CALCIUM ION CONCENTRATIONS AND DNA FRAGMENTATION IN TARGET CELL DESTRUCTION BY MURINE CLONED CYTOTOXIC T LYMPHOCYTES

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Recent studies of murine CTLs suggest that when antigen-specific receptors of these cells interact with ligands on target cells, the CTLs become activated to secrete cytolytic granules that contain a complement (C9)-like pore-forming protein, termed perforin (1–4) or cytolysin (5, 6), and several distinctive cationic serine esterases (7–11). Insertion of perforin into target cell membranes is thought to induce target cells to undergo lysis, as though affected by activated complement. However, other evidence suggests that CTLs induce target cells to undergo self-destruction by means of an endogenous autolytic pathway whose most prominent manifestation is fragmentation of DNA into nucleosome-sized fragments (12, 13). The characteristic fragmentation is probably due to activation of endogenous target cell endonucleases, since similar patterns of DNA fragmentation are evident in those murine lymphocytes that are lysed by corticosteroids (14); and evidence from successive mutations in such cells suggests that the cytolytic effects of these steroids and of CTLs depend upon the same gene product(s) (15). Inasmuch as this distinctive pattern of DNA fragmentation is not seen in cells that are lysed by complement, it seems likely that CTL-mediated and complement-mediated lysis of target cells are not entirely analogous (12, 13).

To learn more about the destruction of target cells by CTLs, we have been studying intracellular Ca^{2+} ion concentrations ($[Ca^{2+}]_i$)[1] in target cells. Ca^{2+} is a critical regulator of diverse metabolic activities, and cells normally maintain an ~10,000-fold gradient in $[Ca^{2+}]_i$ across the cell membrane (~1 mM extracellular and ~200 nM intracellular; references 16–19). Hence, aberrations in cell membranes may be expected to be associated with large changes in $[Ca^{2+}]_i$ and a variety of abnormal metabolic activities. To measure changes in $[Ca^{2+}]_i$, cells were loaded with indo-1, a fluorescent Ca^{2+}-binding dye whose K_D for Ca^{2+} is 250 nM (20), which is close to $[Ca^{2+}]_i$ in normal cells. Upon binding Ca^{2+}, the

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[1] *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular free calcium; ECBS, extracellular buffered salt solution; indo-1 AM, indo-1 acetoxymethyl ester; PE, phycoerythrin; *R*, ratio of fluorescence intensity at 405 vs. 480 nm.

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dye's fluorescent emission spectrum undergoes a blue shift, from a maximum of 480 nm (no Ca^{2+} bound) to a maximum of 405 nm (Ca^{2+} bound) (20). The fluorescence ratio at 405/480 nm can thus provide a sensitive measure of $[Ca^{2+}]_i$ and changes in $[Ca^{2+}]_i$ can be followed by flow cytometry of individual cells independently of variations from one cell to another in total dye concentration or cell size (20, 21).

Our results show that within minutes after interacting with cytotoxic granules that were isolated from CTLs, $[Ca^{2+}]_i$ in target cells is strikingly increased. An increase in $[Ca^{2+}]_i$ was also elicited when target cells were recognized by intact CTLs. However, in CTLs, which have been shown to be refractory to lysis by cytotoxic granules (22) and to be poor targets for other CTLs (23, 24), there was no increase at all in $[Ca^{2+}]_i$ when they interacted with the isolated granules. Ionomycin, a Ca^{2+} ionophore (25), elicited (in the presence of extracellular Ca^{2+}, but not in its absence) a similar increase in $[Ca^{2+}]_i$ and lysed cells. The characteristic cleavage of target cell DNA into nucleosome-sized fragments was also induced by the isolated granules as well as by the ionophore, valinomycin, but not by ionomycin. Overall, the results support the view that lysis of most target cells by cloned murine CTLs is due primarily to target cell membrane perturbations that are functionally equivalent to nonspecific ion channels. The ensuing large increase in $[Ca^{2+}]_i$ is probably responsible for cell lysis, and changes in intracellular cation concentrations also appear to be responsible for DNA fragmentation, probably by activating target cell endonucleases.

Materials and Methods

Reagents. Ionomycin (free acid), valinomycin, A23817, and gramicidin D (all from Calbiochem-Behring Corp., San Diego, CA) were kept as stock solutions in DMSO. The acetoxymethylester and potassium salt of indo-1 were obtained from Molecular Probes (Eugene, OR) and kept as stock solutions in DMSO and distilled water, respectively. Phycoerythrin (PE)-avidin was purchased from Becton Dickinson & Co. (Mountain View, CA) and ^{51}Cr and [^{125}I]deoxyuridine from New England Nuclear (Boston, MA). Dithiothreitol, potassium cyanide, and sodium azide were from Sigma Chemical Co. (St. Louis, MO), Matheson Coleman and Bell (Worwood, OH), and Fluka (Ronkonkoma, NY), respectively. *N*-OH-succinimidobiotin (Sigma Chemical Co.) was dissolved in DMSO, and *N*-succinimidyl-3-(2-pyridyldithiol)proprionate (Pierce Chemical Co., Rockford, IL) was kept in ethanol.

Cells, Media, and Buffers. 2C, 3H2, 3C11, and G4 are murine cloned CTLs that are specific for L^d or D^d (26, 27). They were grown in K medium with 20–30 U/ml human rIL-2 and irradiated P815 or BALB/c spleen cells (H-2^d haplotype). RDM4, S49, Yac 1, and EL-4 are murine T lymphomas, and P815 is a murine mastocytoma (28).

K medium is RPMI 1640 plus 10% heat-inactivated FCS, 4.5 mM Hepes, 1.2 mM L-glutamine, 90 U/ml penicillin, 90 mg/ml streptomycin, and 44 μM β-mercaptoethanol. Relaxation buffer (pH 6.78) contained 100 mM KCl, 3 mM NaCl, 3 mM $MgCl_2$, 1.25 mM EGTA, 10 mM Pipes, and 0.5 mM ATP. Extracellular buffered salt solution (ECBS), pH 7.2, contained 145 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$, 10 mM Hepes, 1 mM NaH_2PO_4, and 5 mM glucose; for some experiments ECBS also contained $CaCl_2$, usually at 1 mM, or EGTA, usually at 5 mM. Tris-EDTA-acetate buffer is 40 mM Tris-acetate, 1 mM EDTA, pH 8; and Tris-EDTA buffer is 10 mM Tris-Cl, 1 mM EDTA, pH 8.

