Lack of lymphocyte-induced DNA fragmentation in human targets during lysis represents a species-specific difference between human and murine cells

cytotoxic T lymphocytes/natural killer/killer lymphocytes/antibody-dependent cellular cytotoxicity/lytic mechanism

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ABSTRACT Significant cytotoxic lymphocyte-induced DNA fragmentation does not occur in four human target cells lysed by human natural killer lymphocytes, killer lymphocytes, or cytotoxic T lymphocytes. These results contrast with the extensive DNA fragmentation that occurs in murine target cells lysed by the analogous effector lymphocytes. Thus, DNA fragmentation, or the lack thereof, represents a species-specific difference between human and murine cells responding to lysis by cytotoxic lymphocytes. The DNase activity observed in murine cells is probably internally activated rather than delivered by the cytotoxic effector cells, since human killer lymphocytes selectively caused DNA fragmentation in murine but not in human target cells lysed by antibody-dependent cellular cytotoxicity.

The mechanism by which cytotoxic lymphocytes lyse their target cells has not yet been fully defined. However, studies of lymphocyte-mediated cytolysis have established several biochemical requirements for lytic processes to occur (1-3) and they have also pointed to several interesting cellular phenomena (4) that potentially play important, if not crucial, roles in such processes. Two of the most intriguing phenomena observed in the context of lymphocyte-induced cytolysis involve the nuclei of murine target cells. Immediately after a murine cytotoxic T lymphocyte (CTL) conjugates with a murine target cell, the nuclear chromatin of the target undergoes gross morphological changes (5). Coinciding in time with this event is the initiation of a rapid DNA fragmentation process in the target cell nucleus that ultimately results in complete breakdown of the DNA into low molecular weight pieces (6-8). The resulting DNA fragments yield a ladder-like appearance when fractionated by gel electrophoresis (7). Because DNA fragmentation is not observed when the same target cells are lysed by other means, such as antibody/complement or freeze-thawing (7, 8), a nuclease has been implicated as a specific part of the lytic mechanism of CTL (7). Whether the nuclease activity observed in the target cell is activated internally or delivered by the lymphocyte effector is not resolved.

Lymphocyte-induced DNA fragmentation processes in the target cells of cytotoxic lymphocytes, other than murine CTL, have recently been described in studies from this laboratory (9). Murine natural killer (NK) lymphocytes were shown to cause DNA fragmentation in murine YAC-1 target cells and human killer (K) lymphocytes were also shown to cause DNA fragmentation in antibody-sensitized murine target cells during lysis by antibody-dependent cellular cytotoxicity (ADCC). Surprisingly, preliminary data presented in the same study indicated that DNA fragmentation does not occur significantly in human tumor cell targets of human CTL and human NK/K lymphocytes. However, DNA fragmentation could have occurred in the two human cells tested in that study but have gone undetected because of the indirect nature of the assay used for detecting low molecular weight DNA. Analysis of total human target cell DNA by agarose gel electrophoresis in the present study directly confirms the lack of significant DNA fragmentation in human target cells undergoing lymphocyte-mediated cytolysis. Thus, the data of the present study challenge the premise that DNA fragmentation is a necessary or even an important aspect of lymphocyte-induced lytic processes.

MATERIALS AND METHODS

Murine and Human Target Cells. All cells were maintained in complete medium (RPMI 1640 supplemented with penicillin, streptomycin, Heps, sodium carbonate, glutamine, glutathione, and 10% fetal calf serum) in a humidified 37°C incubator under 5% CO2/95% air. The murine Pa13 line is a C57BL/6 (H-2^b)-derived "co-helper" lymphocyte clone (10); the murine C14 line is an Abelson murine leukemia virus-transformed murine "null" cell line obtained from Sloan-Kettering (New York). Both the human NK-sensitive (11) K562 erythromyeloid cell line (12) and the human NK-insensitive (9) 8866 B-lymphoblastoid cell line (13) were derived from myelocytic leukemias; the fibroblastic U-1 cell line, derived from human amnion cells, was provided by C. Samuel (University of California, Santa Barbara); the GM 2679 B-lymphoblastoid cell line was derived from *in vitro* infection of human peripheral blood leukocytes with Epstein-Barr virus (14).

