Polyomavirus DNA Is Damaged in Target Cells during Cytotoxic T-Lymphocyte-Mediated Killing

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Target cell DNA damage is an early event in cytotoxic T-lymphocyte (CTL)-mediated killing. It has been hypothesized that this DNA damage may serve as one mechanism of destroying viral genetic material inside infected cells. We directly examined the fate of viral DNA in target cells during CTL-mediated lysis. Polyomavirus DNA in transfected murine P815 mastocytoma targets was digested along with cellular DNA into oligonucleosome-sized fragments, although intact forms, possibly virion-associated DNA, were also present. In infected BALB/3T3 murine fibroblasts, which normally undergo single-stranded nicks when killed by CTL, polyomavirus DNA was converted to relaxed forms in the presence of CTL. These results suggest that the fate of the viral DNA depends on the stage of the viral life cycle and corresponds to the fate of the host cell DNA. Cleavage of the viral genome prior to assembly may thus be an important mechanism in specific antiviral immunity.

The *in vivo* biological role of cytotoxic T lymphocytes (CTL) during the recovery of mice from several viral infections including ectromelia (mousepox) (3), lymphocytic choriomeningitis (LCM) (34), and influenza A (28-33) has been established. In these systems, antiviral protection, as measured by reductions in viral titers in various organs, was adoptively transferred with specifically immune lymphoid cells belonging to the CTL subset. Furthermore, cloned CTL specific for influenza A virus (16) and LCM virus (LCMV) (4) reduce viral titers in mice infected with the respective virus. Oldstone et al. (20) have also demonstrated that LCMV nucleic acids (that is, RNAs) which accumulate during persistent infection are efficiently cleared from most organs (except the brain and kidneys) by LCMV-specific CTL.

It is not yet understood how CTL are able to clear viral infections; mere lysis of an infected target cell could simply facilitate the release of infectious virus. Indeed, this is probably the case for mature, fully assembled virions. However, before assembly of the intact virion, viruses appear to be susceptible to some form of CTL-mediated damage. It has been shown (2) *in vitro* that in addition to lysing syngeneic LCMV-infected targets, LCMV-specific CTL significantly lower viral titers. This effect occurs even when the CTL are added 24 to 48 h after infection and does not appear to be due to the antiviral properties of gamma interferon produced by CTL.

A very early event during CTL-mediated killing of certain target cells is the fragmentation of target cell DNA into oligonucleosomes (8, 24). It has been proposed that a Ca^{2+} - Mg^{2+} -dependent endonuclease which cuts between nucleosomes is responsible for the pattern of DNA fragmentation observed (8). Fragmentation precedes ^{51}Cr release (lysis) by at least 1 h and is not simply a result of cell death, since killing of target cells by heating, freeze-thawing, and lysis with antibody plus complement does not lead to DNA fragmentation. The role of DNA fragmentation in the CTL-

mediated lytic process remains speculative at present. An intriguing possibility is that CTL have evolved a mechanism not only to lyse cells but also to destroy any potentially dangerous genetic material (e.g., viral DNA or RNA) contained within at least certain types of cells.

The purpose of this study was to determine directly the fate of viral DNA in a target cell during CTL-mediated lysis. To compare patterns of fragmentation in cellular versus viral DNA, we wanted a virus (i) which replicates in the cell nucleus (since we are looking at CTL-induced nuclear damage) and (ii) whose DNA structure is similar to that of eucaryotes. We chose polyomavirus as the model system since it fulfills both of these criteria. Polyomavirus DNA is replicated (17) and packaged into virions (18) in the host nucleus (for a review, see reference 1). Additionally, this virus was chosen because its nucleoprotein complexes are similar in structure to eucaryotic chromatin. Specifically, polyomavirus nucleoprotein complexes are organized into nucleosomes (12, 21, 22) and contain histones derived from the host cell (10).

We show in this report that during CTL-mediated killing, a significant proportion of polyomavirus DNA is digested along with murine hematopoietic target cell DNA into oligonucleosomes. Furthermore, we demonstrate that the fate of viral DNA during CTL-mediated killing corresponds to the fate of the host cell DNA.

