Herpes Simplex Virus Type 1 Infection of Activated Cytotoxic T Cells: Induction of Fratricide as a Mechanism of Viral Immune Evasion

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Summary

Herpes simplex virus type 1 (HSV1), a large DNA-containing virus, is endemic in all human populations investigated. After infection of mucocutaneous surfaces, HSV1 establishes a latent infection in nerve cells. Recently, it was demonstrated that HSV1 can also infect activated T lymphocytes. However, the consequences of T cell infection for viral pathogenesis and immunity are unknown. We have observed that in contrast to the situation in human fibroblasts, in human T cell lines antigen presentation by major histocompatibility complex class I molecules is not blocked after HSV1 infection. Moreover, HSV1 infection of T cells results in rapid elimination of antiviral T cells by fratricide. To dissect the underlying molecular events, we used a transgenic mouse model of HSV1 infection to demonstrate that CD95 (Apo-1, Fas)-triggered apoptosis is essential for HSV1-induced fratricide, whereas tumor necrosis factor (TNF) also contributes to this phenomenon but to a lesser extent. By contrast, neither TRAIL (TNF-related apoptosis-inducing ligand) nor perforin were involved. Finally, we defined two mechanisms associated with HSV1-associated fratricide of antiviral T cells: (a) T cell receptor–mediated upregulation of CD95 ligand and (b) a viral "competence-to-die" signal that renders activated T lymphocytes susceptible to CD95 signaling. We propose that induction of fratricide is an important immune evasion mechanism of HSV1, helping the virus to persist in the host organism throughout its lifetime.

Key words: viral immune evasion • apoptosis • activation-induced cell death • herpes simplex virus • antigen presentation

Herpes simplex virus type 1 (HSV1), a member of the subfamily *alphaherpesviridae*, is an important neurotropic virus with a worldwide distribution. After primary infection, HSV1 becomes latent in neurons. From there it can periodically emerge to reinfect skin or other tissues, causing recurrent mucocutaneous lesions in about one third of the human population (1, 2). In rare cases, the virus spreads within the central nervous system to cause life-threatening encephalitis. Numerous reports have demonstrated that T cells are necessary for clearing HSV1 infections and that $CD8^+$ antiviral CTLs play a pivotal role in the elimination of most viruses. However, herpesviruses have evolved delicate strategies to evade the attack of T cells, enabling them to persist and reactivate in the host, usually without causing significant tissue damage (3, 4).

In the course of viral infection, a population of CTL precursor cells with a TCR repertoire strongly skewed toward viral antigens is activated in the draining lymph nodes and, in the case of HSV1 infections, subsequently migrates to the site of viral replication in the epithelium (5). There, HSV virions can productively infect the antiviral CTL because HSV1 has been shown to enter activated T lymphocytes (6, 7) after binding of the viral glycoprotein D (gD)[1] to the herpesvirus entry mediator molecule (HVEM), a novel member of the TNF/nerve growth factor receptor family expressed by activated T cells (8, 9).

HSV1, like other large DNA-containing viruses, employs multiple viral defense mechanisms that cooperate to prevent premature death of the host cell induced by a variety of intracellular and extracellular signals. The virus thereby increases the yield of viral progeny after lytic infection of its main target cells. Whether a particular viral defense mechanism is operative depends on the HSV1-infected cell type

[1]*Abbreviations used in this paper:* AICD, activation-induced cell death; gD, glycoprotein D; L, ligand; MOI, multiplicity of infection; SEB, staphylococcal enterotoxin B; TRAIL, TNF-related apoptosis-inducing ligand; TUNEL, TdT-mediated dUTP-biotin nick-end labeling.

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(10). It has been reported for various cell types other than CTLs that HSV proteins can block apoptosis that has been triggered as a direct response of the host cell to virus infection (cell-autonomous apoptosis) (10–12). In infected human fibroblast cell lines, HSV1 has also been shown to evade attacking antiviral CTLs by preventing the transport of MHC class I molecules to the cell surface (13, 14) and by conferring resistance to CTL-induced antigen-dependent apoptosis (15). As viral replication in CTLs is limited, the contribution of T cell infection to the overall production of infectious HSV1 particles is marginal. This raises two important questions: Are viral defense mechanisms operative in infected T lymphocytes and, more importantly, Why are activated T cells the target of HSV1?

In this paper, we analyze the functional consequences of HSV1 infection for activated antiviral T lymphocytes and show that HSV1 removes the block by which activated CTLs protect themselves from inflicting "self injury" (16–20). On the basis of these data, we propose a scenario of viral immune evasion in which infected antiviral T lymphocytes serve as decoy targets and kill each other (fratricide) instead of infected epithelium, the main cell type producing HSV1 virions.

Materials and Methods

Cells and Media. CV1 African green monkey kidney cells were used for propagation and titration of the virus. For studies of K^b expression and T cell activation, NIH 3T3 murine fibroblast cells or the murine mastocytoma cell line P815 and derived transfectants were employed, namely: P815-K^b expressing the gene for the heavy chain of the murine MHC class I molecule K^b (21), P815-B7 expressing the human B7.1 (22), and P815-K^b-B7, which express both molecules on the cell surface (21). Human embryonic lung fibroblasts (HELs), the human lymphoblastoma cell line Jurkat, and human PBMCs isolated from units of buffy coat supplied from the University of Heidelberg blood bank were used for investigation of HSV1 infection of human cells. CV1, NIH 3T3, P815, and HEL cells were maintained in DMEM supplemented with 10% FCS, 100 IU penicillin, 100 μg/ml streptomycin, and 4 mM l-glutamine (DM medium). Transfectants of P815 cells were maintained in the same medium with a supplement of 0.4 mg/ml G418 (GIBCO BRL). Murine splenocytes and Jurkat cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 IU penicillin, 100 μg/ml streptomycin, 4 mM glutamine, 10 mM Hepes, and 50 μM 2-ME (RP medium). Human PBMCs were propagated in the same medium, except that FCS was replaced with 10% heat-inactivated pooled human AB serum (RP-h medium).

