We investigated the percentage of CD45RA+ and CD45RO+ T cells in peripheral blood and the intracellular glutathione redox balance in these lymphocyte subsets in patients with human immunodeficiency virus type 1 (HIV-1) infection and healthy controls. In HIV-1-infected patients there was a preferential depletion of CD45RA+CD4+ cells, which was most pronounced in symptomatic patients. In CD4+ lymphocytes from HIV-1-infected patients the glutathione abnormalities were clearly most pronounced in the CD45RA+ subset with a marked increase in level of oxidized glutathione and decreased ratio of reduced to total glutathione as the major characteristics. These abnormalities were shown in CD45RA+CD4+ lymphocytes from both symptomatic and asymptomatic patients, whereas similar abnormalities in CD45RO+CD4+ T cells in peripheral blood and the intracellular glutathione redox balance in these lymphocyte subsets in patients with human immunodeficiency virus type 1 (HIV-1) infection. We have recently shown markedly disturbed intracellular glutathione homeostasis in CD4+ lymphocytes from HIV-1-infected patients, particularly in patients with advanced clinical and immunological disease, with elevated levels of oxidized glutathione as the major feature.

Peripheral blood (PB) T lymphocytes are divided into two functionally different subsets based on the reciprocal expression of CD45RO and CD45RA antigens. Responses to recall antigens are confined to T lymphocytes with the CD45RO phenotype. Moreover, as CD45RA+ cells lose CD45RA expression and concomitantly gain CD45RO expression after in vitro activation, it has been postulated that CD45RA and CD45RO expression define naive and memory cells, respectively. Recent studies have suggested that the decline in CD4+ lymphocyte counts during HIV-1 infection is primarily caused by a decrease in numbers of naive CD4+ lymphocytes, because the naive and memory subsets have different functional activities (e.g., activation requirements, proliferation, and cytokine secretion patterns). These changes may have important consequences. Furthermore, it is important to establish if the previously demonstrated glutathione abnormalities in CD4+ lymphocytes during HIV-1 infection merely reflect altered distribution of naive and memory CD4+ lymphocyte subsets. The mechanisms causing this preferential decline in naive T cells are unknown. However, as several lines of evidence suggest that disturbed intracellular glutathione redox balance may be of importance for the increased apoptosis in lymphocytes seen during HIV-1 infection, it is important to investigate if CD45RA+CD4+ lymphocytes differ from CD45RO+CD4+ lymphocytes with respect to intracellular glutathione redox balance in HIV-1-infected individuals. Therefore, in the present study we analyzed the percentage of CD45RA+ and CD45RO T cells in PB and the intracellular glutathione redox balance in these lymphocyte subsets in HIV-1-infected patients and healthy controls.

MATERIALS AND METHODS

Patients and controls. Blood samples were obtained from 14 HIV-1–infected patients (11 homosexual men, 2 women with heterosexual transmission, and 1 hemophilic). Patients were clinically classified according to the revised criteria from Centers for Disease Control and Prevention. Clinical and immunological characteristics of the study group are summarized (see Table 1). Patients with ongoing acute infection at the time of blood collection (2 months before to 1 week after), intravenous drug users, patients abusing alcohol, and patients with serum levels of alanine aminotransferase > 40 U/L or serum creatinine levels > 70 mmol/L were not included in the study. Six patients were receiving zidovudine (median dosage 600 mg/d, range 300 to 900 mg/d) and six patients were receiving Pneumocystis carinii prophylaxis at the time of the study. However, none of these patients had initiated therapy or changed dosage regime during the last 6 months.

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Table 1. Clinical and Immunological Characteristics of the Study Group

<table>
<thead>
<tr>
<th></th>
<th>HIV-1–Infected Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Age in yr, median</td>
<td>38</td>
<td>40</td>
</tr>
<tr>
<td>ranges</td>
<td>25-49</td>
<td>26-51</td>
</tr>
<tr>
<td>Males/ Females</td>
<td>12 (86%) / 2 (14%)</td>
<td>12 (86%) / 2 (14%)</td>
</tr>
<tr>
<td>CD4+ lymphocytes (x10^6/L)</td>
<td>220 ± (150-310)</td>
<td>625 (520-900)</td>
</tr>
<tr>
<td>CD8+ lymphocytes (x10^6/L)</td>
<td>680 ± (450-1000)</td>
<td>400 (290-540)</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>32 ± (20-53)</td>
<td>7 (0-8)</td>
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Six patients were classified as asymptomatic (CDC group A) and eight as symptomatic (six in CDC group B and two in CDC group C) HIV-1–infected patients. Data are given as medians and 25th to 75th percentiles if not otherwise stated.
* P < .01 compared with controls.
† P < .001 compared with controls.

