Decreased Levels of Total and Reduced Glutathione in CD4+ Lymphocytes in Common Variable Immunodeficiency Are Associated With Activation of the Tumor Necrosis Factor System: Possible Immunopathogenic Role of Oxidative Stress

By Pål Aukrust, Asbjørn M. Svardal, Fredrik Müller, Bodil Lunden, Rolf K. Berge, and Stig T. Frøland

We have previously shown chronic immune activation and enhanced generation of reactive oxygen species in common variable immunodeficiency (CVI). In the present study, we examined levels of glutathione, the dominant intracellular thiol, that play an important protective role against oxidative and inflammatory stress in plasma and in monocytes and lymphocyte subsets in 20 CVI patients and in 16 healthy controls. CD4+ lymphocytes from CVI patients had significantly lower levels of both total and reduced glutathione as well as a lower ratio of reduced to total glutathione compared with healthy controls. This decrease in glutathione levels in CD4+ lymphocytes was most pronounced in the CD45RA+ subset. Plasma levels of total glutathione were also significantly decreased in CVI. In contrast, monocytes from CVI patients exhibited increased levels of both total and reduced glutathione compared with blood donor monocytes. CVI patients had significantly raised serum levels of tumor necrosis factor α (TNFα) and TNFα concentration was strongly associated with glutathione depletion in CD4+ lymphocytes. Furthermore, the lowest levels of both total and reduced glutathione were found in a subgroup of CVI patients characterized by persistent immune activation in vivo, decreased numbers of CD4+ lymphocytes in peripheral blood, and splenomegaly. Finally, supplementation of cell cultures with glutathione-monoethyl ester did significantly enhance interleukin-2 production from peripheral blood mononuclear cells in CVI patients. These glutathione abnormalities in CVI indicate increased oxidative stress, particularly in CD4+ lymphocytes, and intracellular depletion of reduced glutathione of the demonstrated magnitude may have profound implications for CD4+ lymphocyte function and the immunodeficiency in CVI.

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Infectious Diseases, Medical Department A, The National Hospital, before Ig substitution was started. Some clinical and immunologic features in the study group are summarized in Table 1. Based on samples and had experienced recurrent sinopulmonary infections chronic rhinosinusitis, 3 had nodular intestinal lymphoid hyperplasia, before to whom were observed at the Section of Clinical Immunology and controls. was a smoker. Informed consent was obtained from all patients and than 50%.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>CVI</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age in yr (range)</td>
<td>42 (21-67)</td>
<td>41 (22-65)</td>
</tr>
<tr>
<td>Males/females</td>
<td>7 (35%)/12 (65%)</td>
<td>6 (38%)/10 (63%)</td>
</tr>
<tr>
<td>CD2+ lymphocytes (x10^3/L)</td>
<td>900 (645-1,060)</td>
<td>1,025 (740-1,150)</td>
</tr>
<tr>
<td>CD4+ lymphocytes (x10^3/L)</td>
<td>340* (250-470)</td>
<td>560 (470-690)</td>
</tr>
<tr>
<td>CD8+ lymphocytes (x10^3/L)</td>
<td>380 (230-600)</td>
<td>350 (220-400)</td>
</tr>
<tr>
<td>Monocytes (x10^3/L)</td>
<td>110 (30-170)</td>
<td>150 (120-170)</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>270 (230-370)</td>
<td>260 (210-290)</td>
</tr>
<tr>
<td>Neopterin (nmol/L)</td>
<td>371 (19-78)</td>
<td>7 (0-9)</td>
</tr>
</tbody>
</table>

Data are given as medians and 25th to 75th percentiles if not otherwise stated.

* P < .01 versus controls.
† P < .001 versus controls.

**MATERIALS AND METHODS**

Patients and controls. We studied 20 patients with CVI, all of whom were observed at the Section of Clinical Immunology and Infectious Diseases, Medical Department A, The National Hospital, Oslo. The diagnosis of CVI was defined as previously described.\(^{12,14}\) All patients had severely reduced IgG levels in at least two serum samples and had experienced recurrent sinopulmonary infections before Ig substitution was started. Some clinical and immunologic features in the study group are summarized in Table 1. Based on previously defined criteria,\(^{14,15}\) 11 patients had splenomegaly, 7 had chronic rhinosinusitis, 3 had nodular intestinal lymphoid hyperplasia, 3 had bronchiectasis, and 2 had autoimmune disorders. At the time of the study, the serum level of alanine aminotransferase was less than 50 U/L and the serum creatinine level was less than 100 µmol/L in all patients. All patients were treated with subcutaneously self-administered Ig, and during the last 3 months before blood collection all had IgG levels greater than 5.0 g/L. Blood samples for the study were drawn just before Ig administration. All patients showed no signs of overt infection at the time of blood collection (3 weeks before to 1 week after). None was receiving antibiotics or immunosuppressive drugs.

