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# CD4+ T LYMPHOCYTE-INDUCED TARGET CELL DETACHMENT

## A Model for T Cell-Mediated Lytic and Nonlytic Inflammatory Processes[1]

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**We have been exploring the hypothesis that T lymphocytes have the potential to mediate immune damage through nonlytic disruption of tissue organization. In this report, we have examined the ability of purified, primary cultures of alloreactive CD4+ T cells to mediate Ag-specific target cell detachment and/or lysis of L cell lines transfected with MHC class II determinants. Using this model, we demonstrate that: 1) MHC class II-specific CD4+ T cells can cause detachment as a distinct event of the E:T interaction, although the pathways or mechanisms involved appear to be different from those utilized by MHC class I-specific CD8+ T cells; 2) detachment and lysis by CD4+ T cells are distinct activities that involve different functional requirements: 3) CD4+ T cell-induced detachment is initiated by direct cell-cell interaction, independent of TNF-α/β; 4) CD4+ T cell-mediated lysis can be accomplished by TNF-α/β-dependent and independent pathways; and 5) the nature of a particular target cell response to alloreactive CD4+ T cell attack reflects its intrinsic susceptibility to one or more potential effector mechanisms.**

Many in vitro studies devoted to understanding the nature and functional ramifications of T lymphocyte-target interactions use individual target cells in suspension. However, in vivo, such immunologic reactions also involve T cells responding to targets that are bound to each other or to an extracellular matrix. Thus, the capacity of T cells, particularly CD8+ CTL, to react with such types of targets is strongly exemplified by their interaction with and rejection of allografts or solid tumors (1–3). To this end, we have recently developed an in vitro model to explore the functional consequences that result from T cell-tissue or tumor interaction (4).

We have previously demonstrated that an early event in the interaction between the CD8+ CTL and an Ag-bearing adherent target is the rapid loss of target cell adhesion to its substratum (4). Detachment normally precedes cell death, but can also occur independently from the lytic event, a finding based on experiments that examined the sensitivity of CD8+ CTL to extracellular $Ca^{2+}$, protein kinase C activation/depletion and inhibition by mAb reactive with functionally relevant cell surface molecules (4, 5). Furthermore, these data suggest that detachment, in contrast to lysis, can be initiated through multiple effector pathways. It is important to emphasize, however, that under physiologic conditions, activation of the T cell Ag receptor is required to initiate both reactivities (5).

The observation that detachment is an early and distinct event of the CD8+ CTL-target cell interaction introduces the possibility that this function could be initiated by other lymphocyte subpopulations, such as CD4+ T cells. In contrast to CD8+ CTL, CD4+ T cells generally respond to, or are restricted by, foreign Ag bound to MHC class II molecules (6). Although CD4+ T cells have been mainly associated with immune regulation, recent studies implicate subsets of CD4+ T cells that have the capacity to mediate effector functions, such as delayed type hypersensitivity and target cell lysis (6, 7). In some (8–11), but not all (12–15) cases, CD4+ T cell-mediated lysis has been attributed to diffusable, T cell-derived, soluble products, including TNF-α and lymphotoxin (TNF-β).

In this report, we have explored the hypothesis that target detachment reflects a general result of a "T cell-target cell" interaction. The experimental paradigm examines the capacity of purified, primary cultures of alloreactive CD4+ T cells to stimulate detachment and/or lysis of L cell lines transfected with MHC class II determinants. Using this model, we demonstrate that: 1) CD4+ T cells can mediate detachment as a distinct consequence of the E:T interaction, although involving different mechanisms or metabolic pathways than those of CD8+ CTL; 2) L cell detachment, but not lysis requires target cell protein synthesis; and 3) CD4+ T cells possess multiple effector mechanisms, and can initiate detachment through direct cell-cell interaction, independent of TNF-α/β; yet, can mediate lysis via both TNF-α/β-dependent and -independent pathways.

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### MATERIALS AND METHODS

#### *Reagents*

The IgG anti-CD8 (53-6.72) (16), anti-CD4 (GK1.5) (17), and anti-I-$A^d$ (MKD6) (18) hybridomas were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and affinity purified for use in the functional assays and for flow cytometry. The IgG anti-CD3 hybridoma (145-2C11) (19) was kindly provided by Dr. Jeffrey A. Bluestone (University of Chicago, Chicago, IL). The IgM anti-CD8 hybridoma (3.155) (20) was obtained from the ATCC and used directly as culture supernatant in combination with rabbit C (Low-

Tox-M; Accurate Chemical Co., Westbury, NY) for negative selection and subsequent isolation of CD4+ T cells. Murine rTNF (sp. act. of $3.0 \times 10^7$ U/mg) and TNF-$\alpha/\beta$ cell-free supernatants (from F128 cell line and F128.G5 clone) were generously provided by Genentech, Inc. (South San Francisco, CA) and Dr. Nancy H. Ruddle (Yale University, New Haven, CT), respectively. mAb to murine TNF-$\alpha$ + TNF-$\beta$ (TN3-19.12) and IFN-$\gamma$ (H22) (21, 22) were kindly provided by Dr. Robert D. Schreiber (Washington University, St. Louis, MO). CHX[3], EM, and AD were obtained from the Sigma Chemical Co. (St. Louis, MO).

