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Arrest of the Cell Cycle Reduces Susceptibility of Target Cells to Perforin-Mediated Lysis

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Abstract Cytotoxic T lymphocytes secrete a pore-forming cytolysin, perforin, that damages membranes of target cells. They also ligate Fas receptors on target cells and provoke apoptotic death. A20 (B lymphoma) and P815 (mastocytoma) cell lines were examined for their susceptibility to perforin-mediated lysis and to Fas-induced apoptosis after blockade of the cell cycle at the G_1/S interface. Cells were arrested at the G_1/S interface by inhibition of DNA synthesis with thymidine or aphidicolin. Subsequently, the treated cells were incubated either with CTL cytotoxic granules or the Fas-specific monoclonal antibody Jo-2. We show that arrest of the cell cycle at the G_1/S interface markedly reduced the susceptibility of target cells to perforin-mediated lysis. In contrast, growth arrest with thymidine or aphidicolin increased susceptibility of A20 and P815 cells to Fas-mediated apoptosis. Susceptibility to lysis by intact CTLs was not affected significantly by blockade of target cells with aphidicolin or thymidine. When cells surviving exposure to perforin-containing granules were isolated on Ficoll density gradients and cell-cycle profiles were examined by flow cytometry, the ratio of G_1 to G_2 cells increased among the survivors exposed to granules in contrast to controls incubated with buffer alone. The data suggest that cells in G_1 phase of the cell cycle are less susceptible to the perforin pathway than cells in G_2 and S phases but are more susceptible to the Fas pathway. J. Cell. Biochem. 69:425–435, 1998.

Key words: perforin; cell cycle; apoptosis; T lymphocyte; DNA synthesis

Cytotoxic T lymphocytes (CTLs) kill target cells via two pathways: 1) a secretory pathway through which the pore-forming cytolysin, perforin, damages target cells [Podack, 1986; Henkart et al., 1984; Kagi et al., 1994a] and 2) the induction of apoptosis mediated through ligation of Fas receptors. Upon recognizing specific antigens on target cell surfaces, CTLs secrete the contents of their granules, which include perforin and granzymes [Pasternack et al., 1986; Masson et al., 1986]. In the presence of calcium, perforin inserts into target cell membranes and polymerizes to form transmembrane pores. Target cells die partly due to colloidal-osmotic swelling and elevation of cytoplasmic Ca^{+2}. CTLs also induce apoptosis of target cells triggered by the interaction of Fas ligand on CTLs with Fas molecules on targets [Kagi et al., 1994a; Lowin et al., 1994; Kagi et al., 1994b].

When CTLs kill target cells, they secrete perforin and expose their own membranes to its cytolytic effects. Nonetheless, CTLs survive the encounter and proceed to kill other available targets. CTLs are highly resistant to perforin and therefore survive exposure to their own secreted perforin. Studies suggest that components of CTL membranes interfere with the insertion of perforin and formation of transmembrane chanels [Verret et al., 1987; Jiang et al., 1988; Shinkay et al., 1988; Jones and Morgan, 1991]. CTLs bind perforin less readily than do other cell lines [Verret et al., 1987; Ojcius et al., 1991]. Some data suggest that perforin assumes different conformations upon interacting with membranes ofsensitive cells as opposed to those of resistant CTLs [Muller and Tschopp, 1994]. The membrane factors protecting CTLs, however, have not been identified.

Abbreviations: CTL, cytotoxic T lymphocyte; FDA, fluorescein diacetate; MTT, 3-[4,5-dimethylthyazol-2-yl]-2,5-diphenytretrazolium bromide; PI, propidium iodide.

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Other work has demonstrated that target cells can mitigate the effects of perforin through various repair processes, including endocytosis of perforin channels and restoration of ionic gradients [Allbritton et al., 1988; Jones et al., 1990]. These repair processes may be enhanced in CTLs and serve to protect them during interactions with target cells.

Susceptibility to Fas-mediated apoptosis has been reported to vary at different stages of the cell cycle. In one report [Nishioka and Welsh, 1994], cells in G_0 or early G_1 were resistant to Fas ligation. However, reentry into the cell cycle due to viral infection rendered them susceptible. In contrast, another study reported that cells arrested in the later part of G_1 were more sensitive to Fas-mediated apoptosis [Komada et al., 1995]. The two studies imply a marked difference between cells in early and late G_1 in their responses to apoptotic signals. The consequences of cell cycle on susceptibility to the Fas pathway suggests that susceptibility to perforin may also be affected. To our knowledge, no examination of the effects of the cell cycle on susceptibility to peforin has been described. In this study, we have arrested cells at the G_1/S interface to determine whether cells at different stages of the cell cycle differ in susceptibility to perforin-mediated cytolysis and to CTL-mediated cytotoxicity.

MATERIALS AND METHODS

Cell Lines

Cell lines were cultured in K-10 medium (RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum, 10 mM Hepes, 50 μM β-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Murine tumor lines A20 (B lymphoma) and P815 (mastocytoma) are described in the *Catalogue of Cell Lines and Hybridoma* (American Type Culture Collection, Rockville, MD). The CTL line, BIID-3 (a gift from Dr. Mark Pasternarck, Massachusetts General Hospital), is an L^d-specific, alloreactive CTL cell line derived from C57BL/6 mice. It was cultured in K-10 supplemented with 10 U/ml of recombinant human IL-2 (rIL-2) (a gift of the Amgen Corp., Thousand Oaks, CA) and stimulated with irradiated (9,000 rads) P815 stimulator cells (1×10^5 cells/ml). CTLL-2, also described in the *Catalogue of Cell Lines and Hybrydoma*, was derived from a cytolytic T lymphocyte line but has lost cytolytic activity, although it contains cytolytic granules and perforin. It was grown in K-10 medium supplemented with 20 U/ml of rIL-2.

