REGULATED EXPRESSION OF GALECTIN-1 AFTER *IN VITRO* PRODUCTIVE INFECTION WITH HERPES SIMPLEX VIRUS TYPE I: IMPLICATIONS FOR T CELL APOPTOSIS

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Apoptosis of cytotoxic T lymphocytes by herpes simplex virus type-1 (HSV-1) has been reported to be a relevant mechanism of viral immune evasion. Galectin-1 (Gal-1), an endogenous lectin involved in T-cell apoptosis, has recently gained considerable attention as a novel mechanism of tumor-immune evasion. Here we investigated whether infection of cells with HSV-1 can modulate the expression of Gal-1. Results show that pro-apoptotic Gal-1, but not Gal-3, is remarkably up-regulated in cell cultures infected with HSV-1. In addition, this protein is secreted to the extracellular milieu, where it contributes to apoptosis of activated T cells in a carbohydrate-dependent manner. Since many viruses have evolved mechanisms to counteract the antiviral response raised by the infected host, our results suggest that HSV-1 may use galectin-1 as a weapon to kill activated T cells and evade specific immune responses.

Herpes simplex virus type-1 and -2 (HSV-1 and -2) can produce acute and latent infections in humans and in experimental animals (1). After infection, HSV replicates at the inoculation site and then migrates along rachydeal and autonomic nerves towards the nuclei of neurons (2-3), where it induces a life-long latent infection. Cell-mediated and humoral immunity have been reported to play key roles in the defense against HSV infection. Immunocytochemical studies in skin biopsies taken from patients with recurrent HSV infections showed infiltration of T lymphocytes around the necrotic lesions (4) and clearance of HSV-2 from recurrent genital lesions has been associated with the local activity of $CD4^+$ T and $CD8^+$ T cells (5). In addition, HSV-1 neurovirulence was enhanced *in vivo* when animals were treated with antibodies against anti-$CD8^+$ T lymphocytes (6).

It has been reported that HSV-1 uses several strategies to evade or subvert the immune response (7-9). Viral protein ICP47 has been reported to bind the transporter associated with antigen processing (TAP), thus blocking presentation of viral peptides to major histocompatibility complex class-I-restricted cells (10). Furthermore, HSV-1 glycoprotein D has been reported to inhibit T-cell proliferation through the inhibition of signaling pathways triggered by the herpes virus entry mediator HVEM (11). After infection with HSV-1, antigen-presenting cells are also impaired in their ability to induce $CD4^+$ T cell activation (12).

Key words: galectins, galectin-1, apoptosis, immune evasion, Herpes simplex virus I,

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Furthermore, fraticidal cell death of T lymphocytes has been proposed to be an important immune evasion mechanism following HSV-1 infection, favoring the persistence of the virus in the host throughout its lifetime (13). However, the molecular mediators and biochemical pathways involved in HSV-1-induced apoptosis have not yet been elucidated in detail. It has been demonstrated that following 1 h infection of epithelial cells, HSV-1 can trigger the synthesis of proteins involved in cellular apoptosis, whereas after 3-6 h post-infection cells start to synthesize a number of proteins which can block the process of cell death (14). Early viral genes are immediately involved in this subtle regulation of promotion and inhibition of apoptosis (15), whereas NF-kappa B (16) and Us3 viral protein kinase (17) are required for apoptosis prevention. In this sense, it has been speculated that HSV-1-infected epithelial cells induce apoptosis of cytotoxic T lymphocytes (CTLs) to avoid immune attack. In fact, recent studies demonstrated the inactivation of CTLs after cell-to-cell contact between CTLs and HSV-infected cells (18).

Galectins are members of a large, growing family of endogenous animal lectins which share sequence similarities in the carbohydrate recognition domain (CRD) and specificity for β-galactoside-containing saccharide ligands (19-21). Despite extensive sequence homology and similarities in sugar-binding specificity, various members of this protein family (such as galectin-3) behave as amplifiers of the inflammatory response and protect cells from apoptosis, while others, such as galectin-1 (Gal-1), have pro-apoptotic and immunosuppressive activity (22-26).