MATERIALS AND METHODS

Viral strains and DNA. Polyomavirus A2 and F101 were generously provided by Luis Villarreal, University of California, Irvine. Mutant polyomavirus DNA, PyA2dIPvuD.X (dIPvuD), was prepared and kindly provided by Bruce Campbell, University of California, Irvine.

Mice. BALB/cBy (*H-2^d* haplotype) and C57BL/6(*H-2^b*) mice, 8 to 24 weeks old, were used as spleen donors. Mice were purchased from the Jackson Laboratory, Bar Harbor, Maine.

Cells and cell lines. Mice were killed by cervical dislocation. Single-cell suspensions were prepared aseptically from spleens in Hanks balanced salt solution by pressing the organs through wire mesh screens and then subjecting them

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to two passages through a 25-gauge needle. The viability of cell preparations was assessed by eosin Y exclusion. P815 murine mastocytoma cells and other murine tumor cells of hematopoietic origin were maintained in tissue culture medium (TCM), composed of RPMI 1640 medium (Whittaker M.A. Bioproducts, Walkersville, Md.) supplemented with 5% heat-inactivated fetal calf serum (Hazelton, Denver, Pa.), L-glutamine, and gentamicin. BALB/3T3 fibroblasts and other murine nonhematopoietic cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated newborn calf serum, L-glutamine, and gentamicin.

Infection and transfection. Various cell lines were infected at a multiplicity of infection of 0.1 PFU of polyomavirus A2 or F101 per cell. Cells were harvested on day 5 postinfection. P815 mastocytoma cells were transiently transfected with dIPvD mutant polyomavirus DNA by the DEAE-dextran and chloroquine phosphate method described for lymphocytes by Campbell and Villarreal (5) and harvested 48 h later. The presence of viral DNA in infected and transfected cells was assessed by Southern blot analysis (27) of Hirt extracts (13) electrophoresed on agarose gels. Infected and transfected cells, as well as their mock-treated counterparts, were used as targets in CTL assays.

Effector cells. Responder spleen cells (7×10^6 /ml) and irradiated (3,000 rad; 1 rad = 0.01 Gy) allogeneic stimulator spleen cells (10^6 per ml) were cultured together in 2 ml of TCM supplemented with 5×10^{-5} M 2-mercaptoethanol (TCM-CTL) in 24-well tissue culture plates at 37°C. On day 5, the cells were harvested from each well by vigorous pipetting. Some of the CTL were assayed on day 5; the rest were maintained in TCM-CTL supplemented with 25% concanavalin A supernatant as a source of interleukin-2 and assayed on day 6 or 7.

^{51}Cr release assay. Target cells were labeled with sodium [^{51}Cr]chromate (ICN Pharmaceuticals, Inc., Irvine, Calif.) ($100 \mu\text{Ci}/10^6$ cells) for 60 min at 37°C and then washed thoroughly. ^{51}Cr -labeled targets (10^4 cells in $100 \mu\text{l}$ of TCM-CTL) were added in duplicate to various numbers of effector cells in $100 \mu\text{l}$ of TCM-CTL in individual wells of 96-well round-bottom microtiter plates (Linbro; Falcon Laboratories, Inc., McLean, Va.). The plates were then centrifuged ($50 \times g$ for 5 min) to establish cell contact and incubated at 37°C for the times indicated. They were then centrifuged at $200 \times g$ for 10 min, and $100 \mu\text{l}$ of supernatant was collected and counted in a gamma spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). The percent specific release was calculated by the following formula: percent specific ^{51}Cr release = $(\text{cpm}_{\text{exp}} - \text{cpm}_{\text{spont}})/(\text{cpm}_{\text{max}} - \text{cpm}_{\text{spont}}) \times 100$. Counts released from targets incubated in the presence of CTL represent the experimental release (cpm_{exp}). Maximal release (cpm_{max}) was determined from supernatants of targets that were lysed in 1% sodium dodecyl sulfate (SDS), and the spontaneous release ($\text{cpm}_{\text{spont}}$) was determined from supernatants of target cells incubated without added effector cells.

