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DISTINCT PATHWAYS OF CD4 AND CD8 CELLS INDUCE RAPID TARGET DNA FRAGMENTATION[1]

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When activated with either Con A, a CD3-specific mAb, or Ag-pulsed B lymphoma (LK35.2) cells, CD4 (Th1) clones quickly induce DNA fragmentation in target cells followed by release of ^{51}Cr-labeled intracellular materials. Both activated CD4 clones and CD8 (CTL) cells fragment target DNA into electrophoretically identical "ladder" pattern made of ~200 bp. The effect of various metabolic inhibitors on the ability of CD4 and CD8 cells to induce target DNA fragmentation was studied. Little effect was observed with the DNA synthesis inhibitor, mitomycin C. The RNA synthesis inhibitor, actinomycin D, and the protein synthesis inhibitor, cycloheximide, strongly inhibited the ability of CD4 cells, but not CD8 cells, to induce target DNA fragmentation. In contrast, target DNA fragmentation by CD8 cells, but not by CD4 cells, was inhibited by cholera toxin. Although cyclosporin A inhibited CD4 cells to fragment target DNA during the early phase (90 min) of E:T interaction, this inhibition was not sustained in the later phase (210 min) of the assay. Zinc ions inhibited the ability of both CD4 and CD8 cells to fragment target DNA. Treatment of effectors and targets with these inhibitors, followed by washings, demonstrated that the action of these inhibitors on effector cells alone is sufficient to inhibit target DNA fragmentation. The strong correlation among these parameters of DNA fragmentation and Cr-release assays supports the hypothesis of programed cell death. Although distinct cytolytic pathways are used by CD4 and CD8 cells to kill targets, both pathways deliver a signal that activates endonuclease(s), fragments target DNA, causes Cr-release, and lyses target cells. Taken together with our previous studies, the present findings demonstrate that activated cytolytic CD4 clones do not use perforin, serine proteases, and TNF as mediators for resistant target DNA fragmentation.

It is now well established that both CD4 and CD8 cells express cytolytic activity when activated by target cells bearing the appropriate Ag (1-8). Both types of T cells can express a TNF-mediated and a TNF-independent cytolytic activities (9, 10). Using ^{51}Cr-release assays, we have shown that different intracellular pathways are used by CD4 and CD8 cells for the expression of the TNF-independent cytolytic activity. It was found that CD4 cells, but not CD8 cells, require de novo synthesis of RNA and proteins for the expression of cytolytic activity. Thus, AcD[3] and Chx inhibited the cytolytic activity of CD4, but not CD8 cells (9, 10). In contrast, ChT blocks the cytolytic activity of CD8, but not CD4 cell. Both CD4 and CD8 cells do not require the synthesis of lymphokines (IL-2, TNF-α, TNF-β, and IFN-γ) for the expression of cytolytic activity toward the TNF-resistant targets (9, 10). Moreover, both resting and activated CD4 cells do not synthesize perforin mRNA and therefore, do not use perforin as a cytolytic mediator to kill target cells (10, 11). Highly concentrated (100-fold) supernatants and cell lysates (30-fold) from activated CD4 clones could not induce detectable target lysis (S.-T. Ju, unpublished observation). In the absence of demonstrable cytolytic mediators for TNF-resistant targets, we focused on the alternative hypothesis that a cytolytic signal is delivered by activated CD4 cells upon contact with target cells. This signal induces programmed cell death of targets as proposed for CD8 cells by Russell (12).

In the present study, we show that both CD4 (Th1) and CD8 (CTL) cells quickly induce target DNA fragmentation. A "ladder" electrophoretic pattern of fragmented target DNA, consisted of ~200 bp units, was generated by CD4 clones and was found identical to that induced by CD8 (CTL) cells. In addition, the different cytolytic pathways of CD4 and CD8 cells used to induce target ^{51}Cr-release are the same pathways leading to target DNA fragmentation as judged by their distinct sensitivity to specific inhibitors. Our study strongly suggests that an emerged common mechanism for target cell destruction, possibly by activating endonuclease for DNA fragmentation, is brought about by different cytolytic pathways of CD4 and CD8 cells.

MATERIALS AND METHODS

Animals. All mice were purchased from The Jackson Laboratories, Bar Harbor, ME. They were maintained with acidified tap water and Purina Chow (Ralston Purina, St. Louis, MO).

Derivation and maintenance of T cells. The derivation of clones F3 (specific to Glu, Lys, Tyr random polymer; I-E^d restricted), D3 (KLH specific; I-E^d restricted), E6 (KLH specific; I-E^d restricted), and C7 (KLH specific; I-E^d restricted) have been described (9, 10). These clones express CD4$^+$,CD8$^-$ phenotype and secrete lymphokines of TH1 type, i.e., IL-2 and IFN-γ. They were maintained in 24-well plates (Costar 2424, Cambridge, MA) by periodic stimulation (every 14 to 28 days) of 0.25 × 10^6 resting clone cells with the appropriate Ag (25 to 40 μg/ml) and 4 × 10^6 irradiated (3000 rad) BALB/c spleen cells, and expanded with IL-2 containing Con A supernatant. Clone

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[3] Abbreviations used in this paper: AcD, actinomycin D; ChT, cholera toxin; Chx, cycloheximide; CsA, cyclosporin A; Mit C, mitomycin C.