Controls in the study were 16 carefully sex- and age-matched healthy, volunteer, unpaid, blood donors (Table 1). All patients and controls had no family history of coronary heart diseases and none was a smoker. Informed consent was obtained from all patients and controls.

**Blood sampling protocol.** Blood samples were drawn between 8 and 10 AM after an overnight fast. For serum sampling, sterile vacuum blood collection tubes without any additives were immediately immersed in melting ice and were allowed to clot for less than 1 hour before centrifugation at 400g for 10 minutes. For determination of various thiols components in plasma, blood was routinely collected into three evacuated tubes placed in melting ice containing dithioerythritol (DTE; final concentration, 50 µmol/L; Sigma) containing dimethylsulfoxide (DMSO; final concentration, 1%, Merck AG, Darmstadt, Germany) containing dithioerythritol (DTE; final concentration, 50 µmol/L; Sigma) was added before freezing. Serum and plasma samples were stored at −70°C until analysis and were frozen and thawed only once.

**Cell separation.** Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep; Nycomed Pharma AS, Oslo, Norway) gradient centrifugation within 1 hour after blood sampling. Mononuclear cells were washed twice in Hank’s 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 × 10^6 PBMC/mL. Further positive selection of cell subsets by monodisperse immunomagnetic beads was performed at 4°C, as previously described.\(^{19,20}\) Briefly, PBMC were mixed with Dynabeads (Dynal, Oslo, Norway) coated with the appropriate antibodies (anti-CD4, Dynabeads M-450 CD4; anti-CD8, Dynabeads M-450 CD8; anti-CD14, Dynabeads M-450 CD14; or anti-CD19, Dynabeads M-450 Pan-B) in a cell-to-bead ratio of 1:10. The mixture was incubated in a test tube on a rocking platform for 30 minutes, and rosetting cells were isolated by application of a samarium cobalt magnet (Dynal) to the side of the test tube. After four consecutive washes in cold PBS/0.3% BSA, the cell pellet was immediately placed in liquid nitrogen and stored. The nonrosetting cells were carefully removed by a Pasteur pipette and received a new round of treatment with appropriate Dynabeads as described above. Thus, from the same portion of PBMC, CD14+*, CD19+*, CD4+*, and CD8+* cells were consecutively isolated.

For isolation of CD45RA+ and CD45RO+CD4+ lymphocytes, mouse anti-CD45RA (clone L48; Becton Dickinson, San Jose, CA) and mouse anti-CD45RO (clone UCHL1; Pharmingen, San Diego, CA) were bound to Dynabeads precoated with rat antimouse IgGl and rat antimouse IgG2a, respectively. CD4+ lymphocytes, isolated as described above, were detached from beads by incubation with goat antimouse Fab antiserum (DetachAbead; CD4/CD8, Dynal) for 1 hour in room temperature. The cells were then divided in two portions, placed at 4°C, and mixed for 30 minutes in a cell-to-bead ratio of 1:20 with the anti-CD45RA- and anti-CD45RO-coated beads, respectively. The positively selected cells were then washed and stored in liquid nitrogen as described above.

The purity of all the obtained cell populations was greater than 98%, as assessed by staining of cytospin preparations of positively selected cells by the alkaline phosphatase anti-alkaline phosphatase procedure\(^{16}\) using a panel of monoclonal antibodies (MoAbs): anti-CD3 (clone SK 7; Becton Dickinson), anti-CD4 (clone SK 3; Becton Dickinson), anti-CD8 (clone SK 1; Becton Dickinson), anti-CD19 (clone HD 37; Dakopatts, Glostrup, Denmark), anti-CD56 (clone T19; Dakopatts), anti-CD14 (clone M6-P9; Becton Dickinson), anti-CD45RA (clone L48; Becton Dickinson), and anti-CD45RO (clone UCHL1).

**Determination of cysteine and glutathione levels in plasma.** Different lymphocyte subsets, and monocytes. After a median storage time of 8 weeks (range, 4 to 16 weeks), thiol analysis was performed. As previously described,\(^{18}\) the amounts of reduced and oxidized thiols were obtained from blood collected into solutions containing mBtB and NEM, respectively. The total amount of thiol components (oxidized + reduced + protein-bound form) was assayed in non-treated plasma in which a solution of sulfosalicylic acid (final concentration, 5%) containing DTE (final concentration, 30 µmol/L) was added before thawing.

