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 S. 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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The steady-state formation of reactive oxygen species (ROS) produced during cell metabolism is normally balanced by a similar rate of consumption by antioxidants. Oxidative stress may result from imbalance in this pro-oxidant-antioxidant equilibrium.1 Glutathione is a cysteine-containing tripeptide (γ-glutamyl-cysteinyl-glycine) that is found in eukaryotic cells at millimolar concentrations as the dominant intracellular thiol and plays an important role in protection against oxidative stress both by reacting directly with some ROS and as a substrate in the glutathione peroxidase catalyzed detoxification of H₂O₂ and organic peroxides.2 In addition, glutathione has a number of other important cellular functions that include activation of thiol requiring enzymes, functioning as an essential factor in the synthesis of DNA precursors, and detoxification of electrophilic metabolites of xenobiotics.3

Adequate concentrations of glutathione are required for a variety of immune functions, including lymphocyte activation,4,5 natural killer cell activation,6 and lymphocyte-mediated cytotoxicity.7 It has been suggested that oxidative stress and deficiency of thiol compounds may play an important pathogenetic role in the development of immunodeficiency in systems ranging from the age-related decline in immunologic activities7 to human immunodeficiency virus (HIV) infection8,9 and immunosuppression associated with critical illness.10

Common variable immunodeficiency (CVI) is a heterogeneous group of B-cell deficiency syndromes characterized by defective antibody production, recurrent sinusopulmonary infections, and a high rate of incidence of lymphoid and gastrointestinal malignancies, nonmalignant lymphoid hyperplasia, and granulomatous inflammation.11,12 There is evidence that the B-cell deficiency in these patients is often associated with abnormalities of T-cell and accessory-cell function and that these abnormalities may be of importance for the defective antibody production and clinical manifestations in CVI.13 The T-cell and accessory-cell dysfunctions include decreased proliferation in response to mitogens and recall antigens, abnormal cytokine production, and abnormal antigen processing and presentation by macrophages.13 Furthermore, we and others have reported evidence of persistent immune activation in vivo,14-16 and a recent report from our group showed enhanced generation of ROS in monocytes in CVI.17 The possibility exists that these cellular abnormalities may result in increased oxidative stress in which disturbed glutathione metabolism may be involved and that this increased oxidative stress may be of importance for the immunopathogenesis in CVI. To explore this hypothesis, glutathione levels in plasma and in different lymphocyte subsets and monocytes were examined in CVI patients and in healthy controls. We also tested whether any abnormalities in glutathione parameters were associated with important immunologic and clinical characteristics of these patients.
Infectious Diseases, Medical Department A, The National Hospital, before Ig substitution was started. Some clinical and immunologic signs of the study, the serum level of alanine aminotransferase was less previously defined criteria,11 patients had splenomegaly, before to
All patients had severely reduced IgG levels in at least two serum controls had no family history of coronary heart diseases and none Oslo. The diagnosis of CVI was defined as previously stated.


<table>
<thead>
<tr>
<th>No. of patients</th>
<th>CVI</th>
<th>Controls</th>
</tr>
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<tbody>
<tr>
<td>20</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Median age in yr (range)</td>
<td>42 (21-67)</td>
<td>41 (22-65)</td>
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<tr>
<td>Males/females</td>
<td>7 (35%)/13 (65%)</td>
<td>6 (38%)/10 (63%)</td>
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<td>CD4⁺ lymphocytes (×10⁶/L)</td>
<td>900 (645-1,060)</td>
<td>1,025 (740-1,150)</td>
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<td>CD4⁺ lymphocytes (×10⁶/L)</td>
<td>340 (250-470)</td>
<td>560 (470-690)</td>
</tr>
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<td>CD8⁺ lymphocytes (×10⁶/L)</td>
<td>380 (230-600)</td>
<td>350 (220-400)</td>
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<td>CD19⁺ lymphocytes (×10⁶/L)</td>
<td>110 (30-170)</td>
<td>150 (120-170)</td>
</tr>
<tr>
<td>Monocytes (×10⁶/L)</td>
<td>270 (230-370)</td>
<td>260 (210-290)</td>
</tr>
<tr>
<td>TNFa (pg/mL)</td>
<td>371 (19-78)</td>
<td>7 (0-9)</td>
</tr>
<tr>
<td>Neopterin (nmol/L)</td>
<td>32.41 (15.1-40.4)</td>
<td>9.1 (5.8-10.1)</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 IgG, 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 thiol components in plasma, blood was routinely collected into three evacuated tubes placed in melting ice containing heparin as an anticoagulant and either monobromobimane (mBrB; Molecular Probes, Eugene, OR) or N-ethylmaleimide (NEM; Sigma Chemical Co, St Louis, MO) as thiol-derivatizing reagents or no addition was made.16 The blood was centrifuged within 15 minutes (400g for 10 minutes at 4°C). Plasma was then transferred to sterile Eppendorf tubes (Eppendorf, Hamburg, Germany) and further centrifuged at 10,000g for 5 minutes at 4°C to obtain platelet-free plasma.