Viruses and Infection. F-US5MHC (recombinant HSV1 expressing murine β2-microglobulin and H-$2K^b$) and F-US5β (expressing β-galactosidase), which are identical with respect to HSV1 genes other than the transgenes, were gifts of D.C. Johnson (McMaster University, Hamilton, Ontario, Canada; 13). HSV1 strain F, from which both recombinant viruses were derived, was obtained from American Type Culture Collection (ATCC). A mutant HSV1 strain F that lacks the transporter associated with antigen processing blocking protein ICP47 (F-ICP47Δ) was a gift of R.L. Hendricks (University of Illinois, Chicago, IL; 23). For production of virus stock supernatant, HSV1-infected CV1 cell cultures were centrifuged at 10,000 *g* and then filtered with a 0.45-mm mesh filter to remove cellular debris before being pelleted for 1.5 h at 43,000 *g*. The viral pellet was resuspended in RPMI with 10% heat-inactivated FCS and then frozen at −80°C to give a concentrated viral stock. Frozen viral stocks were titrated using the endpoint dilution method, tested for the presence of mycoplasma using the mycoplasma detection kit (Boehringer Mannheim), and used within 4 mo of manufacture. To infect cells, virus was added to a minimal quantity of the medium appropriate for various cell types and then cultured for 1 h at 37°C. Subsequently, cells were washed three times with PBS to remove unadsorbed virus before adding fresh medium.

Animals. K^b-specific TCR (Des.TCR)-transgenic mice have been previously described (24). These transgenic animals express the rearranged TCR-α and -β genes derived from CTL clone KB5.C20, which is allospecific for the K^b antigen (25). Perforin-deficient mice (26) were provided by Dr. H. Hengartner (University of Zürich, Zürich, Switzerland). Des.TCR and perforin-deficient mice were crossed and kept under pathogen-free conditions in the animal facility of the German Cancer Research Center (Heidelberg, Germany). Female DBA/2 and C57Bl/6 mice, 4–6 wk old, were purchased from Charles River Labs. and maintained in the animal facility of the University of Heidelberg.

Irradiation. Gamma irradiation of 8,000 rads was found to be equally effective in preventing either cellular (P815 cells and T cell blasts) proliferation or HSV1 replication.

Preparation and Stimulation of CTLs. Mononuclear cells were isolated from murine splenocytes or human peripheral blood by separation over Ficoll-Paque™ columns (Amersham Pharmacia Biotech). To generate murine T cell blasts by polyclonal stimulation, cells were incubated at a density of 2.5×10^6 cells/ml in RP medium with 2.5 μg/ml Con A for 2 d. As $CD8^+$ T cells derived from Des.TCR splenocytes are >90% positive for the transgenic K^b-reactive TCR, and because Des.TCR $CD4^+$ T cells are functionally inert, polyclonal stimulation of Des.TCR splenocytes yields K^b-reactive $CD8^+$ T cells without further purification. For generation of CTLs specifically activated against K^b, mononuclear cells from Des.TCR-transgenic mice were incubated with irradiated P815-K^b-B7 cells at a ratio of 5:1 for 3 d in RP medium.

Purification of Murine $CD8^+$ Lymphocytes. To generate pure activated murine $CD8^+$ cells, Con A–stimulated mononuclear cells were initially depleted of professional APCs by incubation for 1 h on a Sephadex G-10 column (Amersham Pharmacia Biotech). For removal of B cells, the remaining nonadherent cells were incubated with anti–mouse IgG magnetic beads (Paesel & Lorei). Murine $CD8^+$ cells were then isolated by depletion of other cell populations by passing over a CD8 negative selection column (R & D Systems, Inc.). The remaining cells were >95% $CD8^+$ cells.

Preparation and Stimulation of HSV1-reactive Lymphocytes. Murine mononuclear cells were prepared from the spleens of DBA/2 mice infected 6 wk before with 2.5×10^6 PFU of HSV1 strain F injected intraperitoneally in 100 μl PBS. Human mononuclear cells were prepared from the peripheral blood of HSV1-positive donors, and aliquots were frozen at −80°C, one aliquot being retained and cultured in RP-h medium with 4 μg/ml PHA for 24 h to generate activated T cells. Murine splenocytes from DBA/2 mice were activated with 2.5 μg/ml Con A. Stimulator cells were then prepared by infecting murine DBA/2 or human T cell blasts with HSV-1 F-ICP47Δ mutant at a multiplicity of infection (MOI) of 2 and incubating for 24 h before irradiation. Stimulator cells were then incubated with responder cells at a ratio of 1:5 in RP or RP-h medium for 72 h before half of the medium was changed and recombinant IL-2 (human or murine) added at

a concentration of 40 (human) or 90 (murine) U/ml. Medium replenishment was repeated every 3 d. On day 9, cells were removed by Ficoll-Paque™ purification and, when required, $CD4^+$ or $CD8^+$ cells positively selected by biotinylated anti-CD4 or anti-CD8 antibody and streptavidin-coated magnetic particles using the MACS™ system (Miltenyi Biotec). The purified HSV1-reactive T cells were incubated with fresh stimulator cells in the relevant medium with IL-2 for a further 48 h before being used in JAM assays.

Polyclonally stimulated human or mouse T lymphocytes were generated using PHA (4 μg/ml) or Con A (2.5 μg/ml), respectively, as the initial stimulus. Polyclonally activated T cells were cultured as described for HSV1-reactive T lymphocytes except that they were restimulated with PHA and Con A, respectively, in combination with uninfected irradiated stimulator cells.

Preparation and Stimulation of Human CMV-reactive T Cells. For preparation of HCMV-reactive T cells, PBMCs were isolated from HCMV-positive donors and frozen. Monocytes were isolated by adhesion to tissue culture dishes, harvested, and then mixed with mononuclear cells at a ratio of 3:1 in RP-h medium supplemented with 4 μg/ml PHA. After 4 d of cultivation, the stimulated monocytes/macrophages were infected with a clinical strain of human (H)CMV (HCMV-N) at an MOI of 5, washed, and recultivated for a further 3 d before irradiation. Thereafter, these cells were used to stimulate autologous T cells essentially as described for HSV1-reactive T lymphocytes.