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cells were used 12 to 28 days later. Viable cells of all clones were purified over a Ficoll-Hypaque gradient and washed three times with culture medium before use. H-2K-specific CD8 cells were generated by immunizing BALB/c mice i.p., with 8×10^6 BW5147 (abbreviated as BW) thymoma cells. Two wk later, their spleen cells (5×10^6/ml) were cultured with an equal number of irradiated (3000 rad) CBA/J spleen cells for 9 days. Viable cells were purified over a Ficoll-Hypaque gradient, washed three times, and incubated with GK1.5 hybridoma supernatant containing CD4-mAb (13) at 37°C for 30 min. Afterward, cells were washed once and rabbit Complement (Low Tox, Cedarlane; sevenfold diluted) was added and incubated at 37°C for 30 min. Viable cells were purified over a Ficoll-Hypaque gradient, washed three times, and used as anti-H-2K CTL.

Radiolabeling of target cells. A number of targets were used in this study. They are P815 mastocytoma (FcR^+, American Type Culture Collection (ATCC), Rockville, MD), LK35.2 B lymphoma hybridoma (abbreviated as LK; FcR^+, ATCC), BW-5147 thymoma (ATCC), SP2/0 myeloma (ATCC), and TA-3 B lymphoma hybridoma (14). Target cells (0.75×10^6/ml) were labeled with ^3H-thymidine (50 μCi/ml, ICN K&K Laboratories, Inc., Plainview, NY) for 18 h in a 24-well plate. For Ag-dependent assays, appropriate Ag (1 mg/ml) were included for the preparation of antigen-pulsed LK target. These target cells were washed once, resuspended in 10 ml of medium, and incubated at 37°C for 1 h to remove residual ^3H-thymidine. For CD3-mAb- or Con A-dependent assays, washed target cells were diluted with 145-2C11 supernatant containing CD3-mAb (15), or Con A (6.6 μg/ml) and cultured for 20 min before use.

Pelleted target cells were labeled with ^{51}Cr by the addition of radioactive sodium chromate (NEN, Boston, MA), shaken every 10 min for 40 min and then washed three times. Labeled cells were diluted with 145-2C11 supernatant and Con A. The mixtures were cultured for 20 min to prepare CD3-mAb- and Con A-pulsed target cells, respectively.

Determination of cytolytic activity. DNA fragmentation assay was carried out as described by Ostergaard and Clark (16). Various number of effector cells and 1.5×10^4 of target cells were added to 12 × 75 mm polystyrene culture tubes (60818-565 VWR, CA). Cytolytic activity was induced with either CD3 mAb, Con A, or Ag-pulsed LK cells. The total volume of each assay was 0.2 ml. Before incubation in a 37°C, 10% CO_2 incubator, assay mixtures were mildly centrifuged to establish cell contact. At the end of incubation, 1 ml of cold PBS containing 0.2% Triton-X 100 (lysis buffer) was added and the whole content was transferred to a microfuge tube. After centrifugation for 10 min, 0.6 ml of supernatant was carefully removed and radioactivity was determined with a Packard scintillation β-counter (Packard Instrument Co., Downers Grove, IL). Background DNA fragmentation, as determined by culturing target cells in the absence of effector cells, activation agents or inhibitors, was found to be 2 to 15%. Under the conditions used in this study, none of the activating agents and metabolic inhibitors caused a significant increase of nonspecific release. Total fragmentable DNA was defined by the radioactivity released after a 2-h incubation with 1 ml of lysis buffer containing 2 μg/ml pancreatic DNase I (Sigma Chemical Co., St. Louis, MO). The results were expressed as percent specific DNA fragmentation, which were calculated by the formula: (cpm of experimental fragmentation − cpm of background fragmentation)/(cpm of total fragmentable DNA − cpm of background fragmentation). We also used hypotonic lysis buffer containing 10 mM Tris-HCl, 10 mM EDTA, and 0.2% Triton X-100 in a few experiments and obtained nearly identical values of percent specific DNA fragmentation.

^{51}Cr-release assay was carried out as previously described (9, 10). Various number of effector cells and 1.5×10^4 of target cells were added to wells of a flat-bottom 96-well plate (Corning 25860 Corning, NY). Cytolytic activity was induced with either CD3-mAb, Con A, or Ag-pulsed LK35.2 cells. The total volume of each assay was 0.2 ml. After culturing for various times, supernatants (0.1 ml) were collected and counted with a gamma-scintillation counter. The results were expressed as percent specific ^{51}Cr-release = (cpm of test sample − cpm of nonspecific release)/(cpm of total release − cpm of background release). The cpm value of nonspecific release was obtained by culturing target cells with culture medium only. Nonspecific release of ^{51}Cr was found to be 5 to 14% of the total radioactivity. Under the conditions used in this study, none of the activating agents and metabolic inhibitors caused any significant increase of nonspecific release. The cpm value of total release was obtained by lysing the target cells with 1% Nonidet P-40.

