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# Inhibition of murine AIDS by pro-glutathione (GSH) molecules

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**Abstract**

Antioxidant molecules can be used both to replenish the depletion of reduced glutathione (GSH) occurring during HIV infection, and to inhibit HIV replication. The purpose of this work was to assess the efficacy of two pro-GSH molecules able to cross the cell membrane more easily than GSH. We used an experimental animal model consisting of C57BL/6 mice infected with the LP-BM5 viral complex; the treatments were based on the intramuscular administration of I-152, a pro-drug of *N*-acetylcysteine and *S*-acetyl-β-mercaptoethylamine, and *S*-acetylglutathione, an acetylated GSH derivative. The results show that I-152, at a concentration of 10.7 times lower than GSH, caused a reduction in lymph node and spleen weights of about 55% when compared to infected animals and an inhibition of about 66% in spleen and lymph node virus content. *S*-acetylglutathione, at half the concentration of GSH, caused a reduction in lymph node weight of about 17% and in spleen and lymph node virus content of about 70% and 30%, respectively. These results show that the administration of pro-GSH molecules may favorably substitute for the use of GSH as such.


*Keywords:* MAIDS; GSH; Pro-GSH molecules; Antiretroviral

## 1. Introduction

Redox changes mediated by a depletion of glutathione (GSH) occur in the host cell as a result of viral infection and they vary in intensity, duration and mechanism of induction depending on the type of virus. Decreases in GSH have been described in cell cultures experimentally infected with DNA and RNA viruses (Nencioni et al., 2003; Garaci et al., 1997). There is considerable interest in the study of the relationship between the redox status of cells and human immunodeficiency virus (HIV) infection. GSH decreases caused by HIV have been described in human macrophages in which the levels of the antioxidant drop after chronic infection is established (Garaci et al., 1997). Single HIV proteins can exert effects on GSH content, such as gp120 and Tat, which can induce oxidative stress in brain endothelial cells contributing to the development of HIV dementia (Price et al., 2005). Moreover, HIV-infected individuals suffer from systemic oxidative stress. Intracellular levels of GSH are reduced in peripheral blood mononuclear cells and in red blood cells isolated from HIV-infected subjects (Venketaraman et al., 2006).

Some antioxidant molecules such as GSH, glutathione ester and *N*-acetyl-L-cysteine (NAC) are able to suppress HIV expression in chronically infected monocytic cells (Kalebic et al., 1991). More recently, a pyridine *N*-oxide derivative has been described as a potent agent to increase intracellular GSH levels and to induce apoptosis in latently HIV-1-infected cells (Stevens et al., 2006). Furthermore, a novel antioxidant, an NAC derivative, reverses gp120- and Tat-induced oxidative stress in brain endothelial cells (Price et al., 2006).

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In vivo, the administration of high doses of GSH was previously found to reduce viral replication and progression of the disease in murine AIDS and to provide additional antiviral effects to AZT therapy (Palamara et al., 1996; Magnani et al., 1997). Moreover, NAC has been used in HIV infection to prevent the activation of NF-kappa B and the replication of HIV, providing beneficial effects on infected individuals (Nakamura et al., 2002; Aukrust et al., 2003). Furthermore, different pilot studies have shown that the redox profile of patients may be considered as a predictive marker of AIDS progression and that the combined use of antiretroviral and antioxidant drugs may be beneficial (Spada et al., 2002; Malorni et al., 1998). Thus, the control of imbalanced redox status through the administration of antioxidants seems to be advantageous even today when effective therapy based on reverse transcriptase, protease and fusion inhibitors has been installed. NAC and GSH can be used both to replenish intracellular GSH levels altered by viral infections, and to inhibit HIV replication. In addition, NAC and GSH have been demonstrated to restore or increase intra- and extra-cellular GSH levels in T cells and antigen presenting cells (APC) leading to Th1 cytokine production (Eylar et al., 1993; Murata et al., 2002). This latter finding seems particularly important in those infectious diseases in which Th2 predominance is an important aspect in the pathology of the disease as in AIDS. When using GSH as therapeutic agent, the administration of high doses of the molecule is necessary to obtain the desirable effect. It is known that GSH has a brief plasma half life and that it cannot cross the cell membrane but needs to be broken down into amino acids and is then resynthesized to GSH intracellularly, this process often being impaired during viral infections (Wendel and Cikryt, 1980; Lu, 1999). Until now, NAC, the acetylated variant of L-cysteine, has been used as an excellent source of sulphydryl (SH) groups for GSH synthesis and has been the only therapeutic strategy for oxidative stress-associated disorder in HIV infection. The GSH deficiency described in HIV infection along with the inhibitory effect of GSH on HIV and other retroviruses, have prompted researchers to study new pro-GSH molecules able to enter cells more easily. In this article, the effects of two pro-GSH molecules in an experimental murine model for AIDS, already used in our laboratory to test the efficacy of a variety of antiviral strategies (Fraternale et al., 2000, 2001, 2002a,b), are reported. One of the tested molecules, named I-152, is a pro-drug of NAC and β-mercaptoethylamine (MEA). An increase in intracellular GSH by I-152 has been described in human monocyte-derived macrophages (MDMs) (Oiry et al., 2004); moreover, the same cells infected with macrophage-tropic HIV-1/BaL were used to explore anti-HIV effects of I-152 which significantly decreased viral replication, with a 50% effective dose ($ED_{50}$) equal to 3–50 μM regarding the multiplicity of infection (Oiry et al., 2004). The other molecule studied, which is an acetylated GSH derivative (*S*-acetylglutathione, SAG), was shown to restore GSH levels that had been decreased following HSV-1 infection and to delay HSV-1-induced mortality in hr/hr mice (Vogel et al., 2005). Herein, the antiviral effects exerted by these two molecules (I-152 and SAG) are discussed in comparison with GSH which was previously demonstrated to be effective in reducing the typical signs characterizing murine AIDS (Palamara et al., 1996).

