DNA fragmentation: Manifestation of target cell destruction mediated by cytotoxic T-cell lines, lymphotoxin-secreting helper T-cell clones, and cell-free lymphotoxin-containing supernatant (lymphokines)

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ABSTRACT A Lyt-2*, trinitrophenyl-specific, lymphotoxin-secreting, cytotoxic T-cell line, PCI 55, mediates the digestion of target cell DNA into discretely sized fragments. This phenomenon manifests itself within 30 min after effector cell encounter as measured by the release of \(^3\)H counts from target cells prelabeled with \(^3\)H]deoxythymidine and occurs even at very low effector to target cell ratios (0.25:1). A Lyt-1*, ovalbumin-specific, lymphotoxin-secreting T-helper cell clone, 5.9.24, is also able to mediate fragmentation of target cell DNA over a time course essentially indistinguishable from the cytotoxic T lymphocyte-mediated hit. Cell-free lymphotoxin-containing supernatants also cause release of DNA from targets, although they require a longer time course, on the order of 24 hr. In contrast, lysis of cells by antibody plus complement or Triton X-100 does not result in DNA release even after extended periods of incubation (24 hr). All three treatments that result in the release of DNA from cells cause fragmentation of that DNA into discretely sized pieces that are multiples of 200 base pairs. The results thus suggest that cytotoxic T cells, lymphotoxin-secreting helper clones with cytolytic activity, and lymphotoxin all effect target cell destruction by means of a similar mechanism and that observed differences in time course and the absence of target cell specificity in killing mediated by lymphotoxin may simply reflect differences in the mode of toxin delivery.

Reports from our laboratory and others (1–9) have suggested that the cytotoxic T lymphocyte (CTL) lethal hit may require the participation of a soluble cytolitic factor such as lymphotoxin (LT). Russell et al. (2–4) and Cohen et al. (5, 6) have demonstrated that the CTL hit is characterized by the early onset fragmentation and release of target cell DNA, whereas DNA is not fragmented in targets attacked by antibody plus complement (2, 3). Duke et al. (6) have reported that the digestion of DNA proceeds with the generation of fragments that are multiples of approximately 200 base pairs, suggesting the involvement of an endonuclease that cleaves at regular intervals. Since the reported DNA fragmentation by CTL is accompanied by the loss of nuclear membrane integrity (2, 3), a process that begins internally is also suggested.

LT, which was first described in 1968 (10, 11), has been proposed in the past to have some involvement in CTL killing (12–15). However, much evidence seemed to preclude a role for LT in that process, primarily because, unlike CTL, LT lacks any antigen specificity and does not require Ca\(^{2+}\) (16–20). Furthermore, most previous reports had suggested that LT is produced chiefly by Lyt-1* cells, rather than by Lyt-2* CTL (28). Our own laboratory has reported on a series of trinitrophenyl (TNP)-specific, Lyt-2* T-cell lines that produce LT under stimulation by mitogen or TNP-coupled syngeneic splenocytes (21, 22). We have also demonstrated that LT-containing supernatant fluids from these cell lines can kill target cells more rapidly (4 hr) if they are first introduced into the cytoplasm of target cells by using an osmotic pressure-based technique described by Okada and Rechsteiner (23). All of these observations can be explained if CTLs deliver a toxin into their target cells to effect lysis.

In addition, Dennert and Podack (7), and Henkart et al. (8, 9) have reported the occurrence of granules at the periphery of both activated CTLs and natural killer cell clones. Upon interaction with target cells, these granules discharge their contents into the interstitial space, and self-assembling tubules are deposited onto the surface of the target cells. It is possible that a toxin is injected through these tubules.

The series of experiments reported here compares the ability of CTLs, LT-producing T-helper cell clones, cell-free LT-containing supernatant, and antibody plus complement to mediate the fragmentation of target cell DNA. Our findings indicate that, with the exception of antibody plus complement, all of these agents are capable of directing the digestion of target cell DNA into discretely sized fragments.

MATERIALS AND METHODS

Cell Culture. PCI line 55 cells were derived and maintained as described (22). BALB/c clone 5.9.24 cells are Thy-1*, Lyt-1*, L3T4* T helper cells derived from BALB/c mice (H-2b). 5.9.24 and 153-E6, a C57BL/6-derived helper cell clone, were cultivated by seeding 2.5 × 10\(^6\) cells in Click's medium (supplemented with EHA, GIBCO) containing 10% (vol/vol) heat-inactivated fetal calf serum, 20 units of IL-4 cell-derived interleukin 2/ml, 200 μg of ovalbumin/ml and 1.67 × 10\(^5\) syngeneic, mitomycin C-treated (50 μg/ml, 30 min at 37°C) feeder cells/ml.