Coupling Anti-receptor mAb to Cells. The purification of 1B2, an mAb that recognizes the antigen-specific receptor of CTL clone 2C and the coupling of 1B2 to the heterobifunctional cross-linking reagent, *N*-succinimidyl-3-(2-pyridyldithio)proprionate, have been

described (24). 1B2-SPDP was attached to cells as described (24) except that cell surface disulfide bonds were reduced by addition of 50 μM dithiothreitol to 10^6 cells/ml.

Labeling Cells with PE. Cells were washed twice in PBS and resuspended at 2×10^6/ml in PBS, pH 8.0, with 10 μg/ml *N*-OH succinimidobiotin for 45 min at room temperature. The biotinylated cells were then washed twice in PBS, pH 7.2, resuspended at 10^6 cells/100 μl, and 10 μl PE-avidin was added (Becton Dickinson & Co.). After 45 min at room temperature, the cells were washed once in PBS and resuspended in K medium.

^{51}Cr-release Assays. Cell lysis by CTLs, cytolytic granules, or ionophores was measured by determining ^{51}Cr release from ^{51}Cr-labeled target cells (29). The cytolytic activity of intact CTLs was determined in a 4-h assay using 10^4 target cells in 200 μl. Lysis of ^{51}Cr-labeled cells by isolated granules (see below) and by ionophores was carried out in ECBS (with 1 mM Ca^{2+} for assays with granules and various concentrations of Ca^{2+} for assays with the ionophores) for 1 h at 37°C using 5×10^4 ^{51}Cr-labeled cells in 100 μl. Assays were carried out in triplicate (with CTLs) and in duplicate with isolated cytotoxic granules or ionophores. The percentage specific ^{51}Cr release was calculated from $100 \times [(a - b)/(t - b)]$ where a is ^{51}Cr release in the presence of CTLs, granules, or ionophores, b is the spontaneous ^{51}Cr released in the absence of CTLs, granules, or ionophores, and t is the total ^{51}Cr released from target cells with 0.5% NP-40.

Preparation of Isolated Cytotoxic Granules. Cytolytic granules were prepared from mouse CTL clones 3C11, 3H2, or G4 as described (22). Briefly, ~10^9 cells were lysed by nitrogen cavitation (350 psi), centrifuged at 140 *g* for 5 min, and the nuclei-free supernatant was layered (in relaxation buffer) on a discontinuous Percoll gradient formed by 39–60–90% (volume ratio 2:1:1.75) Percoll in relaxation buffer. The gradient was centrifuged at 52,000 *g* in a rotor (model No. SW28; Beckman Instruments, Inc., Palo Alto, CA) for 30 min and fractions were tested for hemolytic activity against sheep RBCs. Granules were isolated from a pool of the peak hemolytic fractions, after the density was raised by adding 0.5 vol of 90% Percoll and the Percoll was removed by centrifugation at 100,000 *g* for 90 min.

DNA Fragmentation. To label DNA, 5×10^6 cells were incubated with 50 μCi of [^{125}I]deoxyuridine in 10 ml of K medium at 37°C under CO_2. After 15 h they were washed twice in PBS. The labeled target cells were resuspended in K medium (if intended for reaction with CTLs) or in ECBS with 1 mM calcium (if intended for reaction with cytolytic granules and ionophores). CTLs or granules were added to 4×10^4 ^{125}I-labeled target cells in 100 μl. After 1 h at 37°C, the cells were lysed by addition of PBS containing 2% SDS and 3 mM EDTA. DNA was then precipitated from the lysate with ethanol and resuspended in TE solution with gel loading buffer, loaded onto a 0.8% agarose gel with TEA buffer, and subjected to electrophoresis overnight at 40 mV. Dried gels were exposed to Kodak X-Omat AR film.

Loading Cells with Indo-1. The acetoxymethylester of indo-1 (indo-1 AM) was stored as a 1-mM stock solution in DMSO in a dessicator at −20°C. It was added at the indicated concentration to cells (at 2×10^6/ml) that had been washed twice and resuspended in RPMI 1640 containing 45 mM Hepes. After 1–1.5 h at 37°C, the cells were washed once in K medium and incubated again at 37°C for 0.5–1.5 h (shorter times were used for cells that were intended to form conjugates with CTLs). Immediately before use, the indo-1–loaded cells were washed with K medium or extracellular buffered salt solution.

The amount of intracellular dye in loaded cells was measured by lysing the cells with 0.02% Triton X-100 and comparing the fluorescence intensity of the released dye with standards (indo-1, as the deesterified dye). Emission spectra (excitation at 350 nm) of the released dye, measured in ECBS containing either 1 mM $CaCl_2$ or 5 mM EGTA, established that the indo-1 AM had been deesterified by the cells. 2C cells loaded with 1 μM indo-1 AM as described above had an intracellular indo-1 concentration of 33 ± 7 pmol/10^6 cells. Other cell lines were loaded with indo-1 AM at concentrations that led to their having approximately the same fluorescence intensity as the 2C cells (e.g., T cells were loaded with 0.5–1.5 μM indo-1 AM and tumor cells with 3–5 μM). As shown by Imboden et al. (30), these intracellular dye concentrations have negligible Ca^{2+}-buffering activity. Spectrofluorimetric measurements were carried out in a spectrophotofluorimeter

(model No. LS-5; Perkin Elmer Corp., Norwalk, CT; or model No. 4-8202; Aminco-Bowman, American Instrument Co., Silver Spring, MD) with cells in buffered salt solution containing either 1 mM $CaCl_2$ or 5 mM EGTA.

CTL–Target Cell Conjugates. To identify CTLs and target cells that were adherent to each other ("conjugates"), the CTLs were biotinylated and labeled with avidin-PE, and the target cells (with or without the attached 1B2 antibody) were loaded with indo-1. Target cells (at 5×10^5/ml) were mixed with CTLs (at 15×10^5/ml) in K medium at room temperature and the mixture was kept undisturbed for 40–60 min at room temperature. At time zero, reaction mixtures were placed at 37°C and they were not agitated until introduced into the flow cytometer. Separate reaction mixtures were prepared for each time point.