For quantification of intracellular glutathione levels, the isolated lymphocyte subsets and monocytes were extracted with 0.3 mL ice-cold 5% sulfosalicylic acid containing 50 µmol/L DTE, and the precipitated protein and the immunomagnetic beads were immediately removed by centrifugation. Storing of cells with immunomagnetic beads did not influence the intracellular glutathione levels (data not shown). Total free glutathione (reduced glutathione + glutathione disulfide + soluble glutathione mixed disulfide; for simplicity...
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referred to as total glutathione level in the text) and reduced glutathione were determined in the acid extract according to a modification of a chromatographic procedure described previously. Measurement of the various thiol species was routinely performed on blinded samples in duplicates.

**Determination of glutamate in serum.** Glutamate was determined in deproteinized serum by an assay based on derivatization with o-phthalaldehyde and fluorescence detection.

**Interleukin-2 (IL-2) production in PBMC.** PBMC were suspended in RPMI 1640 with 2 mmol/L L-glutamine and 25 mmol/L HEPES buffer (GIBCO) supplemented with gentamicin (40 μg/mL) and 10% heat-inactivated pooled human AB+ serum (culture medium) at a concentration of 10⁶ cells/mL. Endotoxin content of the total culture medium was less than 10 pg/mL (Limulus amebocyte lysate test). The cells were placed in flat-bottomed 96-well microtiter trays (Costar, Cambridge, MA) in a final volume of 200 μL/well and stimulated with phytohaemagglutinin (PHA; Murex, Dartford, UK) at a final concentration of 1:500, staphyloccocal enterotoxin B (SEB; Sigma) at a final concentration of 100 ng/mL, or influenza virus A/Singapore/6/86 (INF; a kind gift from The National Institute of Public Health, Oslo, Norway) at a final concentration of 1:1000. The influenza virus was formalin inactivated and preliminary experiments had shown that the final formalin concentration used did not influence the IL-2 production assay (data not shown). The virus preparation had a protein concentration of 2.736 mg/mL, an ovalbumin concentration of 12.75 μg/mL, and a hemagglutinin concentration of 311.0 μg/mL. In some, but not all, experiments, unstimulated PBMC were included. After stimulation, the cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Patients' cells were always cultured in the same microtiter trays as were control blood donors cells. Supernatants were harvested 7 days later and stored at −70°C until analysis. In experiments examining the effect of glutathione supplementation on IL-2 production, glutathione-monoethyl ester (Bachem, Bubendorf, Switzerland) was added to cell cultures in indicated concentrations together with stimulants, and supernatants were harvested after 48 hours.

In all studies of IL-2 production, the anti-IL-2 receptor antibody, human monoclonal anti-CD25, clone 3G10 (Boehringer Mannheim Biochemica, Mannheim, Germany), was added to each well at the initiation of culture at a final concentration of 5 μg/mL to block IL-2 consumption.

**Cytokine enzyme immunoassays (EIAs).** IL-2 and tumor necrosis factor α (TNFα) concentrations were quantified by EIAs provided from Cayman Chemical (Ann Arbor, MI; IL-2) and Medgenix (Fleurus, Belgium; TNFα) according to the manufacturer's guidelines using microtiter wells coated with one (Cayman) or several (Medgenix) MoAbs against distinct epitopes of the actual cytokine. Samples with high IL-2 levels were diluted and reassayed. There was no cross-reactivity in the IL-2 EIA between IL-2 and the anti-IL-2 receptor antibody that was used in the cell culture experiments (data not shown). At our laboratory conditions, the intra-assay and interassay coefficients of variation for both EIAs were less than 8%; the recovery of exogenously added recombinant IL-2 from the cell culture medium and exogenously added recombinant TNFα from human serum was 94% and 96%, respectively; and the detection limit of the EIAs was 5 pg/mL (TNFα) and 60 pg/mL (IL-2; when samples were analyzed in 1:20 dilution).

**Flow cytometry.** PBMC were examined by two-color immuno-phenotyping using peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 (Leu-3a; Becton Dickinson) in combination with fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO (clone UCHL1; Pharmingen) or FITC-conjugated anti-CD45RA (clone L48; Becton Dickinson), respectively. Samples were fixed with 1% paraformaldehyde and analyzed using a FACSscan (Becton Dickinson) with CellQuest software (Becton Dickinson). All samples included staining with isotype-matched control antibodies. List mode files were collected for 25,000 cells from each sample.

