled by yet another factor Vav-1 (Costello et al., 1999). Differences were found in the response of T cells to stimuli that were administered through the T cell receptors (TCR) as compared with stimuli that bypass these receptors. Thus, while activation of T cells, mediated through the T cell receptors, elicited a strong activation of NFκB, activation of T cells by lectins was a relative weak inducer of the NFκB (Tong-Starksen et al., 1987; Emmel et al., 1989).

Results of the present study indicate that chronic activation of T cells, as found in ETH individuals, renders the lymphocyte susceptible to some degree of replication of wt HIV-1 and its isogenic deleted Nef mutants. Additional stimulation of the cells by short-term exposure to PHA further augments the susceptibility of the cells to

### Table 1

| T Cell Activation Markers in PBMCs from ETH and non-ETH Donors, and Comparison to HIV-I Replication in These Cells |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| T cell surface markers (%)                      | HIV replication                                    |
| CD4    | CD3    | CD8    | CD45RA/CD8 | CD45RO/CD8 | CD38/CD8 | CD45RA/CD4 | CD45RO/CD4 | HLA-DR+/CD4 | LAI wt | LAI wt | LAI Nef1 | LAI Nef2 |
| Donor ETH                                      |                                                 |                                                 |                                                 |
| 1      | 45.0   | 88.0   | 37.2       | 60.3       | 58.6     | 19.3       | 37.3       | 64.7       | 11.2   | 48.0   | 3.3     | 3.6     |
| 2      | 44.0   | 78.0   | 36.6       | 70.7       | 40.0     | 13.5       | 41.4       | 64.6       | 10.8   | 64.0   | 1.8     | 2.3     |
| 3      | 35.5   | 78.0   | 36.0       | 63.3       | 51.7     | 35.4       | 37.8       | 66.0       | 13.4   | 26.0   | 2.0     | 2.9     |
| 4      | 34.1   | 86.1   | 44.0       | 63.6       | 71.1     | 20.0       | 27.3       | 84.6*      | 18.1   | 64.0   | 5.0**   | 7.0**   |
| 5      | 21.4   | 90.5   | 62.0       | 70.6       | 60.2     | 52.1       | 22.6       | 88.0*      | 15.8   | 68.0   | 5.6**   | 7.2**   |
| 6      | 43.5   | 94.0   | 42.5       | 61.9       | 52.9     | 31.8       | 20.5       | 86.7*      | 17.6   | 54.0   | 6.7**   | 6.9**   |
| 7      | 39.5   | 71.0   | 33.0       | 77.1       | 15.1     | 25.3       | 54.0       | 48.0       | 11.7   | 40.0   | 0.2     | 0.2     |
| 8      | 36.6   | 72.0   | 41.0       | 65.8       | 23.9     | 18.4       | 34.2       | 67.2       | 9.8    | 32.0   | 2.1     | 2.6     |
| 9      | 36.5   | 79.3   | 42.5       | 77.7       | 22.1     | 30.0       | 43.0       | 54.0       | 12.4   | 32.0   | 0.4     | 0.3     |
| 10     | 37.0   | 81.0   | 48.4       | 92.2       | 14.7     | 17.0       | 40.0       | 68.0       | 12.2   | 40.0   | 1.8     | 2.0     |
| 11     | 35.0   | 72.0   | 38.0       | 83.8       | 13.4     | 28.8       | 59.0       | 51.0       | 11.1   | 32.0   | 0.3     | 0.2     |
| Mean   | 37.1   | 80.9   | 41.9       | 71.5       | 38.5*    | 26.5       | 37.9       | 67.5*      | 13.1   | 45.5   | 2.7     | 3.2     |
| SD*    | 6.5    | 7.8    | 7.9        | 10.1       | 21.3     | 11.0       | 11.9       | 13.9       | 2.8    | 15.0   | 2.2     | 2.7     |
| Donor non-ETH                                  |                                                 |                                                 |                                                 |
| 1      | 36.6   | 58.9   | 27.7       | 86.4       | 12.3     | 19.2       | 70.9       | 37.9       |                                                 |
| 2      | 45.2   | 62.7   | 23.5       | 98.7       | 15.6     | 12.6       | 68.8       | 43.7       |                                                 |
| 3      | 40.4   | 56.3   | 16.9       | 82.7       | 16.4     | 18.9       | 61.3       | 40.2       |                                                 |
| 4      | 42.3   | 61.7   | 33.1       | 82.7       | 23.3     | 20.1       | 65.2       | 50.2       |                                                 |
| 5      | 38.6   | 61.7   | 22.7       | 65.4       | 40.8     | 14.5       | 65.8       | 39.2       |                                                 |
| 6      | 27.9   | 55.5   | 22.9       | 68.8       | 45.7     | 22.0       | 64.6       | 38.1       |                                                 |
| Mean   | 38.5   | 59.5   | 24.5       | 80.8       | 25.7*    | 17.9       | 66.1       | 41.5*      |                                                 |
| SD     | 6.0    | 3.0    | 5.4        | 12.2       | 14.1     | 3.8        | 3.4        | 4.7        |                                                 |