Cytolytic Granules

Perforin-containing cytolytic granules were isolated from BIID-3 cells, as has been described elsewhere [Verret et al., 1987]. Briefly, 1×10^9 cells were lysed by nitrogen cavitation in Relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 1 mM ATP, 10 mM PIPES, pH 6.8) for 15 min at 4°C at a pressure of 280 psi. Nuclei were removed by centrifugation at ~1000*g* for 5 min. The postnuclear supernatant was layered atop a discontinuous Percoll gradient (10 ml 90%; 5.5 ml 60%; 10 ml 39%; Pharmacia, Piscataway, NJ). Gradients were subjected to centrifugation at 20,000 rpm in a Beckman SW28 rotor (g_{av} = 58,000*g*) for 30 min at 4°C. Fractions were collected and assayed by hemolysis for perforin activity [Verret et al., 1987]. Lytic fractions were pooled, their density was augmented by addition of one-half volume of 90% Percoll, and Percoll particles were sedimented by centrifugation at 100,000*g*. The granules, which sedimented as a fine layer above the Percoll, were resuspended in less than 1 ml of Relaxation buffer and stored at -70°C.

CTLL-2 Lysate

Cells were washed and resuspended in sterile phosphate-buffered saline (PBS) containing 1 mM EDTA at 1×10^8 cells/ml and lysed by freezing and thawing. One hundred microliters aliquot (1×10^7 cells) were frozen and stored in microcentrifuge tubes at −70°C. Cell suspensions were thawed rapidly and subjected to centrifugation for 10 minutes at 10,000*g* at 4°C. Perforin activity in the supernatant fluid was quantitated by hemolytic assay [Verret et al., 1987].

Cell Proliferation Assays

Proliferation of cells was assessed by incorporation of ^{3}H-thymidine or by metabolism of MTT, as described in *Current Protocols in Immunology* [Coligan et al., 1991]. Cells (10^4 cells/well) were cultured in microtiter plates with varying concentrations of thymidine (0–10 mM) or aphidicolin (0–28 μg/ml). After 24 or 48 h, ^{3}H-thymidine (1 μCi /well) was added. Cells were harvested after 24 h by filtration with a

multiple cell harvester (Skatron, Sterling, VA), and incorporated radioactivity was measured by liquid scintillation counting. Alternatively, MTT reagent was added and color was developed after 4 h by addition of an HCl solution in isopropanol. Metabolism of MTT was quantitated spectrophotometrically at 630 nm with a microplate reader (Dynatech 5000, Chantilly, VA).

Lysis by Isolated Cytotoxic Granules or CTLL-2 Perforin Containing Lysate

A20 and P815 target cells (3×10^5 cells) were arrested at the G_1/S interface by culture with 5 μg/ml aphidicolin or 2 mM thymidine for 24 h at 37°C. Controls cells were incubated in parallel without the inhibitors. Cells were harvested and washed to remove the inhibitors and then labeled with ^{51}Cr by incubation with 100 μCi ^{51}Cr-sodium chromate (NEN-Dupont, Boston, MA) in 200 μl final volume for 1 h at 37°C. Cells were washed twice and incubated for 1 h in K-10 medium to allow release of unbound ^{51}Cr. Cells were transferred to lysis buffer by washing and resuspending at 1×10^5 cells/ml in lysis buffer (155 mM NaCl, 5 mM $CaCl_2$, 0.4 mg/ml bovine serum albumin (BSA), 10 mM Hepes, pH 7.5). Labeled target cells (10^4 cells in 100 μl) were added to 8 μl of isolated granules or sterile CTLL-2 lysate that were serially diluted, respectively, in Relaxation buffer or 1 mM EDTA in PBS. Maximum release of ^{51}Cr was determined from wells where cells were lysed with 8 μl of 1% Triton X-100 (final concentration 0.1%). Minimum release was determined from wells receiving 8 μl of Relaxation buffer or PBS/EDTA. Plates were incubated at 37°C for 30 min and subjected to centrifugation (~1,000*g* for 5 min). Radioactivity of the supernatant fluid (30 μl) was determined by liquid scintillation counting. Percent specific lysis was calculated as 100 × [(counts per minute released in presence of granules or lysates) − (minimum counts per minute released)]/[(maximum counts per minute released) − (minimum counts per minute released)]. No significant lysis was observed when Ca^{+2} was ommitted or chelated with EDTA, indicating that lysis was dependent on perforin.

In some instances, cells surviving perforin-mediated lysis were isolated on discontinuous Ficoll gradients. Briefly, 3×10^5 A20 cells in 1 ml of lysis buffer (see paragraph above) were incubated with 100 μl of isolated granules or CTLL-2 lysate for 30 min. The mixture was layered atop 1 ml of Ficoll-Paque (Pharmacia) and subjected to centrifugation at 1,500*g* for 15 min at 4°C. Cells at the interface were carefully removed by pipetting, washed in Hepes-buffered saline, and subjected to cell-cycle analysis, as described below (see Flow Cytometry).