## 2. Materials and methods

### 2.1. Virus and mouse infection

The LP-BM5 viral mixture was kindly provided by Robert Yetter (Veterans Administration Hospital, Baltimore, MD) and was maintained in a persistently infected SC-1 cell line as previously described (Mosier et al., 1987). Female C57BL/6 mice that were four weeks old were infected by means of two consecutive intraperitoneal (IP) virus inoculations at a 24-h interval (∼ 1 IU reverse transcriptase, RT). Mice were housed at 22 ± 1 °C with a 12-h light/dark cycle, 60 ± 5% humidity, and 12 air changes/h.

### 2.2. Experimental groups

Seven experimental groups of mice were studied. Two groups of seven mice each were considered as controls: one group was uninfected and untreated (group CTR), another group was infected and untreated (group I).

The treatments with I-152 and SAG started at 24 h after the first virus inoculation, thus coinciding with the second virus inoculation and both molecules were administered intramuscularly in a final volume of 200 μl for five consecutive days weekly. All the treatments were continued for a total of 10 weeks.

I-152 was synthesized as previously described (Oiry et al., 2004) and was used to treat two groups of seven infected mice each: group A was treated with 7 μmol of I-152/mouse (0.09 g/kg) and group B was treated with 30 μmol of I-152/mouse (0.36 g/kg). SAG was obtained from Syncomp GmbH (Frankfurt am Main, Germany) and was used to treat three groups of seven infected mice each: group C was treated with 14 μmol of SAG/mouse (0.23 g/kg), group D was treated with 72 μmol of SAG/mouse (1.15 g/kg) and group E was treated with 143 μmol of SAG/mouse (2.3 g/kg).

### 2.3. Serum immunoglobulin G determination

After 10 weeks of treatment, IgG levels were determined using an ELISA technique.

Polystyrene microtiter plates (Dynex Technologies, Inc., Chantilly, VA) were coated with goat anti-mouse IgG (Sigma BioSciences, St. Louis, MO) diluted 1:100 in 0.135 M NaCl and incubated for 24 h at 37 °C. The plates were washed four times with 0.1% Tween 20 in 10 mM $NaH_2PO_4$, 154 mM NaCl, pH 7.0 (TPBS) and blocked with 5% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. After four washings in TPBS, serial dilutions of murine serum in 50 mM sodium borate, pH 8.5, were added and incubated for 1 h at 37 °C. After four washings in TPBS, 100 μl of goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad, Richmond, CA), diluted 1:1000 in PBS, were added. After incubation for 1 h at 37 °C, serum IgG levels were determined using a colour development solution containing 2.2 mM *o*-phenylenediamine. Absorbance was measured at 492 nm on a Model 2550 enzyme immunoassay (EIA) reader

(Bio-Rad). Absolute serum IgG concentrations were obtained using known concentrations of standard mouse IgG.

### 2.4. Real-time PCR

At 10 weeks after the infection, BM5d DNA content was assayed in spleen and lymph nodes. Total cellular DNA was isolated from spleen and lymph nodes of uninfected (CTR), infected and untreated (I), infected and treated mice; BM5d DNA was quantified by real-time PCR assay as previously described (Casabianca et al., 2004). The copy number (copy no.) of BM5d DNA in target organs was calculated by interpolation of the experimentally determined plasmid standard curve and was normalized to 100 ng of genomic DNA.