Antibodies. Rabbit anti-mouse T-lymphoma antiserum was prepared in this laboratory as described (15). Rabbit anti-human β2-microglobulin (β2m) antiserum was purchased from Olac (Bicester, United Kingdom). The 9H.1 monoclonal antibody (IgG2a) against human β2m was the gift of M. Longnecker (University of Alberta).

Preparation of NK/K Effector Lymphocytes and CTL. Human peripheral blood leukocytes (PBL) from healthy donors were isolated from heparinized blood by centrifugation over Ficoll-Hypaque. Adherent leukocytes were removed by two 1-hr incubations at 37°C as described (15). The remaining nonadherent PBL were used directly as effectors for NK or K cell assays. Total Ficoll-Hypaque purified leukocytes (2 × 10^6) were stimulated with x-irradiated (2500 R; 1 R = 0.258 mC/kg) 8866 cells (2 × 10^6) for 7 days to generate CTL as described (9).

Radiolabeling of Target Cells. For 51Cr release assays, 0.5-1.0 × 10^7 target cells in 200 μl of complete medium were

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; NK, natural killer; K, killer; CTL, cytotoxic T lymphocytes(s); FR, full release; SR, spontaneous release; ER, experimentally released.

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incubated with 100 μCi (1 Ci = 37 GBq) of 51Cr for 1 hr at 37°C and then washed four times. For the DNA fragmentation assays, target cells (1 × 10^6/ml) were incubated with [3H]thymidine at 5–20 μCi/ml for 6–12 hr at 37°C in complete medium, washed three times, allowed to rest for 1–2 hr at 37°C, and finally washed one more time before use. For DNA analysis by agarose gel electrophoresis, target cells were labeled with 125I-deoxyuridine exactly as they were labeled with [3H]thymidine.

**Cytoxicity Assays.** K, NK, and CTL assays were all performed similarly. The appropriate effectors at 2 × 10^5/ml were serially diluted for different effector cell concentrations in 0.5 ml of complete medium in 15-ml plastic conical centrifuge tubes (Falcon). Diluted effectors were added to 0.5 ml of 51Cr-labeled or [3H]thymidine-labeled target cells at 2 × 10^5/ml; in the case of the ADCC assays, targets were incubated for 15 min at 37°C with the appropriate antibody before the addition of effector. After various time intervals (up to 4 hr), the assay mixtures were harvested and processed as follows.

Specific 51Cr release was determined by centrifuging the assay tubes, removing 500 μl of supernatant from each mixture, and finding the cpm experimentally released (ER) into the supernatant by liquid scintillation counting. Full release (FR) controls represent the cpm in supernatants of tubes containing 0.5% Nonidet P-40 in place of effectors. Spontaneous release (SR) controls represent the cpm in supernatants from tubes containing complete medium substituted for the effector cells. SR values were always less than 10% of FR values. The percent specific 51Cr release was calculated as follows: (ER – SR)/(FR – SR) × 100.

Specific DNA fragmentation resulting from lysis was determined as described by Duke et al. (7). Briefly, effector and target cells, or target cells alone, were pelleted in the conical assay tubes, the supernatant was transferred to a scintillation vial, and 1.0 ml of hypotonic 25 mM NaOAc buffer (pH 6.5) was added to each pellet to lyse the cells. The lysate was then centrifuged at 11,000 × g for 20 min to pellet cell fragments and high molecular weight DNA. The supernatants were combined with the corresponding supernatants above and the cpm in soluble [3H]- or 125I-labeled thymidine-labeled DNA were measured by liquid scintillation counting. A formula similar to that above was used for determining percent specific DNA fragmentation, with the FR value determined by total cpm in 1 × 10^5 target cells.

**Agarose Gel Electrophoresis of DNA.** 125I-labeled deoxyuridine-labeled DNA from C14 cells lysed by human K-cell-mediated ADCC and from 8866 cells lysed by human CTL was prepared as follows: effector/target assay mixtures (4 × 10^5 effectors/2 × 10^5 targets) were centrifuged after various incubation periods. The pellets were then suspended in 0.5 ml of 25 mM NaOAc buffer (pH 6.5) to lyse cells, and the DNA was extracted three times with an equal volume of Tris-buffered phenol (pH 7.5), then twice with chloroform/isoamyl alcohol (24:1). The DNA was then concentrated with 1-butanol and extraction with ether twice. After addition of 10× sample buffer (0.1% bromphenol blue/50% glycerol) and heating at 65°C for 10 min, the samples were electrophoresed on a 0.75% agarose gel overnight at 20 V. The gel was dried down onto filter paper and exposed to Kodak XRP film at −70°C in the presence of a DuPont intensifying screen.