DSF assay. Double-stranded DNA fragmentation (DSF) in target cells was measured by a modification of the method of Duke et al. (9). Various numbers of effector cells in $100 \mu\text{l}$ of TCM were added in duplicate to [^{125}I]iododeoxyuridine [^{125}I]IUdR- or [^3H]thymidine-labeled targets (10^4 cells in $100 \mu\text{l}$) in 1.5-ml microcentrifuge tubes. The tubes were centrifuged at $50 \times g$ for 5 min to establish cell contact and incubated at 37°C for various times. After incubation, 0.2 ml of cold saline was added to each tube, and the cells were pelleted by centrifugation ($200 \times g$ for 10 min). The incuba-

tion medium was removed and placed in soda lime glass scintillation vials. Radioactive counts in the incubation media represent fragmented DNA released by the cells. The pelleted cells were lysed with 0.4 ml of hypotonic lysing buffer (TTE; 10 mM Tris, 1 mM EDTA [pH 7.5], 0.2% [vol/vol] Triton X-100), vortexed, and centrifuged ($13,000 \times g$ for 10 min) to separate intact from fragmented chromatin. The $13,000 \times g$ supernatants were withdrawn and placed in scintillation vials. The $13,000 \times g$ pellets (intact chromatin), disrupted by the addition of 10 mM Tris-1 mM EDTA containing 1% SDS and vigorous pipetting, were also placed in scintillation vials. For [^3H]thymidine-labeled targets, scintillation fluor (Optifluor; Packard Instrument Co., Inc., Rockville, Md.) was added to each vial and the radioactivity was measured in a beta counter (Beckman). For [^{125}I]IUdR-labeled targets, counting was performed in the gamma counter. The percent fragmented DNA was calculated by the following formula: percent fragmented DNA = $(\text{cpm}_{\text{frags}}/\text{cpm}_{\text{total}}) \times 100$, where $\text{cpm}_{\text{frags}}$ is the radioactivity in the incubation medium plus the radioactivity in the $13,000 \times g$ supernatant and $\text{cpm}_{\text{total}}$ is $\text{cpm}_{\text{frags}}$ plus the radioactivity in the $13,000 \times g$ pellet. Spontaneous DNA fragmentation was determined from target cells incubated without effector cells.

In some experiments, DNA from unlabeled targets was isolated in this manner for electrophoretic analysis.

Preparation of nuclei. Nuclei were prepared by the method of Miovic and Pizer (19). Cells were suspended in 2.5 ml of TMS buffer (150 mM NaCl, 10 mM MgCl_2 , 10 mM Tris [pH 7.4]). After the addition of Triton X-100 (final concentration, 0.2%) and dithiothreitol (final concentration, 0.2 mM), the cells were disrupted in a Dounce homogenizer (pestle B; Bellco Glass, Inc., Vineland, N.J.) and layered over 2 ml of a 25% glycerol cushion containing 10 mM Tris (pH 7.4), 5 mM MgCl_2 , and 0.2 mM dithiothreitol (TGMD). Centrifugation at $200 \times g$ for 10 min was used to separate the nuclei, which form pellets, from the cytoplasmic fraction, which remains above the TGMD cushion.

Extraction of mouse endogenous endonuclease. Splenic and thymic nuclei contain large quantities of a Ca^{2+} - Mg^{2+} -dependent, Zn^{2+} -inhibitable endonuclease which cuts chromatin between nucleosomes. This enzyme was eluted by a modification of the method of Duke et al. (9). Splenic and thymic nuclei isolated as described above were suspended at 200×10^6 /ml in modified TMS buffer (75 mM NaCl, 10 mM MgCl_2 , and 10 mM Tris) and incubated on ice for 90 min with frequent agitation. The nuclei were then pelleted at $200 \times g$ for 10 min, and the supernatant was withdrawn. After centrifugation at $13,000 \times g$ for 3 min to remove any remaining DNA, the supernatant was used as a source of endogenous endonuclease.