Controls were 14 sex and age-matched healthy, volunteer, unpaid, HIV-1–seronegative blood donors (Table 1). The percentage of smokers was equal in the patient and the control group. Informed consent was obtained from all participants in the study.

Isolation of CD45RA+ and CD45RO+ lymphocyte subsets. PB mononuclear cells (PBMC) were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) gradient centrifugation within 30 minutes after blood sampling. Mononuclear cells were washed twice in Hanks’ balanced salt solution (HBSS; Gibco, Paisley, UK) and finally resuspended in phosphate-buffered saline (PBS) with 0.3% bovine serum albumin (BSA; Calbiochem, La Jolla, CA) at a concentration of 15 x 10^6 PBMC/mL. Further positive selection of cell subsets by monodisperse immunomagnetic beads was done at 4°C as previously described.20,21 Briefly, PBMC were mixed with beads coated with anti-CD4 antibodies (Dynabeads M-450 CD4; Dynal, Oslo, Norway) or anti-CD8 antibodies (Dynabeads M-450 CD8, Dynal) in a cell-to-bead ratio of 1:20 with anti-CD45RA (clone L48, Becton Dickinson, San Jose, CA) and anti-CD45RO (clone UCHL1, Pharmingen) and fluorescein isothiocyanate (FITC) conjugated anti-CD45RA (clone L48, Becton Dickinson). Samples were fixed with 1% paraformaldehyde and analyzed using a FACScalibor flow cytometer (Becton Dickinson). All samples included staining with isotype matched control antibodies. Data were acquired with CellQuest software (Becton Dickinson) and list mode files were collected for 25,000 cells from each sample. Foward and side scatter were used to gate lymphocyte-sized cells and the numbers of CD45RA+ and CD45RO+ cells within the CD4+ and CD8+ subpopulations analyzed. The boundaries between the stained and unstained populations were set using the isotype control settings such that <1% of the events in the control tube were scored as positive.

The absolute numbers of CD45RA+ and CD45RO+ lymphocytes in PB were calculated by multiplying the percentage of CD45RA+ CD4+, CD45RO+ CD4+, CD45RA+CD8+, or CD45RO+CD8+ lymphocytes with the absolute numbers of CD4+ or CD8+ lymphocytes in PB.

Statistical analysis. For comparison of two groups of individuals, the two-tailed Mann-Whitney U test was used. When more than two groups were compared, the Kruskal-Wallis test was used. If a significant difference was found, Fisher’s least significant difference was computed on the ranks to determine the differences between each pair of group. Coefficients of correlation (r) were calculated by the Spearman Rank Test. The calculations were performed using the Statistica (StatSoft, Tulsa, OK) software package. Data are given as medians and 25th to 75th percentiles if not otherwise stated. P values are two-sided and considered significant when <.05.

RESULTS

Distribution of CD45RA+ and CD45RO+ CD4+ lymphocyte subsets in HIV-1–infected patients and in healthy con-
... and asymptomatic HIV-1-infected patients, respectively. Furthermore, these glutathione parameters significantly correlated with levels of oxidized glutathione and ratio of reduced to total glutathione, respectively. Furthermore, these glutathione parameters were significantly inversely correlated with levels of oxidized glutathione and ratio of reduced to total glutathione, respectively.