Recently, we have reported that targeted inhibition of Gal-1 gene expression in the tumor microenvironment suppresses tumor growth and results in heightened T cell- mediated rejection (27), providing evidence of a novel mechanism by which tumor cells evade immune attack (28). Furthermore, we have also reported the ability of Gal-1 and Gal-3 to modulate the outcome of *Trypanosoma cruzi* infection by influencing macrophage and B-cell survival (29-30). In addition, Kiss and colleagues reported the up-regulation of galectin-3 and its ligands following *T. cruzi* infection, an effect which was associated with modulation of adhesion and migration of murine dendritic cells (31). These results suggest that galectins might be important in the control of intracellular infections. Recently, Lanteri and colleagues (32) showed that *in vitro* infection of T cell lines with human immunodeficiency virus type-1 (HIV-1) increases the levels of core 2-*O* glycans, which constitute the preferred ligands for Gal-1, suggesting a potential role for this pro-apoptotic protein in the context of viral infections.

In the search for potential mediators involved in HSV-1-induced T-cell death and immunosuppression, we investigated Gal-1 expression and function after infection of epithelial cells *in vitro* with HSV-1.

MATERIALS AND METHODS

Virus and cells

The MacIntyre strain of HSV-1 was used. The virus was obtained from the Center for Diseases Control (Atlanta, GA), cultured in Vero cells and titrated by the classic plaque-forming units method.

Indirect immunofluorescence

Vero cells (obtained from the National Institute for Microbiology, Argentina) were grown onto glass coverslips placed into Petri dishes with Dulbecco's modified Eagle medium (DMEM) (Gibco) and 10% fetal calf serum, at 37ºC and with an atmosphere of 5% CO_2 Coverslips were fixed in methanol and rehydrated in PBS. Non-specific background was blocked by the addition of 5% normal goat serum in PBS. The first antibodies were either rabbit IgG anti-HSV-1 (Dako) diluted in PBS 1:500 or rabbit anti-Gal-1 or anti Gal-3 polyclonal Abs diluted 1:100 in PBS prepared as described (26). After washing, the secondary antibody (goat anti-rabbit IgG-TRICT conjugated) was added at a dilution of 1:100 in PBS.

SDS-PAGE and Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Miniprotean-II electrophoresis apparatus (Bio-Rad). Non-infected or HSV-1-infected Vero cells were collected in PBS, centrifuged and resuspended in 100 ml of ice-cold lysis buffer containing a protease inhibitor cocktail (Sigma Chemical Co.). Serum-free conditioned medium (SFCM) from non-infected and HSV-1-infected cells were also collected and concentrated by overnight precipitation with 9 vol. of methanol at -20°C in order to investigate the levels of Gal-1 secretion

Protein concentration was measured using the Micro-BCA kit (Pierce). Equal amounts of protein (30 µg) of cell lysates obtained from non-infected or HSV-1-infected cells were loaded into each lane of the gel. Samples were electrophoresed in a 15% separating polyacrylamide slab

gel, transferred onto nitrocellulose membranes and probed with a 1:1000 dilution of the anti-Gal-1 or anti-Gal-3 polyclonal antibodies. Blots were then incubated with a 1 mg/ml concentration of horseradish peroxidase-conjugated anti-rabbit IgG, developed using the ECL detection reagent (Amersham Pharmacia) and exposed to Amersham Hyperfilm for 3 to 5 min. Equal loading was checked by Ponceau S staining or by incubation of the blots with an anti-a tubulin mAb (Sigma).A