Assay of endogenous endonuclease activity. Endogenous endonuclease ($100 \mu\text{l}$), prepared as described above, was incubated with unlabeled isolated BALB/3T3 nuclei (10^4 in $100 \mu\text{l}$ of TMS buffer) in the presence or absence of 5 mM CaCl_2 at 37°C for 90 min. DNA from the incubation media, $13,000 \times g$ supernatant, and the $13,000 \times g$ pellets was isolated as previously described and analyzed by agarose gel electrophoresis.

Agarose gel electrophoresis. Unlabeled DNA from the incubation media, $13,000 \times g$ supernatants, and the $13,000 \times g$ pellets isolated as described above was precipitated with 50% isopropanol and 0.5 M NaCl overnight at -20°C . The precipitates were pelleted by centrifugation at $13,000 \times g$ for 10 min, air dried, and suspended in 10 mM Tris-1 mM EDTA (pH 7.4). Loading buffer containing 15 mM EDTA, 2% SDS, 50% glycerol, and 0.5% bromophenol blue; 10 μg of RNase

per ml was added to samples at a 1:5 (vol/vol) ratio, and the samples were heated to 65°C for 10 min.

DNA samples were run on horizontal 1% agarose (BioRad Laboratories, Richmond, Calif.) gels in a neutral buffer system containing 90 mM Tris, 90 mM boric acid, and 10 mM EDTA. Cellular DNA was visualized by ethidium bromide staining. Gels were blotted to nitrocellulose as described by Southern (27). The filters were air dried, baked for 2 h at 80°C in vacuo, and prehybridized for 18 to 24 h at 65°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 2× Denhardt solution (1× Denhardt solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone). Hybridization was carried out for 24 h at 42°C in a solution containing 50% formamide, 5× SSC, 0.2% SDS, 200 µg of calf thymus DNA per ml plus ³²P-labeled nick-translated (23) polyomavirus DNA cloned into pBR322 at the *Bam*HI site with a minimum specific activity of 10⁸ cpm/µg of DNA. Hybridized filters were washed four times in 2× SSC–0.1% SDS for 5 min at room temperature and then twice in 0.1× SSC–0.1% SDS for 40 min at 50°C. Dried filters were exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.) in the presence of a Cronex Lightning-Plus Intensifying Screen (Du Pont Co., Wilmington, Del.) at –70°C for the times indicated in the figure legends.

RESULTS

Our original plan was to infect murine hematopoietic cells (that is, cells which undergo double-stranded DNA fragmentation before being lysed by CTL) with polyomavirus and use these infected cells as targets of allospecific CTL. DNA isolated from CTL-lysed targets would then be electrophoresed on agarose gels and blotted onto nitrocellulose filters. We know from previous experiments that the cellular DNA should be fragmented into oligonucleosomes. Hybridization with a polyomavirus-specific probe could then be used to distinguish the viral DNA from cellular DNA, and thus the fate of viral DNA in CTL-lysed targets could be monitored.

Several murine hematopoietic cell lines including P815, a mastocytoma; EL-4, a T lymphoma; and YAC-1, a Moloney virus-transformed thymoma were cultured for 5 days after exposure to polyomavirus A2 (the original inoculum of virus was 0.1 PFU per cell). Southern analysis of Hirt extracts (13) of these cells run on agarose gels and hybridized with the polyomavirus-specific probe indicated that the cells either were not infected by or did not allow replication of this virus (data not shown). Similar results were obtained when the mutant F101 strain was tested for its ability to infect murine hematopoietic cells (data not shown). This strain, developed by Fujimura et al. (11), contains a point mutation at position 5258 near the origin of DNA replication and tandem duplications of the sequence that contains the point mutation. The mutation allows strain F101 to replicate in undifferentiated F9 mouse embryonal carcinoma cells which are normally resistant to productive infection by wild-type strain A2. It was hoped that this mutation would also allow strain F101 to replicate in murine hematopoietic cells; however, no viral DNA could be detected in these cells when cultured for 5 days after exposure to this virus.

