Murine Retrovirus-Induced Depletion of T Cells Is Mediated through Activation-Induced Death by Apoptosis

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***ts1*, a mutant of Moloney murine leukemia virus, causes neurologic disorders and acute immunodeficiency associated with the destruction of thymocytes and helper T cells. In this study, we examined whether apoptosis was involved in *ts1*-induced killings of T cells. Neonatal mice were inoculated with *ts1*, and 20 to 23 days postinoculation, when cytopathic effects on T cells normally appear, thymocytes and splenic lymphocytes were isolated and examined. Our results showed that several features of apoptosis were present in *ts1*-infected thymocytes and splenic lymphocytes. Apoptotic fragmented DNA, condensation of the chromatin, and enhanced cell death after stimulation with mitogens which was preventable with protein synthesis inhibitors, all of which are common features of apoptotic cell death, were observed in *ts1*-infected cells. Several other viruses, including human immunodeficiency virus, have been shown to cause apoptotic death of T cells. Here we show for the first time that a murine retrovirus which also induces immunodeficiency can cause apoptotic T-cell death. Future studies with this murine retrovirus may provide important results to help us better understand the mechanisms of retrovirus-induced apoptosis of T cells.**

A mutant of Moloney murine leukemia virus TB, designated *ts1*, has been shown to induce a disease syndrome in neonatal mice that shares important similarities with human immunodeficiency virus (HIV)-induced AIDS. These include thymus and T-cell (primarily CD4⁺ T-cell) tropism (28, 30, 32, 33, 49), functional defects of T cells (49), destruction of T cells (mainly of CD4⁺ T cells) in the late stages of the disease (30, 32, 52), and selective effects on the nervous and immune systems (28, 30, 32, 33, 47–49, 52). We have also demonstrated that the cytopathic effects of *ts1* on T cells are a direct result of virus infection and not secondary to the central nervous system degeneration (51). However, the exact mechanism(s) involved in T-cell killing by *ts1* remains unknown.

HIV-induced AIDS develops primarily because of a progressive decline in the function and number of CD4⁺ T cells (11, 34). However, the mechanism of this destruction of T cells by HIV remains speculative. Several recent reports indicate that apoptosis, or programmed cell death (PCD), may play an important role in this selective killing of CD4⁺ T cells by HIV, both in vivo (3, 13, 22) and in vitro (42). PCD is an active cell suicide mechanism of immense physiological importance in that it is involved in normal negative selection of the T-cell repertoire in the thymus and plays a critical role in the development of tolerance to self antigens (4, 38). However, mature T cells go into PCD in the absence of any costimulatory signals (23). PCD requires cell activation and initiation of protein synthesis and involves the activation of an endonuclease that results in a characteristic nucleosomal fragmentation of the cellular DNA into discrete bands with multiples of 180 to 200 bp (35, 53).

The objective of this study was to determine whether PCD is involved in the in vivo destruction of T cells in *ts1* infections. We investigated whether in vitro activation of T cells, obtained

from *ts1*-infected mice, with polyclonal activators leads to cell death. We also investigated the T-cell response to the superantigen staphylococcal enterotoxin B (SEB).

We examined in vitro proliferative response of splenic T lymphocytes from *ts1*-infected mice at 20 to 23 days postinoculation (dpi) and from age-matched uninfected mice in response to the polyclonal T-cell activators concanavalin A (ConA) and pokeweed mitogen (PWM) and to SEB superantigens. FVB/N mice contain a very high proportion (>80%) of T cells in the spleen, and their CD4⁺/CD8⁺ T-cell ratio (about 4:1) is also higher than average (32). Our method of isolating lymphocytes from spleens yielded cell populations that were >85% T cells; no significant differences were noticed between cellular phenotypes of uninfected and *ts1*-infected mice at this stage (20 to 23 dpi) of disease development, as demonstrated by fluorescence-activated cell sorting (FACS) analysis (32).