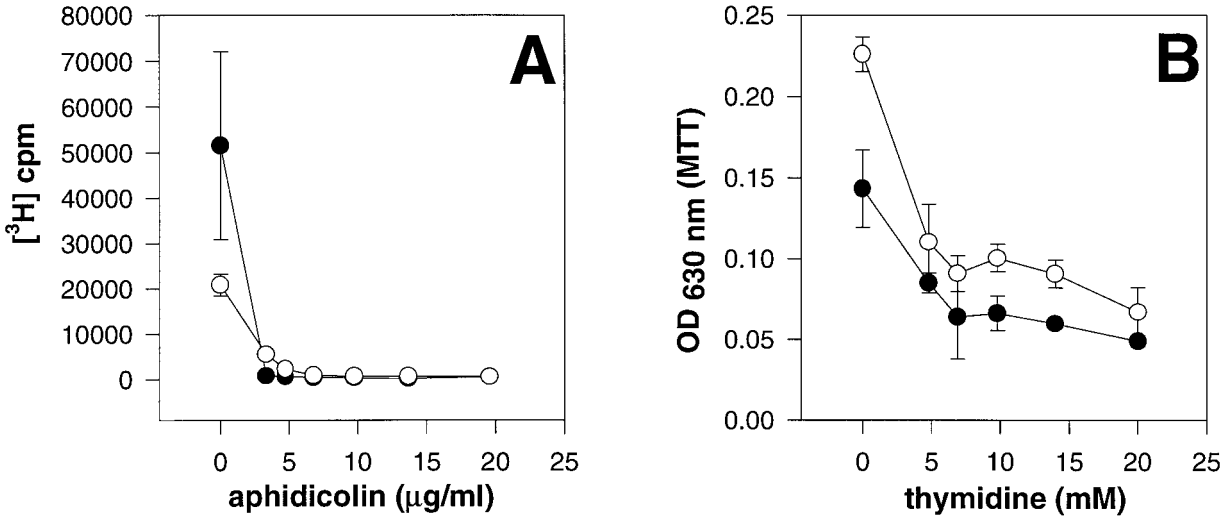
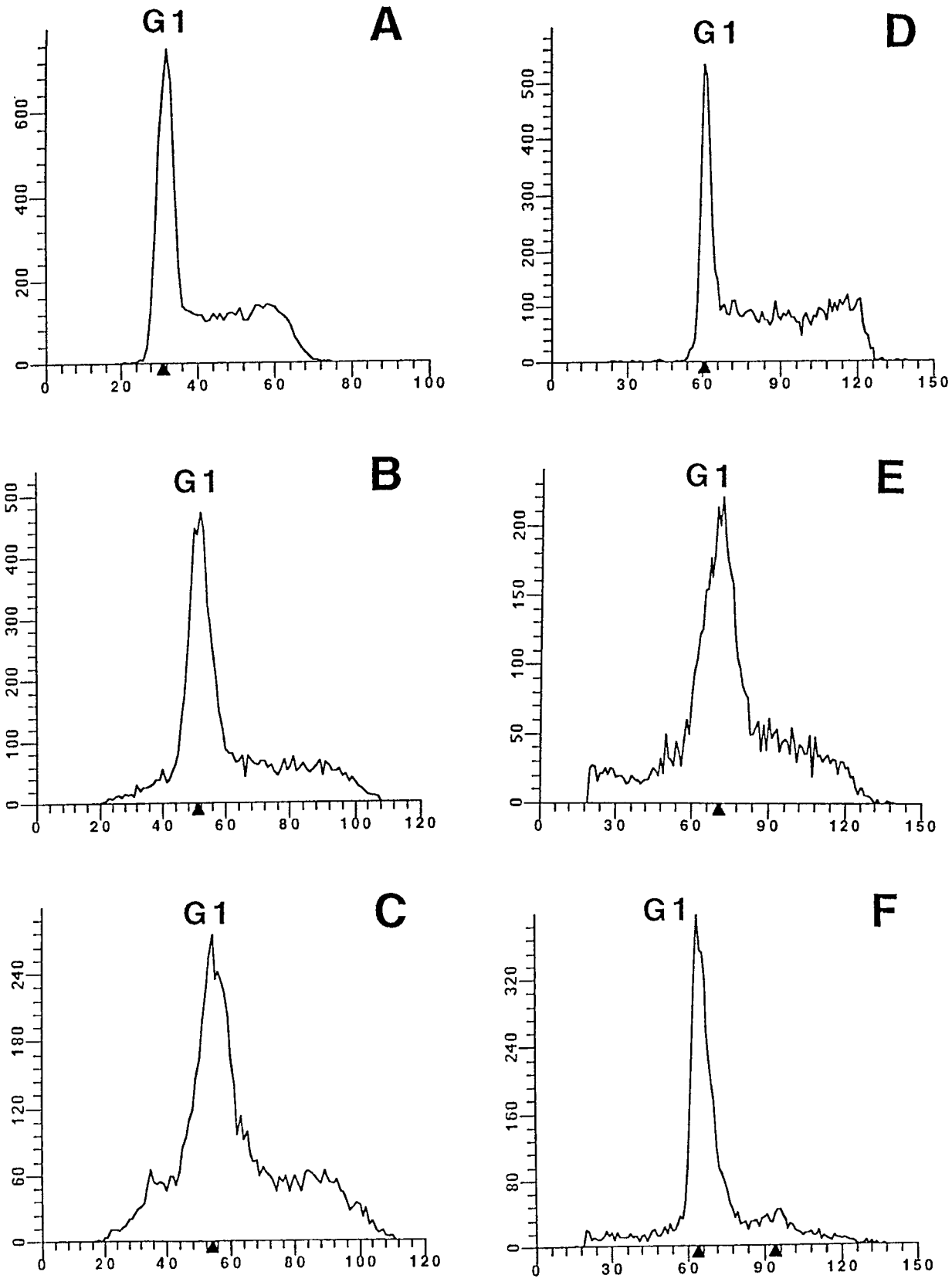


Fig. 1. Proliferation of cells cultured in aphidicolin or thymidine. A20 and P815 cells were cultured for 24 h in thymidine or aphidicolin. As described in Materials and Methods, proliferation of cells cultured in thymidine was measured by metabolism of MTT (**A**); proliferation of cells cultured with aphidicolin was measured by incorporation of ^3H-thymidine (**B**). Filled circles represent the mean proliferation of A20 cells, and open circles represent proliferation of P815 cells (n = 5, ± SD).


G1
A
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200
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0
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Figure 2.

