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Macrophage protection by addition of glutathione (GSH)-loaded erythrocytes to AZT and DDI in a murine AIDS model

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Abstract

Monocyte-macrophages play a central role in HIV-1 infection because they are among the first cells to be infected and because later they are important reservoirs for the virus. Thus, newly designed therapies should take into account the protection of this cell compartment. Herein, we report the results obtained in a murine AIDS model, by the addition to AZT+DDI of a system (GSH-loaded erythrocytes) able to protect macrophages against HIV-1 infection. Five groups of LP-BM5-infected mice were treated as follows: one group was treated by AZT, one group was treated by DDI, one group was treated by the combination of both, another by GSH-loaded erythrocytes, and finally, one by the combination of all three. After 10 weeks of infection the parameters of the disease were studied and the proviral DNA content in different organs and in macrophages of bone marrow and of the peritoneal cavity was quantified. The results obtained show that mice treated with AZT+DDI+GSH-loaded erythrocytes showed proviral DNA content in the brain and in macrophages of bone marrow that was significantly lower than in mice treated with AZT+DDI. This study may help developing strategies aimed at blocking HIV-1 replication in its reservoirs in the body.

Keywords: Murine AIDS; Macrophages; AZT; DDI; GSH-loaded erythrocytes

1. Introduction

Monocyte-macrophages play a central role in HIV-1 primary infection given that they are among the first cells infected, and later because they are important reservoirs for the virus (Zhu et al., 1993; Meltzer and Gendelman, 1992; Gendelman et al., 1989; Chun and Fauci, 1999; Crowe and Sonza, 2000). These cells can support long-term production of HIV particles without a significant alteration of their homeostasis, differently from what happens in activated CD4+

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lymphocytes that are characterized by death upon infection. The capacity to accumulate virions intracellularly, the relative resistance to the cytopathic effects of HIV-1 and their function as antigen-presenting cells, make the cells of the mononuclear phagocyte system important HIV-reservoirs and sources of viral replication at all stages of the disease that may become relevant when the number of T lymphocytes is decreased. Numerous studies have revealed that macrophages, beyond latently infected CD4+ T cells, must be considered as important reservoirs and that the virus that invariably emerges after the cessation of highly active antiretroviral therapy (HAART) emanates from both sources (Crowe and Sonza, 2000; Siliciano, 1999; Chun et al., 2000; Igarashi et al., 2001).

In several organs macrophages are among the most frequently infected cells. In particular, macrophages and microglia cells in the central nervous system of patients with HIV-1-associated cognitive/motor complex were identified as one of the first non-lymphocyte cell lineages that support virus replication (Wiley et al., 1986; Koenig et al., 1986). All these observations suggest that newly designed therapies should include a careful analysis of the levels of HIV-1 infection of the cells of the monocyte-macrophage lineage and that increasing attention should be paid to the antiviral strategies aimed at protecting the macrophage compartment. During the past few years, when a stable state of latency in CD4+ T cells was identified (Chun et al., 1997; Finzi et al., 1999), there was considerable interest in potential approaches toward diminishing the latently infected, resting CD4+ T cells. For example, the use of intermittent IL-2 in patients receiving HAART has been extensively studied (Chun et al., 1999). Now, given the range of cells that are being discovered as cellular reservoirs of HIV, new anti-HIV therapeutic interventions aimed at preventing the persistence and renewal of such reservoirs and/or at inducing cell death in both HIV-infected monocyte-derived macrophages as well as lymphocytes (Lum et al., 2001), might be an important component of future strategies for the treatment of HIV infection.

In previous studies, we have widely demonstrated that erythrocytes can be used as useful carriers of antiretroviral drugs to target specific molecules to macrophage cells infected in vitro with different retroviruses (Magnani et al., 1994, 1995, 1996). Moreover, the addition of drug-loaded erythrocytes to nucleoside analogue therapies affords additive and/or synergistic effects in delaying the progression of murine AIDS and inhibiting proviral DNA content in organs of LP-BM5-infected mice (Fraternale et al., 1996, 1999; Magnani et al., 1998).