For the assay shown in Fig. 1, inbred FVB/N mice obtained from the National Cancer Institute (Harlan Sprague, Inc., Frederickburg, Va.) were inoculated intraperitoneally within 48 h after birth with 0.1 ml of *ts1* (54) virus suspension containing 5×10^6 to 1×10^7 infectious units per ml. The virus was propagated in TB cells, a thymus-bone marrow cell line derived from CFW/D mice (2), and virus titer was determined on 15F cells as described previously (50). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 6% fetal calf serum (FCS) and 4% bovine serum. Control mice received an identical volume of medium only instead of virus. Virus-inoculated or control mice were killed by CO₂ inhalation at 20 to 23 dpi. Their thymuses and spleens were collected, and single-cell suspensions were prepared as described previously (32). In brief, cells were teased apart in cold RPMI 1640 medium supplemented with 5% FCS. Spleen cells were purified over a Ficoll-Hypaque gradient (Sigma Chemical Co., St. Louis, Mo.), and the mononuclear cells at the interphase were collected. Thymus cells were treated with hypotonic saline to lyse erythrocytes and resuspended in complete medium. Cell suspensions were then plated in plastic tissue culture dishes and incubated for 1 h at 37°C. The nonadherent cells, composed primarily of lymphocytes (determined by FACS analysis as described previously

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FIG. 1. Proliferative response of splenic lymphocytes from *ts1*-infected (closed bars) and uninfected control (open bars) mice. Assays were performed as described in the text. Histograms represent means $\pm$ standard deviations of ^3H incorporation measurements in triplicate from four to six separate experiments. MED, medium alone; CHX, cycloheximide.

[32]), were used for further studies. Cell proliferation assays were performed in a final volume of 200 μl (30,000 cells) in 96-well culture plates (Costar Corp., Cambridge, Mass.) as previously described (13). Briefly, splenic mononuclear cells ($10^6/\text{ml}$) were cultured in RPMI-10% FCS at 37°C . Cells were activated by using three mitogens at the following final concentrations: ConA, 3.5 $\mu\text{g}/\text{ml}$; PWM, 5 $\mu\text{g}/\text{ml}$; and SEB, 1 $\mu\text{g}/\text{ml}$. Each sample was cultured in the absence or presence of cycloheximide (100 ng/ml). All mitogens and cycloheximide were purchased from Sigma. After 48 h of incubation, cultures were pulsed with 1 μCi of [^3H]thymidine (2 Ci/mmol; NEN Research, Wilmington, Del.) for 6 h and harvested.

Cells from all uninfected control animals showed enhanced proliferation in response to ConA, PWM, and SEB (Fig. 1). In contrast, *ts1*-infected cells failed to proliferate when these cells were stimulated with ConA, PWM, or SEB (Fig. 1). In fact, the proliferative response of these cells from infected mice was slightly reduced in response to various stimuli. Interestingly, the addition of the protein synthesis inhibitor cycloheximide inhibited proliferation of uninfected control cells in response to all stimuli but had little or no effect on the proliferative responses of *ts1*-infected cells either with (Fig. 1) or without (data not shown) stimulation. A similar failure of response to various stimuli was also observed in the thymocytes from *ts1*-infected mice (data not shown). We have previously shown a similar defective response to ConA by splenic cells from *ts1*-infected BALB/c mice (49). Since B cells were only a small fraction of the total splenic cells and since it has been shown that B cells are not generally affected in *ts1* infections (3, 28), these results indicate that a selective defect in the proliferation of T cells is induced in *ts1* infections.

The viability of cells from *ts1*-infected and uninfected mice was also compared. As shown in Fig. 2, cells from *ts1*-infected animals were killed more quickly than cells from uninfected control mice when they were cultured in normal medium without any stimuli. However, when these cells were stimulated with either ConA (Fig. 2) or PWM (data not shown), the death rate in *ts1*-infected cells was significantly increased (from 28 to 77%), whereas the death rate in the uninfected control cells only slightly increased (Fig. 2).

An essential characteristic of PCD is its dependence on cell activation and new protein synthesis in dying cells (10, 45, 46)

FIG. 2. Cell mortality in splenic lymphocytes from *ts1*-infected (closed bars) and uninfected control (open bars) mice after in vitro culture with medium alone, ConA (3.5 $\mu\text{g}/\text{ml}$), and ConA plus cycloheximide (CHX; 100 ng/ml). Cells were cultured in 96-well plates with various stimuli under conditions similar to those described in the text for the proliferation assays in Fig. 1. Viable cells were counted in a hemocytometer at the same time point as for the proliferation assay by the trypan blue exclusion method (51). In some experiments, the culture supernatants were assayed for virus titers on 15F cells (50). Histograms represent means $\pm$ standard deviations of duplicate measurements of the percentage of cell death from four to six separate experiments.