**Determination of lymphocyte subsets and monocytes in peripheral blood.** The numbers of CD2¹, CD4¹, CD8¹, CD19¹ lymphocytes and monocytes were determined by immunomagnetic quantification, which has been shown to agree well with flow cytometry. Quantification was performed immediately after blood sampling, and the relative distribution of cell subsets was used in the subsequent cell separation when calculating the beads-to-cell ratio (see above).

**Measurement of serum neopterin levels.** Neopterin levels were determined by a commercially available radioimmunoassay method (IMMUtest Neopterin; Henning Berlin GMBH, Berlin, Germany) following the procedure recommended by the manufacturer.

**Statistical analysis.** For comparison of two groups of individuals, the two-tailed Mann-Whitney U Test was used. Coefficients of correlation (r) were calculated by the Spearman Rank Test. The calculations were performed using the STATISTICA (StatSoft, Tulsa, OK) and SOLO (BMDP Statistical Software, Los Angeles, CA) software packages. 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**

**Intracellular glutathione levels in different lymphocyte subsets and monocytes.** Analyses of glutathione levels in PBMC subsets from CVI patients showed several significant differences from normal controls. First, as shown in Fig 1, CD4¹ lymphocytes from CVI patients had significantly decreased levels of total as well as reduced glutathione levels (approximately a 35% decrease of both glutathione species). Second, in contrast to CD4¹ lymphocytes, monocytes from CVI patients exhibited increased intracellular levels of both total (approximately a 50% increase) and reduced glutathione levels (approximately a 20% increase) compared with blood donor monocytes, although the difference in reduced glutathione levels did not reach statistical significance (Fig 1). Third, the ratio of reduced to total glutathione levels, which may reflect the intracellular balance between reduced and oxidized glutathione, was significantly decreased in
CD4+ lymphocytes from CVI patients compared with healthy controls (0.85 [0.82 to 0.89] v 0.90 [0.84 to 0.96], \( P < .02 \)). In addition, 4 CVI patients had ratio of reduced to total glutathione in CD4+ lymphocytes less than 0.75, whereas the lowest value found among controls was 0.83. This ratio tended to be lower also in monocytes among CVI patients, although the difference did not reach statistical significance (0.82 [0.63 to 0.86] v 0.85 [0.74 to 0.96], \( P = .1 \); CVI patients and controls, respectively). In CD8+ and CD19+ lymphocytes from CVI patients, this ratio was not significantly different from control values (data not shown).

Concerning intracellular glutathione levels in CD8+ and CD19+ lymphocytes, no significant differences between CVI patients and controls were found (data not shown). Thus, in further correlation analyses, only glutathione parameters in CD4+ lymphocytes and monocytes were used.

**Circulating levels of glutathione, cysteine, and glutamate.** As in CD4+ lymphocytes, plasma levels of total glutathione were significantly lower in the CVI patients than in controls (approximately a 20% decrease; Table 2). However, there were no significant correlations between plasma levels and intracellular levels of glutathione in any cellular subset neither in patients nor in controls (data not shown). Because decreased cysteine and increased glutamate levels in circulation have been suggested to induce decreased intracellular glutathione levels in mononuclear cells,4 these parameters were also analyzed. However, significantly increased plasma levels of reduced cysteine were shown among CVI patients, and no other significant differences were found between patients and controls (Table 2).

**Glutathione levels in relation to clinical and immunologic manifestations.** We have previously defined a subgroup of CVI patients (CVIHyper) characterized by chronic immune activation, low numbers of CD4+ lymphocytes in peripheral blood, and splenomegaly,14,15 and we hypothesized that this state of sustained immune activation might play a role in the development of disturbed glutathione homeostasis in CVI. In the present study, patients were classified as CVIHyper if they had CD4+ lymphocyte counts less than 400 \( \times 10^6 \) \( \text{L} \), serum neopterin levels greater than 22.0 nmol/L, and splenomegaly (see the Materials and Methods). CVINorm \( (n = 9) \) represents patients with CD4+ lymphocyte counts less than 400 \( \times 10^6 \) \( \text{L} \), serum neopterin levels greater than 22.0 nmol/L, and splenomegaly (see the Materials and Methods). CVINorm \( (n = 11) \) represents the other CVI patients. The bars represent median values.