Herein, we report the results obtained in a murine AIDS model (MAIDS), on the efficacy of two nucleoside analogues (AZT+DDI) and their combination with a system (GSH-loaded erythrocytes), able to protect macrophages, in preventing LP-BM5 infection in the brain and in macrophages in different compartments.

MAIDS develops when susceptible strains of mice are inoculated with the mixture of LP-BM5 (Jolicoeur, 1991; Morse et al., 1992). This syndrome is characterized by severe immunodeficiency, lymphadenopathy, splenomegaly, enhanced susceptibility to infection, and the development of terminal B cell lymphoma (Hartley et al., 1989; Klinken et al., 1988); macrophages may be primary targets for infection by both the ecotropic and defective viral components of the LP-BM5 virus mixture (Cheung et al., 1991).

The choice of glutathione in its reduced form (GSH) was stemmed from previous studies that demonstrated that GSH encapsulated in erythrocytes provides significant additive effects to AZT monotherapy in reducing the proviral DNA content in lymph nodes, bone marrow, spleen and brain of LP-BM5-infected mice (Fraternale et al., 1999).

2. Materials and methods

2.1. Virus and mice 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 were infected by means of two consecutive intraperitoneal inoculations at 24-h interval ($\sim$1 IU reverse transcriptase, RT). Mice were housed at 22 ± 1 °C with a 12-h light/dark cycle, $60 \pm 5\%$ humidity, and 12 air changes/h.

2.2. Animal treatments

Beginning 24 h after the first viral injection, AZT and DDI were administered ad libitum in drinking water at concentrations of 250 and 134 mg l^{-1}, respectively, for the duration of the experiment. GSH was encapsulated into murine erythrocytes at a final concentration of $4.67 \times 10^{-4} \pm 1.00 \times 10^{-4}$ μmol 10^{-6} erythrocytes using a procedure of hypotonic dialysis and isotonic resealing as previously described (Fraternale et al., 1999). Unloaded erythrocytes that are erythrocytes submitted to the procedure of encapsulation without adding GSH, were used as negative controls. GSH-loaded and unloaded erythrocytes were processed to augment macrophage phagocytosis as previously described (Magnani et al., 1992). The membrane of erythrocytes was modified to induce band 3 clustering (band 3 is a transmembrane anion channel protein abundantly present on the erythrocyte membrane). Once band 3 is in clusters the erythrocytes are opsonized by autologous immunoglobulin G (IgG) and complement up to C3b. The opsonized erythrocytes are then selectively recognized by the Fc and C3b receptors present on the macrophage membrane and actively phagocytosed. Band 3 clustering was obtained as follows: erythrocyte suspensions (10% hematocrit) in 1 mM $ZnCl_2$ were treated with 1 mM bis(sulfosuccinimidyl)suberate (BS^3) for 15 min at room temperature and washed once in 10 mM Hepes, 154 mM NaCl, 5 mM Glucose, pH 7.4 (buffer A) containing 10 mM ethanolamine, once in buffer A containing 1% (wt/vol) bovine serum albumin and once in buffer A. These cells were resuspended in autologous plasma and injected. The first administration of erythrocytes was 24 h after the first virus inoculation, thus coinciding with the second virus inoculation. The mice were treated weekly with intraperitoneal injections of GSH-loaded erythrocytes containing 0.134 ± 0.029 μmol of GSH. Treatment was continued for a total of 10 weeks.

2.3. Serum immunoglobulin G determination

Serum IgG levels were determined using an ELISA technique as previously described (Fraternale et al., 1999).