Flow Cytometry. Cells were analyzed in a cytofluorograph (model No. 50H; Ortho Diagnostic Systems Inc., Westwood, MA) linked to a 2150 computer. Indo-1 was excited with the 351–364-nm lines (70 mW) of a 5-W argon ion laser (Coherent Inc., Palo Alto, CA). PE was excited with the 568 nm (50 mW) line of a 5-W krypton laser (Coherent Inc.). For simultaneous measurements of indo-1 and PE, the krypton beam was spatially separated from the argon beam so as to give a signal time difference of 35 μs. For measurements of indo-1 alone, a blue-reflecting dichroic (50% at 455 nm) was used to split the fluorescence emission from the liganded and nonliganded forms of the dye. The 405-nm emission (Ca^{2+} bound) was collected using a 393–409-nm bandpass filter. Forward scatter was collected using a blue-reflecting dichroic (50% at 500 nm). For simultaneous measurements a UV bandpass filter was added to the forward scatter channel to remove scattered krypton laser light. In addition, a red-reflecting dichroic (50% at 540 nm) was introduced to separate the 480-nm indo-1 fluorescence from the PE fluorescence at 576 nm. The PE signal was then passed through a long pass filter (>570 nm) before it was collected.

The UV forward scatter signal was used to gate debris from cells. Cells that met the forward scatter gate were displayed on a cytogram by plotting 405 nm fluorescence against 480 nm fluorescence. The 405 vs. 480 plot served as a gate for the ratio (405/480) histogram, preventing unstained cells or cells with low quantities of indo-1 from being displayed (<1% of unstained cells entered the 405 nm vs. 480 nm gate). The 405/480-nm fluorescence ratio of all cells passing through the two gates was calculated digitally and displayed on the ratio histogram. For the analysis of conjugates, the ratio histogram was replaced by a plot of the 405/480-nm fluorescence ratio vs. the PE fluorescence. This graph was also gated by both the forward scatter histogram and the 405 vs. 480 cytogram (<0.1% of unstained cells and <1% of indo-1–loaded cells, without PE, registered as positive for PE). All points displayed on the ratio vs. PE graph contained cells loaded with indo-1, and all points with PE fluorescence represented target cell–CTL conjugates. Linear axes were used for all data. Cells were analyzed at flow rates of 100–200 cells/s.

For kinetic studies, cells were mixed with cytolytic granules and then introduced into the flowing stream. Approximately 30 s elapsed between introduction of samples and excitation of cells by the laser beam. Control cells (those not challenged with granules) were prepared and analyzed immediately before and after test samples. For time course studies that lasted 10 min or less, samples were passed continuously through the flow cytometer and the data were stored on a 15-Mb hard disk for subsequent analysis in segments of 1-min intervals. For time courses >10 min, 10,000 cells were collected on the ratio graph for each time point. Cells were maintained at 37°C through the analysis. Test and control samples were run one after the other at ~2–3 min apart.

Actual values for $[Ca^{2+}]_i$ were calculated from the 405/580 fluorescence ratio (R) using the following equation: $[Ca^{2+}]_i = K_d *S* (R - R_{min})/(R_{max} - R)$, where S, R_{min}, and R_{max} were determined empirically as described (20). The constants were calculated by measuring PMT voltages obtained when free indo-1 was passed through the flow cytometer (28). R values of 0.28, 0.77, 1.25, 2.0, and 2.5 correspond to $[Ca^{2+}]_i$ of ~60, 300, 800, 2,500, and >10,000 nM, respectively.

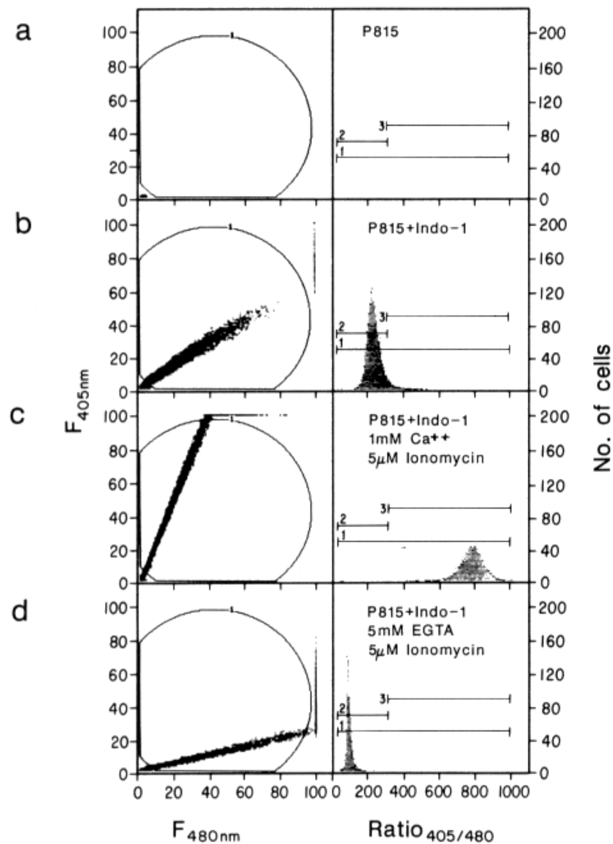

FIGURE 1. Response of the ratio in fluorescence intensities at 405/480 nm to changes in $[Ca^{2+}]_i$. (*a*) Control P815 cells, not loaded with indo-1. (*b*) Indo-1–loaded P815 cells in medium containing 1 mM Ca^{2+} and no ionomycin (mean ratio = 0.77). (*c*) Indo-1–loaded P815 cells in medium containing 1 mM Ca^{2+} and 5 μM ionomycin (mean ratio = 2.5). (*d*) Indo-1–loaded P815 cells in medium containing 5 mM EGTA and 5 μM ionomycin (mean ratio = 0.28). In the left panels, 405-nm fluorescence is plotted against 480-nm fluorescence for individual cells. In the right panels, the same data are presented as histograms of the number of cells vs. the 405/480-nm ratio (multiplied by 300 so as to fit the scale of the graph). Bar *1* in the right panels encompasses the total number of cells used to determine the mean ratio. Bars *2* and *3* are added as an aid in following changes in the mean ratio.

Results and Discussion

Response of the 405/480 nm Fluorescence Ratio to Changes in $[Ca^{2+}]_i$. To determine whether the dye's ester bonds were hydrolyzed by intracellular enzymes, the indo-1–loaded cells were tested with ionomycin, a calcium ionophore, in the presence and absence of extracellular Ca^{2+}. As shown in Fig. 1, the 405/480-nm fluorescence ratio R rose in P815 cells that were incubated in medium containing ionomycin and 1 mM extracellular Ca^{2+}, and decreased when the medium had the extracellular Ca^{2+} replaced by 5 mM EGTA (but was otherwise identical). Before every experiment, target cells were tested in this manner to verify that deesterification of the indo-1 pentaester had occurred.