Flow Cytometry. Standard procedures were used for flow cytometric analysis (27). In brief, for surface immunofluorescence, cells in suspension were washed once with ice-cold wash solution (PBS with 2% BSA and 0.05% sodium azide) before being resuspended with the first antibody in ice-cold blocking solution (PBS with 10% heat-inactivated FCS and 0.2% sodium azide) for 45 min. The cells were then washed in ice-cold wash solution and the staining repeated with a secondary antibody (where relevant). After the final staining step, the cells were washed in ice-cold PBS and then either resuspended in 200 μl blocking solution for measurement or fixed in 4% ice-cold paraformaldehyde for 30 min. Fixed cells were washed twice with ice-cold PBS before being stained by the TUNEL (TdT-mediated dUTP-biotin nick-end labeling) method. For TUNEL staining, fixed cells were permeabilized by ice-cold 0.1% Triton X-100 with 0.1% sodium citrate for 2 min before being washed twice with ice-cold PBS. Fixed permeabilized cells were then incubated for 1 h at 37°C with TUNEL solution (TUNEL staining kit; Boehringer Mannheim) before being washed twice with room temperature PBS. Flow cytometry was performed on a FACScan™ (Becton Dickinson) linked to an Apple Macintosh Quadra 650 using CELLQuest™ software (Becton Dickinson) for data analysis.

Antibodies and Chimeric Proteins. The following reagents were employed for FACS staining: FITC-coupled Désiré-1 as a clonotype-specific mAb to monitor the transgenic TCR (24, 28), and antibodies against murine CD8α (clone Ly-2), H-$2K^b$ (clone AF6-88.5), CD95 (clones DX2 and JO2), CD95L (ligand; clone MFL3), human CD8 (clone RPA-T8), and human CD4 (clone RPA-T4), purchased from PharMingen. Antibody against viral gD was obtained from Advanced Biotechnologies, Inc. The hybridoma producing antibody W6/32, specific for MHC class I, was obtained from ATCC. Secondary antibodies and streptavidin-linked fluorochromes were obtained from Southern Biotechnology Associates. Chimeric proteins consisting of the extracellular portion of murine CD95, human TNF-related apoptosis-inducing ligand (TRAIL)R2, and human TNFR2 combined with the Fc portion of human IgG were produced by transient transfection of COS7 cells with expression plasmids encoding the various proteins in pcDNA3.9 (Invitrogen Corp.) and subsequent purification on protein G–Sepharose columns (Amersham Pharmacia Biotech).

^{51}Cr-Release Assay. P815 cells or $CD8^+$ cells from DBA/2 mice were infected with HSV strain F, F-US5β, or F-US5MHC at an MOI of 10 and incubated for 4 h. Cells were then used as target cells in a ^{51}Cr-release assay as described elsewhere (29). ^{51}Cr was obtained from New England Nuclear.

JAM Test. The JAM assay (30) was performed to measure the degree of cell death. For this purpose, target cells are labeled with tritiated thymidine. If apoptosis occurs in the labeled cell population, DNA fragments will be washed through glass fiber filters during cell harvesting. In contrast, DNA from surviving target cells remains intact and will be captured by the filters. The percent cell death can be calculated by comparing the amount of [^{3}H]thymidine bound to filters in the presence and absence of the apoptosis-inducing event. In brief, proliferating lymphocytes were pulsed overnight with tritiated thymidine at a concentration of 5 μCi/ml (New England Nuclear). Cellular debris was removed by Ficoll-Paque™ purification, and the cells were washed twice with wash medium (RPMI with 5% heat-inactivated FCS) and counted before being used as target cells. Infected or uninfected target cells were incubated for 7 h at a density of 100,000 cells in 200 μl proliferation medium in round-bottom 96-well plates before being harvested and counted. When required, blocking antibodies or chimeric proteins were added 2 h after infection at the specified concentrations. All measurements given represent the mean of six or eight wells, and all experiments were replicated at least once on separate occasions. The highest MOI used was 10 to avoid inhibition of cytotoxic activity by HSV1 as has been previously reported (31).

Apoptosis Induction in Staphylococcus Enterotoxin B–stimulated PBMCs. PBMCs were stimulated with staphylococcal enterotoxin B (SEB) at a concentration of 1 mg/ml for 24 h. Cells were then either infected with HSV1 strain F at an MOI of 10 or mock infected and then incubated for a further 18 h with apoptosis-inducing anti-CD95 mAb. Cells were then stained for CD8 or CD4 and used in the TUNEL assay.

Results

HSV1 Does Not Block MHC Class I Expression on Human T Cells. We hypothesized that infection of T lymphocytes by HSV1 could pose a severe risk for the antiviral immune response because the infected cells could present viral antigen to and thereby kill each other (fratricide). Previous studies have demonstrated that HSV1 efficiently interferes with antigen presentation in human fibroblasts due to its ability to block transport of MHC class I molecules (13, 14). This effect, however, can vary in different cell types. Therefore, we investigated the efficiency of HSV1's blocking of the transport of MHC class I molecules in human T cells compared with human fibroblasts. For this purpose, the human fibroblast cell line MRC-5 and activated human $CD8^+$ lymphocytes were infected with a recombinant HSV1 strain coding for K^b, a murine MHC class I molecule (F-US5MHC). Infection of both cell types was efficient, as shown by FACS™ analysis of gD, a strongly expressed HSV1 glycoprotein (Fig. 1, bottom panels). Expression of endogenous MHC class I molecules and of virus-encoded K^b was analyzed in parallel. Human fibroblasts infected with

F-US5MHC illustrated the effective viral block of MHC class I expression (Fig. 1, left): virus-encoded K^b could not be detected, and the level of endogenous MHC class I molecules was reduced by ~50% 5 h after infection. By contrast, we could detect the viral K^b on the cell surfaces of human $CD8^+$ lymphocytes (Fig. 1, right) and, in addition, we observed no inhibitory effect on the expression of endogenous MHC class I molecules 5 h after infection. These results demonstrate that, in contrast to its effect on human fibroblasts, HSV1 does not inhibit MHC class I expression by human T cells.

Fratricide of Human HSV1-reactive T Cell Lines after HSV1 Infection. It has been demonstrated that freshly activated T cells are resistant to apoptosis and, in addition, that HSV1 itself confers resistance to apoptosis in certain cell types (15, 32). To study the consequences of viral antigen presentation in antiviral T cell populations, we generated different HSV1-reactive T cell lines from seropositive human donors and from mice previously inoculated with HSV1 strain F. As a control, polyclonally stimulated T cells and HCMV-specific T cell lines were employed. HSV1-reactive and control cells expressed comparable levels of CD95 (data not shown). Rapid apoptosis occurred after infection with the HSV1 strain F (MOI of 10) in both murine and human HSV1-reactive T lymphocytes. By contrast, significant cell death was absent in polyclonally stimulated murine and human T lymphocytes and in HCMV-specific human T cell lines (Fig. 2). Infection-mediated apoptosis of the specific antiviral T cell lines suggests that HSV1 infection renders antiviral T cells susceptible to apoptosis and that recognition of viral antigen may be necessary to initiate the cascade of events that lead to fratricide. Like T cell clones or T cell hybridomas, however, T cell lines are especially susceptible to induction of apoptosis and might not reflect the situation in vivo, where freshly activated antiviral CTLs eliminate the invading pathogen. Therefore, we developed a system to investigate the above effects in mice.