2.4. Preparation of peritoneal and bone marrow macrophages

Peritoneal exsudate macrophages were obtained by peritoneal lavage with 5 ml of ice-cold Hank's balanced salt solution supplemented with 10 U ml^{-1} of heparin. The cells were washed twice, resuspended in DMEM (International PBI, Italy) culture medium supplemented with 10% heat-inactivated fetal calf serum, 1% antibiotics, 2 mM glutamine, and overlaid on plastic dishes (35 mm culture plates, Sarstedt, Italy). The plates were incubated in a humidified 5% CO_2 atmosphere at 37 °C over night to allow macrophage adherence. Plates were washed with gentle agitation with warmed DMEM medium to remove non-adherent cells, and a macrophage monolayer was obtained. About 95% of the adherent cells were macrophages as identified by using FITC-conjugated rat monoclonal antibody to mouse F4-80 (Caltag Laboratories Inc., Burlingame, CA); cell analysis was done on a FACScan flow cytometer (Beckton Dickinson).

For the preparation of bone marrow macrophages the two femora were dissected out and cleared of muscular tissue: the upper and lower ends of these bones were cut off at fixed positions and injected with 200 μl of ice-cold Hank's balanced salt solution supplemented with 10 U ml^{-1} of heparin and the cells so obtained were processed as described above for peritoneal macrophages.

2.5. Competitive polymerase chain reaction analysis of LP-BM5 proviral DNA

At 5 days after the last treatment with GSH-loaded erythrocytes, proviral DNA content was assayed in different organs. Total cellular DNA

was isolated from spleen, lymph nodes, and brain of uninfected, infected and infected/treated mice, as previously described (Fraternale et al., 1996). Macrophages, obtained from either peritoneal cavity or bone marrow, were washed twice by centrifugation in 2 ml of NaCl 0.9%. The pellet was resuspended at the concentration of 1500 cells μl^{-1} of lysis buffer containing 1 × PCR buffer (10 mM Tris–HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100), 0.5% Tween 20, 1% NP-40 and 100 $\mu g\ ml^{-1}$ of Proteinase K. The resuspended cells were incubated for 60 min at 60 °C, then 10 min at 95 °C. Five microliter (7500 cells) of sample were used in a 50-μl PCR. BM5d proviral DNA content was quantified by a competitive PCR assay as previously described (Casabianca et al., 1998).

2.6. Lymphocyte proliferative index

[^{3}H] Thymidine incorporation upon lymphocyte stimulation by phytohemagglutinin (PHA) for T cells and lipopolysaccharide (LPS) for B cells was measured as previously described (Rossi et al., 1992).

2.7. Statistical analysis

Statistical analysis of data was performed with the parametric test (t-test) of Microcal ^{TM}Origin^{TM} program (Microcal Software, Inc., Northampton, MA) with a P value <0.05 used to delineate significance.

3. Results

C57BL/6 mice infected with the retroviral complex LP-BM5 were used as animal model of AIDS to evaluate the capacity of AZT+DDI and of their combination with GSH-loaded erythrocytes to protect macrophages in the different body compartments. We studied eight groups of mice: (1) uninfected; (2) infected and untreated; (3) infected and treated with unloaded erythrocytes; (4) infected and treated with AZT; (5) infected and treated with DDI; (6) infected and treated with AZT+DDI; (7) infected and treated with GSH-loaded erythrocytes; (8) infected and treated with AZT+DDI+GSH-loaded erythrocytes.

The results obtained for the group of infected animals treated with unloaded erythrocytes are not reported because they were not different from those obtained for the group of infected and untreated mice.

3.1. Inhibition of BM5d proviral DNA content by AZT+DDI and AZT+DDI+GSH-loaded erythrocytes

In particular, we quantified the proviral DNA content in macrophages obtained from the peritoneal cavity and bone marrow; the results obtained show that all drugs when administered singly exerted a modest action (Fig. 1), while additive effects were obtained with the combination of AZT and DDI which provided an inhibition in about 40% of the infected mice. The difference in proviral DNA content of bone marrow macrophages between the group treated with AZT+DDI and the one treated with AZT+DDI+GSH-loaded erythrocytes was statistically significant ($P=0.02$) (Fig. 1A). Also in the brain (Fig. 1C) the combination of AZT+DDI and GSH-loaded erythrocytes provided a statistically significant reduction ($85\pm10\%$) in proviral DNA content as compared to $47\pm31\%$ provided by AZT+DDI alone ($P=0.018$).