Apoptosis assays

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human volunteers by Ficoll-Paque™ Plus gradient centrifugation, washed and resuspended in RPMI 1640 supplemented with 10% heat-inactivated, pooled normal human AB serum, sodium pyruvate, glutamine and penicillin-streptomycin (RPMI/PHS). PBMCs were stimulated with anti-CD3 mAb to activate T cells, and cultured for 3 days in 24-well, microtiter plates. Fully activated T cells (as measured by the levels of CD69 and CD25 expression) were then exposed for 18 h to supernatants obtained from HSV-1-infected or non-infected Vero cells and processed for apoptosis. To rule out the possibility that HSV-1 may directly interact with human lymphocytes, we added a specific anti-HSV-1 serum to the culture medium to neutralize eventual infectious viral particles. To determine whether HSV-1-induced T-cell apoptosis involved a Gal-1-mediated pathway, activated T cells were also exposed to HSV-1-infected or non-infected cells in the presence of thiodigalactoside (TDG, 30 mM), or a blocking anti-Gal-1 antibody (10 μg/ml) as described (27). Cells exposed to different treatments were finally processed for apoptotic cell detection by propidium iodide (PI) (Sigma Chemical Co.) staining of subdiploid nuclei or by annexin V staining using the FITC -annexin V binding assay (BD Biosciences).

Statistical analysis

Comparison of two groups was made using Student´s *t* test for unpaired data in apoptosis assays. $P<0.05$ was considered statistically significant.

RESULTS

In vitro infection with HSV-1 up-regulates the synthesis and secretion of Gal-1

To investigate the role of Gal-1 during HSV-1 infection, we explored whether the *in vitro* infection with HSV-1 is sufficient to modulate expression of pro-apoptotic Gal-1 in target cells. Monolayers of Vero cells were grown onto glass coverslips and infected at a M.O.I. of 1 pfu of HSV-1/cell. After 2 h of adsorption, cells were washed with PBS and fresh medium was added. At 18 h post-infection (pi) the coverslips were fixed and processed for indirect immunofluorescence (IFI). Fig. 1 illustrates (i): non-infected Vero cells (A) and HSV-1-infected cells (D) treated with normal rabbit serum as primary antibody; (ii): non-infected (B) and HSV-1-infected (E) cells stained with an anti-HSV-1 serum; and (iii): non-infected (C) and HSV-1-infected monolayers (F) treated with an anti-Gal-1 antibody. A productive infection was clearly detected at 18 h p.i. (E). A dramatic up-regulation of Gal-1 expression was found in HSV-1-infected (F) versus non-infected Vero cells (C). Up-regulated expression of Gal-1 was also observed following infection of human fibroblasts with HSV-1 (data not shown).

In another set of experiments, Vero cells monolayers were infected as described above, harvested after 18 h p.i and processed for Western blot analysis. As shown in Fig. 2A, Gal-1 levels were markedly increased in Vero cells after infection with HSV-1 for 18 h (lane 1) as compared with uninfected cells (lane 2). In addition, Gal-1 was also present in concentrated supernatants from HSV-1-infected cells (Fig. 2B, lane 1), but not in uninfected Vero cells (Fig. 2B, lane 2), suggesting that HSV-1 induces release of Gal-1 from infected target cells to exert extracellular biological functions. In contrast, lower levels of Gal-3 were found by Western blot analysis in uninfected Vero cells (Fig. 2C; lane 2) and its expression was not affected after productive infection with HSV-1 for 18 h under the experimental conditions used (Fig. 2C, lane 1).

Herpes simplex virus-1-infected cells promote T-cell apoptosis through a Gal-1-dependent pathway

To gain insights into the functional significance of up-regulated Gal-1, optimal dilutions of supernatants (serum-free conditioned medium: SFCM) from HSV-1-infected or uninfected cells were collected and added to anti-CD3-stimulated T cells (at a 1:10 dilution) to evaluate HSV-1-induced T-cell apoptosis in the absence or presence of thiodigalactoside (TDG; a galectin-specific carbohydrate inhibitor) or a blocking anti-Gal-1 antibody.