Sequential Changes of $[Ca^{2+}]_i$ in Target Cells That Interact with Isolated Cytotoxic Granules. When indo-1–loaded P815 cells were incubated with a high concentration of cytotoxic granules (60 μg granule protein/ml), $[Ca^{2+}]_i$ rose in <0.5 min to extremely high levels (>10 μM), and most of the cells rapidly leaked the dye, as indicated by a marked reduction in fluorescence. In those few cells (~10%) that survived, $[Ca^{2+}]_i$ returned to normal levels. To follow the process more closely, the cells were exposed to sublytic, ~10-fold lower concentrations of the granules. Under these circumstances P815 cells responded in <30 s with a pronounced influx of Ca^{2+}; the $[Ca^{2+}]_i$ rose to saturate the intracellular indo-1 dye in <1 min and then decreased promptly, returning within 3 min to the

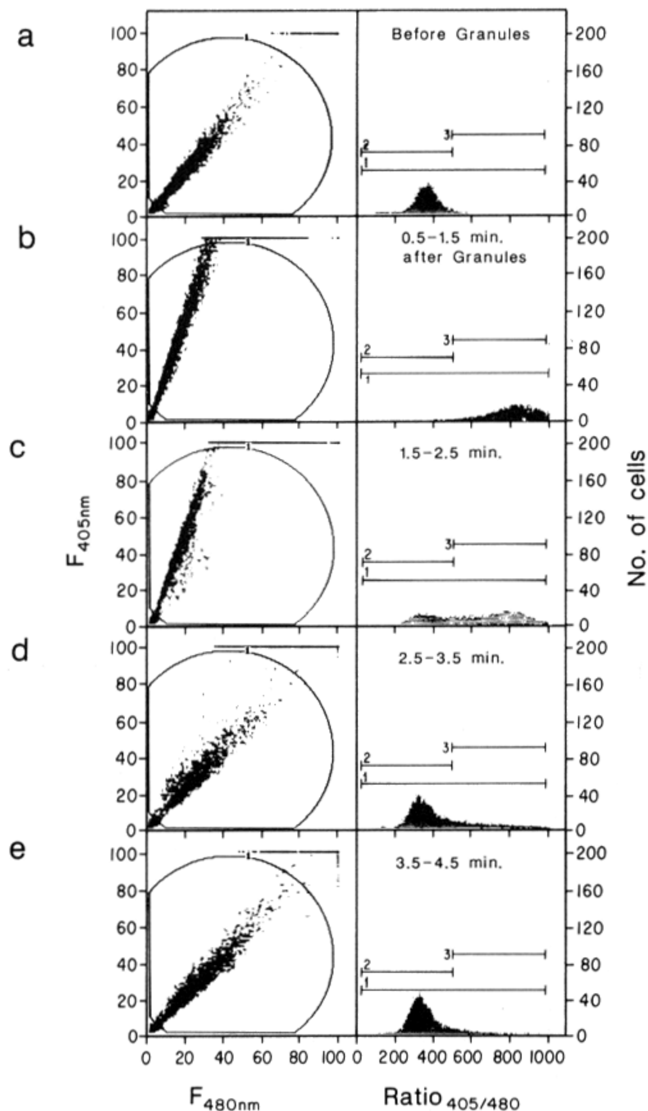

FIGURE 2. Changes in $[Ca^{2+}]_i$ induced by isolated cytotoxic granules. The fluorescence of indo-1-loaded P815 cells was determined before (*a*) and at the indicated times after (*b–e*) the addition of a sublytic quantity of cytolytic granules prepared from 3H2 cells (a cloned murine CTL cell line). This quantity of granules caused <5% ^{51}Cr release from ^{51}Cr-labeled P815 cells. In the left panels, fluorescence at 405 nm is plotted against fluorescence at 480 nm for individual cells. In the right panels, the same data is presented as histograms, plotting the number of cells against 405/480-nm fluorescence ratios, multiplied by 300 (~5,000 cells per ratio histogram). Note the shift in mean ratio with time after addition of granules: the mean ratios were 1.2, 2.6, 2.1, 1.4, and 1.2 for *a–e*, respectively. The total number of cells used to determine these ratios are indicated by bar *1*. For bars *2* and *3* see legend to Fig. 1.

$[Ca^{2+}]_i$ levels of untreated cells (Fig. 2). Other murine tumor cell lines tested (EL4, S49, RDM4, YAC-1) responded similarly; i.e., $[Ca^{2+}]_i$ rose then fell back in <10 min (Fig. 3). The sequential changes suggest that survival of the target cells is associated with the prompt return of the elevated $[Ca^{2+}]_i$ to normal levels; whether the increment in $[Ca^{2+}]_i$ is eliminated by a Ca^{2+} pump or sequestered in organelles is not clear.

$[Ca^{2+}]_i$ Is Not Detectably Increased in CTLs That Interact with Isolated Cytotoxic Granules. The relevance of the granule-mediated increase in $[Ca^{2+}]_i$ to the physiologic destruction of target cells by CTLs is supported by observations on the effects of the isolated cytotoxic granules on CTLs. As was shown previously, cloned CTL cell lines make very poor targets for other cloned CTL cell lines

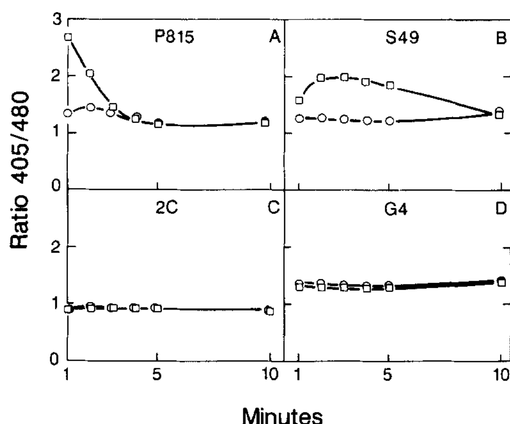

FIGURE 3. Time course of granule-mediated changes in $[Ca^{2+}]_i$ for granule-sensitive (P815 and S49) and granule-resistant cells (CTL cell lines 2C and G4). P815 cells were loaded with indo-1 at 3–5 μM indo-1 AM, and the CTLs were loaded at 1–1.5 μM indo-1 AM. The data in *A* are the same as those in Fig. 2, replotted to show the time course. (○) Cells in absence of granules; (□) cells in the presence of a sublytic quantity (6 μg granule/protein per ml) of granules [see Fig. 2]).

For the CTL cell lines 2C and G4, there is no increase in $[Ca^{2+}]_i$, even when 130 μg granule proteins/ml was added, a dose that is 10-fold higher than required to cause 30% specific ^{51}Cr release from P815 cells. There was also no increase in $[Ca^{2+}]_i$ in the CTL cell lines when the cells were preincubated in 1 mM cyanide and 3 mM Na azide for 2 h before addition of the granules, a procedure that greatly enhances the susceptibility to lysis of all other cells tested (22).