Other organs such as lymph nodes and spleen were examined for proviral DNA content (Table 1): the highest percentages of inhibition with respect to infected mice were obtained in animals treated with AZT+DDI or AZT+DDI+GSH-loaded erythrocytes, but there were not significant differences between the two experimental groups for these other organs.

3.2. Inhibition of lymphoproliferation and hypergammaglobulinemia

Table 2 shows mouse and lymphoid tissue weights at the end of treatment. After 10 weeks of infection, the spleen and lymph node weights of infected mice were about 7 and 36 times higher, respectively than those of uninfected mice. AZT treatment significantly reduced spleen and lymph

node weights that further decreased when DDI was added to AZT therapy. DDI and GSH-loaded erythrocytes did not exert any inhibitory effect when administered singly and addition of GSH-loaded erythrocytes to AZT+DDI administration did not confer additive effects.

IgG serum level represents the polyclonal activation of B cells and in MAIDS hypergammaglobulinemia analyzed by ELISA is used as a sensitive and rapid method to monitor the course of the disease. The results obtained by evaluation of IgG levels in circulation confirmed those obtained by splenomegaly and lymphadenopathy (Table 2).

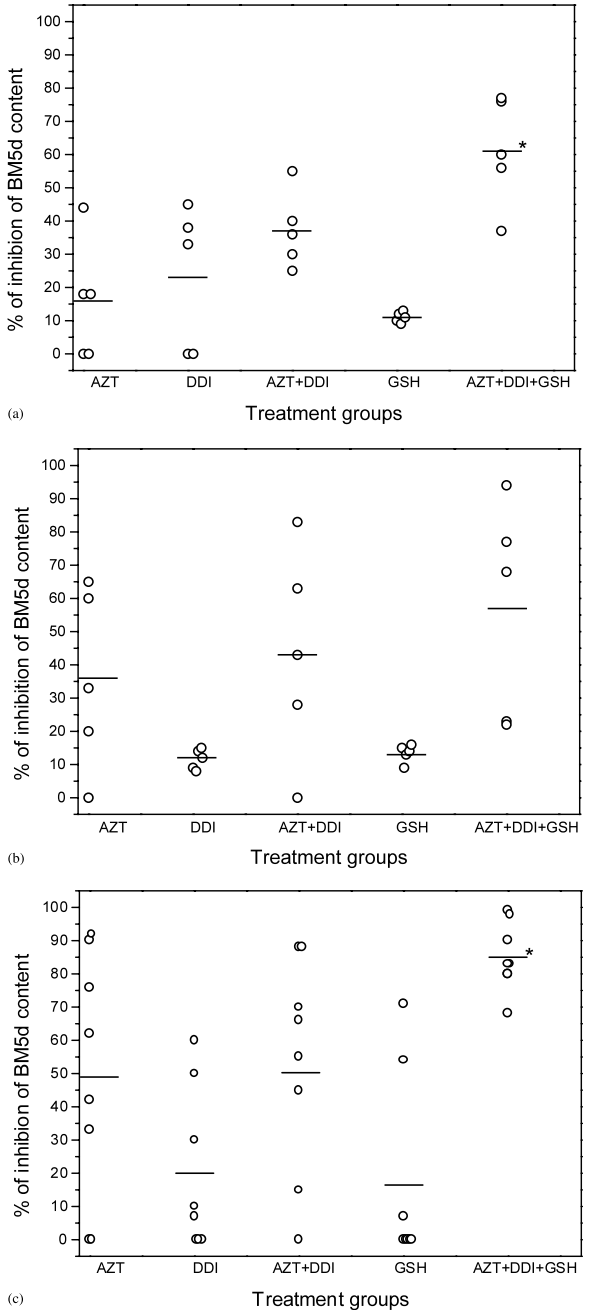

Fig. 1

Table 1
BM5d proviral DNA content in lymph nodes and spleen of C57BL/6 mice infected with LP-BM5 and treated with AZT, DDI, and GSH-loaded erythrocytes