### 2.5. Lymphocyte proliferative index

Spleen T and B lymphocytes were prepared from all experimental groups and distributed in multiwell (96-well) plates at $5 \times 10^5$ cells in a volume of 200 μl in quadruplicate, stimulated with 10 μg/ml PHA or 50 μg/ml LPS and incubated at 37 °C in a 5% $CO_2$ atmosphere. After 70 h, each well received 1 μCi of $^3$[H] thymidine and the incubation was continued for another 8 h. Each sample was then precipitated with 100 μl of 10% (w/v) trichloracetic acid, collected on glass microfibre filters, washed with 3% (w/v) of trichloracetic acid and the amount of acid insoluble radioactivity counted by liquid scintillation counting.

### 2.6. Statistical analysis

Statistical analysis of data was performed with the non-parametric Mann–Whitney test and Kruskal–Wallis test with a $p$ value <0.05 used to delineate significance.

## 3. Results

Mouse AIDS, or MAIDS, is a retroviral disease induced in C57BL/6 mice by the retroviral complex LP-BM5 containing a mixture of murine leukemia viruses, including a replication ecotropic virus (BM5e) that functions as a helper, mink cell cytopathic focus-inducing virus and replication-defective viruses, serving as the agent causing the immunodeficiency (Aziz et al., 1989). Since many of the disease features of the LP-BM5-induced syndrome resemble those occurring in human AIDS, this model is among the animal models frequently used for the evaluation of single and combined drugs efficacy because it is the most suitable for the rapid initial screening of new drugs (Fraternale et al., 2000, 2001, 2002a,b; Mayhew et al., 2005; Beilharz et al., 2004; Dias et al., 2006). Moreover, the costs are relatively low when compared to other animal models. Included among the similarities with human AIDS are hypergammaglobulinemia, splenomegaly, and lymphadenopathy; severely depressed T- and B-cell responses to mitogens, increased susceptibility to infection, disease progression and the development of B-cell lymphomas (Klinman and Morse, 1989; Morse et al., 1989, 1992; Yetter et al., 1988; Cerny et al., 1990). This animal model was previously used to test the efficacy of either GSH or GSH plus AZT administration (Palamara et al., 1996; Magnani et al., 1997); here, we report the results obtained, in MAIDS animal model, by evaluation of I-152 and SAG as pro-GSH molecules. These molecules, being more lipophilic than GSH, are expected to enter the cells more easily. Chemical structures of I-152 and SAG are reported in Fig. 1.

Based on the data already available regarding efficacy and $LD_{50}$ (lethal dose 50) in mice of the two molecules (Oiry et al., 2001, 2004; Vogel et al., 2005) and the effects of GSH

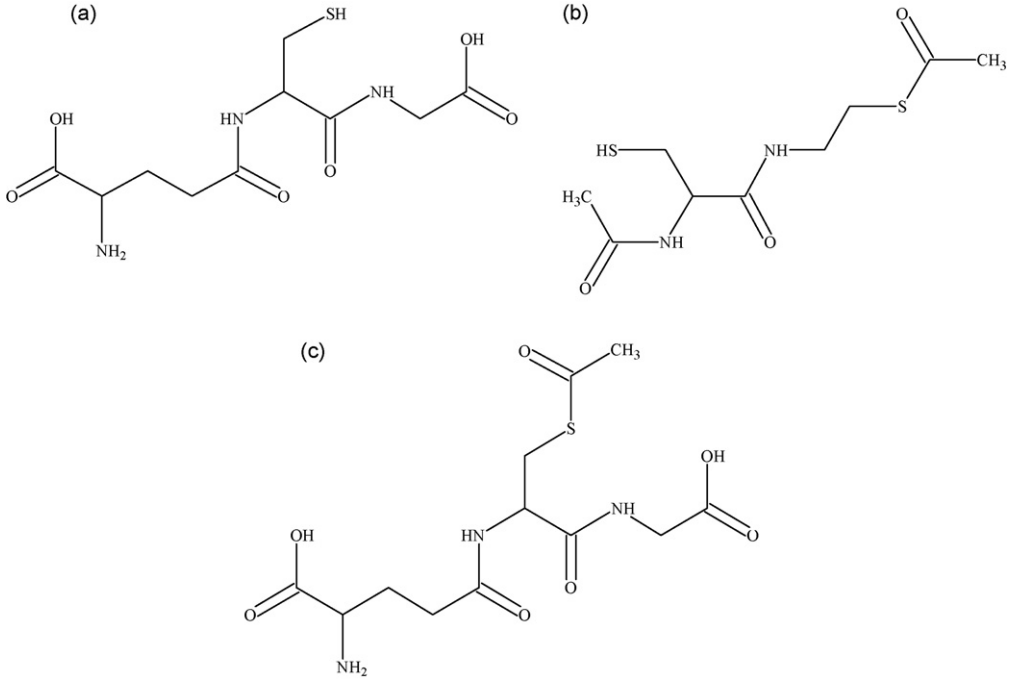


Fig. 1. Chemical structure of reduced glutathione (GSH) (a) and pro-GSH molecules: I-152 (b); *S*-acetylglutathione (SAG) (c).