* High levels of CD45RO+CD4+ cells.
** High levels of replication of HIV-1 deleted Nef mutants.
* SD, values are means ± standard deviation.
* P < 0.001 as compared to CD45RA/CD8.
* P < 0.001 as compared to CD45RA/CD4 (ANOVA).
HIV-1 infection. At present it is unknown which pathways in the chronically activated ETH cells are responsible for these effects.

The susceptibility of lymphocytes to HIV-1 infection may also depend upon the expression of CXCR4 and CCR5 chemokine receptors. CCR5 and CXCR4 are differentially expressed on memory vs naive cells (Bleul et al., 1997). Among CD4+ cells, CCR5 was almost completely confined to the CD45RO+/CD62L+ memory subset but was absent from the CD45RA+/CD62L subset (Lee et al., 1999). In HIV-1-infected ETH individuals the level of both CCR5 and CXCR4 was found to be elevated on CD4+ cells of the ETH group (Kalinkovich et al., 2001 and present study). The elevated levels of both CCR5 and CXCR4, the HIV-1 coreceptors, could contribute to the increased susceptibility of ETH lymphocytes to HIV-1 infection.

How does Nef enhance HIV-1 replication? Some experimental evidence has indicated that the nef gene product is involved in reducing the threshold of T cells for activation by exogenous stimuli (Luria et al., 1991; Niederman et al., 1992; Greenway et al., 1995; Collette et al., 1996; Iafrate et al., 1997; Gulizia et al., 1997). Alternatively, Nef expression may cause a low level of chronic cell activation even without a mitogenic stimulus (Zaunders et al., 1999). In addition, Nef has multiple functions affecting viral replication through mechanisms not necessarily related to T cell activation (Miller et al., 1994; Wang et al., 2000; Geleziunas et al., 2001). It has been shown, for example, that Nef expressed in nonlymphoid cells enhances the reverse transcription of proviral DNA (Skowronski et al., 1993; Baur et al., 1994) and down-regulates several expressed cell-surface receptors, i.e., CD 4 and HLA class I (Garcia and Miller, 1991). It was of interest, therefore, to study replication of HIV-1-deleted Nef mutants in the chronically activated PBMC from ETH individuals as compared with those from non-ETH indi-
viduals. As the Nef protein has several apparent functions mediated by different sequence domains, we chose in this work to delete the nef gene, such that only a short peptide is expressed, lacking of all known functions (Wang et al., 2000). Since we have detected some replication of the nef-deleted virus in ETH lymphocytes, none of the known functions of Nef appears critical for virus replication in these cells. In the cells from ETH donors there was a low but significant replication of the HIV-1-deleted Nef mutants. Stimulation of PBMC from ETH donors by PHA further enhanced replication of the HIV-1-deleted Nef mutants. In parallel, as expected, no replication of wt HIV-1 and the two deleted Nef mutants was observed with unstimulated PBMC from non-ETH donors. Taken together, our results suggest that the virus may respond to several activation states characteristic of T cells. One activation state, defined by the chronically activated lymphocytes present in the ETH population, allows the replication of the wild-type virus and to a lesser degree the deleted Nef mutants. These results are compatible with the notion that Nef enhances the level of T cell activation and consequently it promotes viral replication.

The viruses used in this study only use the CXCR4 coreceptor. Similar results would presumably be expected using the R5 viruses since, as showed in the results, the ETH lymphocytes expressed elevated levels of CCR5 coreceptor, indicating that there is no obvious restriction for infection with the R5 virus. Furthermore, if there will be a restriction, it is probably due to an increased production and secretion of β chemokines by the activated cells that might specifically block R5 virus replication.