As shown in Fig. 3, SFCM derived from HSV-1-infected cells induced high levels of apoptosis (50.17%) on anti-CD3 mAb-stimulated T cells

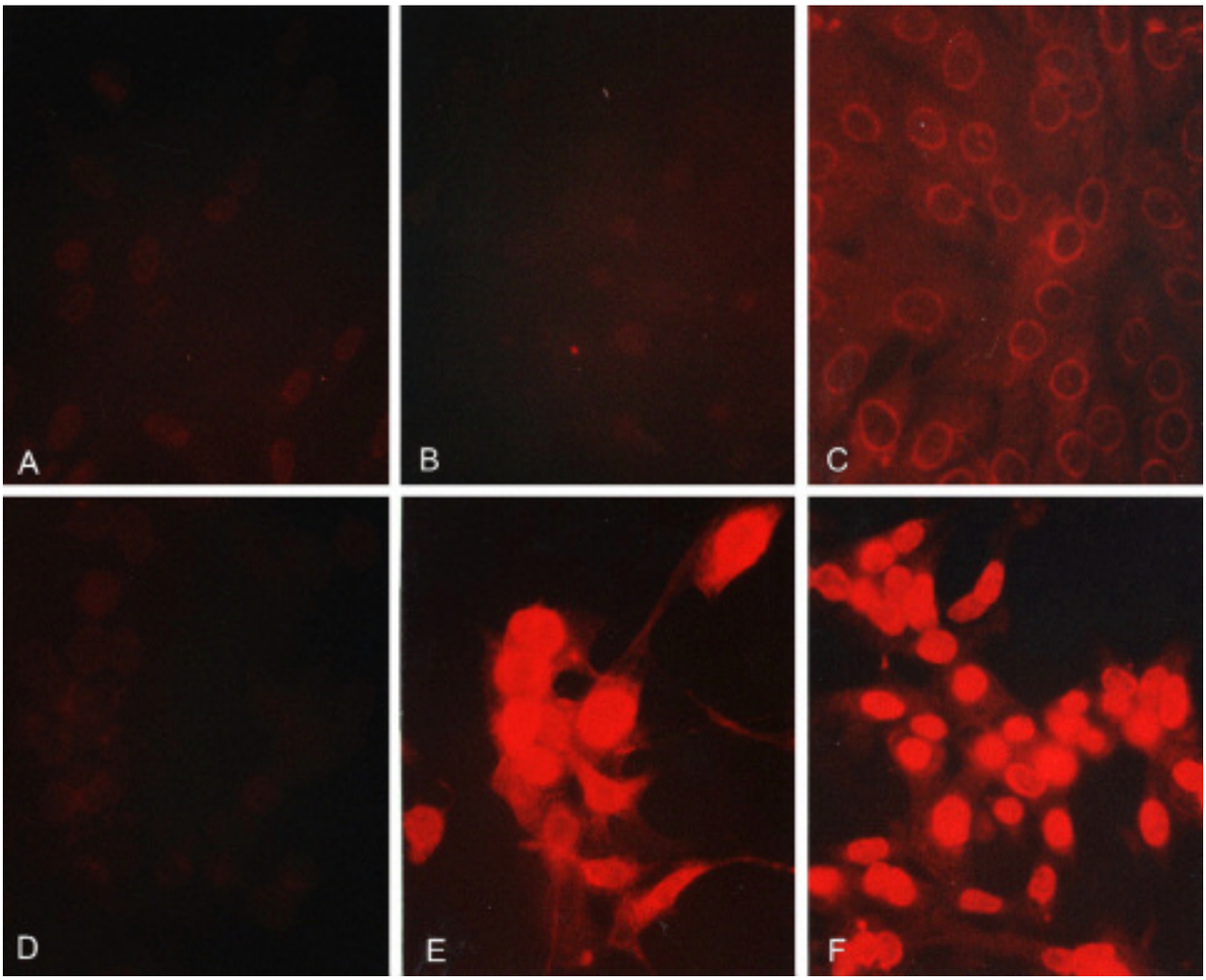

Fig. 1. *Immunodetection of Gal-1 by indirect immunofluorescence staining in mock-infected and HSV-1-infected Vero cells. The figure shows an indirect immunofluorescence staining of non-infected (A, B, C) and HSV-1-infected (D, E, F) Vero cells at 18 h pi. For control purposes, non-infected (A) and HSV-1-infected (D) were incubated with normal rabbit serum as primary antibody. To determine productive infection, non-infected (B) or HSV-1-infected cells (E) were also stained with specific anti-HSV-1 antibody. To analyze modulation of Gal-1 expression, non-infected (C) and HSV-1-infected cells (F) were exposed to a rabbit anti-human Gal-1 polyclonal antibody. The productive infection due to HSV-1 was evident at 18 h p.i (E) compared with the absence of labeling in non-infected cells (B).*

(upper left panel), as shown by propidium iodide (PI) staining of subdiploid DNA content. In contrast, lower levels of apoptosis (24.09 %) were detected in anti-CD3 mAb-stimulated T cells cultured in the presence of SFCM derived from non-infected Vero cells (Fig. 3, second left panel). The pro-apoptotic activity displayed by supernatants from HSV-1-infected cells was partially abrogated when SFCM was preabsorbed with 30 mM TDG (Fig. 3, upper right panel; 19.96%) or a blocking anti-Gal-1 antibody (10 μg/ml) (Fig. 3, lower left panel; 18.76%) before exposure to stimulated T cells suggesting that the pro-apoptotic effect was at least in part associated to the release of biologically active Gal-1 and that the carbohydrate-binding properties of this lectin are involved in this process. As a control, no significant changes in the levels of apoptosis were detected when supernatants from non-infected cells were incubated in the presence of TDG or an anti-Gal-1 antibody before exposure to anti-CD3 mAb-stimulated T cells (Fig. 3, second and lower right panels respectively). As a negative control, anti-CD3 mAb-stimulated T cells, which were cultured for 18 h in the absence of any other