To NEM- and mBrB-treated plasma a solution of sulfosalicylic acid (final concentration, 5%; Merck AG, Darmstadt, Germany) containing dithioerythritol (DTT; 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 × 10⁶ PBMC/mL. Further positive selection of cell subsets by monodisperse immunomagnetic beads was performed at 4°C, as previously described.19 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 IgG1 and rat antimouse IgG2a, respectively. CD4⁺ lymphocytes, isolated as described above, were detached from beads by incubation with goat antimouse Fab antisera (DetectAbead; CD4/C4D; Dynal) for 1 hour in room temperature. The cells were then divided into 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 cytopear preparations of positively selected cells by the alkaline phosphatase anti-alkaline phosphatase procedure20 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,16 the amounts of reduced and oxidized thiols were obtained from blood collected into solutions containing mBrB 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, 50 µmol/L) was added before thawing.

For quantification of intracellular glutathione levels, the isolated lymphocyte subsets and monocytes were extracted with 0.3 M ice-cold 5% sulfosalicylic acid containing 50 µmol/L DTE, and the precipitated protein and the immunomagnetic beads were immediately removed by centrifugation. Staining 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
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-phthaldialdehyde 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 10^6 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, staphylococcal 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 immunophenotyping using peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 (Leu-3a; Becton Dickinson) in combination with fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO (cloneUCHL1; 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 CD2+, CD4+, CD8+, CD19+ 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 radioimmunossay method (IMMULite Test 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, CD4+ 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 CD4+ 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] vs 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] vs 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, 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, 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 × 10^6/L, serum neopterin levels greater than 22.0 nmol/L, and splenomegaly (see the Materials and Methods). CVIHyper (n = 9) represents patients with CD4+ lymphocyte counts less than 400 × 10^6/L, serum neopterin levels greater than 22.0 nmol/L, and splenomegaly. These characteristics were constant when patients were tested and evaluated at least three times over a minimum of 2 years. The 9 CVIHyper patients 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).

When comparing plasma levels, CVIHyper had significantly lower concentrations of both total and oxidized glutathione than other CVI patients (4.76 μmol/L [3.24 to 5.52 μmol/L] vs 6.67 μmol/L [6.05 to 8.10 μmol/L], *P < .05*; and 1.63 μmol/L [1.32 to 1.68 μmol/L] vs 1.90 μmol/L [1.69 to 2.20 μmol/L], *P < .05*; total and oxidized glutathione levels, respectively).

Chronic bacterial infections have been implicated in the generation of increased oxidative stress. 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, we found that CD4+ lymphocytes in CVI patients had a significantly lower

<table>
<thead>
<tr>
<th>Glutathione (μmol/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>
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<tr>
<td>Oxidized</td>
<td>1.7 (1.5-2.1)</td>
<td>2.0 (1.7-2.3)</td>
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<td>Cysteine (μmol/L)</td>
<td>231.4 (288.6-350.2)</td>
<td>206.6 (291.5-355.0)</td>
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<tr>
<td>Oxidized</td>
<td>119.4 (103.5-130.0)</td>
<td>119.4 (109.0-130.1)</td>
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<tr>
<td>Reduced</td>
<td>9.9* (9.2-12.2)</td>
<td>8.4 (7.5-10.4)</td>
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<tr>
<td>Glutamate (μmol/L)</td>
<td>21.2 (16.6-28.1)</td>
<td>22.8 (17.9-29.7)</td>
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</tbody>
</table>