It should be noted that the choice of using round bottom test tubes for DNA fragmentation assays and flat-bottom plates for ^{51}Cr-release assays is based on efficiency and convenience. Essentially identical results were obtained when DNA fragmentation assays were carried out for 90 min in flat-bottom plates and washed out with 0.2% Triton X-100 in PBS to determine the degree of DNA fragmentation. Similarly, the same results were obtained when the 4-h ^{51}Cr-release assays were conducted in round-bottom test tubes. This is not unexpected because these cytolytic assays are dependent on receptor-mediated E:T interaction. In addition, effective E:T interaction is also expected under a high E:T ratio of cloned CD4 and secondary CD8 cells to target. Finally, all experiments were carried out at 37°C in a 10% CO_2 incubator in duplicate with less than 7% variation.

Effect of metabolic inhibitors on target DNA fragmentation. Effector cells were incubated with various amounts of inhibitors for 1 h followed by the addition of target cells that had been pulsed with either Ag, Con A, or CD3-mAb. The percent DNA fragmentation was determined as described above. The inhibitors included Mit C, AcD, Chx, emetine-HCl, CsA, ChT, EGTA, and $ZnCl_2$. CsA was obtained from Sandoz, Basel, Switzerland. All other inhibitors were obtained from Sigma. The concentrations of those inhibitors used were effective but not cytotoxic (as judged by trypan blue exclusion) under the experimental conditions. Thus, Mit C inhibited thymidine incorporation into DNA of activated T cells. The activation-induced de novo synthesis of mRNA and protein for IL-2, TNF-α, TNF-β, and IFN-γ was completely inhibited by AcD, Chx, CsA, and ChT (9, 10). In some experiments, effector cells (10^6/ml) or target cells (0.5×10^6/ml) were cultured with indicated amounts of inhibitors for 1 h, washed twice with medium, and tested for the induction of target DNA fragmentation.

Effect of zinc ions on IL-2 production. Cells from clone F3 (1.5×10^5/well) were activated in the absence or presence of various concentrations of Zn^{2+} with LK cells (1.5×10^4/well) that have been armed with CD3-mAb. Supernatants were collected 5 h later, extensively dialyzed against PBS, sterile-filtered, and assayed for IL-2 activity with CTLL-2 indicator cells (9).

Gel electrophoresis of fragmented DNA. LK cells or CD3-mAb-pulsed LK cells (2×10^6/well) were cultured respectively with anti-H-2K CTL (4×10^6/well) and clone E6 cells (2×10^6/well) in a 24-well culture plate for 120 min. Supernatants were removed by centrifugation and cells were lysed with 0.5 M Tris buffer, pH 9, containing 2 mM EDTA, 10 mM NaCl, 1% (w/v) SDS, and 250 μg/ml proteinase K. After incubating at 48°C for 18 h, DNA samples were extracted with Phenol-$CHCl_3$ according to the method of Goelz et al. (17). Dried DNA extracts were dissolved in 50 μl of Tris buffer containing 330 μg/ml boiled pancreatic RNase A (Sigma) and incubated at 37°C for 2 h. Five μl of each preparations were subject to electrophoresis through a 0.75% agarose gel in TBE buffer (pH 7.8; 45 mM Tris-HCl, 45 mM sodium borate, and 2 mM EDTA). DNA bands were visualized with ethidium bromide. The lack of contribution of fragmented DNA from activated effector cells was determined by culturing effector cells with target cells that had been heated at 56°C for 30 min. Such treatment inhibits target DNA fragmentation without inhibiting activation of cloned T cells (our unpublished observation). Target cells cultured with medium only were also included as controls.

RESULTS

Time course, dose response, and target range of CD4 clone-mediated target DNA fragmentation. Figure 1 shows that cytolytic Th1 clones, upon activation (with Ag + Ia or CD3-mAb), induce target DNA fragmentation in a time- and dose-dependent manner. Target DNA fragmentation became detectable as quickly as 45 min after E:T interaction. Then 2 h later, nearly complete fragmentation of target DNA was observed (Figs. 1 *a* and *b*). Physical evidence of DNA fragmentation was provided in Figure 2, which showed that fragmented target DNA by activated CD4 clone E6 exhibited the "ladder" pattern of ~200 bp unit characteristic of oligonucleosome cleavage. Moreover, the fragmented DNA exhibited an identical pattern to that induced by CD8 (CTL) lymphocytes; suggesting that target DNA fragmentation by both CD4 and CD8 cells is mediated by the same endonuclease(s).