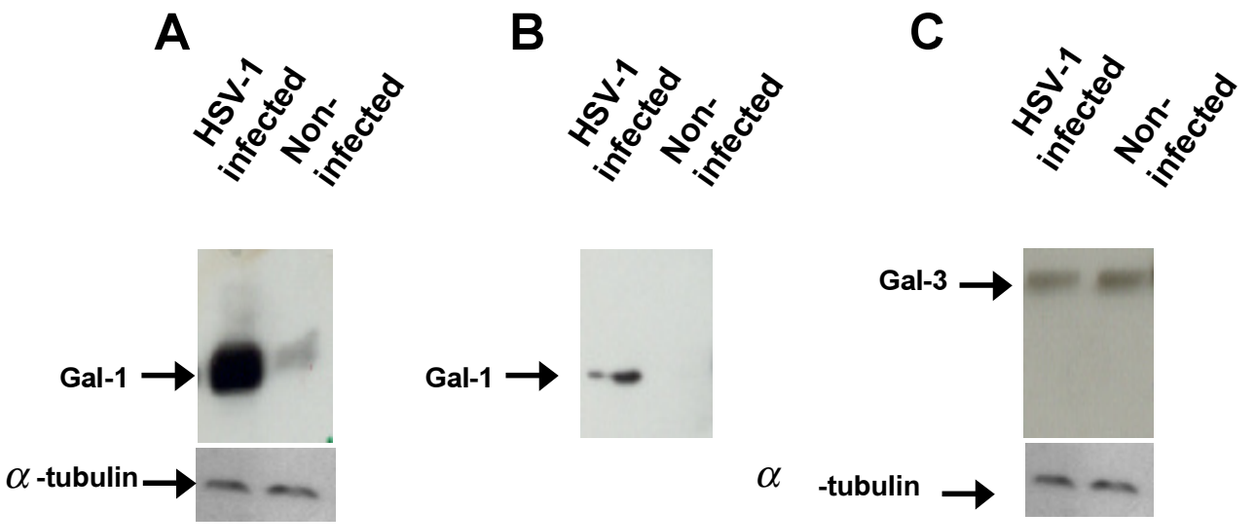

Fig. 2. *Western blot analysis of Gal-1 and Gal-3 in total cell extracts and serum-free conditioned medium from non-infected and HSV-1 infected Vero cells. Herpes simplex virus-1 specifically up-regulates Gal-1 expression (panel A; lane 1 vs lane 2) and induces Gal-1 secretion (panel B, lane 1 vs lane 2) from infected Vero cells, while does not affect Gal-3 expression (panel C, lane 1 vs lane 2). Briefly, cells were infected with HSV-1 as described in Materials and methods. After 18 h, mock infected and HSV-1-infected cells were extensively washed to remove extracellular viral particles and lysates were prepared and processed for Western blot analysis. To determine modulation of Gal-1 and Gal-3 expression, equal amounts of proteins (30 μg) of each cell extract were loaded into each lane, blotted onto nitrocellulose membranes and revealed using rabbit anti-Gal-1 (panel A) or anti-Gal-3 (panel C) specific polyclonal antibodies. Equal amounts of protein were checked using an anti α-tubulin monoclonal antibody (Sigma). To investigate Gal-1 secretion, serum-free supernatants from mock-infected or HSV-1-infected cells were also collected, concentrated and processed by Western analysis (panel B). Gal-3 secretion was not observed in supernatants from mock infected or HSV-1-infected cells under the sensitivity of this method (data not shown).*

stimuli showed low levels of apoptosis (~7-8%) (data not shown). Similar biologically and statistically significant differences were observed when T-cell death was analyzed by annexin V staining (Fig. 3B).

Taken together, these results indicate that HSV-1 modulates expression and secretion of cellular Gal-1 after productive infection *in vitro*, while it does not affect expression of Gal-3, another member of the galectin family with mainly pro-inflammatory activity. Moreover, supernatants from HSV-1-infected cells display pro-apoptotic activity, which was dependent on the carbohydrate-binding properties of this protein. These combined results suggest that up-regulation of Gal-1 may account, at least in part, for augmented T-cell apoptosis observed following HSV-1 infection.

DISCUSSION

Viruses have evolved several strategies to counteract the antiviral immune responses raised by the infected host. These strategies include blockade of components of the antigen processing machinery, inhibition of pro-inflammatory cytokines and induction of T-cell apoptosis (7-9).

Recent studies indicated that tumors can evade T cell mediated responses through Gal-1-dependent mechanisms (27). Interestingly, inhibition of Gal-1 gene expression in tumor tissues resulted in reduced tumor growth and enhanced T cell mediated rejection, suggesting a role for this protein as a novel factor implicated in tumor-immune evasion. The association between these observations and the role of T cell apoptosis as a relevant mechanism of evasion during HSV-1 infection, prompted us to investigate the regulated expression of Gal-1 during HSV-1 infection and its contribution to T cell death.

Galectins have recently gained considerable

attention as novel regulator of T cell homeostasis. Gal-1 has been shown to impair T cell effector functions by promoting growth arrest and apoptosis of activated T cells (25, 33-34), antagonizing T-cell activation (35) and/or blocking pro-inflammatory cytokine secretion (24,36). *In vivo*, therapeutic administration of Gal-1 suppresses T helper (T_H)-1-dependent chronic inflammation in experimental models of autoimmunity by increasing T-cell susceptibility to activation-induced cell death and skewing the balance of the immune response towards a T_H2 profile (26,37). Since HSV-1 has been shown to subvert immune responses by inhibiting T-cell survival and proinflammatory cytokine secretion (7-9), this infection provides an excellent opportunity to correlate the levels of Gal-1 with the outcome of the infection. Studies are currently being conducted in our laboratory to examine the progression of experimental infections in Gal-1-null mutant mice.