L929 cells (H-2\(^a\)), BALB/c 3T3 clone A31 cells (H-2\(^b\)), BWS5147 cells (H-2\(^b\)), and A20 cells (H-2\(^b\)) were cultured as described (1, 22).

LT. LT-containing supernatants used in this study to analyze target cell DNA fragmentation were prepared by stimulating 2 × 10\(^6\) clone 153-E6 cells (Lyt-1*, L3T4*) per ml with ConA-Sepharose (Pharmacia) at a concentration of 25 μg/ml in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal calf serum and incubated for 72 hr at 37°C. ConA-Sepharose and cells were removed from the supernatant by centrifugation at 1000 × g for 10 min, the fluids were filter-sterilized and stored at 4°C.

Abbreviations: CTL, cytotoxic T lymphocyte; LT, lymphotoxin; TNP, trinitrophenyl; MHC, major histocompatibility complex; IFN-γ, interferon γ.

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Antigen Treatment of Target Cells. A20 cells were labeled with ovalbumin by pulsing the cells with 1.0 mg of ovalbumin/ml (ICN) 18 h in advance of the experiment, as described (24). TNP haptenation of target cells has also been described (22). In the case of experiments involving the use of [3H]deoxythymidine or [125I]-labeled deoxyuridine, TNP labeling was done subsequent to radioactive labeling. For the 51Cr experiments, cells were labeled with antigen first.

DNA Release Assay. For cell-mediated DNA release assays, the method of Duke et al. (6) was used. Briefly, effector cells and targets were incubated together for the times shown at 37°C. Cells were pelleted, and the supernatant was collected. Cell pellets were lysed with 25 mM sodium acetate, centrifuged at 18,000 × g for 15 min, and the supernatant was collected. The combined [3H]deoxythymidine counts obtained from these fluids were compared to the unreleasable counts measured in the debris pellet, first subtracting background release from targets incubated alone in medium. LT-mediated DNA release assays were performed by aliquoting 5 × 106 [3H]deoxythymidine-labeled target cells per well into 96-well plates (Costar, Cambridge, MA) in a final volume of 0.2 ml with the indicated dilution of LT and incubating for the time shown at 37°C. Test release values were compared with background release and total release controls and were calculated by using the same formula as is used for the 51Cr release assay.

51Cr Release Assay. This assay was performed as described (1).

Antibody Plus Complement Treatment. Anti-K<sup>k</sup> antibody ([A.B4 × B10.AQR]F<sub>1</sub> anti-A/Sn) was kindly provided by Donal Murphy. [3H]Deoxythymidine-labeled target cells (1 × 10<sup>6</sup>) were incubated with double the final indicated dilution of antibody for 1 h at room temperature, at which point an equal volume of RPMI 1640 medium containing a 1:10 dilution of rabbit low-toxicity mouse complement (Cedarlane Laboratories, Hornby, ON) was added, and cells were incubated for 2 hr at 37°C. Percent release was calculated as for the 51Cr assay.

Agarose Gel Electrophoresis of Released DNA. Exponentially growing target cells (2 × 10<sup>6</sup>) were labeled in 1.0 ml of RPMI 1640 medium [5% (vol/vol) heat-inactivated fetal calf serum] containing 50 μCi (1 Ci = 37 GBq) of [125I]-labeled deoxyuridine (Amersham) for 3 h at 37°C. Cells were washed twice and resuspended at 5 × 10<sup>6</sup> cells/ml. Aliquots of target cell suspension (0.1 ml) were added to polypropylene microcentrifuge tubes (Eppendorf) containing 0.1 ml of effector cell suspension or LT dilution and incubated at 37°C for 2 hr or 24 hr, respectively. Target cells were lysed by adding 50 μl of a 1% Triton X-100 in phosphate-buffered saline, 10 mM EDTA, pH 7.4 (final concentration of 2 mM EDTA, 0.2% Triton X-100) and the tubes were microfuged 10 sec at 13,000 × g to pellet cell debris. Supernatant (0.2 ml) was transferred to a new polypropylene tube and extracted with phenol, and DNA was precipitated by mixing the cells per supernatant (0.2 ml) with 0.4 ml of absolute ethanol, 0.014 ml of 1.0 M MgCl<sub>2</sub>, and 0.014 ml of 3.0 M sodium acetate, pH 5.0 and chilling at −70°C for 3 hr. DNA was pelleted by centrifugation at 13,000 × g for 10 min and dried. DNA was then electrophoresed on 0.9% agarose slabs. Gels were fixed in 7% (wt/vol) trichloroacetic acid (three changes), washed briefly, and dried onto 3 mm chromatography paper (Whatman). Autoradiographs were prepared using XAR-5 x-ray film (Kodak) with intensifying screens (Cronex).