**MATERIALS AND METHODS**

**Human subjects**

The study population consisted of two groups of individuals, 50 HIV-1-negative ETH and 6 HIV-1-negative non-ETH. The ETH individuals participating in this study arrived in Israel as immigrants, between the years 1999 and 2001, and blood was collected during the first week of their arrival.

**PBMC**

Blood from HIV-1-negative individuals was layered onto a Lymph-prep solution (Nycomed, Oslo, Norway) and spun at 2000 rpm for 20 min. The interlayer containing PBMC was collected and washed twice with PBS. PBMC (10^6/ml) were cultured (75-ml flasks, Costar, Cambridge, MA) in RPMI 1640 medium, supplemented with 20% FCS (Gibco Co.), 1 mM glutamine, penicillin (0.6 μg/ml), and streptomycin (60 μg/ml).

**Molecular clones of HIV-1 and deleted Nef mutants**

The infectious molecular clones of the T cell tropic HIV-1 strains LAI and ELI1 have been described (Peden et al., 1991), as have the Nef mutants of these viruses LAInef1, LAInef2, ELI1nef1, and ELI1nef2 (Melanie et al., 1995). The mutations in the nef gene are frameshift mutations at the Xhol site (nef1) or the Bglll site (nef2), resulting in proteins comprising the first 35 amino acids of Nef followed by 11 missense residues for the Nef1 protein and the first 86 amino acids with two missense residues for the Nef2 protein (named deleted Nef mutants).

**HIV infectivity assay**

Viral stocks were generated from the infectious clones by transfection of HEK 293 cells and were used for infection of PBMC as described previously (Shapira-Nahor et al., 1997). Briefly, equivalent quantities of virus, normalized by reverse transcriptase activity (4 × 10^6 RT cpm), were absorbed to 10^5/ml PBMC (prepared as described above) for 2 h at 37°C, and then the cells were diluted in 5 ml enriched RPMI. At approximately 3-day intervals, the cells were fed with RPMI and 10% FCS and samples (1 ml) were taken for p24 antigen determinations.

For lymphocyte activation, cells were cultured in the presence of PHA (2.5 μg/ml, Sigma Chemical Co., St. Louis, MO) for 2–3 days before virus infection (Shapira-Nahor et al., 1997).

**Quantitation of virus replication**

Quantitation of virion-associated p24 antigen released into culture supernatants assessed virus production. Samples of 1 ml were taken from each culture (5 ml) at the indicated times and stored at −70°C until completion of the experiment. Batch assays for p24 antigen were performed by the enzyme-linked immunosorbent assay (ELISA) method (Organon Teknika, Boxtel, NL). Supernatants from uninfected cell cultures were included as negative controls. The concentration of p24 released in the culture supernatants was calculated by interpolation of the optical density (405 nm) values of the viral samples with a standard p24 antigen curve, both measured concurrently with the same ELISA apparatus.

**Immunofluorescent assay and FACS analysis**

Cell suspensions, at a volume of 50 μl (5 × 10^5 cells), were stained with the following labeled monoclonal antibodies (mAbs): fluorescein isothiocyanate (FITC)-anti-CD8, FITC-anti-CD4, phycoerythrin (PE)-anti-CD3, PE-anti-CD38, PE-anti-CD45RA, PE-anti-CD45RO, PE-anti-CCR5, PE-anti-HLA-DR (Pharmining, San Diego, CA), and PE-anti-CXCR4 (R&D Systems Inc., MN). The cells were incubated with FITC and/or PE-conjugated mAbs.
Statistical analysis

The results of HIV-1 infection and T cell activation markers are presented as the mean values ± SD of the mean of a number of independent experiments. The results of CXCR4 and CCR5 are presented as the mean values ± SEM. The data were evaluated statistically using the paired or unpaired Student’s t test and the Mann–Whitney rank sum test; P values < 0.05 were considered significant. Linear correlation analysis was performed using SigmaPlot and SigmaStat (Jandel, San Rafael, CA).

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(10–50 µl) against various surface antigens. After 30 min of incubation at 4°C, the cells were washed twice with PBS containing 0.1% BSA and 0.1% NaN₃. Thereafter, they were fixed in a solution of 1% paraformaldehyde in PBS and stored at 4°C until analysis on a FACScan flow cytometer (Becton-Dickinson) using the Consort 30 program. Double immunofluorescent staining was determined by two-color flow cytometry using the contour-staining program of Consort 30.