Because neither wild-type strain A2 nor mutant strain F101 was able to infect the murine cells of hematopoietic origin, a transient transfection system with mutant polyomavirus DNA, PyA2dlPvuD.X (dlPvuD), was used to introduce polyomavirus DNA into P815 mastocytoma cells (5). Mutant dlPvuD DNA was made by deleting the 137-base-pair *Pvu*II

FIG. 1. ⁵¹Cr release and DNA fragmentation during CTL-mediated killing of mock-transfected or dlPvuD-transfected P815 targets. Ten thousand ⁵¹Cr- or [¹²⁵I]IUdR-labeled mock-transfected (●, ○) or dlPvuD-transfected (▲, △) P815 cells (48 h after transfection) were mixed with 7-day C57BL/6 anti-BALB CTL at various effector-to-target-cell ratios. The percent DNA fragmentation (○, △) and the percent specific ⁵¹Cr release (●, ▲) were determined after a 4-h incubation at 37°C. The spontaneous values for DNA fragmentation were 21% for mock-transfected P815 and 20% for dlPvuD-transfected P815 cells, and the spontaneous values for ⁵¹Cr release were 23% for mock-transfected P815 and 19% for dlPvuD-transfected P815 cells. Each point represents the mean of duplicate cultures. Standard deviations for each point were less than 4% and are not shown.

D region which contains the B enhancer and inserting an *Xho*I linker in place of the deleted sequences. This deletion allows the mutant DNA to replicate in transfected P815 cells (transfected wild-type polyomavirus DNA will not replicate efficiently in these cells [5]). P815 cells were transfected with dlPvuD DNA or mock-transfected and incubated at 37°C for 40 to 48 h. Approximately 2 × 10⁶ of these cells were then labeled with ⁵¹Cr or [¹²⁵I]IUdR or left unlabeled (for the electrophoretic analysis) for use as targets in CTL assays. To avoid any possible interference with viral DNA replication by the radionucleotide, [¹²⁵I]IUdR was used in this assay because this compound results in relatively high radioactive precursor incorporation in just 1 h as compared with the overnight incubation required for adequate [³H]thymidine labeling.

Allospecific C57BL/6 anti-BALB/c CTL killed both mock-transfected and transfected P815 as measured by ⁵¹Cr release and induced extensive double-stranded DNA fragmentation in these targets as quantitated by the DSF assay (Fig. 1). Levels of CTL-induced lysis and DNA fragmentation were virtually identical in the two targets; thus, the presence of dlPvuD polyomavirus DNA in target cells neither enhances nor inhibits the normal lytic process. Additionally, levels of spontaneous ⁵¹Cr release and DNA fragmentation in the transfected and mock-transfected targets were equivalent. This observation indicates that transfection with dlPvuD polyomavirus DNA does not cause lysis or induce DNA fragmentation in and of itself.

The quantitative assay for DNA fragmentation does not distinguish between effects on viral or cellular DNA (although we assume that the majority of radioactive counts represent cellular DNA). To distinguish between these two DNA types, DNA (cellular and viral) isolated from transfected and mock-transfected P815 cells incubated in the presence or absence of CTL was electrophoresed on agarose gels. Figure 2 is a representative ethidium bromide-stained

FIG. 2. Agarose gel electrophoresis of mock-transfected and dlPvuD-transfected P815 cells after treatment with CTL. Electrophoresis of DNA isolated from 100,000 unlabeled (A) dlPvuD-transfected P815 cells or (B) mock-transfected P815 cells incubated alone (lanes 1 to 3 and 7 to 9) or with 7-day C57BL/6 anti-BALB/c CTL at an effector-to-target-cell ratio of 1:1 (lanes 4 to 6 and 10 to 12) at 37°C for 4 h is shown. DNA was isolated from the incubation media (lanes 1, 4, 7, and 10), the $13,000 \times g$ supernatant (lanes 2, 5, 8, and 11), and the $13,000 \times g$ pellet (lanes 3, 6, 9, and 12). Marker DNA was strain A2 DNA (lane 13). Abbreviations: R, relaxed circular form; L, linear form; S, supercoiled form of closed-circular polyomavirus DNA. Electrophoresis in 1% agarose was carried out for 16 h at 40 V. DNA was visualized with ethidium bromide.