(at a concentration of 325 μmol/mouse (4.5 g/kg)) on murine AIDS (Palamara et al., 1996), the doses reported in Section 2 were selected for testing. In detail, the intraperitoneal $LD_{50}$ in a chronic treatment with I-152 (30 days) had been reported equal to 700 mg/kg (Oiry et al., 2001). Therapeutic protocol based on the intramuscular administration of 30 μmol of I-152 per mouse (0.36 g/kg), i.e. half of the $LD_{50}$, for 5 days a week for a total of 10 weeks, did not give any toxic effect (unpublished results). For SAG, it had been observed that oral doses of up to 3.5 g/kg body weight were well tolerated in mice (unpublished results). Preliminary experiments performed to establish toxicity of chronic intramuscular administration of SAG showed that 143 μmol of SAG per mouse (2.3 g/kg) did not exert any toxicity (unpublished results).

We studied the signs of MAIDS-related symptoms including splenomegaly, lymphadenopathy, hypergammaglobulinemia and proliferative response of spleen cells to mitogens at week 10 after LP-BM5 MuLV inoculation. Furthermore, we investigated the BM5d DNA content by real-time PCR in the spleen and lymph node cells of mice belonging to all experimental groups.

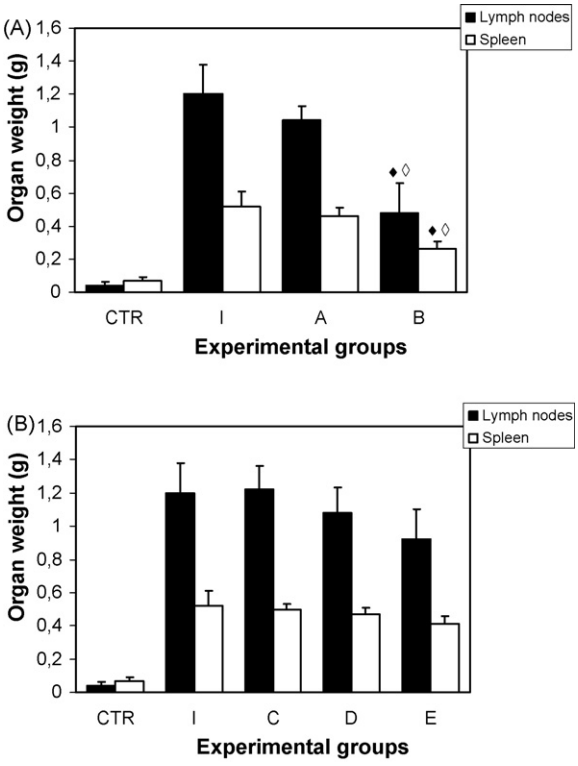


Fig. 2. Splenomegaly and lymphadenopathy in C57BL/6 mice infected with LP-BM5 and treated with I-152 (panel A) and SAG (panel B). CTR: uninfected and untreated; I: infected and untreated; (A) infected and treated with I-152 at the dose of 7 μmol/mouse (0.09 g/kg); (B) infected and treated with I-152 at the dose of 30 μmol/mouse (0.36 g/kg); (C) infected and treated with SAG at the dose of 14 μmol/mouse (0.23 g/kg); (D) infected and treated with SAG at the dose of 72 μmol/mouse (1.15 g/kg); (E) infected and treated with SAG at the dose of 143 μmol/mouse (2.3 g/kg). Both molecules were administered intramuscularly five days a week for a total of 10 weeks. Values are mean ± S.D. of seven animals and were obtained at 10 weeks post infection. $^{\blacklozenge}p < 0.05$ vs. I; $^{\lozenge}p < 0.05$ vs. A.