In contrast to the rapid target DNA fragmentation, ^{51}Cr-release was essentially undetectable for the first 90 min (Figs. 1 *a* and *b*). A significant degree of ^{51}Cr-release was observed at 3 h after E:T interaction and complete ^{51}Cr-release required >5 h after cell interaction (data not shown). This pattern of early DNA fragmentation with subsequent ^{51}Cr-release was observed with different tar-

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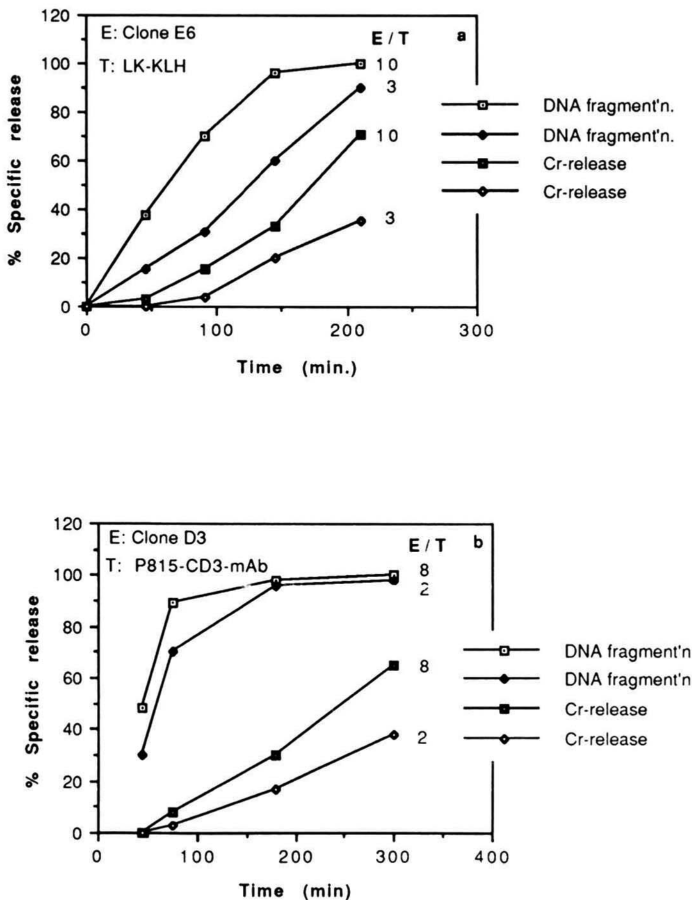

Figure 1. CD4 clones induce early target DNA fragmentation, followed by ^{51}Cr-release. In *a*, E6 cells were cultured with Ag-pulsed target, LK-KLH. In *b*, D3 cells were cultured with P815 cells that had been pulsed with CD3-mAb through Fc-FcR interaction. Various E:T ratios were used. At various times after culture, target DNA fragmentation and ^{51}Cr-release were determined as described in *Materials and Methods*. In all cases, the nonspecific ^{51}Cr-release is <8% for LK and <14% for P815. The nonspecific DNA fragmentation for LK is <7% for LK and <15% for P815.

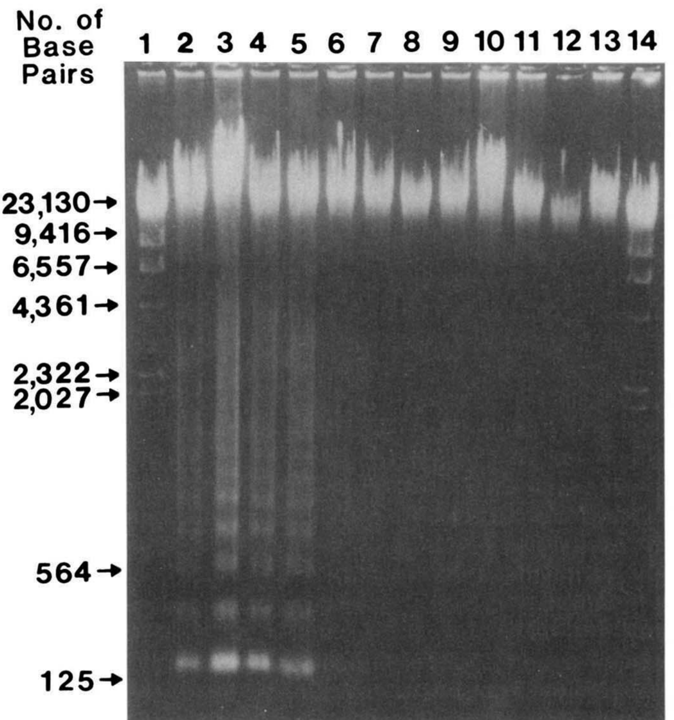

Figure 2. DNA extracts from CD3-mAb-pulsed LK target (*lanes 10* and *11*), clone E6+CD3-mAb-pulsed LK target (*lanes 2* and *3*), and clone E6+heat-killed, CD3-mAb-pulsed LK target (*lanes 6* and *7*) were subject to agarose gel-electrophoresis (0.75%) and stained with ethidium bromide. Also, DNA extracts from LK target (*lanes 12* and *13*), anti-H-2K CTL+LK (*lanes 4* and *5*), and anti-H-2K CTL+heat-killed LK (*lanes 8* and *9*) were included for comparison. Standard was a *Hin*dIII digest of λ-phage DNA (*lanes 1* and *14*).

gets and different clones that were activated with Con A (Table I).