	% of inhibition of BM5d proviral content	
Experimental group	Lymph nodes	Spleen
AZT	67±24	26±37
DDI	41±25	14±10
AZT+DDI	82±29*	47±31*
GSH	33±31	17±15
AZT+DDI+GSH	75±14*	66±17*

Total cellular DNA (40 ng) was used in a competitive PCR assay for proviral DNA detection performed at 5 days after the last treatment with GSH-loaded erythrocytes (Casabianca et al., 1998). The percentage of inhibition refers to the infected mice. Values are shown as mean±S.D. of eight animals and were obtained 10 weeks post infection. The number of copies of proviral DNA in the lymph nodes and spleen of infected mice was $1.9 \times 10^6 \pm 0.4 \times 10^6$ and $3.0 \times 10^6 \pm 0.65 \times 10^6 \mu g^{-1}$ DNA, respectively.

* $P < 6 \times 10^{-4}$ versus untreated infected group.

Fig. 1. Inhibition of BM5d proiral DNA content in A bone marrow macrophages; B peritoneal caity macrophages; and C brain by AZT, DDI, AZTDDI, GSH-loaded erythrocytes and AZTDDIGSH-loaded erythrocytes. AZT and DDI were administered in drinking water at the concentration of 250 and 134 mgl1, respectiely. GSH was encapsulated in erythrocytes as described in Section 2 at a final concentration of 4.671041.00104 mol106 erythrocytes. Proiral DNA content was detected 5 days after the last treatment with GSH-loaded erythrocytes. A total of 40 ng of cellular DNA were used in a competitie PCR assay for proiral DNA quantification Casabianca et al., 1998. The percentage of inhibition refers to the infected mice and was calculated on fie animals panel A, B and eight animals panel C. The number of copies of proiral DNA in the peritoneal and bone marrow macrophages of infected animals was 1.1941060.200106 and 9.9391052.310105 g1 DNA, respectiely. The number of copies of proiral DNA in the brain was 6.7501050.725105 g1 DNA. P0.03 ersus IAZTDDI.

Table 2
Splenomegaly, lymphadenopathy and hypergammaglobulinemia in C57BL/6 mice infected with LP-BM5 and treated with AZT, DDI and GSH-loaded erythrocytes

Experimental group	Body weight (g)	Lymph node weight (g)	Spleen weight (g)	IgG (mg/100 ml)
Uninfected	21.5±1.1	0.04±0.01	0.09±0.01	151±23
Untreated	23.7±0.9	1.46±0.41	0.67±0.09	1957±477
AZT	21.4±0.9	0.62±0.45*	0.36±0.14*	1000±200*
DDI	23.4±1.6	1.49±0.41	0.63±0.17	1515±249
AZT+DDI	21.6±1.2	0.33±0.14*	0.25±0.08*	605±227*,**
GSH	22.1±1.5	1.24±0.50	0.55±0.12	1247±347
AZT+DDI+GSH	21.2±2.0	0.36±0.10*	0.28±0.05*	467±114*,**

Values are shown as mean±S.D. of eight animals and were obtained at 10 weeks post infection. AZT was administered in drinking water at a concentration of 250 mg l^{-1}. DDI was administered in drinking water at a concentration of 134 mg l^{-1}. GSH was encapsulated in erythrocytes as described in Section 2 at a final concentration of $4.67 \times 10^{-4} \pm 1.00 \times 10^{-4}$ μmol 10^{-6} erythrocytes.
* $P < 5 \times 10^{-3}$ versus untreated.
** $P \leqslant 10^{-2}$ versus AZT.

3.3. Mitogenic response of splenocytes

As previously reported, at 10 weeks after infection, T- and B- cell lymphocytes from infected mice had almost completely lost their ability to proliferate following activation with a mitogenic agent (Fraternale et al., 1999). Splenocytes of mice treated with either AZT+DDI or AZT+DDI+GSH-loaded erythrocytes had a proliferative response to mitogens almost comparable with that of control uninfected mice, while the drugs administered singly had no effect (Fig. 2).