### 3.1. Effect of pro-GSH molecules on spleen and lymph node weight

Disease progression was assessed by spleen and lymph node weights that were significantly increased in infected and untreated mice versus those uninfected and untreated (Fig. 2, groups I and CTR respectively). In Fig. 2 (panel A), the results obtained by evaluation of spleen and lymph node weight in mice treated with I-152 are reported. We can note that the higher dose of I-152 (group B) was highly effective in reducing both spleen and lymph node weight ($p = 6 \times 10^{-4}$ versus group I), whereas no significant differences in both organs were observed when the lower dose of the molecule (group A) was used ($p > 0.05$). When the two dosage levels of I-152 were compared, significant statistical results both in spleen and lymph nodes were obtained ($p = 6 \times 10^{-4}$). Not significantly different was the reduction in spleen and lymph node weights caused by the highest concentration of SAG (group E) ($p = 0.096$ and $p = 0.073$ versus group I in spleen and lymph nodes respectively) (Fig. 2 panel B). The other concentrations of SAG (groups C and D) tested did not provide any reduction in spleen and lymph node weight.

### 3.2. Effect of pro-GSH molecules on hypergammaglobulinemia

The serum levels of IgG are markers of the progression of murine AIDS. As shown in Fig. 3, mice that were inoculated with LP-BM5 MuLV had increased levels of IgG in serum. This parameter was not influenced by the treatments tested ($p > 0.05$), not even by 30 μmol of I-152/mouse (0.36 g/kg) (group B) that induced a significant reduction in lymph node and spleen mass (Fig. 3).

### 3.3. Effect of pro-GSH molecules on BM5d DNA content in spleen and lymph nodes

The etiologic agent of murine AIDS (MAIDS) is a replication defective BM5d virus. Infected mice have increased levels

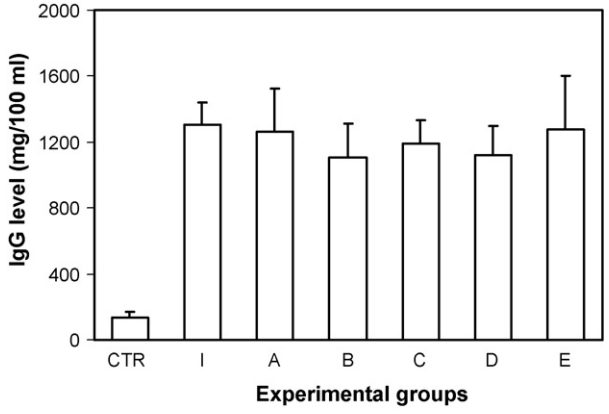


Fig. 3. Serum IgG levels in C57BL/6 mice infected with LP-BM5 and treated with I-152 (groups A, B) and SAG (groups C–E) as described in the legend to Fig. 2. IgG concentration was determined as described in Section 2. Values are mean ± S.D. of seven animals and were obtained at 10 weeks post infection.

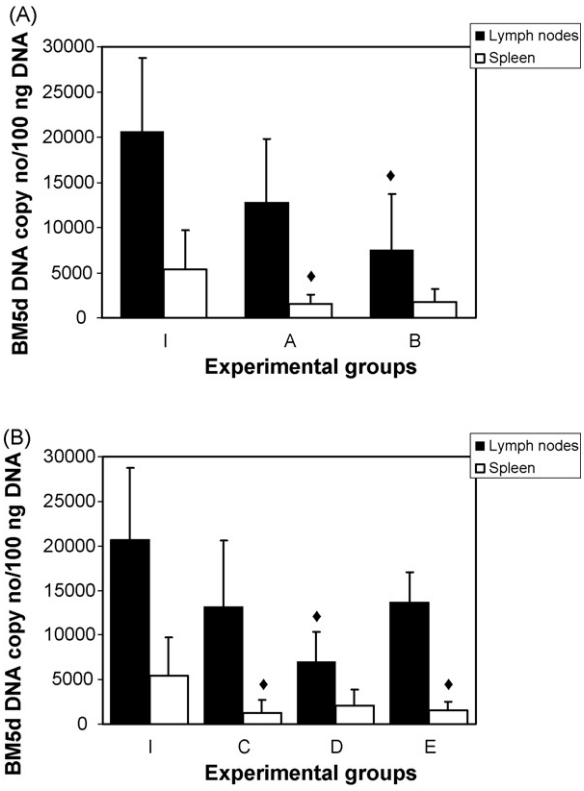


Fig. 4. BM5d DNA copy no. in C57BL/6 mice infected with LP-BM5 and treated with I-152 (panel A) and SAG (panel B) quantified by real-time PCR assay (Casabianca et al., 2004). The treatments are described in detail in the legend to Fig. 2. Values are mean ± S.D. of seven animals and were obtained at 10 weeks post infection. ♦$p < 0.05$ vs. I.

of BM5d DNA in lymph nodes and spleen. Herein, we are reporting the results of the quantification of the BM5d DNA level in infected and infected/treated mice to investigate the direct effect of pro-GSH molecule treatments on the BM5d virus level and MAIDS development. To this aim, we used a specific and reliable real-time PCR assay previously optimized (Casabianca et al., 2004). In Fig. 4 the BM5d DNA copy numbers per 100 ng DNA in infected and treated versus infected and untreated mice are shown.