Effects of metabolic inhibitors on target DNA fragmentation by CD4 and CD8 cells. We have recently shown that AcD and Chx inhibited the ability of CD4 cells, but not CD8 cells, to cause target ^{51}Cr-release. In contrast, ChT only inhibited target ^{51}Cr-release by CD8 cells. CsA weakly inhibited ^{51}Cr-release and its significance was difficult to assess (10). In this study, these and additional inhibitors were used to examine the relationship between DNA fragmentation and ^{51}Cr-release. Both Ag-specific and CD3-mAb-induced target DNA fragmentations were examined and the results were similar (Table II). The DNA synthesis inhibitor, Mit C, did not significantly inhibit target DNA fragmentation by either CD4 or CD8 cells. AcD and Chx strongly inhibited the induction of target DNA fragmentation by CD4 clones, but not by CD8 cells. In contrast, target DNA fragmentation by CD8 cells, but not CD4 clones, was inhibited by ChT. The data demonstrate that CD4 and CD8 cells use different pathways to express their cytolytic function as defined by either DNA fragmentation or ^{51}Cr-release from targets. Two inhibitors, EGTA and Zn^{2+}, inhibited target DNA fragmentation by both CD4 and CD8 cells; suggesting that both Ca^{2+} and target endonuclease activity are required (18–20).

Interestingly, CsA consistently inhibited more than 50 to 85% of target DNA fragmentation by control CD4 cells. Our early study has shown that CsA only caused weak inhibition (<30%) of target ^{51}Cr-release (9). This difference could be due to the fact that DNA fragmentation was assayed at 90 min after E:T cell interaction as oppose to 4 to 5 h for ^{51}Cr-release assay. When DNA fragmentation was assayed at 3.5 h after cell interaction, it was found that CsA cannot sustain its inhibitory power and the percent inhibition was reduced to the level comparable to that of ^{51}Cr-release assay (10). In contrast, both AcD and Chx strongly inhibited target DNA fragmentation at both 90 min and 3.5 h after cell interaction (Table III).

Inhibitors act on effector cells to inhibit target DNA fragmentation. We next determined whether inhibitors act on CD4 cells or target cells to block target DNA fragmentation. Both effector and target cells were cultured with indicated amounts of inhibitors for 1 h, quickly washed with a large volume of medium, and then tested by DNA fragmentation assays. The results are shown in Table IV. Inhibitors of DNA fragmentation such as AcD, Chx, and CsA, were found to act on effector cells because treatment of CD4 effector cells with these agents inhibited target DNA fragmentation. In contrast, such treatment on target cells did not inhibit target DNA fragmentation.

The quick washes with large volume of medium and the short period of DNA fragmentation assays may explain why pretreatment with the reversibe inhibitor Chx was still able to inhibit CD4 clone-mediated DNA fragmentation. To firmly establish that protein synthesis of

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TABLE I
Early DNA fragmentation with subsequent Cr-release were observed in various targets[a]

Target	Clone	Percent DNA Fragmentation		Percent Cr-Release	
		90 min	4 hr	90 min	4 hr
BW	F3	37	100	2	49
P815	F3	75	100	5	34
LK	F3	38	100	7	64
P815	E6	65	100	3	48
TA3	E6	12	43	1	10
Sp2/0	E6	10	69	3	4

[a] The E/T ratio was 10 in all cases. Con A (3.3 μg/ml) was used as activation agent. The spontaneous release values for DNA fragmentation are: 6% for BW, 14% for P815, 8% for LK, 4% for TA3, and 14% for Sp2/0. The spontaneous release values for Cr-release are: 7% for BW, 12% for P815, 5% for LK, 4% for TA3 and 7% for Sp/20. All experiments were carried out in duplicate with less than 7% variation.

TABLE II
DNA fragmentation activity of CD4 and CD8 cells is sensitive to different metabolic inhibitors[a]

Inhibitor	Quantity[b]	Percent DNA Fragmentation		Anti-H-2K (CD8)	
		E6(CD4) LK-CD3 -mAb	D3(CD4) LK-KLH	BW	LK-KLH
Medium		39	100	51	79
MitC	10 μg/ml	NT[c]	100	53	74
AcD	2.5 μg/ml	0	17	43	69
Chx	2.5 μg/ml	3	17	51	88
CsA	3 μM	13	20	61	93
ChT	0.5 μg/ml	34	89	17	39
EGTA	3 mM	NT	25	NT	25
$ZnCl_2$	0.75 mM	8	20	10	18

[a] The E/T ratio for anti-H-2K CTL was 5. All others were 10. DNA fragmentation was determined at 75 to 100 min after reaction. The spontaneous release values for LK-CD3, LK-KLH and BW were 11, 9, and 13%, respectively.
[b] Effector cells were cultured with indicated concentrations of inhibitors in 0.1 ml for 1 h before the addition of 0.1-ml targets.
[c] NT, Not tested.