**RESULTS**

A DNA fragmentation analysis is shown in Fig. 1 for four human cell types (8866, GM 2679, K562, and U) and one murine cell type (PA13) lysed by either human NK/K lymphocytes, human CTL, antibody plus complement, or temperature extremes. Cell lysis was monitored as radioactivity released from 51Cr-labeled targets after a 3- to 4-hr incubation at 37°C with cytotoxic lymphocytes or after a 2-hr incubation at 37°C following lymphocyte-induced lysis. The DNA fragmentation accompanying lysis was monitored simultaneously as radioactivity released into the supernatant by an 11,000 × g centrifugation of NaOAc-treated [3H]thymidine-labeled target cells. Only murine target cells showed a significant level of DNA fragmentation (>95%) and only when lysed (96% specific 51Cr release) by human K lymphocytes mediating ADCC. Lysis of the PA13 cells by lymphocyte-independent mechanisms resulted in <10% specific fragmentation and >80% specific 51Cr release in all cases. The maximum level of lymphocyte-induced DNA fragmentation for any of the human cells lysed by cytotoxic lymphocytes was low by comparison with murine target cells. For example, at 55% 51Cr release from human GM 2679 cells lysed by ADCC, there was a corresponding DNA fragmenta-

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**Fig. 1.** Fragmentation of target cell DNA as a function of cell lysis. hu, Human; mu, murine; Ab+C, antibody plus complement. DNA fragmentation and 51Cr release were determined at 2 hr for nonlymphocyte-mediated killing and at 4 hr for lymphocyte-mediated killing. Effector/target ratios were 50:1 in all experiments with lymphocyte effectors. Minimum FR value: [3H], 34,000 cpm; [51Cr], 20,000 cpm. SR values were <90% of FR values in all cases. Data shown represent average duplicate samples ± 3% or less for 51Cr release values and ± 10% or less for DNA fragmentation values.
tion amounting to only 14%. The three other human cell lines, lysed by the same or different lymphocyte mechanisms, showed even less specific DNA fragmentation at comparable or greater levels of \(^{31}\text{Cr}\) release. These data not only confirm our preliminary observations with K562 and 8866 cells (9) but also extend these observations to include the additional human cell types, U and GM 2679. Thus, significant lymphocyte-induced DNA fragmentation is lacking in at least four human cell types, suggesting that human cells differ in this regard from murine cells.

Potentially, significant DNA fragmentation might occur in human cells but at slower rates than observed for murine cells. To assess this possibility, a kinetic comparison was made between lysis \(^{31}\text{Cr}\) release and DNA fragmentation in murine (Pa13) and human (8866) target cells reacting with the same type of cytotoxic effector cell population. As illustrated in Fig. 2A, lysis of murine target cells by ADCC with human K cells resulted in nearly complete (>80%) fragmentation of the target cell genome after incubation times of up to 4 hr. However, in the same experiment, human targets (Fig. 2B) showed virtually no DNA fragmentation (<5%) when allowed to react with the same effector type even though the specific \(^{31}\text{Cr}\) release was identical with that observed with murine targets. The same differences between human and murine cells were also observed after nearly complete lysis by NK or CTL effectors (ref. 9; data not shown).

One possibility that these experiments do not completely rule out is that DNA fragmentation occurring in human cells may be largely undetectable by the indirect assay method used in the experiments thus far. Much larger-sized less-soluble DNA fragments might arise in human as compared with murine nuclei, for example, and these might not be efficiently released from human nuclei under the conditions used here for detection. Therefore, \(^{125}\text{I}\)-labeled thymidine-labeled DNA from human and murine target cells lysed by cytotoxic lymphocyte effectors was directly analyzed and compared by agarose gel electrophoresis. An autoradiogram of one such gel is shown in Fig. 3. In the left two lanes of this gel, the electrophoretically fractionated DNA isolated from fully viable murine C14 cells (lane 4) is compared with that from another sample of these cells after a 1-hr incubation with human K cells, resulting in 18% \(^{31}\text{Cr}\) release (lane 3). Although lysis of these cells was far from complete, DNA fragmentation is still evident in the lower two-thirds of lane 3 but not lane 4. After longer incubation periods with effectors, most of the C14 target cell DNA is converted to lower molecular weight fragments (ref. 9; data not shown). The relatively incomplete DNA fragmentation pattern shown in Fig. 3 is indicative of the sensitivity of this method for detecting low levels of DNA fragmentation when cell lysis is relatively incomplete or when the duration of the assay is relatively short. However, human 8866 target cells still showed no evidence of minimal DNA fragmentation, even when lysed to a 3-fold greater extent than C14 cells and after a 2- to 3-fold longer assay period, thus confirming the results of Figs. 1 and 2.