In particular, as compared to infected and untreated mice (Fig. 4 panel A), 30 μmol of I-152/mouse (0.36 g/kg) (group B) displayed no significant values in the spleen ($p = 0.056$), while reaching significant results in lymph nodes ($p = 6 \times 10^{-3}$). Conversely, 7 μmol of I-152/mouse (0.09 g/kg) (group A) displayed no significant effects in lymph nodes ($p = 0.056$) while reaching significance in the spleen ($p = 0.03$). Furthermore, the content of BM5d DNA in group A was not significantly different from that of group B both in lymph nodes and spleen ($p = 0.09$ and 0.84, respectively).

With SAG, a reduction of BM5d DNA content was observed (Fig. 4 panel B) both in spleen and lymph nodes, but these effects were not dose-dependent. For SAG, inhibition range of BM5d DNA copy numbers respect to group I was 35–65% in lymph nodes and 62–77% in spleens. These inhibition values were calculated by the following formula: 100 − [(mean BM5d DNA copy number in each experimental group × 100)/mean BM5d DNA copy number in infected group]. In detail, BM5d DNA content in spleen showed significant differences of groups C and E versus group I ($p = 0.016$ and $p = 0.032$, respectively), whereas no significant differences were observed in lymph nodes ($p = 0.1$ and $p = 0.16$). Furthermore, there was no significant difference in the BM5d level in the spleen of group D versus group I ($p = 0.2$), whereas a significant result was obtained in lymph nodes ($p = 2.3 \times 10^{-3}$). Also, following SAG treatment, no significant differences in BM5d DNA levels in lymph nodes and spleen were observed when the three groups were compared (Kruskal–Wallis test, $p > 0.05$).

### 3.4. Lymphocyte proliferative index

The response of T and B lymphocytes to mitogen stimulation was significantly decreased in C57BL/6 mice infected with the LP-BM5 virus complex (Beilharz et al., 2004). To determine whether T and B lymphocytes responded differently to PHA and LPS respectively following treatments with pro-GSH molecules, spleen cells were isolated from uninfected, infected and untreated and infected/treated mice and their proliferation was determined by in vitro incorporation of $^{3}$[H] thymidine. The results, reported in Fig. 5, show that I-152 at the dose of 30 μmol/mouse (0.36 g/kg) (group B), was effective in restoring the ability to proliferate following LPS in B cells ($p = 0.029$) (Fig. 5 panel A), whereas it exerted a modest effect in restor-

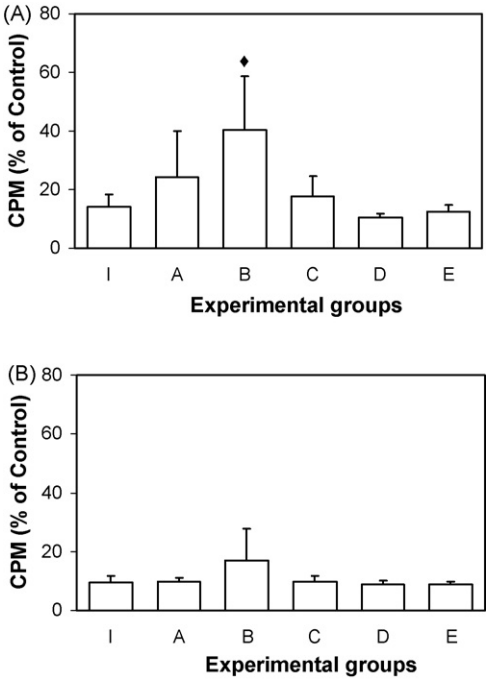


Fig. 5. Lymphocyte proliferative response to LPS (B cells) (panel A) and PHA (T cells) (panel B) observed in LP-BM5-infected animals, not treated or treated as described in the legend to Fig. 2. Spleen lymphocytes were obtained 10 weeks after infection and were assayed for $^{3}$[H]thymidine incorporation after 3 days of culture with mitogens as described in Section 2. The values are mean ± S.D. of five animals. CPM values ± S.D. in the controls were 20, 283 ± 4210 $(10^{6}$ cells$)^{-1}$ for T cells and 13,544 ± 2860 $(10^{6}$ cells$)^{-1}$ for B cells. Results are shown as the percentage of $^{3}$[H]thymidine incorporation in infected mice as compared with the control uninfected mice. ♦$p < 0.05$ vs. I.

ing the proliferative index of T cells induced by PHA (Fig. 5 panel B). On the contrary, SAG treatment did not influence the parameter examined (Fig. 5).