Our results indicate that after the infection of epithelial cells with HSV-1, Gal-1 synthesis is remarkably increased. Moreover, this protein is also secreted to supernatants of infected cells. Release of biologically active Gal-1 to culture medium of HSV-1-infected cells was clearly associated with an induction of apoptosis of human activated T lymphocytes. Participation of a Gal-1-mediated mechanism in this apoptotic effect was confirmed, since it could be inhibited by addition of TDG (a β-galactoside-related sugar) or a blocking anti-Gal-1

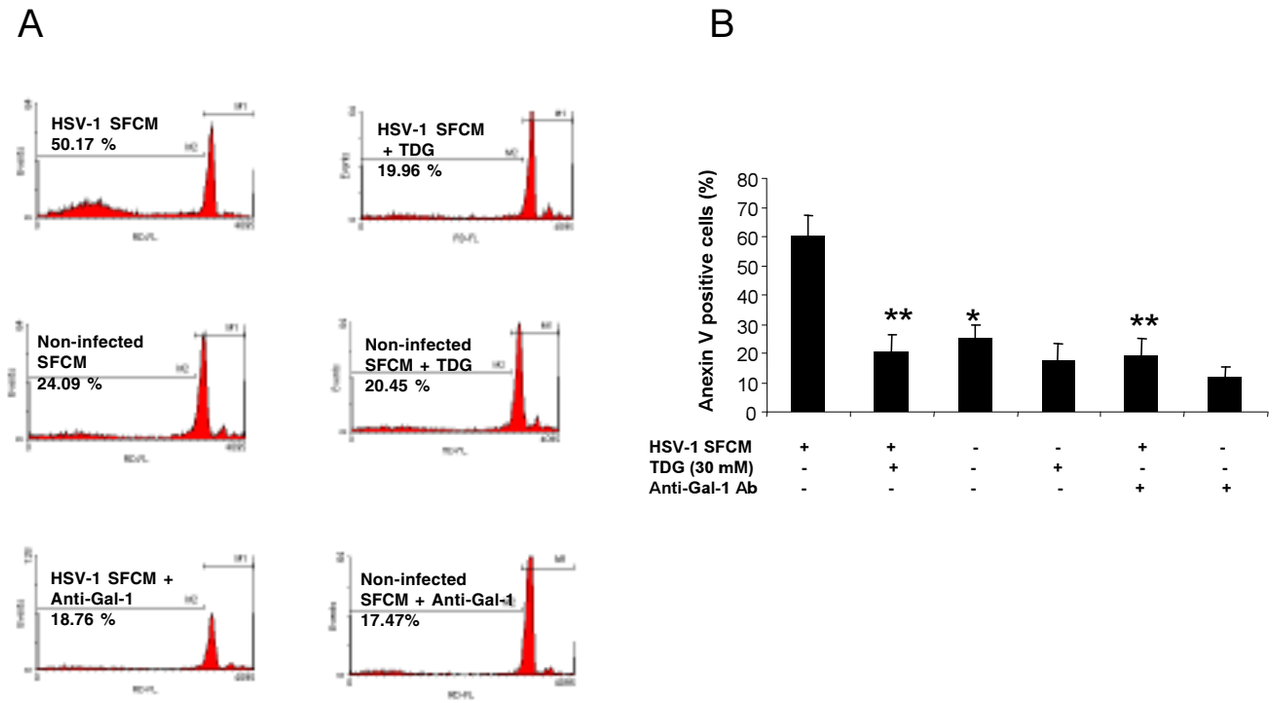

Fig. 3. *Supernatants from HSV-1-infected cells induce apoptosis of activated T cells through a Gal-1-dependent pathway. Peripheral blood mononuclear cells were activated with anti-CD3 monoclonal antibody for three days as described in Materials and methods to mimic peptide-MHC cross-linking of the TCR/CD3 complex. Fully activated T cells were further exposed for 18 h to supernatants derived from HSV-1-infected (upper left panel) or non-infected (second left panel) Vero cells, in the absence or in the presence of 30 mM thiodigalactoside (TDG), a specific galectin inhibitor (upper right panel) or a blocking anti-Gal-1 antibody (10 μg/ml; lower left panel). Apoptosis was then assessed by analyzing the frequency of cells with subdiploid nuclei using propidium iodide (PI) staining (A) or by measuring the level of phosphatidilserine exposure using FITC-anexin V (B) as described in Materials and methods. As a control, anti-CD3 mAb-stimulated T cells cultured for 18 h in the absence of any other stimuli showed low levels of apoptosis (~ 7-8%). Similar results were obtained after T-cell activation with PHA instead of an anti-CD3 mAb. Data are representative of three independent experiments using T cells from different donors. *P< 0.05 supernatants from HSV-infected vs supernatants from mock infected cells; **P< 0.05 supernatants of HSV-1-infected cells cultured in the presence of TDG or anti-Gal-1 vs supernatants of HSV-1-infected cells cultured in the absence of anti-Gal-1 antibody or TDG.*

antibody to the culture medium. These results were not due to the direct effects of HSV-1 interaction with human lymphocytes since a specific anti-HSV-1 serum had been added to the culture medium to neutralize any eventual infectious viral particles. Moreover, we also rule out the possibility that Gal-1 present in the serum added to the culture medium could be responsible of the effects described, since serum-containing medium was replaced by serum-free medium after viral