except in rare cases in which apoptosis is induced by cytotoxic T lymphocytes in which synthesis of new proteins or mRNA may not be necessary (reviewed in reference 30). Addition of cycloheximide, which prevents PCD (13, 36), prevented enhanced cell death in ConA-stimulated and *ts1*-infected cells (Fig. 2). Addition of cycloheximide to *ts1*-infected but unstimulated cells also reduced cell mortality, although the difference was not as dramatic as in the mitogen-stimulated cells (data not shown). In contrast, the death rate among control cells did not change appreciably with or without cycloheximide (Fig. 2). Since thymocytes and CD4^+ T cells are depleted in *ts1* infection in vivo (30, 32, 52) and the cell populations examined here contained predominantly CD4^+ T cells (32), these results indicate that T-cell killing by *ts1* probably requires new protein synthesis. We have previously shown by using chimeric viruses that enhanced killing of thymocytes is an intrinsic property of *ts1* (51). Recently, we have also shown that *ts1* causes apoptotic cell death in primary thymocyte cultures (unpublished data). Therefore, the CD4^+ T-cell and thymocyte depletion observed in *ts1* infection in vivo is probably mediated by an activation-induced cell death pathway.

The development of apoptosis in *ts1*-infected T cells was further confirmed by the presence of characteristic nuclear and ultrastructural changes. Induction of apoptosis is usually associated with morphologic changes such as cell shrinkage (pyknosis) and chromatin condensation and is visualized as nucleosome-sized DNA multimers of 180 to 200 bp forming a typical stepladder pattern after separation on agarose gels (9, 29). Gel electrophoresis of the DNA from the thymuses and spleens, performed soon after isolation of mononuclear cells from sample obtained in vivo, clearly showed a typical stepladder pattern in the *ts1*-infected mice; little or no fragmentation of the DNA was observed in the age-matched control mice (Fig. 3). However, after 48 h of incubation, control cells also showed some DNA fragmentation, although *ts1*-infected cells produced higher level of fragmentation at the same time point

FIG. 3. Agarose gel electrophoresis of cellular DNA of thymocytes and splenic lymphocytes obtained from 20- to 23-dpi *ts1*-infected or age-matched control mice. Cells (5×10^6) from thymuses or spleens were collected by centrifugation at $300 \times g$ for 5 min and lysed in 1.5 ml of lysing buffer (20 mM Tris-HCl [pH 7.6], 4 mM EDTA, 3% sodium dodecyl sulfate, 0.5 mg of proteinase K per ml) at 50°C for 1 h. RNase (20 $\mu\text{g}/\text{ml}$; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was then added, and incubation was continued for 1 h. The lysates were centrifuged at $12,000 \times g$ for 10 min, and the supernatants were deproteinized by extraction once in phenol and twice in chloroform-isoamyl alcohol (24:1) and then precipitated with ethanol at -20°C . The resulting pellets were dried and resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) buffer. The DNA was mixed with sample buffer (1 $\mu\text{g}/\text{ml}$) containing ethidium bromide and subjected to electrophoresis in a 1.8% agarose gel. Lanes: 1, 100-bp stepladder weight marker; 2, thymocytes from control mice; 3, thymocytes from infected mice; 4, splenic lymphocytes from control mice; 5, splenic lymphocytes from infected mice.

(data not shown). The induction of DNA fragmentation in normal thymocytes as early as 8 h after incubation has been reported (9, 43). Although the lag time before the apoptotic process begins is variable within a cell population, the transition from normal to apoptotic takes place with extreme rapidity. However, cell death as measured by vital dye uptake follows long after DNA fragmentation (6). Therefore, it is not unusual to notice some degree of DNA fragmentation in any primary cultured cells after some time even though the cells may look viable by dye exclusion. Nonetheless, the distinctive stepladder pattern of DNA was obvious in *ts1*-infected cells at all times, thus indicating apoptosis.