## 4. Discussion

Oxidative stress has been implicated in the pathogenesis of a variety of infections, including HIV-1 (Herzenberg et al., 1997). It has been demonstrated that therapeutic intervention aimed at normalization of oxidative disturbances in HIV infection might be of interest in addition to HAART, the standard protocol followed to treat HIV-infected patients (Nakamura et al., 2002; Aukrust et al., 2003; Spada et al., 2002; De Rosa et al., 2000). Indeed, GSH and some pro-GSH molecules are able to interfere with different steps in the biological cycle of the virus (Stevens et al., 2006; Oiry et al., 2004; Vogel et al., 2005; Palamara et al., 2004). Effective GSH replenishment has been described in HIV infection by oral administration of NAC, an effective precursor of cysteine for tissue GSH synthesis. NAC also appears to enhance T cell function in HIV-infected patients suggesting that this molecule might represent a useful adjunct therapy not only to increase protection against oxidative stress, but also to improve immune function (Spada et al., 2002). This second aspect is becoming important now that recent findings indicated that it is possible to drive T-cell responses toward Th1 or Th2 type response when changing the redox state of antigen presenting cells (Murata et al., 2002; Fraternale et al., 2006; Iijima et al., 2003). On this matter there are contrasting findings. On one hand, it was found that augmenting intracellular soluble thiol pools in splenocytes with NAC, blocked the induction of IFN-gamma and increased the production of IL-4, contributing to alterations in the balance between Th1 and Th2 responses in lung diseases (Monick et al., 2003), while thiol antioxidants inhibit the formation of the IL-12 heterodimer (Mazzeo et al., 2002). On the other hand, it was found that NAC and GSH decreased IL-4 production by stimulated T cells and acted on B cells reducing production of IgE and IgG isotypes involved in Th2 response (Jeannin et al., 1995); moreover, GSH depletion inhibited Th1-associated cytokine production and/or favored Th2-associated responses (Murata et al., 2002; Dobashi et al., 2001). For the reasons listed, we suggest that the administration of GSH in the viral infection might be advantageous as an antiviral agent and as an immunomodulator, in particular in those diseases in which there is an imbalance between Th1 and Th2 responses. However, it is known that therapy based on GSH administration presents several problems such as the necessity to use very high doses of the drug as a consequence of its brief half-life in circulation and its difficulty in crossing the cell membrane (Wendel and Cikryt, 1980; Lu, 1999). Consequently, there is great interest in new pro-GSH molecules able to replenish GSH that is depleted by the viral infection (Stevens et al., 2006; Price et al., 2006; Oiry et al., 2004; Vogel et al., 2005; Palamara et al., 2004). In a model of murine immunodeficiency, we have examined the effects of I-152, a prodrug of NAC and MEA, and of SAG, a derivative of GSH that had been already reported to increase intracellular SH groups (Oiry et al., 2001, 2004; Vogel et al., 2005). These molecules could be of interest in HIV infection and in other diseases to counteract oxidative stress. In our previous works, involving the same animal model, high doses of GSH (325 μmol/mouse) (4.5 g/kg) were found able to reduce spleen and lymph node weight by about three and six times, respectively, compared to infected and untreated mice; moreover, the levels of serum IgG in GSH-treated animals were about half when compared to infected and untreated mice (Palamara et al., 1996). Herein, we report that I-152, when administered at a concentration of 30 μmol/mouse (0.36 g/kg), i.e. 10.7 times lower with respect to the GSH dose already tested, caused a significant reduction in lymph node and spleen weights but had no effects on hypergammaglobulinemia. As already observed for GSH (Palamara et al., 1996), I-152 also exerted different effects on different organs and cells as demonstrated by the restoration of LPS-stimulated proliferation of B cells up to 40% of uninfected group as compared to a poor proliferation index of T cells.

Moreover, a very significant reduction of BM5d DNA content in the lymph nodes of animals treated with higher concentration of I-152 and in the spleen of mice treated with lower concentration of the drug was obtained. This anomalous trend of results is only apparent because the statistical analysis performed to compare group A versus B, does not reveal significant differences suggesting that there is no statistically significant dose response.