A Transgenic Mouse Model of HSV1 Infection as a Tool to Further Dissect the Mechanisms Involved in HSV1 Fratricide. Molecular analysis of freshly activated antiviral CTLs is hampered by the fact that T cells reactive to a particular virus occur only at a low frequency in the peripheral T lymphocyte pool. To circumvent this problem, we employed a transgenic mouse model of HSV1 infection for further analysis of HSV1-induced fratricide. In this model, TCR-transgenic mice that express a T cell repertoire strongly skewed toward K^b (Des.TCR mice) serve as the hosts and F-US5MHC virions encoding K^b as a viral neoantigen are used as infectious particles. First, we tested whether the transgenic TCR can recognize viral K^b on murine cells as a target structure. For this purpose, we infected the murine mastocytoma cell line P815 (MHC haplotype $H\text{-}2^d$) with F-US5MHC. As demonstrated by analysis of gD, the P815 cells were efficiently infected with F-US5MHC (Fig. 3 A, right). However, these cells showed only weak expression of K^b in comparison to stable transfectants constitutively expressing K^b under the control of a strong promotor (P815-K^b cells) (Fig. 3 A, left). Nonetheless, the K^b-reactive CTLs from Des.TCR

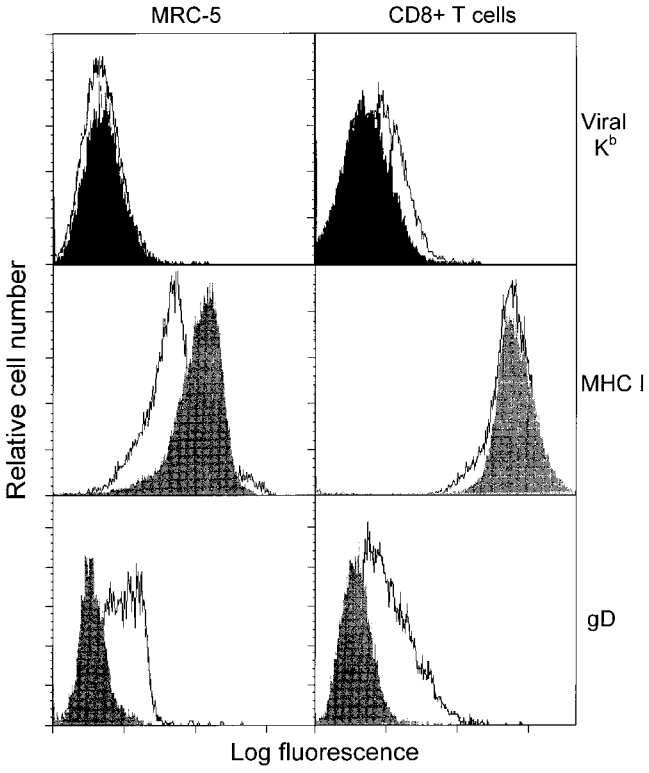

Figure 1. Levels of virus-encoded K^b and endogenous MHC class I molecules on human fibroblasts and $CD8^+$ T cells infected with HSV1. The human fibroblast cell line MRC-5 (left) and human Con A–activated PBMCs (right) were infected with K^b-encoding HSV1 strain F-US5MHC at an MOI of 10 (unfilled curve), HSV-1 strain F at an MOI of 10 (black curve), or mock infected (gray curve). After incubation for 5 h, cells were stained for viral K^b, endogenous MHC class I molecules, and viral gD using a primary mAb followed by an isotype-specific FITC-labeled secondary reagent. PBMCs were subsequently stained with a PE-conjugated anti-CD8 mAb, and data were electronically gated for $CD8^+$ cells. The x-axis shows fluorescence intensity (log scale, four decades), and the y-axis depicts the relative cell number. The results shown are representative of three independent experiments.

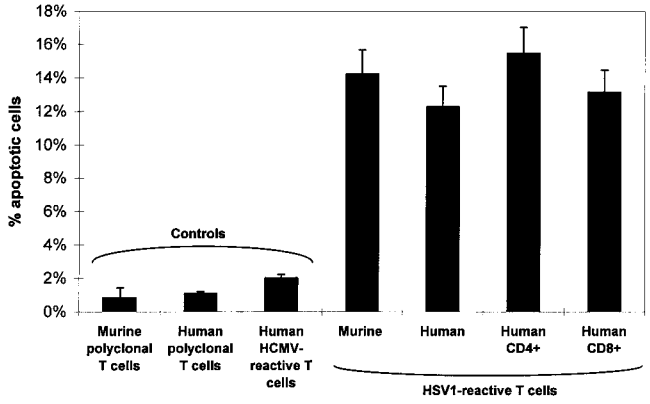

Figure 2. Fratricide of HSV1-reactive human and murine T cell lines. HSV1-reactive T cell lines from mice and human donors were isolated as described in Materials and Methods. The expanded T cells were infected with HSV1 strain F at an MOI of 10 and analyzed in a JAM assay 5 h after infection to measure the degree of cell death. As controls, polyclonally activated human and murine T cells and HCMV-reactive T cell lines were treated in the same way. One of three independent experiments is depicted.