**Table 2. Circulating Levels of Glutathione, Cysteine, and Glutamate in CVI Patients and Controls**

<table>
<thead>
<tr>
<th>Glutathione (( \mu \text{mol/L} ))</th>
<th>CVI ( (n = 20) )</th>
<th>Controls ( (n = 18) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>6.1* (4.2-7.4)</td>
<td>7.6 (6.3-9.1)</td>
</tr>
<tr>
<td>Oxidized</td>
<td>1.7 (1.6-2.1)</td>
<td>2.0 (1.7-2.3)</td>
</tr>
<tr>
<td>Cysteine (( \mu \text{mol/L} ))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>316.4 (288.6-350.2)</td>
<td>306.6 (291.5-355.0)</td>
</tr>
<tr>
<td>Oxidized</td>
<td>119.4 (103.5-130.0)</td>
<td>119.4 (109.0-130.1)</td>
</tr>
<tr>
<td>Reduced</td>
<td>9.9* (9.2-12.2)</td>
<td>8.4 (7.5-10.4)</td>
</tr>
<tr>
<td>Glutamate (( \mu \text{mol/L} ))</td>
<td>21.2 (16.6-28.1)</td>
<td>22.8 (17.9-29.7)</td>
</tr>
</tbody>
</table>

Data are given as medians and 25th to 75th percentiles.

* \( P < .03 \) versus controls.

When comparing plasma levels, CVIHyper had significantly decreased levels of total as well as reduced glutathione in CD4+ lymphocytes compared with other CVI patients (Fig 2), with approximately 40% (total glutathione) and approximately 50% (reduced glutathione) lower levels than the corresponding levels in healthy controls. Also, the ratio of reduced to total glutathione in CD4+ lymphocytes and monocytes was lower in the CVIHyper subgroup compared with the rest of the CVI patients, although the differences did not reach statistical significance (\( P = .11 \) and \( P = .06 \), respectively). In contrast, the increased intracellular glutathione levels found in monocytes from CVI patients could not be related to any of the defined CVI subgroups (data not shown).

Chronic bacterial infections have been implicated in the generation of increased oxidative stress.17 However, in the present study, we could not find any significant associations between the occurrence of neither chronic rhinosinusitis nor bronchiectasis and the demonstrated glutathione abnormalities (data not shown).

**Glutathione parameters in isolated CD4+CD45RA+ and CD4+CD45RO+ lymphocyte subsets.** We analyzed the proportion of CD45RA+ and CD45RO+ CD4+ lymphocytes by flow cytometry in 10 CVI patients and 10 healthy controls, and, in accordance with a recent report,26 we found that CD4+ lymphocytes in CVI patients had a significantly lower...
CD45RA⁺/CD45RO⁺ ratio than did healthy controls (0.42 [0.16 to 0.45] vs 0.98 [0.57 to 1.17], P < .02), with the lowest levels found in the CVIhyper group (data not shown). To investigate if the glutathione abnormalities in CD4⁺ lymphocytes merely reflect increased proportions of CD4⁺CD45RO⁺ cells in CVI, we examined glutathione levels in isolated CD4⁺CD45RO⁺ and CD4⁺CD45RA⁺ subsets in 6 CVIhyper patients and in 6 controls. We found that, although CVIhyper patients had only a modest decrease in glutathione levels in CD4⁺CD45RO⁺ lymphocytes, CD4⁺CD45RA⁺ cells from these patients had a marked decrease in levels of both total and reduced glutathione compared with cells from healthy controls (Table 3). In fact, in CD4⁺CD45RA⁺ lymphocytes from CVIhyper patients, there was approximately a 70% (reduced species) and 60% (total species) decrease in glutathione levels (Table 3). Furthermore, as can be seen in Table 3, among healthy controls intracellular levels of total and reduced glutathione were markedly higher in CD4⁺CD45RA⁺ than in CD4⁺CD45RO⁺ cells (approximately 125% and 110% higher, total and reduced glutathione, respectively). In contrast, in the CVI group, the levels of these glutathione species in these two subsets were almost equal, although there was a moderate decrease in ratio of reduced to total glutathione in CD45RA⁺ compared with CD45RO⁺ cells (Table 3).

**Glutathione status in relation to circulating levels of TNFα.** Among proinflammatory cytokines, TNFα seems to be of particular importance for the regulation of intracellular glutathione levels, and we therefore investigated if CVI patients had increased serum levels of TNFα as a marker for activation in the TNF system. We found a significant increase in circulating levels of immunoreactive TNFα in CVI patients compared with serum levels in controls (Table 1). Furthermore, among CVI patients, the serum level of TNFα was significantly inversely correlated with both total and reduced glutathione levels of CD4⁺ lymphocytes (Fig 3) and with the ratio of reduced to total glutathione in CD4⁺ lymphocytes and monocytes (r = - .75, P < .001; r = - .72, P < .001; CD4⁺ lymphocytes and monocytes, respectively).