Lysis by Intact CTLs

Tumor cells arrested at the G_1/S interface were labeled with ^{51}Cr (see previous section). Labeled target cells (10^4 cells/well) and a varying number of BIID-3 cells ($1 \times 10^4 - 2 \times 10^5$ cells) were combined in 200 µl of K-10 medium in microtiter wells and incubated for 4 h at 37°C. After centrifugation at 1,000*g*, radioactivity released in 100 µl of supernatant fluid was counted and percent specific lysis calculated as described in the previous section. Maximum lysis was determined from wells containing 0.1% Triton X-100 and minimum release from wells containing labeled target cells but no CTLs.

Fas-Mediated Apoptosis

Fas-mediated apoptosis was triggered by culturing cells in K-10 medium with Fas-specific monoclonal antibody Jo-2 (PharMingen, San Diego, CA). Cells (3×10^5 cells in 10 ml K-10 medium) were cultured with 2 mM thymidine or 5 µg/ml aphidicolin for 24 h. Jo-2 was added to a final concentration of 12 ng/ml. Cells were incubated for another 24 h prior to analysis. Viable, necrotic, and apoptotic cells were determined by flow cytometry, as described in the next section.

Flow Cytometry

Flow cytometry was performed on a FACSort equipped with argon-ion laser (Becton-Dickinson Immunocytometry Systems, San Jose, CA). For cell-cycle analysis, cells were fixed at 4 °C with 70% ethanol for >2 h, pelleted by centrifugation, and then resuspended in 1 ml of PBS, containing 1 mg/ml glucose, 1 mg/ml RNase A (Sigma Chemicals, St. Louis, MO), and 50 µg/ml propidium iodide (Sigma Chemicals). Flow cytometry was performed after 1 h incubation in propidium iodide. Ten thousand events were recorded, stored, and analyzed by ModFit-LT and Cell Quest softwares (Becton-Dickinson). Doublets were excluded with appropriate gates. Although a total of 10,000 cells was analyzed per sample, the number of gated events differed between samples due to variable frequency of doublets.

Apoptotic and necrotic cells were evaluated by incorporation of fluorescein diacetate (FDA) and propidium iodide (PI) [Ross et al., 1989]. Cells were washed and incubated at 37°C for 10 min with 5 µg/ml FDA and 10 µg/ml PI in 4.5 ml K-10 medium. Viable cells incorporate FDA but exclude PI. Apoptotic cells exclude both fluorophores. Necrotic cells incorporate PI while excluding FDA. Data were analyzed using Cell Quest software.

RESULTS

Cell-Cycle Arrest With Aphidicolin and Thymidine

Cells cultured in the presence of thymidine or aphidicolin undergo proliferative arrest. Figure 1 depicts the effect of these two drugs on A20 and P815 cells. The abolishment of ^3H-thymidine incorporation showed that proliferation of both cells lines ceases in aphidicolin above 5 µg/ml (Fig. 1A). As shown by metabolism of MTT, thymidine at 2 mM blocked cell growth of the two lines (Fig. 1B). Based on these results, the lowest concentrations of thymidine (2 mM) and of aphidicolin (5 µg/ml) that arrested proliferation were selected. Cells arrested at these concentrations for 24 h were analyzed by flow cytometry for cell-cycle profiles. Figure 2 shows that A20 and P815 cells were arrested predominantly in the G_1 phase of the cell cycle at these concentrations of thymidine and aphidicolin. Profiles of A20 and P815 cells blocked with either thymidine or aphidicolin indicate a reduction of S-phase cells and near abolition of G_2-phase cells (Table I). Broadening of the G_1 peak suggests that some cells were proceeding with DNA replication. Nonetheless, the majority of cells were in G_1.

Fig. 2. Cell-cycle profiles of cells cultured in 2 mM thymidine or 5 µg/ml aphidicolin. Cell-cycle profiles of a freely cycling population of A20 cells (**A**) were compared to profiles of cells arrested with (**B**) thymidine or with (**C**) aphidicolin. Also, freely cycling P815 cells (**D**) were compared to P815 arrested with (**E**) thymidine or with (**F**) aphidicolin. Cells were cultured in thymidine or aphidicolin for 24 h, and the cell-cycle profiles were evaluated by flow cytometry as described in Materials and Methods. The abscissa are arbitrary units of DNA content; cell numbers are represented on the ordinate. Distribution of cells in the three phases of the cell cycle is presented in Table I.

Granule-Mediated Lysis of Cells Arrested in G_1

To determine whether cells arrested in G_1 differed from cycling populations in sensitivity to perforin, cells were arrested in G_1 either with thymidine or with aphidicolin, labeled with ^{51}Cr-sodium chromate, and subjected to lysis with CTL cytotoxic granule as described in Materials and Methods. In these experiments, cells arrested in G_1, whether with aphidicolin or

TABLE I. Cell-Cycle Profiles of Cells Cultured in 2 mM Thymidine or 5 μg/mlAphidicolin

Phase of cell cycle	Percentage of cells ± SD (n = 4)					
	A20			P815		
	Controls	Thymidine	Aphidicolin	Controls	Thymidine	Aphidicolin
G_1	37.9 ± 2.3	65.9 ± 5.9	74.2 ± 10.6	37.6 ± 1.1	77.6 ± 3.7	63.6 ± 3.9
S	50.2 ± 2.9	34.0 ± 5.7	23.1 ± 7.7	51.3 ± 1.3	22.0 ± 3.7	32.8 ± 10.1
G_2	11.9 ± 1.0	0.1 ± 0.2	2.4 ± 4.2	11.2 ± 0.4	0.4 ± 0.3	4.2 ± 0.7