RESULTS

Target-Cell DNA Release Is Mediated by PCI Line 55 Cells but not by Antibody Plus Complement. While mixed lymphocyte reaction cells have been reported by two other groups (2–6) to mediate the release of target cell DNA, more precisely defined populations of CTLs have not been tested for this effect. PCI line 55 is a TNP-specific, Lyt-2' CTL line, as evidenced by its ability to mediate 51Cr release from targets in an antigen-specific, class I major histocompatibility complex (MHC)-restricted fashion (Fig. 1A). In addition, this cell line secretes LT when appropriately stimulated with antigen or mitogen. PCI line 55 also mediates DNA release with the same characteristic specificity (Fig. 1B). Peak levels of DNA release were obtained after only 2 hr of incubation, thus adding support to the contention that the CTL lethal hit leads to an early onset release of target cell DNA.

To assess the potential for antibody-initiated complement lesions to effect target cell DNA release, 51Cr-labeled or [3H]deoxythymidine-labeled BWS517 cells were incubated at 37°C for 2 hr with the indicated dilutions of anti-K<sup>k</sup> antibody ([A.B4 × B10.AQR]F<sub>1</sub> anti-A/Sn). The data indicate (Fig. 1C) that antibody plus complement causes the release of high levels of 51Cr from target cells more rapidly than do CTLs. In contrast, no appreciable DNA release is evident at 2 hr as measured by scintillation counting of [3H]DNA (Fig. 1D). This confirms previous observations that killing mediated by CTLs can be clearly distinguished from killing mediated by antibody plus complement on the basis of this criterion.

DNA Release Mediated by T-Helper Cell Clone 5.9.24. 5.9.24 is a BALB/c-derived, Lyt-1+, ovalbumin-specific, class II MHC-restricted T-helper cell clone with cytolytic activity (24). It produces LT when stimulated by either mitogen or antigen coupled to syngeneic I<sup>a</sup> cells, or antigen presented by syngeneic spleen cells (25). To determine whether clone 5.9.24 cells could mediate target cell DNA

![Fig. 1.](image-url) 51Cr release and [3H]deoxythymidine release from targets mediated by PCI 55 cells and by antibody plus complement. (A, B) PCI 55 (anti-TNP/H-2<sup>b</sup>) effector cells. (C) BWS517-TNP (H-2<sup>b</sup>). (D) A20-TNP (H-2<sup>b</sup>). All [3H]release assays were conducted for 2 hr at 37°C. (C, D) (A) % 51Cr release from BWS517 targets. (C) % [3H]deoxythymidine release from BWS517 targets. All 51Cr assays were conducted for 4 hr at 37°C. 51Cr tests were performed in quadruplicate. Standard errors for 51Cr test values were always less than 2.0. (A.B4 × B10.AQR)F<sub>1</sub> anti-A/Sn antibody (Ab) was used in these experiments. Rabbit low-toxicity mouse complement was added to test wells at a final dilution of 1:20, and wells were incubated for 2 hr at 37°C.
release, the cells were incubated at various ratios for a 2-hr period at 37°C with ovalbumin-pulsed A20 B-lymphoma cells. The results of an experiment in which an effector to target ratio of 5:1 was used are presented in Fig. 2 along with 6 hr $^5$Cr release assay results. The amount of killing was dose dependent over a range of effector to target cells (1:1 to 10:1; data not shown). The T-helper cells caused considerable release of DNA from the A20-ovalbumin target. The ability to mediate this phenomenon was clearly restricted to target cells that expressed ovalbumin and Ia antigen of the H-2d haplotype. These same restrictions are observed in the mediation of $^5$Cr release from targets. These results, therefore, demonstrate that a T-helper cell clone is capable of performing a function that has been generally assigned to class I MHC-restricted CTLs.

Comparison of the Time Course of Target Cell DNA Release Mediated by PCI Line 55 and Clone 5.9.24. The rates at which DNA release proceeds in target cells lethally hit by PCI line 55 and helper clone 5.9.24 were considered. DNA release was first detectable at 30 min, regardless of whether the lethal hit was delivered by PCI line 55 or clone 5.9.24 (Table 1). The amount of $[^3]$Hdeoxythymidine released resulting from the hit varied in general with the ratio of effectors to targets. The amount of early detectable DNA release also varied between target cells attacked by PCI 55 and 5.9.24, such that higher levels of release were always obtained from PCI 55 stricken targets. Despite this difference, the two populations appear to exhibit equivalent lethal hit kinetics.