4. Discussion

HAART has provided extraordinary clinical benefits in HIV-infected patients in lowering morbidity and mortality; however, replication-competent HIV-1 has been recovered from resting memory T cells, seminal cells, and monocytes of patients on HAART, possessing plasma HIV RNA levels below 50 copies ml^{-1} (Zhang et al., 1998; Finzi et al., 1997). The major obstacle to eradication of HIV is due to the latent virus reservoirs. Cells within anatomic sanctuary sites, such as the brain, may harbor replication-competent HIV-1. Potent HAART drugs such as protease inhibitors are effective in inhibiting replication of HIV-1 within chronically infected cells but, because of the presence of blood–brain barrier, they can not reach adequate concentrations in the CNS, where chronically infected cells of macrophage lineage may be responsible for continuing low-level local production of HIV (Kravcik et al., 1999). The important role of macrophages both in the HIV-1 primary infection, and its further evolution, because they function as reservoirs for the virus, has prompted the search for drugs and/or strategies to protect this cell compartment.

In this paper we investigated the capacity of two nucleoside analogues (AZT+DDI) and of their combination with GSH-loaded erythrocytes in protecting the macrophage compartment; GSH-loaded erythrocytes consisted of autologous erythrocytes in which GSH was encapsulated; the erythrocyte membrane was then modified so as to increase the recognition of these cells by macrophages (Magnani et al., 1992). The results obtained show that AZT+DDI treatment was very effective in reducing splenomegaly, lymphadenopathy and hypergammaglobulinemia (about 63, 77 and 69%, respectively, compared to infected mice); moreover, the same combination was able to almost completely restore the proliferative response of T and B splenocytes to mitogens. AZT administered singly was modestly effective, as described previously (Fraternale et al., 1996), while DDI did not exert any inhibitory effect. DDI provides a very limited protection in the MAIDS model, also at concentrations higher than those

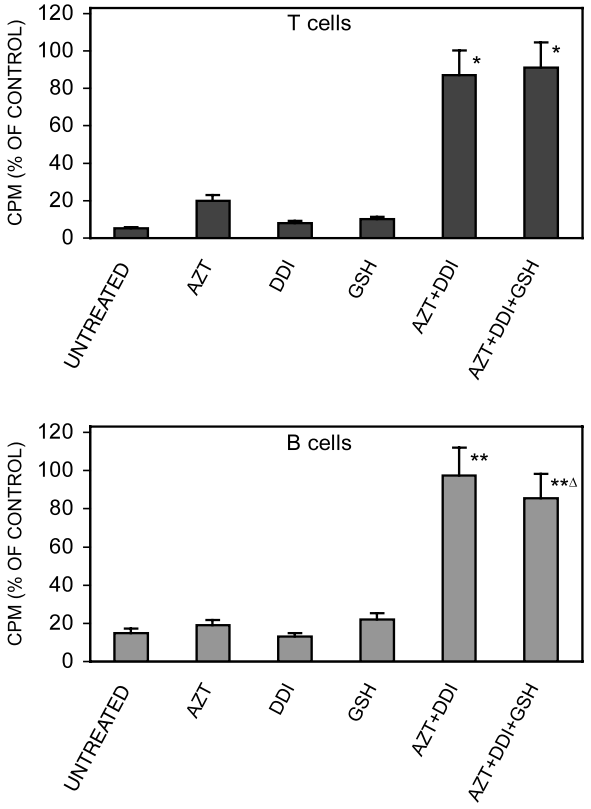

Fig. 2. Lymphocyte proliferative response to PHA (T cells) and LPS (B cells) observed in C57BL/6-infected mice, untreated, or treated as described in the legend to Fig. 1. Spleen lymphocytes were obtained 10 weeks post infection and were assayed for [^{3}H] thymidine incorporation after 3 days of culture with mitogens. Values represent the mean ± S.D. of eight animals. Counts per minute ± S.D. in the controls were $6.2 \times 10^4 \pm 0.7 \times 10^4$ for T cells and $3.93 \times 10^4 \pm 0.4 \times 10^4$ for B cells. $^*P \leqslant 10^{-5}$; $^{**}P \leqslant 2 \times 10^{-2}$ (versus untreated). $^{\Delta}$not significantly different (versus AZT+DDI).

used in the experiments described in this paper (Harvie et al., 1996). As the plasma concentration of DDI in infected animals treated with the drug at a concentration of 134 mg l^{-1} was 3–5 μM (data not shown), we may infer that the limited effect of DDI is not due to insufficient bioavailability.