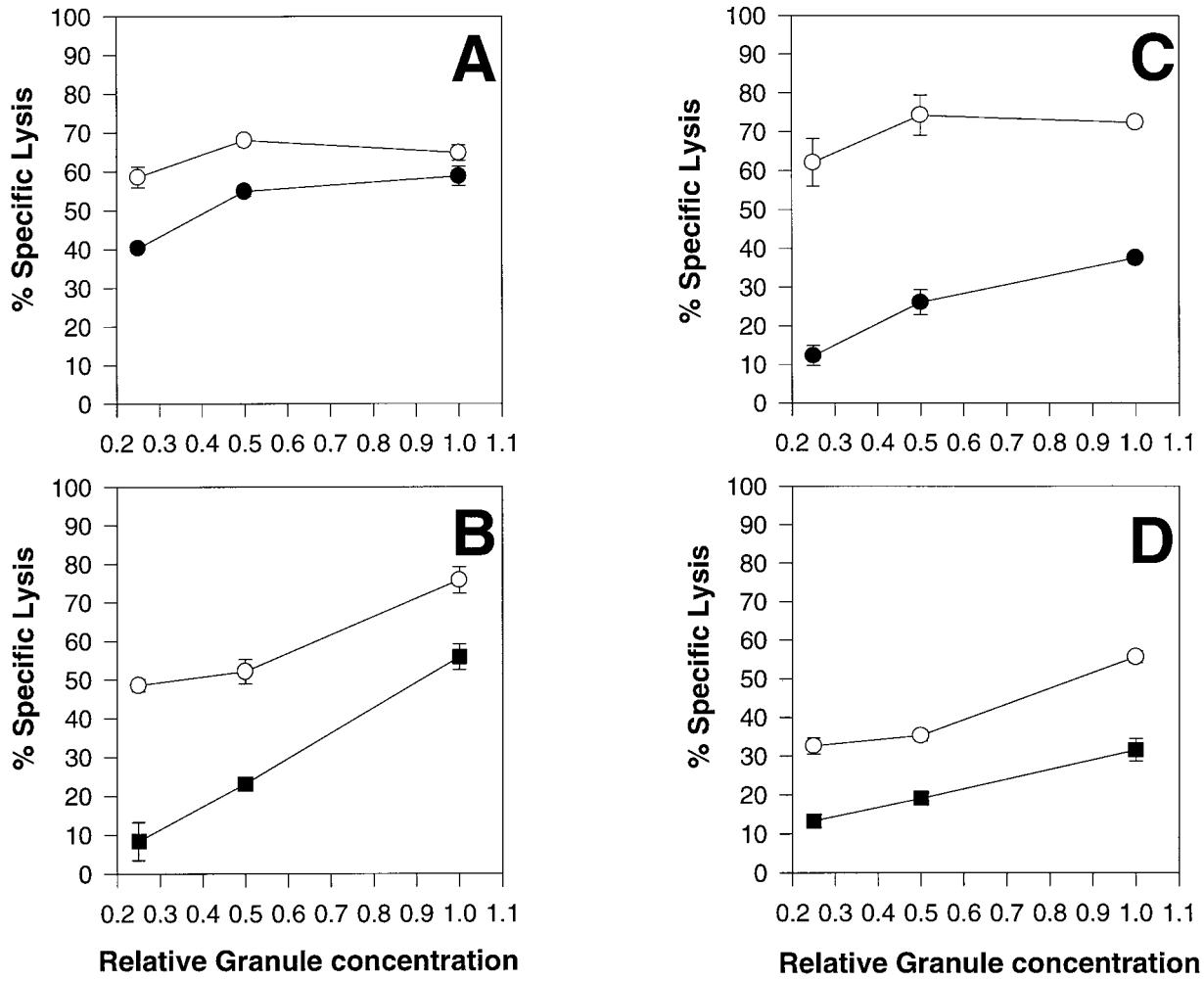

Fig. 3. Granule-mediated lysis of cells arrested in G_1. A20 (**A,B**) and P815 (**C,D**) cells were cultured in 2 mM thymidine or 5 μg/ml aphidicolin. Lysis of the cells by isolated CTL granules was evaluated as described in Materials and Methods. The ordinate (% specific lysis) represents the mean ± SD of triplicate measurements. Filled circles represent lysis of thymidine-blocked A20 (A) or P815 (C), and filled squares represent lysis of aphidicolin-blocked A20 (B) or P815 (D). Open circles represent lysis of control A20 or P815 not blocked with inhibitors. Controls in A,B and in C,D differ, as experiments were performed on different days with different preparations of granules.

thymidine, were lysed less efficiently than cycling controls (Fig. 3), indicative of reduced susceptibility to perforin. The difference between cycling controls and blocked cells was most pronounced at suboptimal concentrations of granules. Since the extent of lysis approaches saturation at higher granule concentrations, the disparity between lysis of cycling and arrested cells narrowed at those concentrations. Granule-mediated lysis was due to action of perforin since no lysis was observed when Ca^{+2}, required for perforin-mediated lysis, was omitted or with addition of EDTA (data not shown). Background lysis of thymidine- or aphidicolin-

blocked cells was less than 7% and did not differ significantly from that of cycling controls.

Fas-Mediated Apoptosis of Cells Arrested in G_1

P815 and A20 cell lines expressed high levels of Fas on their surfaces, as judged by flow cytometry of cells stained with fluoresceinated Jo-2 (data not shown). Cells were triggered to undergo apoptosis by culture with the monoclonal antibody Jo-2. An appropriate antibody concentration (12 ng/ml), causing less than 10% apoptosis, was selected for subsequent experiments after titration with varying concentrations of Jo-2. P815 and A20 cells arrested with thymidine or aphidicolin were more sensitive to Fas-mediated apoptosis than were cycling cultures (Fig. 4). As shown, the percentage of apoptotic cells increased markedly among cells blocked at the G_1/S interface either with thymidine or aphidicolin. This effect contrasts with the reduced susceptibility to granule-mediated lysis shown in Figure 3. Apoptotic cells were identified by flow cytometry as cells that did not incorporate significant PI and FDA. When cells were cultured with Jo-2 for greater than 24 h, late apoptotic cells were indistinguishable from necrotic cells as judged by the incorporation PI.