Palamara et al. (1996) previously founded a lower reduction of virus content in the organs of animals treated with 325 μmol of GSH/mouse (4.5 g/kg) (about 38% and 56% reduction in lymph nodes and spleen respectively when compared to infected and untreated mice). SAG did not reduce splenomegaly and lymphadenopathy, but it provided an effect on BM5d DNA content in spleen and lymph nodes. As already observed for I-152 treatment, the apparent anomalous trend of results regarding BM5d DNA content (Fig. 4) is explained by statistical analysis showing no significant differences when comparing experimental groups treated with different drug doses. SAG treatment did not affect either serum IgG levels or the proliferation index of B and T cells.

Precise modes of action of I-152 and SAG are not well-known today, and if we suspect a major role for GSH, the ability of these two compounds to generate GSH is different. SAG as a GSH derivative is believed to enter cells directly, then it is converted to GSH in a ratio of 1:1 by the cytoplasmic enzyme thioesterase; I-152 is a prodrug of NAC and MEA, expected to liberate, after metabolization, two potential pro-GSH compounds providing a more abundant source of soluble thiol pools that can be immediately used for GSH synthesis. Furthermore, the lipophilic nature, the reduced size of I-152 and its bioavailability when compared to SAG may favor its entry through cell membrane. These observations are supported by the results obtained in murine peritoneal macrophages used to evaluate their intracellular SH content after I-152 and SAG treatments. In fact, the levels of SH content found in these cells after I-152 treatment were 1.6 times higher than those found after SAG treatment (unpublished results). Despite the significant reduction of lymph node and spleen mass found in animals treated with I-152, we have not observed any reduction in IgG levels. This is a deviation from all the antiviral treatments previously tested in this animal model (Fraternale et

al., 2000, 2001, 2002a,b; Palamara et al., 1996; Magnani et al., 1997). We can observe that high levels of IgG in murine AIDS and AIDS have different sources. In fact, in humans hypergammaglobulinemia represents antibody production versus HIV-1; while a role of B cells and their expansion have been described in MAIDS (Huang et al., 1991). The different effect exerted by pro-GSH molecules may be due to a specific effect of sulphydryl groups replenished by the compounds on B and other immune cells. These conclusions are supported by the results obtained in parallel experiments conducted on murine macrophages in which the modulation of GSH content is different according to the pro-GSH molecule used. All the antioxidant treatments tested, including I-152, caused an increase in IgG levels which, however, were different in the profile of subgroups leading to a shift versus Th1 or Th2 response (unpublished results). This observation can explain the finding of high levels of IgG in LP-BM5-infected mice treated with I-152.

The molecules studied exert different effects also on spleen lymphocytes: I-152 partially increased the ability of B spleen lymphocytes to incorporate $^{3}$[H]-thymidine in the presence of lipopolysaccharides (LPS) which was markedly reduced in infected animals (Fig. 5), while SAG had no effects on mitogen-induced proliferation of spleen lymphocyte subpopulations. Other treatments previously investigated always reduced plasma IgG concentration and restored B-cell response to LPS (Fraternale et al., 2000, 2001, 2002a,b; Palamara et al., 1996; Magnani et al., 1997). As already observed for other antioxidant molecules such as NAC, that has been reported to exert different immunomodulatory activity according to the cells involved in the immune response (Jeannin et al., 1995; Verhasselt et al., 1999; Giordani et al., 2002), pro-GSH molecules may have different mechanisms of action according to the type of immune cell and its redox state. However, the precise mechanism(s) underlying the complex effects of these pro-GSH agents on different immune cells have not yet been fully elucidated.

The results obtained from this study has prompted us to suggest that the molecules tested may inhibit MAIDS by both blocking retroviral replication and exerting immunomodulatory role.

## 5. Conclusions

The results reported in this paper confirm those obtained in our previous studies and those of others showing that the addition of antioxidant molecules to antiretroviral drugs could be advantageous in the treatment of HIV and other retroviral infections (Magnani et al., 1997; Aukrust et al., 2003; Spada et al., 2002; Malorni et al., 1998; Eylar et al., 1993) and suggest that the use of more lipophilic pro-GSH molecules actually increases the efficacy of GSH in its antiviral effect. For these reasons, it would be interesting to investigate, in MAIDS, the potential additive or synergistic effects of the pro-GSH molecules tested, in combination with antiretroviral drugs, such as azidothymidine and didanosine which were already demonstrated to be effective in reducing murine AIDS (Fraternale et al., 2001, 2002a,b).

Thus, I-152 and SAG could be considered novel agents with potent antioxidative and antiviral properties, providing a rationale for their combination with antiretroviral drugs. Further studies in other experimental models would be useful to investigate the potential of novel GSH pro-drugs in HIV infection therapy and to better understand their immunomodulatory role in viral infections.

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