Electron microscopic examination of the thymuses and spleens from *ts1*-infected mice also revealed increased nuclear chromatin condensation typical of apoptotic cells. This feature in the infected thymus was noticed as early as 20 to 23 dpi (Fig. 4A) and gradually increased in intensity as the disease progressed (data not shown). Control mice (Fig. 4B) only rarely showed similar chromatin condensation. However, no chromosomal abnormalities were observed in the infected mice for the first 10 days after virus inoculation. The number of pyknotic cells in *ts1*-infected thymus was also increased after the first 10 to 15 dpi (data not shown). It was reported previously that the numbers of mitotic cells decrease while the numbers of pyknotic cells increase in *ts1*-infected thymus after the first 10 dpi and that a decrease in thymic weights in *ts1* infection correlated with an increase in the number of pyknotic cells in the thymus (39). Splenic cells from *ts1*-infected mice also showed signs of similar chromatin changes after 20 dpi. Increased pyknosis has also been associated with other virus-induced T-lymphocyte apoptosis (reviewed in references 6 and 17). It is possible that as *ts1* disseminated between 10 to 20 dpi in our

studies, PCD was induced in a greater number of T cells, leading to the ultimate development of severe immunodeficiency (32, 52).

T cells, mainly immature thymocytes and CD4^+ T cells, are the primary targets for *ts1* (32, 33, 39, 47, 51, 52). Interestingly, in *ts1* infections, these cells along with the neural cells are eventually killed, leading to immunodeficiency and neurologic disorders. In this regard, *ts1* is quite similar to HIV (25, 52). Apoptosis probably plays an important role in HIV-induced T-cell depletion (3, 13, 22). Here we show that apoptosis probably also plays a very significant role in *ts1*-induced T-cell depletion in vivo. Several features, summarized in Table 1, clearly indicate that activation-induced death of T cells, at least partially, is responsible for the ultimate destruction of T cells in *ts1* infection.

The selective defect in the proliferative response of T cells to mitogens and superantigens from *ts1*-infected mice is probably related to the induction of an active cell death process by these stimuli. Although cell death was observed in the absence of any stimuli in *ts1*-infected cells, addition of a T-cell mitogen, e.g., ConA, significantly accelerated the cell death process (Fig. 2). Apoptosis normally is accompanied by an increase in the cytosolic Ca^{2+} level (19, 20). Mitogens like ConA can stimulate intracellular Ca^{2+} levels (44). However, along with Ca^{2+} , treatment with ConA also induces diacylglycerol, which in turn activates protein kinase C (PKC) (41). PKC has been shown to inhibit DNA fragmentation by apoptosis (20). Moreover, ConA by itself does not stimulate apoptosis in normal T cells (20). Therefore, the apoptosis observed here in the *ts1*-infected T cells was not likely due to the direct effect of ConA alone. Defective cell proliferation and accelerated cell death after mitogen treatment in *ts1*-infected T cells indicate that somehow the virus infection stimulated the cascade of events that induced the generation of endonuclease and DNA fragmentation leading to apoptosis (6). Inhibition of this *ts1*-induced cell death by the addition of the protein synthesis inhibitor cycloheximide further substantiates the notion that new protein synthesis is involved in this apoptotic cell death. Whether *ts1* actually acts as a PKC antagonist, or whether *ts1*-infected cells are already activated in vivo so that further activation by mitogens in vitro drives these cells into PCD, remains unclear. Interestingly, however, a report favoring the latter hypothesis in the case of feline immunodeficiency virus infection of T cells has been published (24).

Another way that T cells can go into PCD is by separate ligation of major histocompatibility complex (MHC) class II-restricted CD4 and the T-cell receptor (TCR) (23). Responses to self MHC class II-dependent TCR mobilization was investigated by using SEB superantigens. These superantigens bind to MHC class II molecules and interact with particular TCR V_β molecules that are expressed by many T cells and induce proliferation of these cells (16). The in vitro proliferative defects and enhanced death of T cells from *ts1*-infected mice due to TCR mobilization by these superantigens probably indicate that these cells were primed in vivo for apoptosis upon further stimulation (Fig. 1). Similar responses by CD4^+ T cells from HIV-infected individuals have been reported by Groux et al. (13). These authors have also hypothesized that it is possible that the T cells in vivo may not have any intrinsic defect in HIV infection, but that an abnormal antigen-presenting cell function may account for the T-cell apoptosis. We cannot, however, rule out either of these two possibilities. Another possibility is that the superantigens may have induced increased virus expression from the infected T cells that was responsible for the accelerated cell death. Recently, staphylococcal superantigens have been shown to induce HIV expression in CD4^+ T cells (5). Since treatment of *ts1*-infected T cells with SEB