(23, 24) and they are highly resistant to the lytic effects of the isolated cytotoxic granules (22). We therefore determined whether the cytotoxic granules can affect CTL $[Ca^{2+}]_i$. As shown in Fig. 3, where two indo-1–loaded CTL cell lines (2C and G4) are compared with two indo-1–loaded tumor cell lines (P815 and S49), $[Ca^{2+}]_i$ rose promptly and then returned to normal levels in the P815 and S49 cells, but did not change at all in the 2C and G4 cells. There was no change in the CTL $[Ca^{2+}]_i$ even when 20-fold higher concentrations of granules (130 μg granule protein/ml) were used or when the CTLs were preincubated for 2 h in 1 mM KCN and 1 mM NaN_3; the latter conditions deplete cells of ATP and greatly enhance the susceptibility of other cells to lysis by the cytotoxic granules (22). Thus granule-mediated increases in target cell $[Ca^{2+}]_i$ and cytolysis are closely correlated.

Effect of Intact CTLs on Target Cell $[Ca^{2+}]_i$. CTLs adhere to and form transient conjugates with many cells, but usually lyse only those adherent cells whose antigens they recognize. Accordingly, to determine if intact CTLs have the same effect as the isolated granules on target cell $[Ca^{2+}]_i$, we used EL-4 cells as target cells and cells of the cloned 2C cell line as the intact CTLs. The haplotype of the MHC of EL-4 cells is $H\text{-}2^b$; hence these cells are not normally recognized and lysed by 2C cells, which are specific for a class I molecule of the $H\text{-}2^d$ haplotype (L^d, see reference 32). However, by attaching to EL-4 cells an mAb (termed 1B2) that is specific for the antigen-specific receptor of 2C cells, the altered EL-4 cells (termed EL-4/1B2) are converted into targets that are specifically recognized and lysed by 2C cells (29). Thus, by loading EL-4 and EL-4/1B2 cells with indo-1, and labeling 2C cells with biotin and then avidin-PE, it was possible to follow $[Ca^{2+}]_i$ in target cells when they were either unconjugated or conjugated with CTLs in two kinds of conjugates: those in which, as EL-4/1B2 cells, they could be specifically recognized and lysed, and those in which, as unmodified EL-4 cells, they were not recognized or lysed (control conjugates). (The attachment of biotin and avidin-PE to the CTLs did not interfere with their ability to kill their specific target cells [P815 cells, which express L^d; data not shown]; and

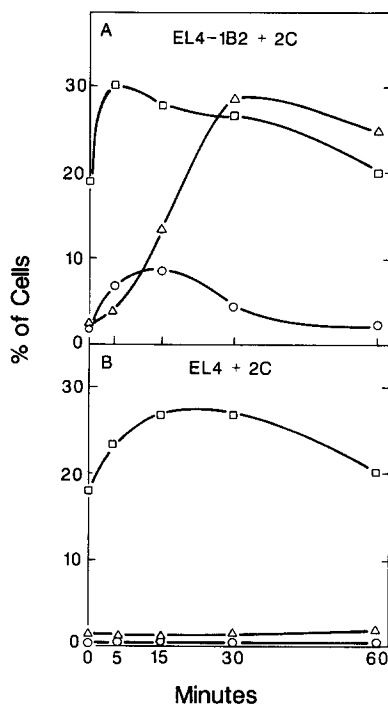

FIGURE 4. Time course of changes in $[Ca^{2+}]_i$ in target cells as they interact with CTLs. EL-4 cells (H-2^b), with (EL4-1B2) and without (EL-4) the attachment of monoclonal anti-receptor antibody 1B2 to their surface, were loaded with indo-1 and incubated with CTLs (2C cell line) that had been biotinylated and labeled with PE-avidin in order to distinguish target cells that were or were not adherent to ("conjugated" with) the CTLs. Shown here are the changes with time in the properties of three populations of target cells: (□) conjugated target cells with normal $[Ca^{2+}]_i$ ($R < 1.25$); (△) unconjugated target cells with elevated $[Ca^{2+}]_i$ ($R > 1.25$); and (○) conjugated target cells with elevated $[Ca^{2+}]_i$ ($R > 1.25$). The 1B2 antibody is specific for the antigen-receptor of 2C cells; EL4-1B2 cells (but not EL-4 cells) are recognized and killed by 2C cells (29). The EL4-1B2 cells with an elevated $[Ca^{2+}]_i$ ($R > 1.25$) had a mean $[Ca^{2+}]_i$ of ~3,000 nM while those with a normal $[Ca^{2+}]_i$ had a mean $[Ca^{2+}]_i$ of ~250 nM. The CTL to target ratio was 3:1.

loading target cells with indo-1 did not interfere with their susceptibility to lysis by intact CTLs or isolated granules [data not shown]).

The time required for $[Ca^{2+}]_i$ to rise in target cells that were recognized by CTLs is indicated in Fig. 4, which plots, as a function of time, the changing proportions of three populations of target cells: (*a*) conjugated target cells with normal $[Ca^{2+}]_i$ (ratio [*R*] of fluorescence intensities at 405/480 nm is < 1.25); (*b*) unconjugated target cells with elevated $[Ca^{2+}]_i$ ($R > 1.25$); and (*c*) conjugated target cells with elevated $[Ca^{2+}]_i$ ($R > 1.25$). The total percentage of conjugated cells was only slightly higher in the specific mixture of 2C plus EL-4/1B2 cells (20–37%, populations a+c; Fig. 4*A*) than in the control mixture of 2C plus EL-4 cells (20–27%, populations a+c; Fig. 4*B*). However, there was an elevated $[Ca^{2+}]_i$ (405/480-nm ratio >1.25) in a strikingly high proportion of EL-4/1B2 cells that interacted with 2C cells. For instance, after 30 min, 33.5% of these target cells had an elevated $[Ca^{2+}]_i$ (populations b+c; Fig. 4*A*), whereas the proportion of various control target cells with an elevated $[Ca^{2+}]_i$ was only 1.3–1.6% (populations b+c; Fig. 4*B*); the controls included EL-4/1B2 cells in the absence of 2C cells and EL-4 cells in the presence and absence of 2C cells. The different times at which the peak frequencies were reached suggest that $[Ca^{2+}]_i$ is initially normal in most of the conjugated, specifically recognized target cells (EL-4/1B2) and then increases markedly in these cells shortly before or after the CTLs dissociate from them. Entirely concordant results were obtained in other experiments, using target cells (P815 cells) that express the L^d antigen and that were recognized and lysed by 2C CTLs. That an increase in target cell $[Ca^{2+}]_i$

occurs has also been suggested by Tirosh and Berke (33) and shown in preliminary observations by fluorescence imaging (34).[2]