TABLE III
CsA inhibits early but not late phase of target DNA fragmentation[a]

Clone	Assay Time (Min)	Percent DNA fragmentation[a]			
		Medium	AcD	Chx	CsA
E6	90	37	1	1	15
E6	210	75	11	7	69
C7	90	38	4	NT	8
C7	210	56	17	NT	50
D3	90	50	8	2	30
D3	210	61	27	25	62

[a] Clones were cultured with inhibitors for 1 h, followed by the addition of LK target cells that have been pulsed with CD3-mAb. Target DNA fragmentation was determined after the indicated time of culture. The spontaneous release was 5% for 75-min culture and 14% for 210-min culture.
[b] The concentrations of inhibitors were the same as those in Table II.

TABLE IV
Metabolic inhibitors act on effector cells to inhibit target DNA fragmentation

Inhibitor[a]	Percent DNA Fragmentation	
	E6-pretreated[b]	LK-pretreated[c]
Medium	36	37
AcD (2.5 μg/ml)	2	45
Chx (2.5 μg/ml)	2	44
Emetine (5 μg/ml)	9	39
CsA (3 μM)	16	35
Zn^{2+} (2 mM)[d]	20	38

[a] The concentrations used for treatment were shown in parentheses.
[b] Clone E6 (0.5×10^6/ml) was cultured for 1 h with indicated concentrations of inhibitors, washed twice, and tested for DNA fragmentation activity on labeled LK target cells in a 90-min assay. CD3 mAb was used as activation agent.
[c] Labeled LK target (0.5×10^6/ml) was cultured for 1 h with indicated concentrations of inhibitors, washed twice, and tested for DNA fragmentation by clone E6 in a 90-min assay. CD3-mAb was used as activation agent. The spontaneous release was 8% and did not changed significantly in treated groups.
[d] The minimum concentration needed to demonstrate inhibitory effect on E6 under the experimental condition.

CD4 clone cells is needed for target DNA fragmentation, another protein synthesis inhibitor emetine was tested under identical conditions. The results showed that treatment of clone E6 with emetine effectively inhibits target DNA fragmentation. Treatment of target cells with emetine did not inhibit target DNA fragmentation (Table IV).

It has been proposed that Zn^{2+} inhibits DNA fragmentation by inhibiting target endonuclease activity (20). However, Zn^{2+} treatment of effector cells, but not target cells, followed by quick and extensive washings, resulted in weak but significant inhibition of DNA fragmentation (Table IV). Therefore, the possibility that Zn^{2+} inhibits T cell activation for cytolytic activity was examined. Indeed, the concentrations of Zn^{2+} that inhibited target DNA fragmentation and ^{51}Cr-release also inhibited the activation of effector cells as determined by IL-2 production (Fig. 3). Under the experimental conditions described in Table IV, the effect of Zn^{2+} on target cells may be quickly reversed by washings such that the minimum concentration of Zn^{2+} needed to inhibit endonuclease activity can

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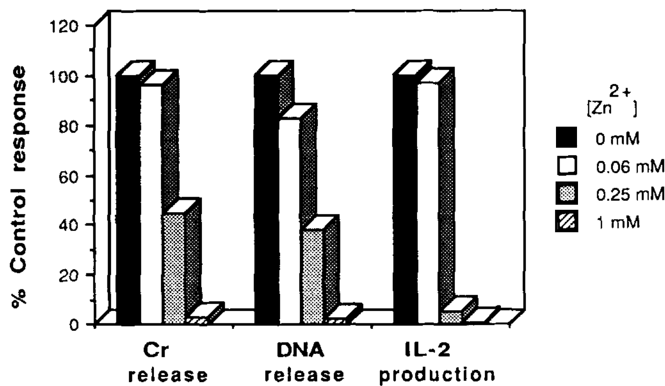

Figure 3. Zn^{2+} inhibits T cell activation as determined by IL-2 production, target DNA fragmentation, and ^{51}Cr-release. Clone F3 cells (1.5×10^5/well) were cultured in the presence of various concentrations of $ZnCl_2$ with LK cells (1.5×10^4/well) that have been pulsed with CD3-mAb. The target cells were either labeled with ^{51}Cr (for ^{51}Cr-release), or ^{3}H-thymidine (for DNA fragmentation), or unlabeled (for IL-2 determination). The effect of Zn^{2+} was expressed as percent control response. The control response in the absence of Zn^{2+} was 75% for ^{51}Cr-release, 56% for DNA fragmentation, and 18 U/ml for IL-2 production.

not be maintained. Thus, although the data clearly indicate that Zn^{2+} inhibits T cell activation and subsequent cytolytic functions including the activity of DNA fragmentation, it does not exclude the possibility that Zn^{2+}, when presents in appropriate concentrations, also inhibits DNA fragmentation by inhibiting target endonuclease activity.