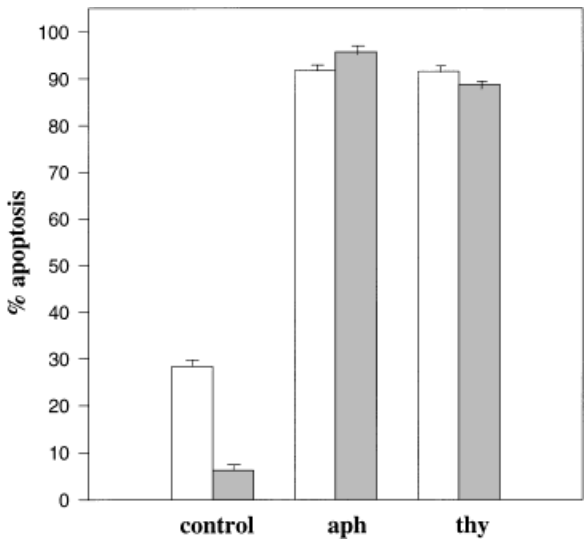

Fig. 4. Fas-mediated apoptosis of P815 and A20 cells arrested in G1. A20 (open bars) and P815 (hatched bars) cells were arrested in 2 mM thymidine (thy) or 5 μg/ml aphidicolin (aph) and then cultured with a fas-specific monoclonal antibody (Jo-2). Apoptotic cells were determined by flow cytometry as described in Materials and Methods.

CTL-Mediated Lysis of Cells Arrested in G_1

The results presented in Figures 3 and 4 show that growth arrest of target cells alter their sensitivity to granule-mediated lysis and to Fas-triggered apoptosis. These observations suggested that sensitivity to CTL-mediated cytotoxicity may also be affected by growth arrest. Susceptibility of cells arrested in G_1 to lysis by intact CTLs was evaluated in 4 h chromium-release assays. BIID-3 CTLs were incubated with chromium-labeled A20 and P815 target cells that had been arrested by culture with aphidicolin or thymidine. Lysis of arrested targets was compared to that of cycling controls. Preincubation with thymidine caused a slight increase in susceptibility of A20 cells, but aphidicolin blockade caused a slight reduction in susceptibility of this line to CTL-mediated lysis (Fig. 5A,B). Lysis of P815 cells blocked with aphidicolin and thymidine did not differ significantly from that of controls (Fig. 5C,D). Lysis by intact CTLs involves recognition of surface antigen, activation of CTLs, and delivery of cytotoxic factors. Thus, multiple factors may be involved in explaining the stark contrast between the responses to killing by isolated granules and Jo-2 and to cytolysis by intact CTLs.

Survivors of Granule-Mediated Lysis

We have demonstrated that cells arrested in G_1 were less susceptible to perforin. To eliminate the possibility that a drug-induced artifact underlies these observations, we undertook an alternative approach that was independent of thymidine and aphidicolin. Cells were lysed with perforin-containing granules, and survivors were isolated on Ficoll gradients (see Materials and Methods). Distributions within the cell cycle were examined by flow cytometry. When compared to controls incubated in parallel with buffer lacking granules, survivors from cells incubated with granules were enriched in G_1 phase cells and contained significantly fewer G_2 cells (Fig. 6; Table II). Enrichment of G_1 cells was also observed when A20 cells were incubated with lysates of CTLL-2 in the presence of Ca^{+2}. G_1 phase cells died less readily in cycling populations than other cells. These observations are consistent with the observed resistance to perforin of cells arrested with thymidine or aphidicolin.

DISCUSSION

In examining the mechanisms by which CTLs are resistant to lysis by their own cytotoxic factor, perforin, we have isolated a tumor line made partially resistant to perforin after transfection with CTL-derived cDNA and selected by lysis with perforin-containing CTL extracts [manuscript in preparation]. Cells surviving exposure to perforin were initially not growing and resumed their normal growth after 72 h. Along these lines, we sought to determine whether tumor cells arrested in distinct phases of the cell cycle differ in their susceptibility to perforin. Here we have presented data showing that cells in G_1 are less susceptible to perforin-mediated lysis than those in S and G_2 phases.

When treated with CTL granules, A20 and P815 cells blocked with thymidine or aphidicolin were lysed less efficiently than cycling controls. This unexpected result showed that G_1 cells and especially those blocked at the G_1/S interface are less sensitive to perforin-mediated lysis. Due to its poor stability, purified perforin was not used for these experiments. Perforin rapidly loses activity when refrigerated at 4°C and is inactivated by freezing. Preparations of cytotoxic granules, on the other hand, were stable and retained consistent activity when stored at -70°C. Lysis by isolated cytotoxic granules was clearly due to formation of polyperforin pores [Podack, 1986; Henkart et al., 1984]. No lysis was observed in the absence