**Glutathione parameters in relation to stimulated IL-2 production.** Decreased IL-2 production has been suggested to play an important role in the immunopathogenesis of CVI. Furthermore, decreased intracellular levels of reduced glutathione seems to impair IL-2 production in lymphocytes, although the reports are somewhat conflicting. To further elucidate the relationships between cellular redox status and IL-2 production, PBMC from the same sample as used in glutathione analyses were activated by PHA, SEB, and INF to simultaneously determine IL-2 production in cell supernatants from 15 CVI patients and 14 healthy controls. We found that IL-2 production in SEB- and INF-stimulated PBMC, but not in PHA-stimulated cells, was considerably decreased in CVI patients (SEB, 28.35 ng/mL [22.45 to 38.36 ng/mL] vs 7.89 ng/mL [4.12 to 17.10 ng/mL], P < .005; INF, 0.85 ng/mL [0.58 to 1.15 ng/mL] vs 0.23 ng/mL [0.10 to 0.42 ng/mL], P < .001; PHA, 2.84 ng/mL [1.98 to 3.95 ng/mL] vs 2.19 ng/mL [1.23 to 3.06 ng/mL], P = .15, controls and CVI patients). This significant decrease in IL-2 production was also shown when calculating the IL-2 production per number of CD4⁺ lymphocytes in cell culture (data not shown).

When analyzing the relationships between IL-2 production and glutathione parameters we first found that, among CVI patients, but not among controls, reduced to total glutathione ratio for CD4⁺ lymphocytes and monocytes was significantly correlated with SEB and INF, but not with PHA-stimulated IL-2 production (Table 4). In a second set of experiments, we examined the effect of glutathione-monoethyl ester, which is readily transported into cells and deesterified intracellularly, on SEB- and PHA-stimulated IL-2 production in PBMC from 5 CVIhyper patients and 5 healthy controls. In CVI hyper patients, glutathione-monoethyl ester supplementation increased in a dose-dependent manner both

<table>
<thead>
<tr>
<th>CD4⁺CD45RA⁺ lymphocytes</th>
<th>Reduced glutathione (nmol/10⁶ cells)</th>
<th>Total glutathione (nmol/10⁶ cells)</th>
<th>Ratio of reduced to total glutathione</th>
<th>CD4⁺CD45RO⁺ lymphocytes</th>
<th>Reduced glutathione (nmol/10⁶ cells)</th>
<th>Total glutathione (nmol/10⁶ cells)</th>
<th>Ratio of reduced to total glutathione</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVIhyper (n = 6)</td>
<td>0.36* (0.24-0.49)</td>
<td>1.16 (0.60-1.87)</td>
<td></td>
<td>0.45 (0.33-0.63)</td>
<td>1.40 (1.07-1.48)</td>
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<td></td>
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<tr>
<td>Controls (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td>0.67 (0.46-0.91)</td>
<td>0.90 (0.84-0.93)</td>
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</table>

Data are given as medians and ranges. Glutathione levels in CD4⁺CD45RA⁺ lymphocytes were only analyzed in 5 controls and 4 CVIhyper patients. For definition of CVIhyper, see the legend to Fig 2. * P < .03 versus controls.
Table 4. Correlations (r) in Patients With CVI (n = 15) Between Stimulated IL-2 Production in PBMC and the Ratio of Reduced to Total Glutathione in CD4+ Lymphocytes and Monocytes

<table>
<thead>
<tr>
<th>Ratio of Reduced to Total Glutathione in</th>
<th>CD4+ Lymphocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEB-stimulated IL-2 production</td>
<td>.70*</td>
<td>.75*</td>
</tr>
<tr>
<td>INF-stimulated IL-2 production</td>
<td>.68*</td>
<td>.78*</td>
</tr>
<tr>
<td>PHA-stimulated IL-2 production</td>
<td>.22</td>
<td>.16</td>
</tr>
</tbody>
</table>

* P < .01.

SEB- and PHA-stimulated IL-2 production, although the effect on PHA stimulation did not reach statistical significance (Fig 4). In contrast, the effect of glutathione-monoethyl production in cells from blood donors was only modest (<15% increase for both stimuli, data not shown).

DISCUSSION

The present study shows several glutathione abnormalities in CD4+ lymphocytes from CVI patients compared with cells from healthy controls, with significantly decreased intracellular levels of both reduced and total glutathione as well as a significant decrease in the ratio of reduced to total glutathione levels in CVI. Although the absolute decrease in intracellular levels of reduced glutathione was relatively modest (approximately 35% compared with healthy controls), intracellular depletion at this magnitude of this thiol species may have profound implications for CD4+ lymphocyte function.