DISCUSSION

In this study, we showed that both cytolytic CD4 (Th1) and CD8 (CTL) T lymphocytes quickly disintegrate target cell chromosomal DNA into fragments, cause the release of ^{51}Cr-labeled intracellular material and the eventual cell death (as determined by cell disintegration under microscope). Although both types of T cells induce target DNA fragmentation, the expression of the activity in CD4 and CD8 cells can be distinguished by means of several metabolic inhibitors. DNA synthesis is not required because Mit C does not inhibit DNA fragmentation. The DNA fragmentation activity of CD4 cells, but not CD8 cells, is strongly suppressed by the RNA synthesis inhibitor, AcD, and the protein synthesis inhibitor, Chx. In contrast, ChT strongly inhibits DNA fragmentation of targets by CD8 cells, but causes little inhibition of this activity of CD4 cells. This reciprocal pattern of inhibition indicates that CD4 and CD8 cells use different pathways for the expression of DNA fragmentation activity. Our data demonstrete CD4 cells, but not CD8 cells, require de novo synthesis of RNA and proteins to induce target DNA fragmentation. The sensitivity of CD8 cells to ChT suggests that ADP-ribosylation of G proteins, increase of adenyl cyclase activity and $[cAMP]_i$ may interfere with the expression of DNA fragmentation activity of CD8 cells, but not of CD4 cells. It is possible that the putative "signals" or "mediators" for target DNA fragmentation are short-lived in CD4 cells but long lasting and preexisting in CD8 cells. The same conclusion has been made based on essentially the same result of the effect of these inhibitors on target ^{51}Cr-release (10).

Similar to previous findings with CD8 cells (12, 16), target DNA fragmentation occurred rapidly after contact with CD4 effector cells. As early as 45 to 60 min after cell interaction, DNA fragmentation was detected. Most targets tested were sensitive to CD4 cytolytic activity and showed DNA fragmentation before ^{51}Cr-release. In general, the more resistant targets manifested less DNA fragmentation and ^{51}Cr-release. A complete dissociation of DNA fragmentation from ^{51}Cr-release was not found. Moreover, DNA fragmentation and ^{51}Cr-release were induced in a variety of targets by different CD4 clones using three different activation methods. In addition, the ability of various inhibitors to block DNA fragmentation by both CD4 and CD8 cells was in parallel with the effect of these inhibitors on ^{51}Cr-release (10). Both CD4 and CD8 cells fragmented target DNA into identical electrophoretic pattern of units of ~200 bp; suggesting that the same endonuclease activity was activated by distinct cytolytic pathways of CD4 and CD8 cells. These observations demonstrate a strong cause-result relationship between DNA fragmentation and ^{51}Cr-release and support the hypothesis that a common self-destruction program in targets, mediated by activated endonuclease(s), can be induced by distinct cytolytic pathways of CD4 and CD8 cells.

In a previous study, we observed that CsA weakly inhibited ^{51}Cr-release from targets by CD4 cells by an average of 30% in a 5-h assay (9). This degree of inhibition appeared insignificant when compared with more than 70% inhibition obtained with AcD and Chx. However, in the present study, CsA was found to inhibit up to 85% of DNA fragmentation by CD4 cells at 90 min after cell contact. This inhibition appeared temporary because a substantial DNA fragmentation (>90% of control) occurred 3.5 h later. This observation satisfactorily accounts for the weak inhibition of target ^{51}Cr-release by CsA. CsA could slow down the cytolytic activity of the effector by partial inhibition of the production or delivery of cytolytic signal(s), or by inhibiting only one of several possible cytolytic pathways that induce early target DNA fragmentation and subsequent ^{51}Cr-release.

When effector and target cells were separately treated with inhibitors, washed and then tested for target DNA fragmentation, it was found that inhibitory action on effector cells alone is sufficient to inhibit target DNA fragmentation. The same conclusion was obtained in a previous study using ^{51}Cr-release as measurement of cytolytic activity (9). The limitation of this protocol is that an inhibitor whose activity quickly disappears by washings can not be tested. Such is the case for EGTA. In the present study, the inhibitory activity of Zn^{2+} was greatly reduced after washings, but the residual inhibitory effect of Zn^{2+} on effector CD4 cells could still be detected. Furthermore, T cell activation for the production of IL-2 was inhibited in the presence of noncytotoxic levels of Zn^{2+}. Although these observations indicate that Zn^{2+} inhibits T cell activation and cytolytic activity, it does not exclude the possibility that target cell endonuclease activity can also be inhibited (18). Active participation of target cells with respect to increase of $[Ca^{2+}]_i$, DNA fragmentation, and cell death has been implicated in CTL cytotoxicity (19–20).