FIG. 4. Electron microscopic examination (magnification, $\times 8,000$) of thymuses from 20- to 23-dpi *ts1*-infected mice (A) and age-matched uninfected control mice (B). Electron microscopy was performed on cells and whole organs as described previously (37). Briefly, tissues were fixed in 4% glutaraldehyde in phosphate-buffered saline for 2 h, postfixed in osmium tetroxide for 1 h, and dehydrated through ascending concentrations of ethanol and propylene oxide. Samples were then embedded in Epon araldite. Finally, sections were cut on a leukotriene ultramicrotome, stained with uranyl acetate and lead citrate, and examined on a Philips electron microscope. Several thymocytes show various stages of chromatin condensation in *ts1*-infected thymus. The arrow in panel A points to a typical apoptotic condensed chromatin.

produced rapid cell death, whether this cell death was due to increased *ts1* expression remains unclear.

Although functional defects as well as quantitative depletion (through apoptosis) of $CD4^+$ T cells have been shown in patients with HIV infections (1, 13, 25), it remains unclear

whether virus replication was necessary for the induction of these defects. We have previously shown that *ts1* is less efficient than the wild-type (WT) at processing precursor envelope gPr80^{env} into gp70 and Prp15E (40). We have also shown that in astrocytes, the cytopathicity of *ts1* is associated with higher

TABLE 1. Features of apoptotic death of T cells after *ts1* infection^a

Mouse group	Depletion of thymocytes and CD4 ⁺ T lymphocytes ^b	No. of pyknotic cells/high-power field in the thymus ^c	Chromatin condensation	DNA fragmentation	Enhanced cell death after stimulation	Inhibition of cell death after addition of protein synthesis inhibitor
Control	No	<2	No	No	No	No
<i>ts1</i> infected	Yes	>18	Yes	Yes	Yes	Yes

^a Data are from 20- to 23-dpi *ts1*-infected mice.^b From references 30 and 32.^c From reference 39.

viral expression and increased intracellular accumulation of nonprocessed gPr80^{env} (37). It is noteworthy that both CD4⁺ T cells and thymocytes are more conducive for growth of *ts1* than the WT virus (32, 51) and that gPr80^{env} is inefficiently processed and accumulated in the immature (thymocytes) as well as mature T cells in *ts1* infection (33a). Furthermore, by using primary cultures of thymocytes, we also recently found that apoptosis, increased virus replication, and accumulation of gPr80^{env} were all associated in *ts1*-infected cells compared with WT-infected cells (33a). Several reports have indicated a possible relationship between unprocessed precursor envelope gp160 and a cytopathic effect on CD4⁺ T cells in HIV infection (7, 15). Crise and Rose (7), who have shown an increased accumulation of an intracellular complex of gp160 and CD4⁺ in T cells, have hypothesized that this complex may induce defective cell signals which could be responsible for the observed cell death in HIV infection. Defective signal transduction has been suggested as a common pathway for cellular dysfunction in HIV infection (reviewed in reference 18). It is possible that in *ts1*-infected T cells, the retention of unprocessed precursor envelope polyprotein gPr80^{env} alone or in the form of a complex with other cellular proteins may induce imbalanced signal transduction or that the increased accumulation of gPr80^{env} itself may physically block nuclear transport; both of these events may ultimately lead to apoptosis.

Several cytokines, including tumor necrosis factor alpha, interleukin-1 (IL-1), and IL-2 have been shown to play important roles in apoptosis (8, 24, 26). Deng and Podack (8) showed that the proto-oncogene *bcl-2* can actually prevent apoptosis in IL-2-dependent T-cell lines. Recently, Zhong et al. indicated that *bcl-2* can inhibit apoptosis of the neural cells by multiple agents (55). Others have suggested that deregulation of one or several cytokines may be responsible for CD4⁺ T-cell depletion in HIV infection (14). We are currently investigating the role of cytokines and *bcl-2* in *ts1* infection. Our preliminary data indicate an upregulation of tumor necrosis factor alpha, IL-1, and IL-4 in *ts1* infection both in vivo and in vitro (unpublished data). However, the significance of these cytokine changes in *ts1*-induced apoptosis remains to be solved.

Several viruses other than HIV, including feline immunodeficiency virus (24), feline leukemia virus (29), and chicken anemia virus (14), have also been shown to cause apoptosis in T cells. As far as we know, this is the first report of a murine retrovirus inducing activation-induced death by apoptosis in T cells. Further studies in this murine model may provide important results that will help in better understanding the complex pathways involved in T-cell killing by retroviruses.

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