Glutathione deficiency in plasma, PBMC, CD4+, and CD8+ lymphocytes has previously been shown in HIV-sero-positive patients, and decreased antioxidant levels have been implicated in the immunopathogenesis of HIV infection. However, the present findings represent the first demonstration of glutathione abnormalities in CVI patients. Although B-cell deficiency is the hallmark of CVI, certain immunologic similarities with HIV infection have also previously been reported in subgroups of CVI patients, eg, decreased numbers of circulating CD4+ lymphocytes, functional abnormalities of T lymphocytes, and persistent immune activation in vivo.

The intracellular level of glutathione is subject to tight regulation through maintenance of a precise redox equilibrium. The demonstrated decrease in reduced to total glutathione ratio in CD4+ lymphocytes may therefore indicate a disturbance in redox status in CVI in these cells. Indeed, it has been suggested that the ratio of reduced to total glutathione is a more important parameter for cellular antioxidant defense against enhanced ROS generation than other glutathione parameters and that decrease in this ratio may be an important indirect parameter of enhanced ROS generation possibly reflecting increased oxidative stress.

We confirm recent studies showing a decreased CD4+CD45RA+/CD4+CD45RO+ ratio in CVI. It has been suggested that altered proportion of CD4+CD45RA+ cells could be related to some abnormalities found in CD4+ lymphocytes from CVI patients. However, with regard to the decreased glutathione levels, we show that, whereas glutathione levels in CD4+CD45RO+ cells were only slightly decreased in CVI patients compared with cells from healthy controls, these patients had markedly decreased levels of both reduced and total glutathione in CD4+CD45RA+ lymphocytes. Interestingly, we also found that, in healthy controls, but not in CVI patients, CD4+CD45RO+ lymphocytes had considerably lower levels of both reduced and total glutathione compared with CD4+CD45RA+ lymphocytes. It has been suggested that decreased levels of bcl-2 proto-oncopogene and its product in CD4+CD45RO+ lymphocytes from healthy controls, by sensitizing these memory cells to apoptotic stimuli, are of import it for the short life span of these cells compared with naive CD4+CD45RA+ lymphocytes. It seems that bcl 2 protein is preventing apoptosis by reducing the intracellular oxidative stress. It is tempting to hypothesize that the markedly decreased glutathione levels in CD4+CD45RA+ lymphocytes in CVI might contribute to the in vivo decrease of this CD4+ lymphocyte subpopulation in these patients.

The reason for decreased levels of glutathione in CD4+ lymphocytes among CVI patients is unknown. However, the association between glutathione deficiency and persistent immune activation, previously shown by us to be related with increased ROS production in CVI, suggests that glutathione depletion in CD4+ lymphocytes may be caused by inflammatory stress leading to enhanced ROS generation and consumption of reduced glutathione.

Increased serum levels of TNFα have not previously been reported among CVI patients, and the present study may seem in some conflict with previous reports from us and others. These discrepancies most probably reflect differences between different ELISAs in detecting circulating TNFα levels. Indeed, we have recently suggested that the ELISA used in the present study may better detect TNFα in complex with soluble TNF receptors than other ELISAs. Furthermore, a recent study in our laboratory has shown increased circulating levels of soluble TNF receptors in these patients (manu-
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found that TNFα stimulation of T-cell lines and endothelial cells decreases total glutathione levels in these cells by causing enhanced ROS generation, which in turn leads to consumption of reduced glutathione.⁴¹ Thus, increased TNFα activation may, at least in part, be responsible for the glutathione abnormalities in CD4⁺ lymphocytes among CVI patients. Furthermore, decrease in intracellular levels of reduced glutathione markedly increase the proinflammatory cellular responses to TNFα stimulation,⁴² and the TNFα activation with enhanced ROS generation and depletion of glutathione levels may thus represent a vicious circle leading to increased levels of oxidative stress in CVI patients, particularly in CD4⁺ lymphocytes.

Although glutathione depletion among CVI patients most probably reflects increased inflammatory stress, other mechanisms may also be involved. Cysteine availability is rate-limiting for glutathione synthesis and lymphocytes seem to depend on extracellular cysteine concentrations for glutathione synthesis.⁴⁷ However, although malabsorption, which may involve loss of sulphur-containing amino acids,⁴⁸ is often found in CVI patients,¹¹ we could not find any decrease in plasma cysteine levels among CVI patients. In fact, levels of reduced cysteine were significantly increased in these patients. Furthermore, the regeneration of reduced glutathione from the oxidized form is dependent on the glutathione reductase system,⁴⁰ and defect in this reductase system (eg, decreased activity of glutathione reductase, NADPH, or enzymes in the pentose-phosphate pathway)²,4⁰ may also be of importance for disturbed glutathione homeostasis in CD4⁺ lymphocytes from CVI patients. Nonetheless, inflammatory stress might also affect these enzyme systems, eg, increase in ROS generation may result in loss of NADPH.⁴⁹