gel in which a majority of the visible DNA probably represents cellular DNA. A low level of oligonucleosome-sized fragments (representing about 20% fragmentation in the DSF assay) was observed in the incubation media and $13,000 \times g$ supernatant of transfected or mock-transfected P815 targets without CTL (spontaneous); however, the majority of the DNA was large and was associated with the $13,000 \times g$ pellet. In transfected and mock-transfected P815 targets incubated in the presence of CTL, there was a striking increase in the amount of DNA released into the incubation medium as oligonucleosome-sized fragments (representing approximately 80% fragmentation in the DSF assay). Southern analysis of this gel with the ^{32}P -labeled polyomavirus-specific probe revealed that a significant proportion of polyomavirus DNA was also digested into oligonucleosomes and released into the incubation medium during CTL-mediated killing (Fig. 3). Various amounts of intact supercoiled, linear, and relaxed forms of polyomavirus DNA were also observed in the incubation medium of transfected P815 cells incubated with CTL. The intact forms of polyomavirus DNA may be derived from virions. On the other hand, fragmented viral DNA probably represents unpackaged DNA, which is readily accessible to enzymatic digestion.

We have demonstrated that there are differences in the characteristics of DNA digestion in the targets of CTL at both the lineage and species level (K. S. Sellins, R. C. Duke, and J. J. Cohen, *FASEB J.* 2:A468, 1988). In contrast to murine cells of hematopoietic origin, most murine nonhematopoietic (fibroblast or epithelial) cell lines undergo single-stranded nicks in their DNA during CTL-mediated killing. Furthermore, we and others (14) have shown that the

FIG. 3. Autoradiograph of dlPvuD polyomavirus DNA in transfected P815 target cells. This is an autoradiograph of lanes 1 to 6 of the gel shown in Fig. 2. The DNA was transferred to a nitrocellulose filter and hybridized to 10×10^6 cpm of ^{32}P -labeled polyomavirus-specific probe. Polyomavirus DNA was visualized by autoradiography after 7 days.

fate of the target cell DNA during CTL-mediated lysis is determined by the target cell and not by the CTL. We were therefore interested in determining the fate of polyomavirus DNA in murine fibroblast targets. Specifically, we asked whether the pattern of viral DNA digestion corresponds to that of the host cell DNA or whether infection with virus might allow the induction of double-stranded DNA fragmentation of viral or even cellular DNA during CTL-mediated lysis.

FIG. 4. ^{51}Cr release and DNA fragmentation during CTL-mediated killing of uninfected and strain A2-infected BALB/3T3 targets. Ten thousand $10,000$ ^{51}Cr - or $[^3\text{H}]$ thymidine-labeled uninfected (●, ○) or A2-infected (▲, △) BALB/3T3 cells were mixed with 5-day C57BL/6 anti-BALB/c CTL at various effector-to-target-cell ratios. The percent DNA fragmentation (○, △) and the percent specific ^{51}Cr release (●, ▲) were determined after an 8-h incubation at 37°C. The spontaneous values for DNA fragmentation were 1% for uninfected BALB/3T3 cells and 1% for A2-infected BALB/3T3 cells, and the spontaneous values for ^{51}Cr release were 25% for uninfected BALB/3T3 cells and 30% for A2-infected BALB/3T3 cells. Each point represents the mean of duplicate cultures. Standard deviations for each point were less than 10% and are not shown.

FIG. 5. Autoradiograph of strain A2 DNA in infected BALB/3T3 target cells. Electrophoresis of DNA isolated from 10,000 unlabeled A2-infected BALB/3T3 cells incubated with 7-day C57BL/6 anti-BALB/c CTL at an effector-to-target-cell ratio of 100:1 (lanes 1 to 3) or incubated alone (lanes 4 to 6) at 37°C for 4 h is shown. DNA was isolated from the incubation media (lanes 1 and 4), the 13,000 $\times$ *g* supernatant (lanes 2 and 5), and the 13,000 $\times$ *g* pellet (lanes 3 and 6). Electrophoresis in 1% agarose was carried out for 16 h at 40 V. The DNA was transferred to a nitrocellulose filter and hybridized to 10^7 cpm of 32 P-labeled polyomavirus-specific probe. Polyomavirus DNA was visualized by autoradiography after a 10-day exposure.