**DISCUSSION**

Rapid and extensive DNA fragmentation has been observed in murine cells lysed by either murine CTL (6-9), murine NK lymphocytes, or human K lymphocyte-mediated ADCC (9). As shown previously (6-9) and as shown by the DNA gel pattern shown in Fig. 3 (lane 3), fragments of low molecular weight DNA begin appearing soon after contact is made between lymphocyte effector cells and murine target cells. Complete lysis is accompanied by extensive fragmentation of the target cell DNA into low molecular weight pieces that sharply resolve into a ladder-like banding pattern on agarose gels, as demonstrated previously (7, 9). The DNA fragmentation process observed in murine cells is specifically lymphocyte-mediated since other means of cell lysis—antibody plus complement, freeze-thawing, or heating—do not lead to appreciable DNA fragmentation (Fig. 1 and refs. 7 and 8).
Human cells sharply contrast with murine cells with respect to cytotoxic lymphocyte-induced DNA fragmentation, as shown in the present study. Namely, there is a pronounced lack of DNA fragmentation in human target cells during their lysis by either NK/K lymphocytes or CTL. Only a small fraction of low molecular weight DNA appeared during the lysis of two human cell types (GM 2679 and U; Fig. 1) and virtually no low molecular weight DNA appeared during lysis of two other human cell types (8866 and K562; Figs. 1–3). These results were valid for cytolysis mediated by either human NK/K lymphocytes or human CTL. A direct analysis of the DNA from 8866 target cells by agarose gel electrophoresis confirmed that extensive lymphocyte-induced DNA fragmentation does not occur in these cells (Fig. 3). It is concluded that extensive DNA fragmentation is not a general requirement for lymphocyte-mediated cytolysis, at least in the case of human cells. Thus, the importance of DNA fragmentation for murine target cell lysis is brought into question.

The data presented here support the hypothesis that cytotoxic lymphocyte-induced DNA fragmentation in murine cells is a secondary rather than a primary cytolytic event. The same cytotoxic effector cell population, i.e., human K cells, was shown to cause significant and rapid DNA fragmentation in murine but not in human target cells (Figs. 1–3) even though these targets exhibited nearly identical kinetics of 31Cr release (Fig. 2A and B). Thus, the cytolytic process induced by a single type of effector lymphocyte is "uncoupled" in these experiments from the DNA fragmentation process and, further, the occurrence of DNA fragmentation depends on the species of the target cell, at least in the case of lymphocytes, since most of the target cells examined in this study were of lymphocyte lineage. In the simplest interpretation of these experiments, lymphocyte-induced DNA fragmentation in murine targets is a secondary effect of the lytic mechanism(s) of cytotoxic lymphocytes, and the nuclease activity observed in murine cells is internally activated by the lytic process rather than delivered by the effector cell.

In more complicated interpretations of these data, an effector cell-delivered nuclease is inactive for some reason in human but not in murine target cells, or fundamentally different lymphocyte-induced lytic pathways in human and murine target cells account for their lysis. However, the simpler interpretation above is probably correct because the basic lytic processes induced in target cells by all cytotoxic lymphocytes have similar biochemical requirements and kinetic parameters (1–4). The similarity between these lymphocyte-induced lytic processes is also evident in the present study by the fact that DNA fragmentation was consistently observed in murine but not in human target cells regardless of the type or species of the cytolytic effector cell. In other words, effector lymphocytes of any type predictably had the same effect on the DNA in murine or human target cells. Thus, it appears that fundamentally different lytic mechanisms do not account for the striking species-specific difference in DNA fragmentation observed here between human and murine target cells.

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