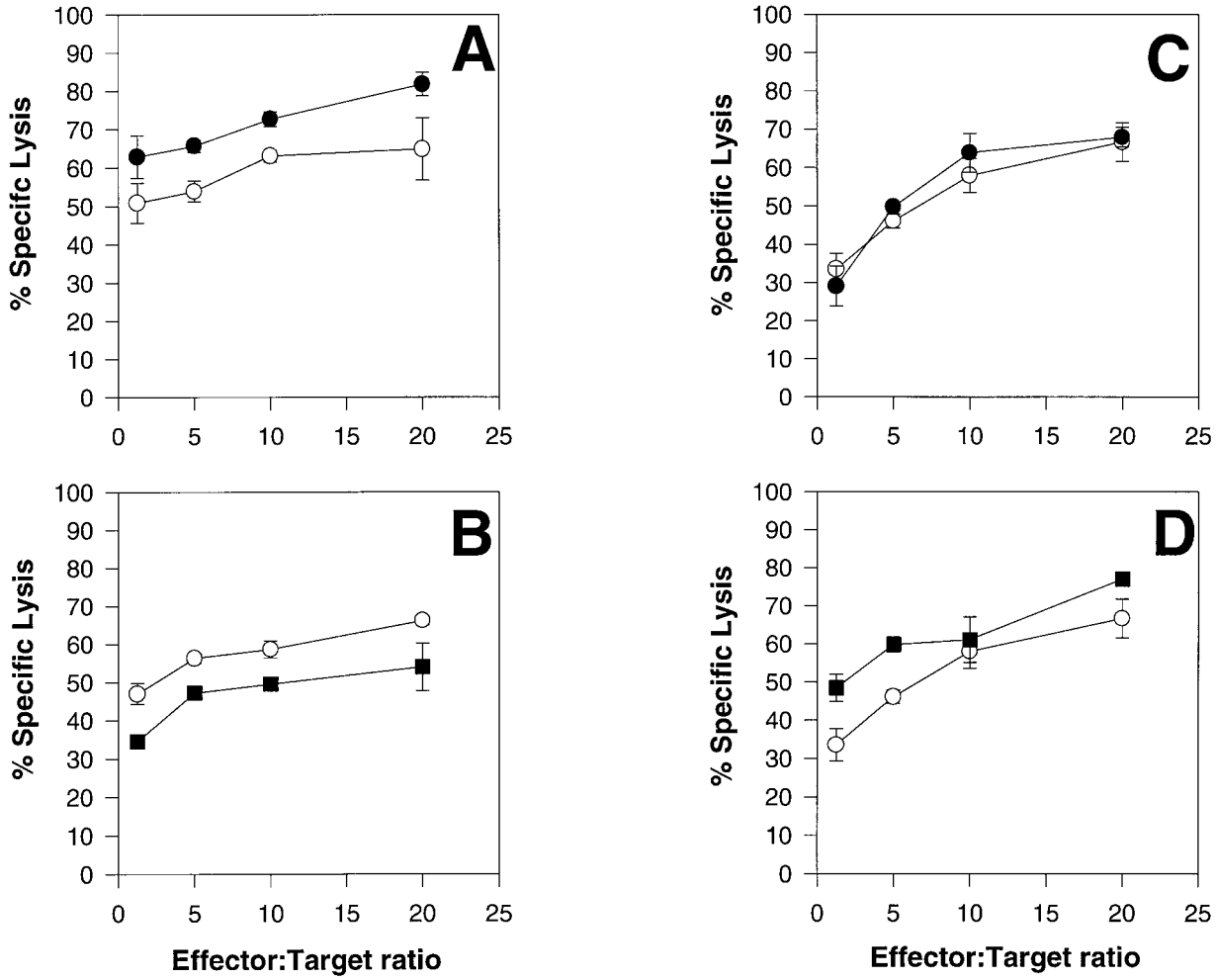

Fig. 5. Lysis by intact CTLs of A20 and P815 cells arrested in G1. A20 (**A,B**) and P815 (**C,D**) cells were cultured in 2 mM thymidine or 5 μg/ml aphidicolin. Target cells were incubated with varying numbers of CTLs (BIID-3), and the extent of lysis was determined as described in Materials and Methods. Ordinates (% specific lysis) represent the mean ± SD of triplicate wells. Filled circles represent lysis of thymidine-blocked A20 or P815, and filled squares represent lysis of aphidicolin-blocked A20 or P815. Open circles represent lysis of control A20 or P815 not blocked with inhibitors.

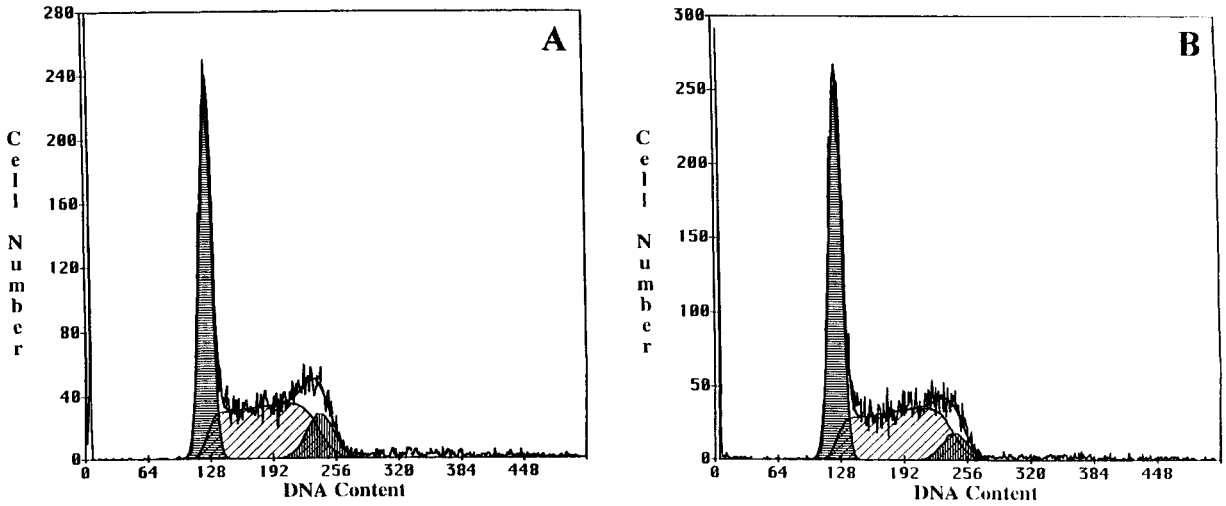

Fig. 6. A20 cells were incubated with lysis buffer without granules (**A**) or with CTL granules (**B**), and survivors were separated on Ficoll gradients, as described in Materials and Methods. Cell-cycle profiles of survivors were determined by flow cytometry and quantitated using Modfit LT software, as described in Materials and Methods. Percentages of cells in the three phases of the cycle are presented in Table II.