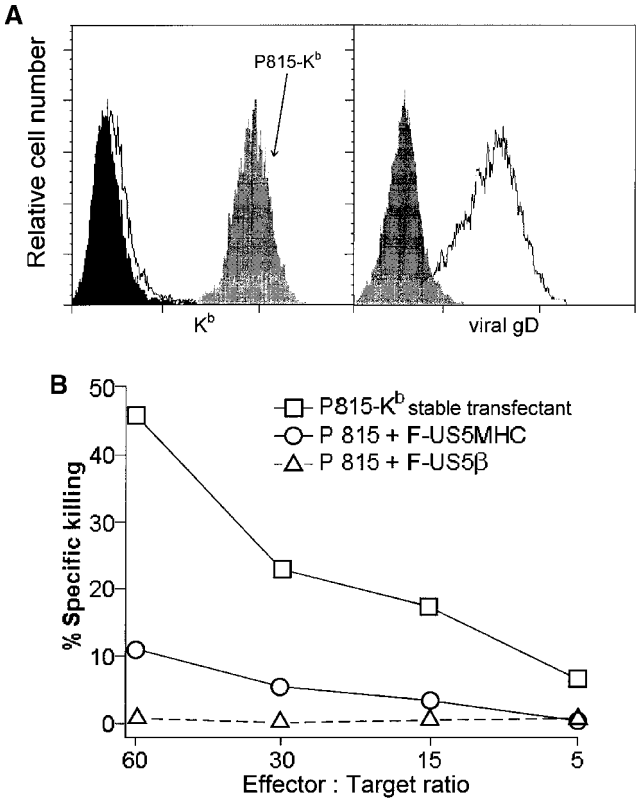

Figure 3. CTLs from Des.TCR mice recognize viral K^b as a target structure. (A) FACS™ analysis of K^b expression on target cells. P815 cells were infected with either F-US5MHC (unfilled curve) or F-US5β (black curve) at an MOI of 10 or mock infected (gray curve). After incubation for 5 h, the cells were stained for K^b (left) or gD (right). As a positive control, uninfected P815-K^b transfectants strongly expressing K^b (gray curve marked P815-K^b) were stained for K^b. The x-axis shows fluorescence intensity (log scale, three decades), and the y-axis depicts the relative cell number. (B) In parallel, target cells were used in a ^{51}Cr-release assay. The x-axis shows the E/T ratio, whereas the y-axis depicts specific lysis (%). Results are representative of three independent experiments.

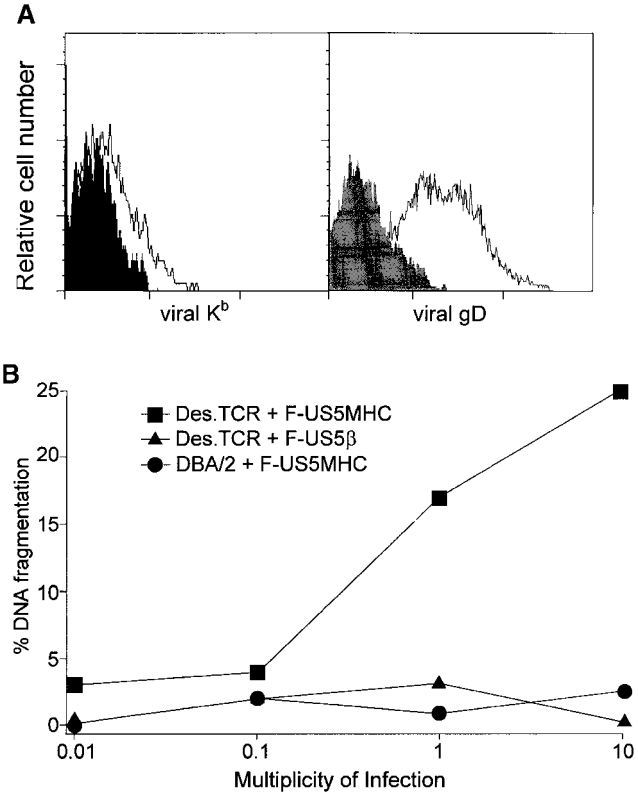

Figure 4. F-US5MHC infection and fratricide of Des.TCR cells. (A) Levels of viral K^b and glycoprotein gD on activated murine T cells infected with F-US5MHC (unfilled curve). As a control, cells were infected with F-US5β (black curve) or mock infected (gray curve). After incubation for 5 h, the cells were stained for viral K^b and gD using a primary mAb followed by an isotype-specific FITC-labeled secondary reagent. Splenocytes were subsequently stained with PE-conjugated anti-CD8 mAb, and data were electronically gated for $CD8^+$ cells. (B) Fratricide of Des.TCR cells after infection with F-US5MHC. For the JAM assay, cells were labeled with tritiated thymidine and then infected either with F-US5MHC or F-US5β as a control and incubated for 7 h before harvesting and measuring $[^3H]$thymidine. The y-axis gives the degree of fragmentation of labeled target cell DNA in response to cytotoxic attack, whereas the x-axis depicts the MOI. One representative experiment of three is shown.

mice could recognize and kill F-US5MHC–infected P815 cells (Fig. 3 B). The obtained lysis was relatively weak in comparison to lysis of P815-K^b cells, reflecting the weak K^b expression of F-US5MHC–infected P815 cells. In contrast, we observed no lytic activity against target cells infected with a recombinant HSV1 strain encoding lacZ (F-US5β) as a control. Thus, the transgenic TCR recognizes virus-encoded K^b as a target structure on infected cells.

We then investigated whether fratricide also applied to F-US5MHC–infected T lymphocytes from Des.TCR mice. After F-US5MHC infection, we detected viral K^b on the cell surfaces of activated murine T cells (Fig. 4 A). Consistent with this observation, we detected rapid apoptosis in the JAM assay in activated T cells from Des.TCR mice after infection with F-US5MHC but not after F-US5β infection (Fig. 4 B). As a further negative control, we used Con A–activated T lymphocytes from DBA/2 mice. In these cells, expression of HSV1-encoded genes was as efficient as in activated Des.TCR lymphocytes (data not shown), but the cells did not show an enhanced rate of DNA fragmentation after F-US5MHC infection (Fig. 4 B). In addition, we analyzed whether expression of the K^b antigen by means other than F-US5MHC infection results in a significant increase in death of Des.TCR lymphocytes. For this purpose, activated but uninfected Des.T cells were incubated with P815-K^b transfectants that express much higher levels of K^b than F-US5MHC–infected cells. After 7 h, only a slight increase in DNA fragmentation (i.e., 3%) occurred in comparison to that in activated Des.TCR cells that had been incubated with K^b-negative P815 cells (data not shown). Thus, the observed fratricide was dependent on HSV1 infection and occurred not only in long-term–cultured T cell lines but also in freshly activated CTLs, which comprise a high proportion of antigen-reactive T cells. These results demonstrate that Des.TCR mice, in combination with F-US5MHC, represent a suitable model with which to analyze the underlying mechanisms of HSV1-induced fratricide.