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

* *P < .03* versus controls.
CD45RA+/CD45RO+ ratio than did healthy controls (0.42 to 0.45, \( P < .02 \), with the lowest levels found in the CVI\text{hyp}r 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 CVI\text{hyp}r patients and in 6 controls. We found that, although CVI\text{hyp}r 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 CVI\text{hyp}r 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,\(^{27}\) 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 (\( r = -0.75, P < .001 \); \( r = -0.72, P < .001 \); 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.\(^{19-20}\) Furthermore, decreased intracellular levels of reduced glutathione seems to impair IL-2 production in lymphocytes,\(^{11,32}\) although the reports are somewhat conflicting.\(^{33,34}\) 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] v 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] v 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] v 2.19 ng/mL [1.23 to 3.06 ng/mL], \( P = .15 \); controls and CVI patients, respectively). 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,\(^{20}\) on SEB- and PHA-stimulated IL-2 production in PBMC from 5 CVI\text{hyp}r patients and 5 healthy controls. In CVI\text{hyp}r patients, glutathione-monoethyl ester supplementation increased in a dose-dependent manner both

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Table 3. Intracellular Glutathione Levels in Isolated CD4+CD45RO+ and CD4+CD45RA+ Lymphocytes in CVI\text{hyp}r Patients and in Healthy Controls

<table>
<thead>
<tr>
<th></th>
<th>CVI\text{hyp}r (n = 6)</th>
<th>Controls (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>CD4+CD45RA+ lymphocytes</td>
<td></td>
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<tr>
<td>Reduced glutathione (nmol/10⁶ cells)</td>
<td>0.36* (0.24-0.49)</td>
<td>1.16 (0.60-1.87)</td>
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<tr>
<td>Total glutathione (nmol/10⁶ cells)</td>
<td>0.53* (0.50-0.64)</td>
<td>1.40 (0.67-1.48)</td>
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<td>Ratio of reduced to total glutathione</td>
<td>0.67 (0.46-0.91)</td>
<td>0.90 (0.84-0.93)</td>
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<tr>
<td>CD4+CD45RO+ lymphocytes</td>
<td></td>
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<tr>
<td>Reduced glutathione (nmol/10⁶ cells)</td>
<td>0.45 (0.08-0.63)</td>
<td>0.55 (0.31-0.91)</td>
</tr>
<tr>
<td>Total glutathione (nmol/10⁶ cells)</td>
<td>0.54 (0.36-0.68)</td>
<td>0.63 (0.41-1.01)</td>
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<tr>
<td>Ratio of reduced to total glutathione</td>
<td>0.82 (0.23-0.93)</td>
<td>0.94 (0.80-1.00)</td>
</tr>
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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 CVI\text{hyp}r patients. For definition of CVI\text{hyp}r, see the legend to Fig 2. * \( P < .03 \) versus controls.

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Fig 3. Correlations (r) in CVI patients (n = 20) between serum levels of immunoreactive TNFα and the reduced glutathione levels in CD4+ lymphocytes. The TNFα levels were also significantly inversely correlated with total glutathione levels in CD4+ lymphocytes among these patients (\( r = -0.52, P < .05 \)).
been implicated in the immunopathogenesis of HIV infection through B-cell deficiency is the hallmark of CVI, certain other 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-oncogene and its product in CD4+CD45RO+ lymphocytes from healthy controls, by sensitizing these memory cells to apoptotic stimuli, are of importance 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.

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 EIA’s in detecting circulating TNFα levels. Indeed, we have recently suggested that the EIA used in the present study may better detect TNFα in complex with soluble TNF receptors than other EIA’s. Furthermore, a recent study in our laboratory has shown increased circulating levels of soluble TNF receptors in these patients (manu-

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</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.