Several murine nonhematopoietic lines including BALB/3T3, a fibroblast derived from BALB/c mice, were efficiently infected with wild-type strain A2 and with strain F101 (data not shown). ^{51}Cr - or $[^3\text{H}]$ thymidine-labeled A2-infected or uninfected BALB/3T3 fibroblasts were used on day 5 after infection as targets of C57BL/6 anti-BALB/c CTL in an 8-h assay (Fig. 4). Although relatively high (and roughly equivalent) levels of ^{51}Cr release were obtained with uninfected and infected BALB/3T3 targets, no double-stranded DNA fragmentation could be detected in either target by the DSF assay. Again, the majority of the radioactive counts detected by the DSF assay represent cellular DNA, and effects on viral DNA would probably not be detected by this assay. To determine whether viral DNA was affected, we used unlabeled infected and uninfected BALB/3T3 cells as targets of allospecific CTL. DNA in the incubation media, the 13,000 $\times$ *g* supernatant, and the 13,000 $\times$ *g* pellet was precipitated, run on a 1% agarose gel, and blotted onto nitrocellulose. Hybridization to the polyomavirus-specific probe (Fig. 5) revealed that the polyomavirus DNA in the infected fibroblasts was not degraded into oligonucleosome-sized fragments when these cells were killed by CTL. Polyomavirus DNA in the incubation media from both control and CTL-lysed infected targets was in the relaxed circular form. Supercoiled, linear, and relaxed circular viral DNA was detected in the 13,000 $\times$ *g* pellets of both types of targets. Consistent with our previous observation that fibroblast nuclear chromatin undergoes single-stranded nicks when the cells are killed by CTL (K. S. Sellins, R. C. Duke, and J. J. Cohen, *FASEB J.* 2:A468, 1988), polyomavirus DNA in the 13,000 $\times$ *g* supernatant from CTL-lysed targets, but not from

FIG. 6. Autoradiograph of strain A2 DNA isolated from infected BALB/3T3 nuclei treated with thymus or spleen endonuclease. Electrophoresis of DNA isolated from 500,000 unlabeled A2-infected BALB/3T3 nuclei in 100 μl of TMS (day 5 postinfection) incubated with 100 μl of either spleen enzyme plus 5 mM CaCl_2 (lanes 1 to 3), thymus enzyme plus 5 mM CaCl_2 (lanes 4 to 6), TMS plus 5 mM CaCl_2 (lanes 7 to 9), spleen enzyme (lanes 10 to 12), or thymus enzyme (lanes 13 to 15) for 90 min at 37°C is shown. DNA was isolated from the incubation media (lanes 1, 4, 7, 10, and 13), the 13,000 $\times$ *g* supernatant (lanes 2, 5, 8, 11, and 14), and the 13,000 $\times$ *g* pellet (lanes 3, 6, 9, 12, and 15). Electrophoresis in 1% agarose was carried out for 1 h at 100 V. The DNA was transferred to a nitrocellulose filter and hybridized with 5×10^6 cpm of ^{32}P -labeled polyomavirus-specific probe. Polyomavirus DNA was visualized by autoradiography after a 24-h exposure.

controls, was converted from the supercoiled form to the relaxed circular form (a process which would occur by nicking of the polyomavirus DNA).

It was conceivable that the BALB/3T3 nuclear environment somehow rendered the polyomavirus DNA resistant to double-stranded cutting. To test this possibility, we examined the effect of a Ca^{2+} - Mg^{2+} -dependent endonuclease (similar to that thought to be involved in CTL-mediated killing) (9) on polyomavirus DNA in infected fibroblast nuclei. When nuclei from A2-infected BALB/3T3 fibroblasts were treated in the presence of 5 mM CaCl_2 with spleen or thymus nuclear extract containing a Ca^{2+} - Mg^{2+} -dependent endonuclease which cuts between nucleosomes, both the fibroblast DNA (data not shown) and the polyomavirus DNA were fragmented into oligonucleosomes (Fig. 6). No fragmentation was observed when the nuclei were treated with enzyme in the absence of Ca^{2+} .