Mechanisms of Fratricide. There are two known main antigen-dependent pathways by which CTLs can kill virus infected-cells: killing by the perforin/granzyme system and killing by molecules that belong to the TNF cytokine family (33). Nearly all of the cytotoxicity mediated by $CD8^+$ CTLs is due to the perforin/granzyme system. Therefore, we analyzed whether perforin was involved in HSV1-induced fratricide. For this purpose, CTLs from either Des.TCR mice or Des.TCR mice lacking perforin (Des.TCR × perforin$^{-/-}$) were infected with F-US5MHC. Surprisingly, we detected identical levels of apoptosis in the JAM assay, indicating that HSV1-induced fratricide was perforin independent (Fig. 5 A). To further dissect the molecular events underlying HSV1-induced apoptosis, we investigated whether apoptosis-inducing members of the TNF cytokine family, namely TNF, CD95L, and TRAIL, play a role in HSV1-induced fratricide by blocking the activity of the respective ligands with receptor–Fc fusion proteins. In addition, we determined whether antigen recognition is necessary to trigger the apoptogenic signaling cascade. To this end, we used an anti-K^b mAb to inhibit the interaction between the transgenic K^b-reactive TCR and the K^b molecule (Fig. 5 B).

We found that upon infection of activated Des.TCR CTLs with F-US5MHC, inhibition of the CD95–CD95L interaction substantially prevented apoptosis, blocking of TRAIL had virtually no effect, and inhibition of TNF partially abrogated apoptosis. Interestingly, blocking of the TCR–K^b interaction also prevented apoptosis after F-US5MHC infection of activated T lymphocytes from Des.TCR mice.

Given the importance of the CD95–CD95L interaction in HSV1-induced fratricide, we next examined the expression of CD95L and its receptor on infected $CD8^+$ lymphocytes (Fig. 6). Interestingly, CD95L was upregulated on activated Des.TCR $CD8^+$ T cells only after productive infection with F-US5MHC but not after infection with F-US5β, implying that triggering of the transgenic TCR by K^b resulted in higher surface expression of CD95L on activated CTLs. Supporting these data, levels of CD95L remained unchanged on HSV1-infected nontransgenic murine T cells, which are not antigen specific. Thus, HSV1 infection per se does not induce higher expression of CD95L on activated T lymphocytes 5 h after HSV1 infection. In contrast to CD95L, the expression of its receptor was not significantly altered, regardless of whether the HSV1-infected T cells recog-

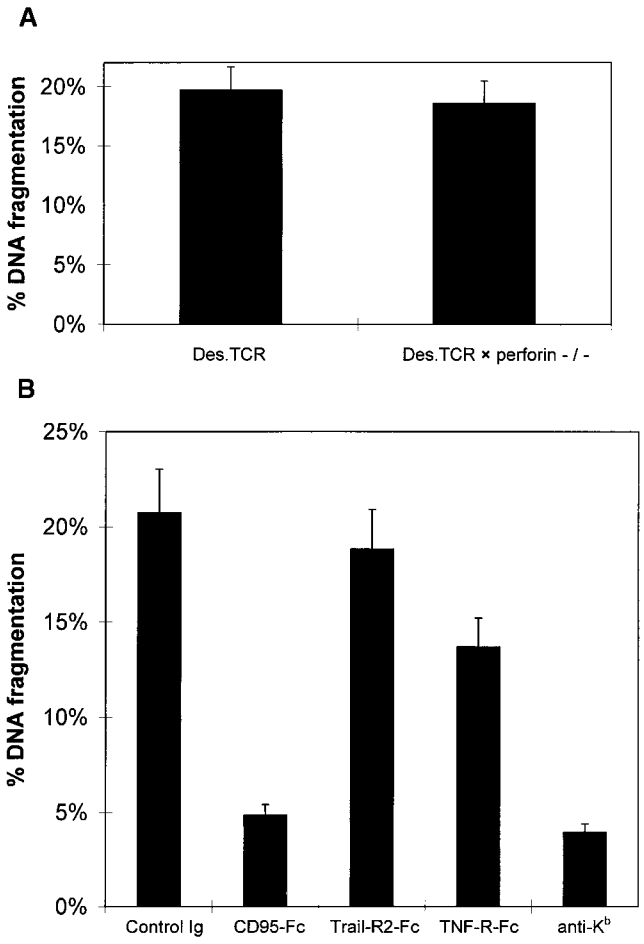

Figure 5. HSV1-induced fratricide is primarily mediated by CD95 signaling and requires a further signal through TCR–MHC interaction. (A) CTLs from Des.TCR mice or Des.TCR perforin$^{-/-}$ transgenic mice were infected with F-US5MHC and then used in a JAM assay to measure DNA fragmentation. (B) JAM tests using chimeric human Fc–murine CD95 to block CD95L, chimeric human Fc–murine TRAILR2 to block TRAIL, and chimeric human Fc–TNFR2 to block TNF. In addition, K^b was blocked with an mAb against K^b (clone AF6-88.5). One representative experiment of three is shown.

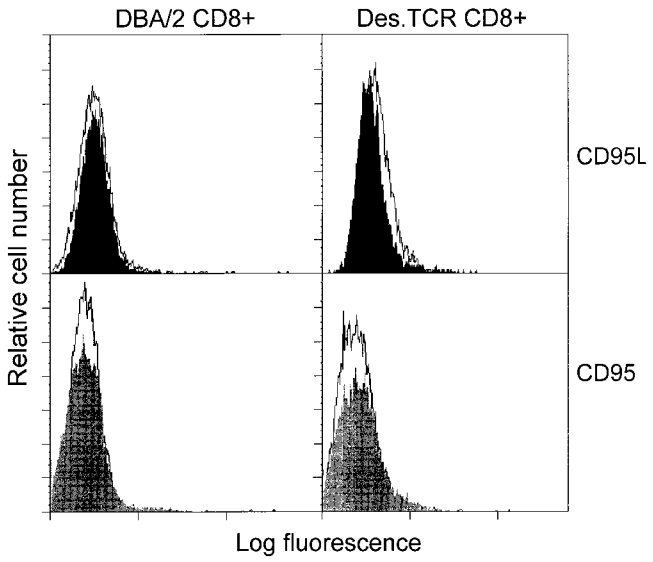

Figure 6. Expression of CD95L and its receptor on Con A–stimulated $CD8^+$ T cells from Des.TCR and DBA/2 mice after HSV1 infection. Top panels: activated T cells from DBA/2 mice (left) or Des.TCR mice (right) were infected with either F-US5MHC (unfilled curve) or F-US5β (black curve) at an MOI of 10, and cells were stained for CD95L after 5 h. Bottom panels: activated T cells from DBA/2 mice (left) or Des.TCR mice (right) were infected with HSV1 strain F (unfilled curve) at an MOI of 10 or mock infected (gray curve) and stained for CD95 after 5 h. All cells were stained with a PE-conjugated anti-CD8 mAb, and data were electronically gated for $CD8^+$ cells. The x-axis shows fluorescence intensity (log scale, three decades), and the y-axis depicts the relative cell number. The results shown are representative of three independent experiments.