In 4 asymptomatic HIV-1-infected patients and 4 healthy controls the glutathione redox status was also analyzed in CD45RA+CD4+ lymphocytes isolated by negative selection. Also in negatively selected CD45RA+CD4+ lymphocytes we found that asymptomatic HIV-1-infected patients had significantly higher levels of oxidized glutathione and significantly decreased ratio of reduced to total glutathione than healthy controls (data not shown). Similar to positively selected cells, no significant differences were found between CD45RA+CD4+ lymphocytes from asymptomatic HIV-1-infected patients and healthy controls in levels of either reduced or total glutathione (data not shown).

As can be seen from Figs 1 and 3, among healthy controls intracellular levels of both total and reduced glutathione were markedly higher in CD45RA+ than in CD45RO+CD4+ lymphocytes (~120% [P < .001] and ~100% [P < .001] increase, reduced and total glutathione, respectively). Furthermore, although levels of oxidized glutathione were low in both CD45RO+ and CD45RA+CD4+ lymphocytes from healthy controls, the level of this glutathione species was ~100% higher (P < .005) in the CD45RO+ than in the CD45RA+ subset (Figs 1 and 3). In contrast to healthy controls, we could not show any significant differences in these glutathione parameters between CD45RA+ and CD45RO+CD4+ lymphocytes from HIV-1-infected patients (Figs 1 and 3).

Relationships between glutathione levels in CD45RA+ and CD45RO+CD4+ lymphocytes and other immunological parameters in HIV-1-infected patients. In HIV-1-infected patients we found that numbers of CD4+ lymphocytes in PB were significantly inversely correlated with levels of oxidized glutathione and significantly positively correlated with ratio of reduced to total glutathione in CD45RA+CD4+ lymphocytes (r = -.81, P < .001 and r = .73, P < .005; oxidized glutathione and ratio of reduced to total glutathione, respectively). Furthermore, these glutathione parameters were also correlated with serum levels of TNF-α although the correlation with ratio of reduced to total glutathione did not reach statistical significance (r = .55, P < .04 and r = -.50, P = .06; oxidized glutathione and ratio of reduced to total glutathione, respectively). Neither levels of reduced glutathione in CD45RA+CD4+ lymphocytes nor any of the glutathione parameters in CD45RO+CD4+ lymphocytes...
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Fig 1. Intracellular levels of reduced glutathione (A), total glutathione (B), ratio of reduced to total glutathione (C), and oxidized glutathione (D) in isolated CD45RA"CD4" lymphocytes from 5 patients with asymptomatic HIV-1 infection, 8 patients with symptomatic HIV-1 infection, and 12 healthy controls. Bars represent median values.

Fig 2. Correlation between percentage of CD45RA+ cells of total CD4+ lymphocyte counts and intracellular levels of oxidized glutathione in CD45RA"CD4" lymphocytes in 13 HIV-1-infected patients. The levels of oxidized glutathione in CD45RA"CD4" lymphocytes were also significantly inversely correlated with the absolute numbers of this lymphocyte subset ($r = -0.73, P < 0.005$). Also decreased ratio of reduced to total glutathione, but not decreased levels of reduced glutathione, in CD45RA"CD4" lymphocytes were significantly correlated with decreased proportion as well as decreased absolute numbers of CD45RA"CD4" lymphocytes (data not shown).
were significantly correlated with CD4⁺ lymphocyte counts or TNF-α levels (data not shown).

Thus, the glutathione abnormalities in CD45RA⁺CD4⁺ lymphocytes in HIV-1 infection are not only associated with advanced clinical disease, but also significantly correlated with decreased numbers of CD4⁺ lymphocytes in PB and increased TNF-α activation reflecting advanced immunodeficiency.

**Intracellular glutathione levels in isolated CD45RA⁺ and CD45RO⁺CD8⁺ lymphocyte subsets.** In a recent study we could not show any significant abnormalities in glutathione metabolism in CD8⁺ lymphocytes from HIV-1-infected patients except for a slight increase in ratio of reduced to total glutathione.8 To elucidate if glutathione abnormalities in CD8⁺ lymphocytes during HIV-1 infection are masked by an altered distribution of naive and memory CD8⁺ lymphocyte subsets, we analyzed the proportion of CD45RA⁺ and CD45RO⁺CD8⁺ lymphocytes in PB and the intracellular glutathione parameters in these CD8⁺ lymphocyte subsets in 6 HIV-1-infected patients (4 asymptomatic and 2 symptomatic patients, CDC group B) and in 6 healthy controls.