Cells with Elevated $[Ca^{2+}]_i$ Undergo Lysis. Is the elevated $[Ca^{2+}]_i$ induced by isolated granules and by intact CTLs a sufficient cause of cell lysis? The Ca^{2+}/Mg^{2+} ionophore A23817 has been shown to be cytolytic if the extracellular medium contains added Ca^{2+} (35). We therefore tested ionomycin, a more selective Ca^{2+} ionophore (25, 26), in the same way. Ionomycin (at 4–5 μM) caused ~70% specific ^{51}Cr release (within 1 h) from ^{51}Cr-labeled YAC-1 cells when the extracellular Ca^{2+} was 5 mM, but it caused no specific ^{51}Cr release when extracellular Ca^{2+} was 0.08 mM or less (data not shown). Similar results were obtained with P815 and RDM4 cells. It appears therefore that a markedly elevated $[Ca^{2+}]_i$ can be cytolytic.

It is notable that CTLs were just as readily lysed as the various tumor cell lines by ionomycin plus extracellular Ca^{2+}. Hence the resistance of CTLs to lysis by isolated granules (and by other CTLs) is not due to an unusual capacity to dispose of excess levels of $[Ca^{2+}]_i$.

DNA Fragmentation. Ca^{2+}/Mg^{2+}-dependent endonucleases that are associated with chromatin in thymocytes (37) and in other mammalian cells (38) have been described. Hence we examined the ability of the isolated granules and ionophores to induce DNA fragmentation. As shown in Fig. 5, in YAC-1 cells (which express L^d and are thus specific targets for 2C cells), the characteristic degradation of DNA into nucleosome-sized fragments was elicited by 2C cells and by isolated granules (at ~6–7 μg granule protein/ml); it was also caused by valinomycin, a K^+ ionophore (at 1.5 and 15 μM) (39), but not by the Ca^{2+} ionophores (ionomycin and A23817) or by gramicidin (a Na^+/K^+ ionophore; see reference 40). Though the DNA fragmentation elicited by valinomycin must obviously result in target cell destruction, during the period of observation (1 h), valinomycin caused only minimal specific release of ^{51}Cr (3 and 12.8% at valinomycin concentrations of 1.5 and 15 μM, respectively). While more ^{51}Cr may well have been released over a longer period of observation, these findings and those made with ionomycin (extensive ^{51}Cr release but no detectable DNA fragmentation) imply that changes in various intracellular cation concentration can affect differently the rates at which diverse manifestations of target cell death become evident.

Concluding Remarks. The recent finding of similarities between the lysis of mutant cell lines by CTLs and corticosteroids (15) can be taken to mean that, like corticosteroids, the cytolytic components of CTLs act primarily not at the target cell membrane but within target cells by activating endogenous autolytic activities (15, 41). However, the cytolytic activity of a glucocorticosteroid receptor–like antigen at the surface of susceptible cells (42). Likewise, the lysis of thymocytes by corticosteroids depends upon extracellular Ca^{2+} (43). These findings and those reported here suggest that the two principal models proposed (41) for target cell destruction by CTLs (i.e., pore formation in cell membranes and activation of an endogenous suicide pathway [see references 15, 41]), are not inconsistent with each other. Both are in accord with the view that the

[2] While this manuscript was being completed, an elegant study by M. Poenie, et al. (46) appeared showing a similar marked increase of target cell $[Ca^{2+}]_i$ by the entirely different approach of fluorescence imaging.

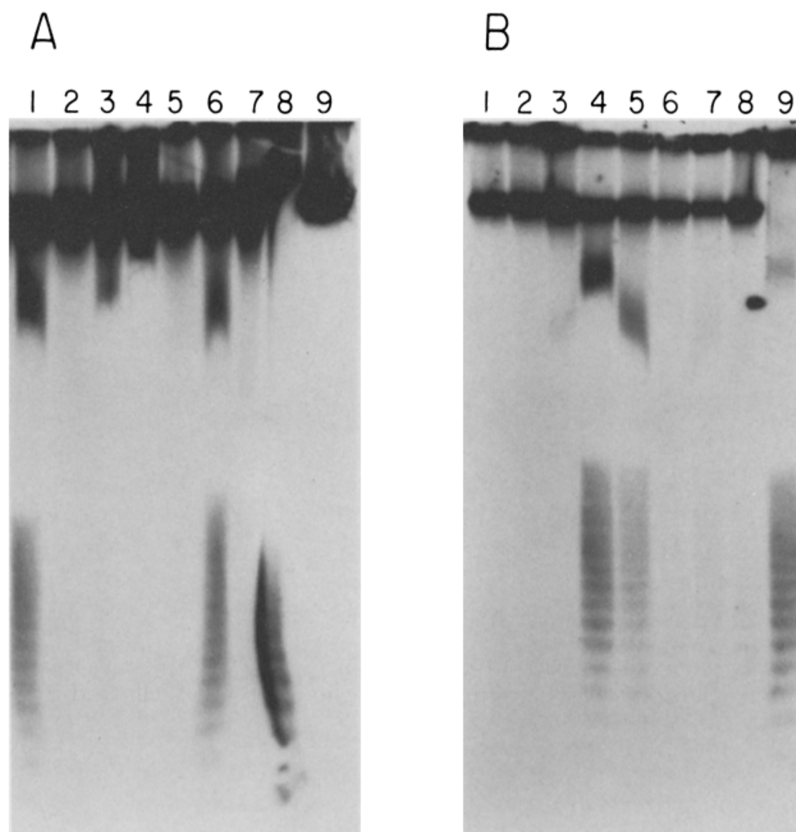

FIGURE 5. DNA fragmentation in YAC-1 cells exposed to CTLs, cytotoxic granules, and some ionophores. YAC-1 cells in which DNA was labeled with [^{125}I]iododeoxyuridine were incubated with CTLs (2C cells that specifically lyse YAC-1) or isolated cytotoxic granules, or various ionophores for 1 h (see Materials and Methods). The cells were then lysed and DNA was precipitated and subjected to electrophoresis in 0.8% agarose gel.