Cell-Free LT Containing Supernatant Causes DNA Release. Both PCI 55 cells and clone 5.9.24 cells secrete the soluble factor LT under appropriate antigen stimulation. To determine whether such a cytotoxic factor was responsible for the DNA fragmentation, LT-containing supernatant was used that had been generated from C57BL/6-derived, ovalbumin-specific, Lyt-1+ helper T-cell clone, 153-E6. Dilutions of LT-containing supernatant were incubated with $[^3]$Hdeoxythymidine-labeled BW5147 targets to assess the potential of LT to mediate the release of target cell DNA (Table 2). The supernatant was able to cause substantial target cell DNA release over an extended time course that was first detectable after 18 hr of coculture. The amount of LT-induced DNA release at 24 hr ranged from 6.2 to 55.9%, depending on the quantity of LT to which target cells were exposed.

Analysis of DNA Released from Targets Exposed to PCI 55 and Cell-Free LT-Containing Supernatant. The target cells were labeled for 3 hr with $[^3]$Hdeoxyuridine, washed, and incubated with varying amounts of LT supernatant or PCI 55 effectors for 24 hr or 2 hr, respectively, and the DNA was analyzed by agarose gel electrophoresis. Targets attacked by either TNP-specific CTLs or LT-containing fluids generated fragments of DNA that separated in a laddered pattern, with a minimum size of roughly 200 base pairs. The results of two separate experiments are presented in Fig. 3. As can be seen in the autoradiographs, both PCI 55 and LT were able to fragment target cell DNA, and both the cellular and the cell-free mediators generated similar patterns of fragmentation, with maximal sizes of greater than 23 kilobases and a definite cutoff at approximately 200 base pairs. DNA from target cells that had been attacked by LT was present predominantly as fragments at the lower limit of the defined molecular weight range. In most cases, no DNA was released from the nuclei of $[^3]$Hdeoxyuridine-labeled targets that had been incubated with medium alone, nor with antibody plus complement. When labeled DNA was obtained, as in lane F (Fig. 3), it was unfragmented, high molecular weight DNA. The DNA released from ovalbumin-pulsed cells attacked by 5.9.24 also demonstrated the same range of size and the same distinctive laddering pattern (data not shown). The data, therefore, serve to strengthen the hypothesis that LT is involved in cell-mediated lethal hit mechanisms.

![Fig. 2. $^5$Cr release and $[^3]$Hdeoxythymidine ($^3$H) release from target cells mediated by T-helper cell clone 5.9.24. $^5$Cr assays were conducted for 6 hr at 37°C. DNA fragmentation assays were conducted for 2 hr at 37°C. The effector to target ratio was 5:1.](image)

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Time course of target cell DNA release mediated by cell-free LT-containing supernatant. Target cells were BW5147 cells labeled with 4.0 µCi of $[^3]$Hdeoxythymidine/ml for 18 hr at 37°C. Results shown are taken from two separate experiments. All tests were performed in quadruplicate. Standard errors for test values were all under 2.0. One unit of LT cytotoxic activity is defined as that which inhibits 50% of maximal growth of L929 target cells in 72 hr.
**DISCUSSION**

Published research by two groups (2–6) has demonstrated that mixed-lymphocyte reaction cells mediate the fragmentation of target cell DNA in a rapid time course. The present study extends these findings to a TNP-specific cytotoxic T-cell line, a helper T-cell clone with cytolytic activity, and cell-free, helper-T cell-generated LT-containing supernatant. Fragmentation was evidenced both by the releaseable [*H]thymidine counts from target cells, and by agarose gel electrophoresis of isolated target cell DNA that had been labeled with 25-labeled deoxyuridine. Target cells lysed by antibody plus complement do not release DNA, indicating that the mechanism of killing employed by CTL, cytolytic helpers, and LT is distinct from that of complement-mediated lysis. Russell et al. (4) have shown that 125I-labeled deoxyuridine-labeled DNA from target cells lysed with antibody plus complement consists entirely of large molecular weight DNA.

The kinetics of target cell DNA fragmentation mediated by the CTL line PCI 55 was virtually indistinguishable from that mediated by T-helper clone 5.9.24, and thereby reinforces the hypothesis that the two cells kill by a similar or identical mechanism. Although variations in the intensity of fragmentation, particularly at the early time points, were observed, the relatively lower amount of early onset target cell DNA fragmentation observed following attack by 5.9.24 may simply reflect a difference in target cell susceptibility.