nized antigen or not. Thus, an enhanced level of CD95L expression is relevant for HSV1-induced fratricide, whereas upregulation of CD95 expression is not required. Altogether, these findings demonstrate that the CD95 system is the main effector system of HSV1-induced fratricide and, in addition, that the virus-induced increase of CD95L expression is dependent on TCR–antigen interaction.

Sensitization of HSV1-infected T Cells for CD95-mediated Apoptosis Due to a Viral "Competence-to-Die" Signal. Virus-induced upregulation of CD95L is necessary but not sufficient for induction of fratricide, as freshly activated T cells are normally resistant to CD95 signaling. Therefore, we determined whether HSV1 renders activated T cells susceptible to CD95 triggering after infection. For this purpose, we used the superantigen SEB to stimulate PBMCs. SEB activation is similar to normal activation with antigen because it also requires APCs and a CD28 costimulation signal and yields activated T cells that are resistant to apoptosis induction via CD95 (34). After SEB activation, PBMCs were infected with HSV1 or, as a control, left uninfected and subsequently challenged with apoptosis-inducing anti-CD95 mAb (Fig. 7). We found that HSV1-infected $CD8^+$ and $CD4^+$ T cells are highly susceptible to CD95-mediated apoptosis, whereas the majority of uninfected T lymphocytes and T lymphocytes treated with UV-inactivated virus particles (mock infection) remained resistant to CD95 ligation. Similar data were obtained when PHA was used instead of SEB to stimulate T lymphocytes (data not shown). These results demonstrate that triggering of CD95 alone is not sufficient for induction of fratricide. Only in the course of productive HSV1 infection is an additional viral competence-to-die signal generated that renders short-term–activated T cells sensitive to CD95-mediated apoptosis.

Discussion

In this paper, we analyzed the functional implications of HSV1 infection of activated T cells. We observed that, in contrast to human fibroblasts, MHC class I expression on HSV1-infected human T lymphocytes is not disrupted. This supports the notion that the efficiency of HSV1's interference with transport of peptides into the lumen of the endoplasmic reticulum varies not only in different species (13, 35, 36) but also in different cell types of the same species (13). As a consequence of unaltered presentation of viral antigens by MHC class I molecules, antiviral CTL populations infected with HSV1 are rapidly eliminated. In theory, this could either be due to suicide of individual CTLs or, alternatively, fratricide (killing of each other). We prefer the latter because antigen recognition by infected T cells was required for induction of apoptosis, and it has been shown that TCR molecules are unable to interact with MHC–peptide complexes on the same cell (19).

To identify the molecules involved in virus-induced fratricide, we used a transgenic mouse model of HSV1 infection. CTLs from mice expressing a K^b-reactive transgenic TCR (Des.TCR mice) were infected with a recombinant HSV1 strain coding for K^b (F-US5MHC). Blocking experiments revealed that HSV1-induced fratricide is mainly triggered by the CD95–CD95L system. Intriguingly, blocking CD95 did not totally prevent cell death, as TNF also contributed to fratricide, although to a lesser extent. In contrast, TRAIL, another member of the TNF family, was not involved. Similarly, perforin did not play a role in HSV1-induced fratricide, because T lymphocytes from Des.TCR × perforin$^{-/-}$ mice were as susceptible to apoptosis as T cells from Des.TCR mice. Blocking of the TCR–antigen interaction reduced fratricide to the same extent as blocking CD95. As an explanation, we found that activated CTLs from Des.TCR mice further upregulated CD95L molecules on the cell surface within a few hours after infection with K^b-encoding HSV1. Such a rapid upregulation may be due to recruitment of preformed CD95L molecules rather than to de novo synthesis (37, 38). This result, together with the fact that rapid cell death was not induced in human T cell lines reactive with CMV, suggests that signaling through

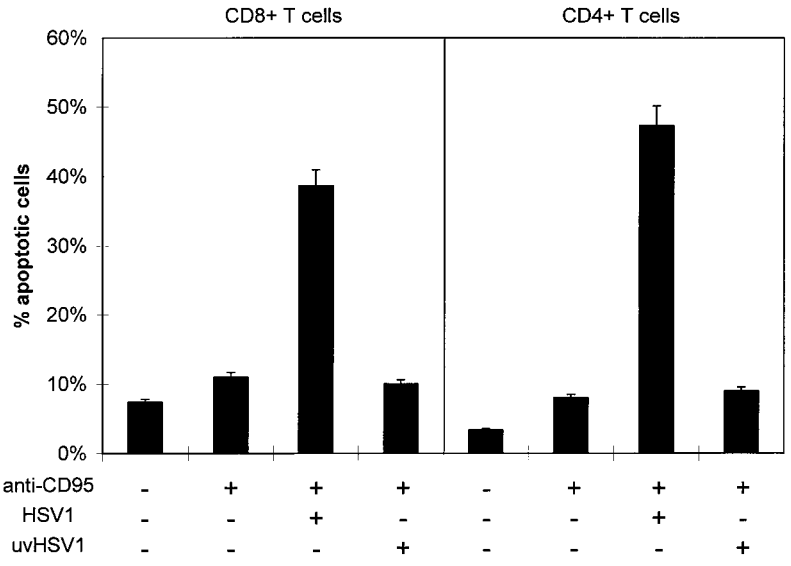

Figure 7. HSV1 infection renders T cells susceptible to CD95-mediated apoptosis. Human PBMCs were activated with SEB for 24 h before being either infected with HSV1 strain F or left uninfected. As a further control, cells were infected with UV-inactivated HSV1 particles (mock infection). Subsequently, cells were incubated with apoptosis-inducing anti-CD95 mAb. After 18 h, cells were stained with Cy-Chrome–conjugated anti-CD8 or anti-CD4 mAb, fixed, and TUNEL stained as described in Materials and Methods. The results shown are representative of three independent experiments.

the TCR complex after recognition of viral antigen is a prerequisite of HSV1-induced fratricide and may be the basis of viral immune evasion. In this aspect, it resembles FasL- and TNF-mediated mechanisms that induce peripheral tolerance and maintain physiological lymphocyte homeostasis (39).