(*A*) Lanes *1* and *6*, granules from the CTL 3H2 cells (6.5 μg granule protein/ml, sufficient to cause ~70% specific lysis of YAC-1 cells in a 4-h ^{51}Cr-release assay); lanes *2* and *7*, buffered salt, control medium (ECBS) for lanes *1–6*; lane *3*, 1 μM ionomycin; lane *4*, 10 μM ionomycin; lane *5*, 0.65 μg 3H2 granule protein/ml; lane *8*, 2C cells (E/T ratio = 10:1); lane *9*, K medium as control for lane *8*.

(*B*) Lane *1*, 10 μM A23817; lane *2*, 10 μM ionomycin; lane *3*, 30 μM gramicidin; lane *4*, 15 μM valinomycin; lane *5*, 1.5 μM valinomycin; lane *6*, 0.15 μM valinomycin; lane *7*, "relaxation" buffer as control for lanes *8* and *9*; lane *8*, 6.5 μg/ml 3H2 granule protein (inactivated by repeated cycles of freezing and thawing); lane *9*, granules (from CTL G4 cells), sufficient to cause ~70% lysis of YAC-1 cells.

cytolytic components of CTLs act primarily on the target cell's membrane, causing changes that are functionally equivalent to the formation of nonspecific ion channels. Cell lysis may well be a consequence of the ensuing sustained increase in target cell $[Ca^{2+}]_i$, and cleavage of DNA into nucleosome-sized fragments, which is most likely due to activation of target cell endonuclease(s), is probably also the result of changes in intracellular cation concentrations.

Though this study is focused on the lysis of target cells by cytolytic granules isolated from cloned CTL cell lines, some recent observations indicate that there

may also be granule-independent mechanisms by which some CTLs lyse some target cells (44). Thus, extracellular Ca^{2+} is not required for the lysis of certain target cells by some CTLs (33) and some actively cytotoxic human CTLs appear to lack the components that are characteristically found in cytolytic granules (perforin and high levels of T cell-specific serine esterases; see reference 45). Accordingly, it will be of considerable interest to determine whether or not DNA fragmentation and the increase in target cell $[Ca^{2+}]_i$ described here are also evident in target cells when they are lysed by those CTLs that appear to lack or not to secrete cytotoxic granules.

Summary

To investigate the destruction of target cells by murine CTLs, we examined intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and DNA fragmentation in target cells. Changes in $[Ca^{2+}]_i$ were followed by flow cytometry by loading the cells with indo-1, a Ca^{2+}-binding fluorescent dye, and determining the ratio of fluorescence intensities at 405 nm (emission maximum for Ca^{2+}-bound dye) over 480 nm (emission maximum for the free dye). Within minutes after interacting with the cytolytic granule fraction that had been isolated from CTLs, $[Ca^{2+}]_i$ in target cells was strikingly increased. A pronounced increase in $[Ca^{2+}]_i$ was also observed in target cells when they were specifically recognized by intact CTLs. Since ionomycin, a Ca^{2+} ionophore, caused a similar increase in $[Ca^{2+}]_i$ and lysed cells (provided that extracellular Ca^{2+} was present), it appears that a sustained high level of $[Ca^{2+}]_i$ is cytolytic. In contrast with other cells, CTLs, which have been shown to be refractory to granule-mediated lysis and to be poor targets for other CTLs, did not manifest an elevation in $[Ca^{2+}]_i$ when they were similarly loaded with indo-1 and treated with isolated granules. The characteristic cleavage of target cell DNA into nucleosome-sized fragments was also induced by isolated granules as well as by valinomycin, a K^+ ionophore, but not by ionomycin. The results support the view that lysis of most target cells by cloned CTLs is due primarily to target cell membrane changes that are fundamentally equivalent to the formation of nonspecific ion channels. The resulting large increase in $[Ca^{2+}]_i$ is probably responsible for target cell lysis; and changes in intracellular ion concentrations also appear to be responsible for DNA fragmentation, probably by activating endogenous target cell endonucleases.

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References

1. Podack, E. R., and P. J. Konigsberg. 1984. Cytolytic T cell granules. *J. Exp. Med.* 160:695.
2. Podak, E. R., J. D. Young, and Z. A. Cohn. 1985. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T cell granules. *Proc. Natl. Acad. Sci. USA.* 82:8629.
3. Young, J. D., Z. A. Cohn, and E. R. Podack. 1986. The ninth component complement

and the pore-forming protein (perforin 1) from cytotoxic T cells: structural, immunological, and functional similarities. *Science (Wash. DC)*. 233:184.

4. Young, J. D., C. F. Nathan, E. R. Podack, M. A. Palladino, and Z. A. Cohn. 1986. Functional channel formation associated with cytotoxic T cell granules. *Proc. Natl. Acad. Sci. USA*. 83:150.
5. Henkart, P. A., P. J. Millard, C. W. Reynolds, and M. P. Henkart. 1984. Cytolytic activity of purified cytoplasmic granules from rat large granular lymphocyte tumor. *J. Exp. Med.* 160:75.
6. Henkart, P. A. 1985. Mechanisms of lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* 3:31.
7. Pasterack, M. S., and H. N. Eisen. 1985. A novel serine esterase expressed by cytotoxic T lymphocytes. *Nature (Lond.)*. 314:743.
8. Pasternack, M. S., C. R. Verret, M. A. Liu, and H. N. Eisen. 1986. Serine esterase in cytolytic T lymphocytes. *Nature (Lond.)*. 322:740.
9. Masson, D., M. Nabholz, C. Estrade, and J. Tschopp. 1986. Granules of cytolytic T lymphocytes contain two serine esterases. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1595.
10. Young, J. D., L. G. Leong, C. C. Liu, A. Damiano, D. A. Wall, and Z. A. Cohn. 1986. Isolation and characterization of a serine esterase from cytolytic T cell granules. *Cell.* 47:183.
11. Masson, D., and J. Tschopp. 1987. A family of serine esterases in lytic granules of cytolytic T lymphocytes. *Cell.* 49:679.
12. Russell, J. H., V. R. Masakowski, and C. B. Dobos. 1980. Mechanisms of immune lysis. I. Physiological distinction between target cell death mediated by cytotoxic T lymphocytes and antibody plus complement. *J. Immunol.* 124:1100.
13. Russell, J. H., V. Masakowski, T. Rucinsky, and G. Phillips. 1982. Mechanisms of immune lysis. III. Characterization of the nature and kinetics of the cytotoxic T lymphocyte-induced nuclear lesion in the target. *J. Immunol.* 128:2087.
14. Cohen, J. J., and R. C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* 132:38.
15. Ucker, D. S. 1987. Cytotoxic T lymphocytes and glucocorticoids activate an endogenous suicide process in target cells. *Nature (Lond.)*. 327:62.
16. Deutsch, C., and M. A. Price. 1982. Cell calcium in human peripheral blood lymphocytes and the effect of mitogen. *Biochim. Biophys. Acta.* 687:211.
17. Campbell, A. K., R. A. Daw, M. B. Hallet, and J. P. Luzio. 1981. Direct measurement of the increase in intracellular free calcium ion concentration in response to the action of complement. *Biochem. J.* 194:551.
18. Gorecka-Tisera, A. M., K. W. Snowdowne, and A. B. Borle. 1986. Implications of a rise in cytosolic free calcium in the activation of RAW-264 macrophages for tumor cell killing. *Cell. Immunol.* 100:411.
19. Farber, E. L. 1982. Biology of disease. Membrane injury and calcium homeostasis in the pathogenesis of coagulative necrosis. *Lab. Invest.* 47:114.
20. Grynkiewicz, G., M. Poenie, and R. Tsien. 1985. A new generation of Ca^{++} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440.
21. Rabinovitch, P. S., C. H. June, A. Grossman, and J. A. Ledbetter. 1986. Heterogeneity among T cells in intracellular free calcium responses after mitogenic stimulation with PHA or anti-CD3. Simultaneous use of Indo-1 and immunofluorescence with flow cytometry. *J. Immunol.* 137:952.
22. Verret, C. R., A. A. Firmenich, D. M. Kranz, and H. N. Eisen. 1987. Resistance of cytotoxic T lymphocytes to the lytic effects of their toxic granules. *J. Exp. Med.* 166:1536.