Since crude supernatant was used in these experiments, it is not possible to rule out the involvement of lymphokines other than LT in the phenomenon reported here. Because the supernatant was generated from mitogen-stimulated helper T-cell clones, tumor necrosis factor and cytolyisin, produced by macrophages (26) and natural killer cells (9), respectively, are probably absent from our preparations. The two most likely of the described lymphokines to be involved are interferon-γ (IFN-γ) and LT, both of which are present in clone 153-E6 supernatant, as determined by functional assays and in the case of LT by RNA blot hybridization analysis with an LT DNA probe (N.H.R., K. M. McGrath, C. B. Li, and P. Gray, unpublished results). Other results in our laboratory show that recombinant IFN-γ fails to cause DNA release and that IFN-γ does not enhance the ability of LT-containing supernatant to fragment DNA. In addition, previously reported studies from our laboratory have shown that LT inhibits proliferation of all of the target cells used in these experiments more effectively than does recombinant-derived IFN-γ (21), and in fact IFN-γ was completely ineffective against L929, BW5147, and P815. We have recently shown that recombinant-derived tumor necrosis factor, which is absent from our LT preparations but has common functional characteristics, can also cause DNA release from target cells (D.S.S., K. M. McGrath, and N.H.R., unpublished results). On the basis of those studies we favor the involvement in these experiments of LT in the fragmentation of target cell DNA.

Differences in the time course of killing probably reflect differences in the mode of toxin delivery. Despite the longer time course required by cell-free LT-containing supernatant to bring about target cell DNA fragmentation, there are similarities to the cell-mediated hit that suggest a common mechanism. For example, DNA fragmentation in target cells stricken by PCI 55 or 5.9.24 precedes by hours the release of 51Cr label, thus suggesting that the target cell destruction begins internally. We have shown that the vast majority of cells exposed to lymphotoxin continue to exclude the vital dye trypan blue at a point in time (24 hr, data not shown) when considerable DNA fragmentation has occurred. By comparison, cells killed by antibody plus complement failed to yield measurable DNA fragments as long as 22 hr after the majority of target cells stained positively with trypan blue. Insensitivity of nuclei to Triton X-100 in normal or antibody-plus-complement-treated targets was observed by Russell et al. (4) and was attributed to the relatively higher density of protein on the nuclear membrane. This observation also explains the frequent absence of unfragmented large molecular weight DNA in the medium controls for the agarose gel electrophoresis experiments.

Another feature common to the cytolytic cell and LT-mediated hit is the cleavage of target cell DNA into discretely sized pieces that are multiples of approximately 200 base pairs. The pattern of fragmentation in LT-attacked target cells was somewhat different than that resulting from targets attacked by CTL, the fragment size of the former being restricted to the lower molecular weight range. This effect is probably attributable to the longer period of time for which LT-treated targets are exposed. Whether or not DNA fragmentation results from a direct attack by LT or represents a secondary effect of the attack is not clear from these studies. The primary event may occur as membrane damage, which manifests itself first at the nuclear membrane, resulting in the exposure of unprotected DNA to an endonuclease within the cell. Russell et al. (4) have presented micrographs that indicate an early onset breakdown of nuclear membrane integrity. A second possibility is that LT serves to activate an endonuclease within the target cell. The third least likely possibility is that LT itself is an endonuclease. Preliminary evidence in our laboratory suggests that this is not the case.

We reported (22) that a population of T lymphocytes that display a surface phenotype generally ascribed to CTL can secrete lymphokines when appropriately stimulated by antigen or mitogen. In addition, T-cell clones that have been shown to behave as helper T lymphocytes (24) and that display the surface phenotype characteristic of helper cells...
are able to directly mediate antigen-specific, MHC-restricted cytotoxicity in a short term assay. The Lyt-1+ cells tested in these experiments are restricted to class II MHC antigens, and the Lyt-2+ cells are restricted to class I elements, as has been commonly described (19, 27). The observations reported here imply that a certain amount of flexibility with respect to function resides in the T-lymphocyte population, as has been suggested (24). The results furthermore suggest that the mechanism of killing employed by CTL, cytolytic helper T lymphocytes, and cell-free cytolytic factors is similar, if not identical, and that apparent differences between these three killing phenomena may be the result of differences in the nature of the initial T-cell-target-cell interaction and the mode of toxin delivery.

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