As can be seen in Table 2, although the HIV-1-infected patients had significantly raised numbers of CD8⁺ lymphocytes in PB (Table 1), this rise was only found in the CD45RO⁺ subset. In fact, there was a decline rather than a rise, in both proportion and absolute numbers of CD45RA⁺CD8⁺ lymphocytes among HIV-1-infected patients, reflecting altered distribution of CD45RA⁺ and CD45RO⁺ subsets among CD8⁺ lymphocytes in HIV-1 infection.

When analyzing the glutathione redox status in isolated CD45RA⁺ and CD45RO⁺CD8⁺ subsets we found a significant decrease in ratio of reduced to total glutathione (20% decrease) and in particular, a marked increase in level of oxidized glutathione (−400% increase) in the CD45RA⁺ subset in HIV-1-infected patients (Table 3). In fact, all HIV-1-seropositive patients had higher levels of oxidized glutathione in CD45RA⁺CD8⁺ lymphocytes than all controls. In contrast, no significant differences between HIV-1-infected patients and controls were found in the CD45RO⁺ subset (Table 3). Furthermore, as can be seen from Table 3, in healthy controls the CD45RO⁺ subset had significantly higher levels of oxidized glutathione than the CD45RA⁺ subset (−200% increase, P < .05). In healthy controls the CD45RO⁺ subset also tended to have lower ratio of reduced to total glutathione than the CD45RA⁺ subset (Table 3).
may be involved in the dysregulated cytokine production
nistic pathogens as well as the continuously mutating HIV-
may have important immunological consequences including
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ied.32-36
The results of the present study indicate in accordance
with other recent reports,13,14,30,31 that naive CD4+ as well as
naive CD8+ lymphocytes are preferentially lost as total
CD4+ lymphocyte counts decline during HIV-1 infection. How-
However, discrepant results have been reported possibly re-
flecting methodological differences and differences in the
degree of immunodeficiency among patient groups stud-
In fact, it appears as found in the present study, that
naive cells,21 it appears established that the T-cell memory
subset and that the naive T
resides within the
subset.5,15,29
The results of the present study indicate in accordance with other recent reports,13,14,30,31 that naive CD4+ as well as naive CD8+ lymphocytes are preferentially lost as total CD4+ lymphocyte counts decline during HIV-1 infection. However, discrepant results have been reported possibly reflecting methodological differences and differences in the degree of immunodeficiency among patient groups studied.32-36 In fact, it appears as found in the present study, that the preferential loss of CD45RA+ T cells is most prominent in advanced clinical and immunological disease.14,37 Nevertheless, a loss of naive T lymphocytes in HIV-1 infection may have important immunological consequences including inability to mount responses to novel antigens, eg, opportunistic pathogens as well as the continuously mutating HIV-1 itself.17 Furthermore, as naive and memory T lymphocytes appear to differ in their potential for cytokine synthesis,9,15,16 altered distribution of naive and memory T lymphocytes may be involved in the dysregulated cytokine production observed in HIV-1-infected individuals.38 Interestingly, it has been reported that naive CD8+ lymphocytes are potent sources of macrophage inflammatory protein 1α and RANTES,39 and these chemokines have very recently been shown to suppress HIV replication in vitro, possibly reflecting the "soluble anti-HIV factor(s)" derived from CD8+ lymphocytes.40
We have previously shown increased levels of oxidized glutathione and decreased ratio of reduced to total glutathione as the major intracellular glutathione abnormalities in CD4+ lymphocytes during HIV-1 infection.5 The present study clearly shows that these previous findings do not merely reflect altered distribution of naive and memory subsets in CD4+ lymphocytes from HIV-1-infected individuals. On the contrary, although glutathione abnormalities were present in both CD45RO+ and CD45RA+CD4+ lymphocytes, they were clearly most pronounced in the CD45RA+CD4+ lymphocyte subpopulation of which a decreased proportion was found in HIV-1-infected patients. Furthermore, although abnormalities in CD45RO+CD4+ lymphocytes were only shown in advanced clinical disease, significant abnormalities were found in CD45RA+CD4+ lymphocytes from both asymptomatic and symptomatic HIV-1-infected patients.
In contrast, our previous report of isolated CD8+ lymphocytes from HIV-1-seropositive patients showing a normal glutathione redox status except for a slight increase in ratio of reduced to total glutathione,5 seems partly to reflect altered distribution of naive and memory CD8+ lymphocytes. In fact, although there was an increase in the CD45RO+ subpopulation comprising normal glutathione redox status, there was a decrease in proportion of CD45RA+CD8+ lymphocytes with raised levels of oxidized glutathione and decreased ratio of reduced to total glutathione in HIV-1-infected patients. These findings underscore the importance of examining isolated naive and memory T cells in addition to CD4+ and CD8+ lymphocytes when studying T cells in HIV-1 infection.
The precise mechanisms leading to CD4+ lymphocyte depletion in general and in particular, to the preferential depletion of CD45RA+ T cells in HIV-1 infection are still unknown. A loss of thymopoietic capacity has been sug-
gested as an important factor. Our present findings of marked glutathione redox abnormalities in CD45RA+CD4+ lymphocytes significantly correlated with decreased numbers of CD45RA+CD4+ lymphocytes in PB and increased TNF-α activity may also represent important, pathogenic factors in this preferential depletion of naive T lymphocytes during HIV-1 infection. Several lines of evidence suggest that disturbed intracellular glutathione redox homeostasis might be involved in the increased apoptosis of T lymphocytes during HIV-1 infection, particularly when associated with increased TNF-α activity. However, caution should be used when drawing conclusions from correlation analyses, and other mechanisms leading to depletion of naive T cells may also be involved in HIV-1-infected individuals. For example, recent in vitro studies have suggested that Fas antigen stimulation is of importance for the increased apoptosis of T lymphocytes during HIV-1 infection. Although this mechanism may also possibly be influenced by intracellular glutathione redox status, the significance of intracellular redox status on Fas-mediated apoptosis has recently been questioned.