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-seropositive 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-oncogene and its product in CD4+CD45RO+ lymphocytes from healthy controls, by sensitizing these memory cells to apoptotic stimuli, are of importance 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 EIA’s in detecting circulating TNFα levels. Indeed, we have recently suggested that the EIA used in the present study may better detect TNFα in complex with soluble TNF receptors than other EIA’s. Furthermore, a recent study in our laboratory has shown increased circulating levels of soluble TNF receptors in these patients (manu-

![Graph](https://via.placeholder.com/150)

**Fig 4.** The effect of different doses of glutathione-monoethyl ester supplementation on PHA- and SEB-stimulated IL-2 production in PBMC from 5 CVI patients in the CVI+ group (see Fig 2). Data are given as median and ranges. *P < .05 v IL-2 production without glutathione-monoethyl ester supplementation. (□) Medium; (●) 2 mmol/L; (●) 5 mmol/L; (●) 15 mmol/L.
GLUTATHIONE ABNORMALITIES IN CVI

found that TNFα stimulation of T-cell lines and endothelial
activation of the TNF system is strongly associated with
script submitted), which also indicate activation of the TNF
system in CVI. The present findings demonstrate that this
activation of the TNF system is strongly associated with
glutathione depletions in CD4+ lymphocytes. It has been
found that TNFα stimulation of T-cell lines and endothelial
cells decreases total glutathione levels in these cells by caus-
ing enhanced ROS generation, which in turn leads to con-
sumption of reduced glutathione.23,46 Thus, increased TNFα
activation may, at least in part, be responsible for the glu-
thione abnormalities in CD4+ lymphocytes among CVI pa-
patients. Furthermore, decrease in intracellular levels of re-
duced glutathione markedly increase the proinflammatory
cellular responses to TNFα stimulation,29 and the TNFα acti-
vation with enhanced ROS generation and depletion of gluta-
thione levels may thus represent a vicious circle leading to
increased levels of oxidative stress in CVI patients, particu-
larly in CD4+ lymphocytes.

Although glutathione depletion among CVI patients most
probably reflects increased inflammatory stress, other mech-
nisms may also be involved. Cysteine availability is rate-
limiting for glutathione synthesis and lymphocytes seem to
depend on extracellular cysteine concentrations for glutathi-
one synthesis.47 However, although malabsorption, which
may involve loss of sulphur-containing amino acids,48 is
often found in CVI patients,13 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 glutath-
one 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 en-
zymes 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 con-
trols. 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 gen-
eration such as that shown in CVI17 may consume reduced
 glutathione in some cell types (eg, T lymphocytes). On
the other hand, at least some radical defences can be en-
bhanced by increased ROS generation.41,50,52 and the present
study supports the notion that glutathione levels are differ-
ently 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 mono-
cytes, 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 re-
reflecting increased oxidative stress could have significant
immunologic consequences. Several immune functions such as
cytotoxic T-cell activation,5 maintenance of normal numbers
of circulating CD4+ lymphocytes,5 and, of particular inter-
est, generation of Ig secreting cells,3,4 seem to be 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 prolifera-
tive response.29 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,25 which is compat-
ible with decreased intracellular levels of reduced glutathi-
one involving tyrosine phosphorylation upstream of the pro-
duction of inositol trisphosphate and release of calcium from
internal stores.56 Although mitogen-stimulated proliferation
may also be impaired,36 it seems that the inhibitory effect of
 glutathione depletion is most pronounced after stimulation
through the TCR/CD3 receptor,26 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.18-30 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 re-
cent studies have shown enhancing effects of antioxidants
including glutathione on IL-2 production in human T cells11
and PBMC,29 possibly in part mediated by decreased eicos-
oid production.32 Furthermore, studies analyzing glutathi-
one 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 apo-
apoptosis upon activation in vitro49 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+ lym-
phocytes 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. Further-
more, chronic immune activation and particularly activation
of the TNF system seems to be important in the development
of the demonstrated glutathione abnormalities. The demon-
strated enhancing effect of glutathione-monoethyl ester sup-
plementation 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.

ACKNOWLEDGMENT

We thank Audun Haylandskjær and the Laboratory for Immunohistochemistry and Immunopathology (LIIPAT), Rikshospitalet for excellent technical assistance.

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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

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