It has been shown that uninfected T cells acquire a transient state of resistance to CD95-mediated signaling after activation lasting several days (32). Therefore, we have compared the susceptibility of HSV1-infected versus uninfected T lymphocytes to CD95-mediated apoptosis after SEB stimulation. As previously reported, SEB stimulates both $CD4^+$ and $CD8^+$ T cells (40) and confers resistance to CD95-induced apoptosis in freshly activated T cells (34). We found that subsequent HSV1 infection renders SEB-activated $CD4^+$ and $CD8^+$ T cells highly susceptible to induction of apoptosis through engagement of CD95 by mAbs. In contrast, abortive infection with UV-inactivated HSV1 did not confer susceptibility to CD95 signaling. This result implies that viral proteins expressed in the course of productive infection facilitate activation-induced cell death (AICD). Such a competence-to-die signal (41) has recently been demonstrated by stimulating uninfected T lymphocytes with variant peptide ligands that do not induce T cell effector functions (e.g., cytokine secretion). This does not result in upregulation of death receptors on "competent" T lymphocytes but renders them susceptible to death ligands on neighboring cells. However, the molecular basis of this phenomenon has yet to be defined, although tyrosine phosphorylation of intracellular proteins could play a role (42).

Taken together, these data suggest that apoptosis of activated T cells requires two distinct signals: one that leads to upregulation of CD95/CD95L and another that removes the block of CD95-signaling pathways at the time of CD95 triggering. AICD is triggered by repeated antigenic stimulation (43–45). Accordingly, HSV1 proteins could lower the AICD threshold, the critical number of triggered TCRs during TCR–peptide–MHC interaction. It is unlikely that the viral proteins target expression or function of antiapoptotic proteins of the Bcl family like Bcl-x_L or Bcl-2, because it has been reported that AICD is not prevented by these molecules (46). Alternatively, HSV1 proteins could restore the formation of the death-inducing signaling complex (DISC), which has been shown to lack FLICE (FADD-like IL-1β–converting enzyme, now referred to as caspase-8; reference 47) in freshly activated T cells that are resistant to AICD (48). This could be accomplished by downregulation of the antiapoptotic cellular FLIP (FLICE-inhibitory protein), which competes for caspase-8 recruitment to the DISC (49–54). Thus, the viral proteins involved in facilitating AICD and the molecular basis of this effect remain to be elucidated.

Fratricide has also been implicated as an important factor in the pathogenesis of AIDS (55). T cells from HIV-1–infected individuals show upregulation of CD95/CD95L and enhanced susceptibility to CD95-mediated killing (56–59). Moreover, HIV-1–derived proteins (Tat, gp120) can enhance CD95L expression (55, 60). Recently, it has been demonstrated that Nef, another HIV-1 protein, forms a signaling complex with the TCR. This results in T cell activation and upregulation of CD95L expression, thereby circumventing the need for antigen recognition (60). In this way, HIV may cause global immunodeficiency, which finally results in opportunistic infections and death of the infected individuals.

We propose a different mechanism underlying fratricide in active HSV1 infections (Fig. 8). In HSV1 lesions, which usually remain focused to an area of epithelial cells, free virions can infect invading antiviral CTLs by binding to herpesvirus entry mediator (HVEM) molecule. Subsequently, viral antigens are efficiently presented on infected antiviral CTLs. Neighboring CTLs that recognize viral antigen upregulate CD95L. In addition, HSV1 lifts the block for AICD. As a consequence, infected antiviral CTLs could be better targets than infected epithelial cells, in which antigen presentation via MHC class I molecules is prevented by viral proteins. Such a cell type–specific regulation of viral defense mechanisms could turn antiviral CTLs into decoy targets, thereby enhancing local spread of the virus in epithelial cells.

The elimination of antiviral CTLs by antigen-dependent fratricide could be a general immune evasion mechanism employed by viruses that can infect and replicate in activated T cells, although they mainly propagate in nonlymphoid cells. In contrast, viruses that are dependent on T cells for virion production (e.g., human herpesviruses 6 and 7) may have to prevent T cell death to yield enough infectious particles.

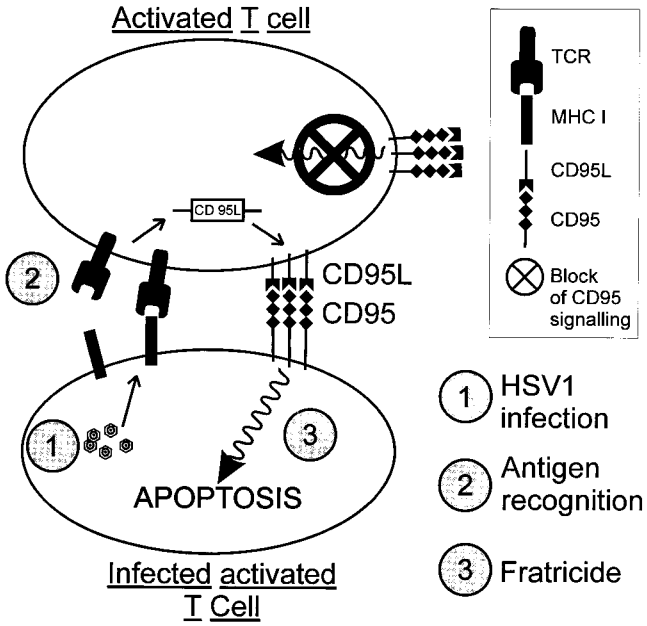

Figure 8. HSV1-induced fratricide of antiviral CTLs as a mechanism of viral immune evasion. Antiviral CTLs activated in the draining lymph nodes enter the epithelial lesions, where they are infected by HSV1 virions (1). Due to inefficient activity of the TAP (transporter associated with antigen processing) blocker in T lymphocytes, viral antigens are presented via the MHC class I molecules and recognized by neighboring CTLs (2). In addition, HSV1 lifts the block for CD95-mediated death signals, resulting in fratricide (3).

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