23. Luciani, M. F., M. S. Brunet, F. Denizot, and P. Golstein. 1986. Self sparing of long-term in vitro cloned or uncloned cytotoxic T lymphocytes. *J. Exp. Med.* 164:962.
24. Kranz, D., and H. N. Eisen. 1987. Resistance of cytotoxic T lymphocytes to lysis by a clone of cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 84:3375.
25. Liu, C., and T. E. Hermann. 1978. Characterization of ionomycin as a calcium ionophore. *J. Biol. Chem.* 253:5892.
26. Pasternack, M. S., M. V. Sitkovsky, and H. N. Eisen. 1983. The site of action of Nα Tosyl-L-Lysyl chloromethylketone (TLCK) on cloned cytotoxic T lymphocytes. *J. Immunol.* 131:2477.
27. Reilly, E. B., D. M. Kranz, S. Tonegawa, and H. N. Eisen. 1986. A functional γ gene formed by the known γ gene segments is not necessary for antigen-specific responses of some murine cytotoxic T lymphocytes. *Nature* (*Lond.*). 321:878.
28. American Type Culture Collection Catalogue of Cell Lines and Hybridomas. 5th ed. 1985. Rockville, MD.
29. Kranz, D. M., S. Tonegawa, and H. N. Eisen. 1984. Attachment of anti-receptor antibody to non-target cells renders them susceptible to lysis by a clone of cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 81:7922.
30. Imboden, J. B., A. Weiss, and J. D. Stobo. 1985. Transmembrane signalling by the T3-antigen receptor complex. *Immunol. Today.* 6:328.
31. Allbritton, N. L. 1987. The role of calcium in the destruction of target cells by cytotoxic T cells. Ph.D. Thesis. Massachusetts Institute of Technology, Cambridge, MA. 239 pp.
32. Kranz, D. M., D. H. Sherman, M. V. Sitkovsky, M. S. Pasternack, and H. N. Eisen. 1984. Immunoprecipitation of cell surface structures of cloned cytotoxic T lymphocytes by clone-specific antisera. *Proc. Natl. Acad. Sci. USA.* 81:573.
33. Tirosh, R., and G. Berke. 1985. T-lymphocyte-mediated cytolysis as an excitatory process of the target. I. Evidence that the target cell may be the site of Ca^{++} action. *Cell. Immunol.* 95:113.
34. Tsien, R. Y., and M. Poenie. 1986. Fluorescence ratio imaging: a new window into intracellular tonic signaling. *Trends in Biochem. Sci.* 11:450.
35. Kaiser, N., and I. S. Edelman. 1978. Calcium dependence of ionophore A23817-induced lymphocyte cytotoxicity. *Cancer Res.* 38:3599.
36. Nakashima, R. A., R. S. Dordick, and K. D. Garlid. 1982. On the relative roles of Ca^{++} and Mg^{++} in regulating the endogenous K^+/H^+ exchanger of rat liver mitochondria. *J. Biol. Chem.* 257:12540.
37. Nakamura, M., Y. Sakaki, N. Watanabe, and Y. Takagi. 1981. Purification and characterization of the Ca^{++} plus Mg^{++} dependent endodeoxyribonuclease from calf thymus chromatin. *J. Biochem.* 89:143.
38. Hewish, D. R., and L. A. Burgoyne. 1973. The calcium dependent endonuclease activity of isolated nuclear preparations. Relationships between its occurrence and the occurrence of other classes of enzymes found in nuclear preparations. *Biochem. Biophys. Res. Commun.* 52:475.
39. Pressman, B. C. 1976. Biological applications of ionophores. *Annu. Rev. Biochem.* 45:501.
40. Anderson, O. S. 1984. Gramicidin channels. *Annu. Rev. Physiol.* 46:531.
41. Golstein, P. 1987. Cytotoxic T-cell melodrama. *Nature* (*Lond.*). 327:12.
42. Gametchu, B. 1987. Glucocorticoid receptor-like antigen in lymphoma cell membranes: correlation to cell lysis. *Science* (*Wash. DC*). 236:456.
43. Kaiser, N., and I. S. Edelman. 1977. Calcium dependence of glucocorticoid-induced lymphocytolysis. *Proc. Natl. Acad. Sci. USA.* 74:638.

44. Berke, G. 1987. An appraisal of some current thoughts on cytolytic T-lymphocyte killing mechanisms. *Ann. Inst. Pasteur Immunol.* 138:289.
45. Ferguson, W. S., C. R. Verret, E. B. Reilly, M. J. Iannini, and H. N. Eisen. 1988. Serine esterase and hemolytic activity of cytotoxic cloned human T lymphocytes. *J. Exp. Med.*
46. Poenie, M., R. Y. Tsien, and A.-M. Schmitt-Verhulst. 1987. Sequential activation and lethal hit measured by $[Ca^{++}]_i$ in individual cytolitic T cells and targets. *EMBO (Eur. Mol. Biol. Organ.). J* 6:2233.