TABLE II. Cell-Cycle Profile of A20 Cells Surviving Exposure to CTL Granules

	Percentage of cells ± SD (n = 7)	
Phase of cell cycle	A20 controls	A20 treated with granules
G_1	43.1 ± 5.9	49.2 ± 6.5
S	44.8 ± 6.7	45.7 ± 8.4
G_2	11.9 ± 1.3	5.1 ± 2.9

of Ca^{+2}. Furthermore, lysis with whole granules represents the physiological situation, where target cells are exposed to the entire contents of granule, including both perforin and granzymes. Granzymes themselves have no lytic activity, although it has been proposed that they can pass through polyperforin channels and induce fragmentation of chromosomal DNA [Henkart et al., 1993]. To confirm the influence of the cell cycle on susceptibility to perforin, we also undertook experiments that did not depend on aphidicolin and thymidine. Cells surviving exposure to perforin were isolated on density gradients, and cell-cycle profiles were compared to controls. The data showed enrichment for cells in G_1 and depletion of G_2 phase cells and indicate that cells in G_1 have an advantage over other cells in the cycle when challenged with perforin.

Some variability in the response of tumor cell lines to thymidine and aphidicolin was observed. Both compounds interfere with DNA synthesis and consequently entry of cells into S phase, although through different mechanisms: aphidicolin directly inhibits DNA polymerase [Sheaff et al., 1991], while elevated levels of thymidine inhibit biosynthesis of essential deoxycytidine [Fox et al., 1980]. Conceivably, the uptake of the two compounds by these cell lines may vary and result in different cytosolic concentrations. The variable cytosolic concentrations of the compounds may allow some cells to proceed into S phase, while others are arrested. Nonetheless, the cell lines became significantly less sensitive to perforin when they were arrested with either of the two compounds.

CTLs also utilize an alternate perforin-independent pathway by inducing apoptosis through ligation of Fas receptors. Reports indicate that cells in G_0 and early G_1 were resistant to the induction of apoptosis. Nishioka and Welsh [1994] found that fibroblasts in G_0 were resistant to CTL-induced apoptosis but became susceptible when provoked by viral infection to enter the cell cycle. Komada and coworkers [1995] also observed that leukemia cells arrested in early G_1 with thymidine or phorbol esters were resistant to Fas-mediated apoptosis. They also noted that, within cycling populations, cells in G_1b underwent apoptosis more readily. We have observed that the A20 and P815 tumor lines blocked with thymidine or aphidicolin were much more sensitive to Fas-mediated apoptosis as opposed to their decreased susceptibility to perforin. However, Komada et al. [1995] found that a leukemia line

was resistant to Fas-mediated apopotosis when blocked with thymidine, while A20 and P815 became more sensistive in our hands. The basis for these different observations is unclear. Conceivably, the leukemia line was arrested in early in early G_1 (G_1a), while A20 and P815 were arrested in late G_1 (G_1b). Our data are consistent with other reports that T cells in G_1a are also resistant to Fas-induced apoptosis, while those in G_1b are more sensitive [Meinkrantz and Schlegel, 1995]. The fact that the cells arrested at the G_1/S interface increase in susceptibility to Fas while decreasing in susceptibility to perforin may allow the two pathways to complement each other. Accordingly, a factor that reduces sensitivity of target cells to perforin renders them more susceptible to the alternate killing pathway of CTLs. This is reminiscent of the complementarity of CTLs and natural killer (NK) cells, whereby cells that cease to express class I MHC antigens become more susceptible to NK cells even as they are no longer killed by CTLs.

CTLs lyse targets arrested in G_1/S essentially to the same extent that they kill cycling controls. This seems paradoxical in light of the observed decrease in susceptibility to perforin and increase in sensitivity to Fas signals. Since blockade at G_1/S affects sensitivity to the Fas and perforin pathways in opposite directions, Fas-mediated death may be compensating for the perforin pathway. Alternatively, CTLs may deliver saturating doses of perforin onto target cells and overcome resistance due to blockade. This assertion is supported by the fact that, at high granule concentration, differences in lysis disappear between blocked and cycling cells.

The demonstration that perforin-knockout mice become more susceptible to lymphocytic choriomeningitis virus infections and lose their ability to reject certain transplanted tumors underscores the importance of perforin and of granule-mediated lysis for destruction of target cells [Kagi et al., 1994a]. CTL killing of target cells lacking Fas receptors depends essentially on the perforin pathway [Kagi et al., 1994b]. It is plausible that, in tumors that are Fas-insensitive and killed principally via the perforin pathway, a subset of cells arrested in the cell cycle may survive CTL attack and re-emerge later. The resistance of cells in late G_1 may also be pertinent to the resistance of CTLs to their own secreted perforin. The proliferative status of CTLs during attack on targets may be important to their survival. However, this remains to be demonstrated. CTLs may not be proliferating while attacking target cells and, during this process, are protected in part from secreted perforin. This will require further investigation. The molecular basis for the resistance to perforin of cells in G_1 will have to be clarified. Changes in the composition of the plasma membrane or in metabolism may modify the susceptibilty of cells to perforin. Complement complexes are eliminated from cell surfaces by endocytosis [Carney et al., 1986; Ramm et al., 1983]. Reduced levels of intracellular ATP also render cells more sensitive to perforin [Verret et al., 1987]. Conceivalbly, cells at the G_1/S interface may more effectively repair perforin damage via endocytosis of lesions and restoration of ionic gradients.

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