The results obtained by the quantification of proviral DNA content show that AZT and DDI administered singly exerted an inhibitory effect mainly in the lymph nodes and less so in the spleen; however, significant additive effects were obtained if they were combined. If we examined proviral DNA content in the peritoneal and bone marrow macrophages and in the brain, we noted that AZT and DDI studied either alone or in combination did not exert the same inhibitory effects as in the lymphoid organs.

As already reported (Fraternale et al., 1999), the treatment with GSH-loaded erythrocytes did not reduce spleen and lymph node weight and IgG levels in comparison with infected untreated animals and did not provide additive effects to the combination of AZT and DDI. This finding could be explained by the fact that the combination of AZT and DDI is already greatly efficacious in suppressing MAIDS at the sites of spleen and lymph nodes, so that addition of GSH-loaded erythrocytes did not confer an additional benefit. If, however, this system able to selectively protect macrophages (i.e. GSH-loaded erythrocytes) was added to AZT+DDI regimen, we observed additive effects in the reduction of viral load in those compartments (bone marrow, brain), where macrophages play a central role. The results suggest that nucleoside analogues such as AZT or DDI alone cannot efficiently protect those sites in which macrophages have a central role such as the brain, even if there is a good penetration of some nucleoside analogues, as has been described in HIV-infected patients (Daluge et al., 1997). Moreover, erythrocytes represent a valid strategy to selectively deliver drugs to the macrophage compartment to combine with antiretroviral drugs included in HAART. The combination of AZT+DDI with GSH-loaded erythrocytes is the most effective of all antiretroviral combination therapies that we tested so far in MAIDS for protecting the brain; in fact, the highest percentage of inhibition obtained in this organ (60 ± 21 with respect to control infected mice) had been achieved with the combination of AZT and GSH-loaded erythrocytes (Fraternale et al., 1999). The present findings may seem important since it is known that HIV-1 replication within the CNS may continue in the presence of HAART because of the poor penetration across the blood–brain barrier of anti-HIV-1 drugs currently used. Erythrocytes, being able to cross the blood–brain barrier, represent a valid system to improve drug delivery and entrapment in CNS.

Moreover, recent findings have ascertained that HIV-infected macrophages in the absence of their productive infection may affect the astrocytic function with consequences for neuronal homeostasis and alteration of the blood–brain barrier (Balestra et al., 2001). In particular the selective delivery of GSH to macrophages seems interesting as it has been demonstrated that HIV-1 infection induces a significant decrease in intracellular GSH levels in human macrophages (Garaci et al., 1997; Mialocq et al., 2001). Moreover, recently it has been suggested that oxidative stress, which occurs in brain tissues of patients undergoing HIV infection, plays an important role in the pathogenesis of neuroAIDS (Mollace et al., 2001). For these reasons, HIV-infected macrophages represent a target for new antiretroviral GSH-replenishing drugs (Mialocq et al., 2001). GSH (and GSH-replenishing drugs) are interesting also because different mechanisms for GSH antiviral action have been proposed; these are: (a) selective decrease of the levels of specific glycoproteins, such as gp 120, which are particularly rich in disulfide bonds (Palamara et al., 1996); (b) inhibition of the activation of NF-kB (Mihm et al., 1995); (c) inhibition of reverse process (Kameoka et al., 1996).

In conclusion, erythrocytes represent a valid carrier system to selectively deliver antiretroviral molecules to macrophages; in combination with nucleoside analogues, they show, in a murine model of AIDS, significant additive antiretroviral effects in those compartments where macrophages play a key role. Erythrocytes might be used as carriers of GSH to achieve better efficacy, lower toxicity and better macrophage targeting of the drug.

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