Cohen and colleagues (22) have proposed that DNA fragmentation is induced as a result of CTL activation of endogeneous DNA endonucleases in targets. The activity of these enzymes requires both Ca^{2+} and Mg^{2+}, and is inhibited by Zn^{2+}. Thus, increase of $[Ca^{2+}]_i$ of target cells after E:T interaction may provide an activation signal for

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these enzymes (18–21). Unfortunately, some cytolytic granule preparations from CTL clones also induce increase of $[Ca^{2+}]_i$ and subsequent DNA fragmentation in target (20, 22); making it difficult to determine whether target death was brought about as a result of DNA fragmentation or perforin-induced osmotic shock, or both. It was also found that the granzyme A of granules in CD8 clones has DNA fragmentation activity upon incubation with nuclei (23). When combined with purified perforin, DNA fragmentation of target cells was induced, but at much a slower rate than that induced by intact CD8 cells. It is possible that perforin first lyses target cells and then granzyme A acts on nuclei and induces DNA fragmentation; a process inconsistent with early DNA fragmentation preceeding cell death. Other investigators have reported that isolated granules and purified perforin do not have DNA fragmentation activity (24–26). Moreover, CsA is a strong inhibitor of granule exocytosis (27) but does not inhibit target DNA fragmentation and ^{51}Cr-release by CD8 cells (Table II) (10, 14). Perhaps more important is the fact that CD4 cells quickly fragment target DNA even though they do not express perforin and secrete very weak serine protease activity upon activation (9–10). Taken together, these observations suggest that a mechanism(s) other than granzyme A and perforin of CD8 granules is present in CD4 (and CD8) cells that induces target DNA fragmentation. Other studies have suggested that TNF-α or TNF-β of CD4 clones (28) and the TNF-related Leukalexin from CD8 cells (29) are the soluble mediators for target DNA fragmentation. The rate (>6 to 20 h) of DNA fragmentation by these mediators is slow in comparison with effector-mediated DNA fragmentation. In addition, the targets used in this study are resistant to high concentrations of TNF-α and TNF-β (9). That target DNA fragmentation does not require TNF-α and TNF-β is supported by the observation that even though both ChT and CsA efficiently shut off the production of TNF-α and TNF-β (10), target DNA fragmentation by CD4 clones is not persistently and significantly inhibited (Tables II and III).

DNA fragmentation has been observed in many cytotoxic systems such as cell-death induced with cytotoxic drugs (30), hormones (31), irradiation (32), growth-factor withdrawal (33), and activation of T hybridomas (34). These systems differ from the cytotoxicity of CD4 and CD8 cells in two major aspects. First, their cytolytic pathways inflict damage to themselves instead of targets of interacting cells. Second, the rate of DNA fragmentation (>4 to 20 h) is considerably slower than that mediated by CD4 and CD8 cells (75 min). Among these systems, corticosteroid toxicity on thymocytes (35) and activation-induced death of T hybridomas of CD4 origin (36) are sensitive to the same inhibitors (i.e., AcD, Chx, CsA, EGTA, and Zn^{2+}) that blocked CD4 cytolytic activity on target cells. This raises the possibility that a common cytolytic pathway is activated by corticosteroid on thymocytes, by immobilized CD3-mAb on T hybridomas, and by Ag-pulsed presenting cells on mature CD4 cells. In the latter case, the activated CD4 cells induce death of the APC (4). It is speculated that this pathway is preferentially used for activation-induced cell-death for the purpose of negative selection of thymocytes in thymus (32, 37). Along with maturation and differentiation, T cells become resistant to activation-induced suicide and develop cytolytic activity toward interacting targets. Loss of endogenous endonucleases, activation of protein kinases by Ag, and IL-2- (or IL-4) induced protection may confer the mature CD4 cells resistance to activation-induced DNA fragmentation (Figs. 1 and 2) (33, 38, 39). Thus, under the high E:T condition of peak immune response, this cytolytic activity can kill physiologic targets, such as activated B cells and Ia^+ macrophages bearing the appropriate Ag. This process controls the ceiling of a specific immune response and permits activated T cells progressing to memory T cells.

Finally, the remarkably rapid prelytic DNA fragmentation and high degree of ^{51}Cr-release by CD4 cells suggest that, similar to CD8 cells, CD4 cells also possess a protective effector-function against (Ia-bearing) cells infected with viruses, intracellular parasites or bacteria. This is significant in view of the fact that many class II Ag-restricted, viral Ag-specific CD4 clones have been demonstrated to have cytolytic activity (3, 40). Moreover, CD8 cells have been shown to induce nicking and fragmentation of polyoma viral DNA in parallel with target (host) DNA fragmentation (41). Whether the DNA fragmentation activity of CD4 cells can act on extranuclear DNA of these infectious agents to mediate prelytic halt of viral replication (42), remains to be established.

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