The present study also shows that in healthy controls the CD45RO+CD4+ lymphocytes have markedly lower levels of both total and reduced glutathione and also increased levels of oxidized glutathione, than the CD45RA+CD4+ lymphocytes as we have previously suggested. These findings, demonstrating that the intracellular glutathione levels in healthy individuals seem to be markedly different in CD45RA+, compared with CD45RO+CD4+ lymphocytes, extend our knowledge of potentially important differences between human CD45RO+ and CD45RA+CD4+ lymphocytes. Furthermore, the altered glutathione redox status in CD45RO+, compared with CD45RA+CD4+ lymphocytes, together with the recent demonstration of decreased Bcl-2 expression in these memory cells, might be of importance for the increased susceptibility to apoptosis normally found in human memory T lymphocytes.

A preferential loss of CD45RA+CD4+ lymphocytes in PB may not be restricted to HIV-1 infection. For example, we and others have shown this phenomenon in patients with common variable immunodeficiency (CVI). In fact, there are several immunological similarities between HIV-1-infected patients and subgroups of CVI patients including decreased numbers of CD4+ lymphocytes and increased numbers of CD8+ lymphocytes in PB and persistent immune activation in vivo. Interestingly, we have recently found that CD45RA+CD4+ lymphocytes from CVI patients are also characterized by marked glutathione abnormalities, and these glutathione redox disturbances were correlated with increased TNF-α activity. Thus, the mechanisms leading to altered glutathione homeostasis in naive T cells in HIV-1-infected individuals may not be unique for this infection, but may possibly reflect a state of persistent immune activation, particularly in the TNF system, also occurring in other clinical disorders.

In conclusion, the markedly disturbed glutathione homeostasis in naive T cells in HIV-1-infected patients may have profound consequences contributing to both the progression of immunodeficiency and clinical manifestations in these patients. The results of the present study represent further justification for attempts at therapeutical intervention with drugs with effect on the disturbed glutathione metabolism in HIV-1-infected patients.

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Markedly disturbed glutathione redox status in CD45RA+CD4+ lymphocytes in human immunodeficiency virus type 1 infection is associated with selective depletion of this lymphocyte subset

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