In contrast to the glutathione deficiency found in CD4⁺ lymphocytes, monocytes from CVI patients had increased intracellular glutathione levels compared with healthy controls. The reason for this is unknown, and we could not relate this finding to any particular clinical and immunologic category of patients. It seems that glutathione metabolism may be differently regulated in different cell types.⁴¹ As discussed above, it is hypothesized that increased ROS generation such as that shown in CVI¹⁷ may consume reduced glutathione in some cell types (eg, T lymphocytes).² On the other hand, at least some antioxidant defences can be enhanced by increased ROS generation.⁴¹,⁵⁰,⁵ⁱ and the present study supports the notion that glutathione levels are differently regulated in monocytes and CD4⁺ lymphocytes, as we have previously suggested.⁵² Nevertheless, the ratio of reduced to total glutathione tended to be lower also in monocytes, and this ratio was significant inversely correlated with serum levels of TNFα, suggesting oxidative stress also in monocytes from CVI patients.

The demonstrated glutathione abnormalities possibly reflecting increased oxidative stress could have significant immunologic consequences. Several immune functions such as cytotoxic T-cell activation,⁶ maintenance of normal numbers of circulating CD4⁺ lymphocytes,⁵ and, of particular interest, generation of Ig secreting cells,²,⁴ are partly dependent on adequate glutathione levels. Furthermore, it is well documented that T cells from some CVI patients have subnormal DNA synthesis in response to recall antigens.¹³ It has been shown that a decrease in reduced glutathione levels in T lymphocytes by 10% to 30%, which is in the order found in CD4⁺ lymphocytes from CVI patients in the present study, almost completely blocks the T-cell antigen receptor (TCR)/CD3-stimulated calcium flux and proliferative response.⁵³ Interestingly, a recent study has indicated a defect in the early phase of T-cell activation after triggering of the TCR in a subgroup of CVI patients,⁵⁴ which is compatible with decreased intracellular levels of reduced glutathione involving tyrosine phosphorylation upstreams of the production of inositol trisphosphate and release of calcium from internal stores.⁵⁵ Although mitogen-stimulated proliferation may also be impaired,⁵⁶ it seems that the inhibitory effect of glutathione depletion is most pronounced after stimulation through the TCR/CD3 receptor,⁵⁶ supporting the findings in the present study in which glutathione redox disturbances were significantly associated with impaired IL-2 response to INF and SEB stimulation, but no significant association was found with the PHA response.

Impaired IL-2 production seems to be of importance in the immunopathogenesis of CVI.²⁰-³⁰ In the present study, we showed that supplementation with glutathione-monoethyl ester enhanced stimulated IL-2 production in PBMC from CVI patients. These results may seem in conflict with certain other studies finding that IL-2 production in T cells or T-cell lines induced by mitogens is not inhibited even by severe depletion of intracellular glutathione.³³,³⁴ However, some recent studies have shown enhancing effects of antioxidants including glutathione on IL-2 production in human T cells³¹ and PBMC,³² possibly in part mediated by decreased eicosanoid production.² Furthermore, studies analyzing glutathione effects on IL-2 synthesis in cell lines or cells from healthy donors by manipulating glutathione levels in vitro may not reflect the situation in the present study analyzing cells from CVI patients with glutathione abnormalities, unphysiologically high ROS generation, and activation of the TNFα system present in vivo. Such cells may undergo apoptosis upon activation in vitro³⁶ and this may indirectly and negatively influence IL-2 production. Finally, it seems that long-term in contrast to short-term exposure to oxidative stress may impair IL-2 production in T cells.³⁷

In conclusion, the present study shows for the first time glutathione abnormalities both in plasma and in CD4⁺ lymphocytes and monocytes, but not in B lymphocytes, among CVI patients. Our findings may suggest increased oxidative stress in CD4⁺ lymphocytes among CVI patients. The results support the notion that T-cell and monocyte abnormalities, particularly abnormalities in CD4⁺ lymphocytes, may be of importance in the immunopathogenesis of CVI. Furthermore, chronic immune activation and particularly activation of the TNF system seems to be important in the development of the demonstrated glutathione abnormalities. The demonstrated enhancing effect of glutathione-monoethyl ester supplementation on IL-2 production should lead to further stud-
ies examining the influence of glutathione-replenishing agents in vivo and in vitro on immunologic and clinical manifestations in CVI patients. Such studies might elucidate the pathogenetic significance of the present findings.

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