adsorption to the cells. Furthermore, according to our results, it is evident that HSV-1 infection dramatically increases Gal-1 expression, while the synthesis of Gal-3 is not significantly affected in comparison with non-infected cells, suggesting the ability of HSV-1 to specifically modulate Gal-1 gene expression. The intracellular pathways involved in this regulated expression are currently under investigation.

The multivalent properties of galectins make these proteins suited for cell adhesion functions, suggesting that they could also be engaged in host-pathogen interactions. In fact, β-galactoside-related carbohydrates (polygalactose epitopes) are commonly present in pathogen-associated glycoconjugates leading to the suggestion that galactose-binding proteins may act as host receptors for bacteria, fungi and parasites and there are now several examples in the literature of apparent direct or indirect galectin-dependent specific host-pathogen interactions (reviewed in 38). For example, Moddy *et al* (39) demonstrated that galectin-3 specifically binds to *T. cruzi* trypomastigotes and modulates adhesion of the parasite to extracellular matrix glycoproteins, such as laminin in a carbohydrate-dependent manner (39). Furthermore, Sato and colleagues (40-41) showed that galectins-3 and -9 can interact with *Leishmania major* through binding to specific polygalactose epitopes on lipophosphoglycans (LPG) and galectin-1 can favor attachment of HIV-1 to target cells (42). In agreement with these findings, recent work showed that [GalNAc β1-4GlcNAc] (LacdiNAc)-glycans constitute a specific parasite pattern for galectin-3-mediated immune recognition (43). Thus, galectins may function as potential pattern recognition receptors (or danger signals) transmitting the information of microbial invasion to immune cells.

To conclude, in the present study we describe the up-regulation of Gal-1 after *in vitro* lytic infection of cells with HSV-1. Furthermore, HSV-1 induces release of bioactive Gal-1 from epithelial cell cultures, which contributes to apoptosis of activated T cells. Although these results cannot be extrapolated "*prime facie*" to the situation of the natural infection produced by HSV-1 in humans, it is attractive to hypothesize that during the acute phase of HSV-1 infection, the virus may trigger a Gal-1-mediated immunosuppressive effect towards effector T lymphocytes using a novel, but evolutionarily conserved, immune evasion mechanism, as has been proposed for tumour cells.

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