These results suggest that the fate of viral DNA during CTL-mediated lysis corresponds to the fate of the host cell DNA; that is, in cells which undergo extensive double-stranded DNA fragmentation when killed by CTL, the viral DNA undergoes similar damage, whereas in cells whose DNA undergoes single-stranded nicking, nicking is also observed in the viral DNA.

DISCUSSION

The mechanism by which CTL clear a viral infection is not understood. We (7) and others (6, 25) have hypothesized that CTL have evolved a killing mechanism designed not only to lyse virus-infected cells but also to destroy potentially dangerous (infective) genetic material (that is, viral DNA and possibly RNA) contained within the cell. Specifically, perhaps to ensure that such genetic material is destroyed, the CTL induce very rapid DNA (and possibly RNA) fragmen-

tation before cell lysis occurs in murine hematopoietic cells. The studies presented herein have demonstrated that polyomavirus DNA in transfected P815 targets is digested along with cellular DNA into oligonucleosomes during CTL-mediated killing. This fragmented viral DNA probably represents unpackaged DNA that is readily accessible to enzymatic digestion by the endonuclease proposed to be involved in CTL-mediated killing. In addition to fragmented viral DNA, various amounts of intact supercoiled, linear, and relaxed polyomavirus DNA were detected in transfected, CTL-lysed P815 targets. Shelton et al. (26) have demonstrated that in infected CV-1 nuclei, virions are the source of simian virus 40 form I, II, and III DNAs, which are resistant to micrococcal nuclease digestion. The intact forms which we observe may represent virion-associated DNA that is protected from endonuclease digestion. Experiments to test this hypothesis are currently in progress.

This explanation is consistent with previous studies by other investigators which have suggested that the fate of the virus during CTL-mediated killing depends on its life cycle stage. For example, viral PFU and the number of infectious centers were reduced following incubation of vaccinia virus-infected targets with vaccinia virus-specific CTL *in vitro* (33). However, in these experiments, the antiviral effect was dependent upon the timing of addition of CTL: when the CTL were added 1 to 4 h postinfection, the PFU and the number of infectious centers were reduced; addition of the CTL 4 to 8 hours postinfection did not result in these reductions, perhaps owing to the inability of CTL-mediated lysis to inactivate fully assembled virions. Howell and Martz (15) were also unable to demonstrate CTL-induced inactivation of intact intracellular reovirus virions. Therefore, it appears that the ultimate fate of the virus depends on the stage of its life cycle at the time of CTL interaction with the infected target cell; the protein coat of the virion seems to render the virus immune to CTL-mediated damage. Lysis of a cell containing packaged virions would simply result in release of infectious virus; however, CTL induce DNA fragmentation much earlier than lysis (8, 25), potentially ensuring that the viral genetic material will be destroyed rather than packaged.

Consistent with our observations that CTL induce only single-strand nicks in murine nonhematopoietic cells (K. S. Sellins, R. C. Duke, and J. J. Cohen, *FASEB J.* 2:A468, 1988), polyomavirus DNA in infected BALB/3T3 fibroblasts is not digested into oligonucleosomes, but undergoes single-stranded nicking. Thus, the fate of viral DNA corresponds to the fate of host cell DNA during CTL-mediated killing.

The relative role of DNA damage in the reduction of viral titers and in the clearance of virus during recovery from *in vitro* and *in vivo* infections remains to be determined. It also remains to be seen whether damage to viral nucleic acids is a generalizable phenomenon important in CTL-mediated immunity against other viruses. CTL-induced DNA damage may be particularly important in the destruction of proviral DNA. Since this viral DNA is unpackaged and integrated into the host genome, we predict that this DNA should be completely susceptible to CTL-induced DNA damage.

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