*Animals.* All experiments were performed using male CBA/J (H-$2^k$) and DBA/2J (H-$2^d$) mice obtained from The Jackson Laboratory (Bar Harbor, ME).

*Cell lines.* The adherent cell lines included L929 (H-$2^k$) and the transfected L cell lines, RT 1.1.12 (Ia$^-$ control), RT 2.3.3H-D6 (I-A$^d$) and RT 4.15HP (I-A$^k$) (23), kindly provided by Dr. Ronald N. Germain (National Institutes of Health, Bethesda, MD). The transfectants, maintained in culture medium containing HAT (15 $\mu$g/ml hypoxanthine, 0.2 $\mu$g/ml aminopterin, and 5 $\mu$g/ml thymidine), were derived from the thymidine kinase negative L cell subline, DAP.3 (23). The suspension grown cell lines included P815 (H-$2^d$), and A20.1.11 (H-$2^d$), an MHC class I and II Ag-bearing B lymphoma (24) kindly provided by Dr. Thomas J. Braciale (Washington University, St. Louis, MO). The expression of MHC class II Ag on the transfected L cell lines was measured by an enzyme immunoassay and flow cytometry. Flow cytometric analysis consistently revealed the alloAg transfected L cells to be 100% positive for the appropriate MHC class II molecule.

### *Effector Cells*

All experiments were performed using alloreactive T cells produced in primary MLC. Such MLC-derived alloimmune T cells were generated in 5-day bulk cultures, then isolated by Ficoll-Hypaque density gradient centrifugation and used as effectors. For functional analysis of MHC class II Ag-specific CD4+ T cells, contaminating macrophages and B cells were removed from the resting splenocyte population before the MLC by nylon wool fractionation and CD8+ T cells were eliminated by mAb + C lysis, respectively. Briefly, adherent cell-depleted suspensions were treated with the 3.155 hybridoma (60 min at 4°C), followed by incubation with C (60 min at 37°C). Viable cells were recovered by density gradient fractionation, and the purity of the cell population assessed by flow cytometry before and after the MLC. MHC class I Ag-specific CD8+ T cell activity was examined from H-$2^d$ anti-H-$2^k$ lymphocytes in unfractionated MLC populations. Because their functional activity and Ag specificity are similar to "classical" CD8+ CTL, we will refer to these unfractionated MLC cells as CTL effectors. Ag specificity for both CD4+ T cells and CTL effectors was confirmed in detachment/lytic assays using various targets.

### *Surface Marker Analysis*

Cells were mixed with the appropriate primary mAb (anti-CD3, CD4, CD8, I-A$^d$), followed by incubation with FITC-conjugated, affinity-purified goat anti-rat, mouse (Southern Biotechnology, Birmingham, AL) or hamster Ig (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). After staining, preparations were fixed in 1% paraformaldehyde and evaluated by flow cytometry using a FACS 440 (Becton Dickinson, Mountain View, CA) for the percent positive cells and the mean channel of fluorescence.

### *Treatment of Effectors or Targets with CHX, EM, or AD*

Effectors (CTL, CD4+ T cells) and targets (transfected L cells, A20.1.11) were exposed to each drug at concentrations optimal for inhibition of protein or RNA synthesis by 90 to 98%. Protein or RNA synthesis was measured by incorporation of $^{35}$S-methionine (>800 Ci/mM; ICN Radiochemicals, Irvine, CA) or [5-$^3$H]-uridine (>20 Ci/mM; New England Nuclear (NEN, Boston, MA), respectively, into TCA-precipitable macromolecules, via a modification of the method previously described (25). Thus, based on dose-response experiments, CHX, EM, and AD were used in the functional assays at 10, 3 to 5, and 1 $\mu$M, respectively. In addition, effectors and targets were pretreated with EM for 60 min at 37°C, washed to remove residual drug, and then assayed. Control preparations included cells which were incubated in the absence of drug, but containing drug vehicle (≤0.01% DMSO) and/or exposed to the drug, but washed immediately.

[3] Abbreviations used in this paper: CHX, cycloheximide; EM, emetine; AD, actinomycin-D.

### *Detachment and Lytic Assays*

The method used to measure target detachment is essentially as described previously (4, 5). L cell lines were seeded at $2 \times 10^4$ cells/well in sterile 96-well, flat-bottomed microtiter plates 16 to 24 h before radiolabeling for 90 min with either chromium-51 ($Na_2{}^{51}CrO_4$; > 400 Ci/g, used at 250 $\mu$Ci/ml) (NEN) or $^{125}$IUdR (2200 Ci/mM used at 20 $\mu$Ci/ml) (ICN). Radiolabeled monolayers were washed, incubated with effectors ($4 \times 10^5$ cells/well), in the absence or presence of drug, at 37°C for up to 18 h. After culture, each well was subjected to six vigorous cycles of negative and positive pressure by a Pasteur pipette on the fluid to detach loosely bound target cells. The cell suspension was collected, mixed with 1 ml PBS with 1 mM EDTA (for $^{51}$Cr) or 1 ml PBS with 1 mM EDTA containing 0.3% Triton X-100 (for $^{125}$IUdR), centrifuged and divided into two tubes. Each tube was counted and the percent specific release for both total (i.e., supernatant + pellet) and soluble (i.e., supernatant only) radioactivity determined (4). In the data, however, percent specific release of total radioactivity, which reflects detachment and lysis, will be referred to as target cells "detached," whereas soluble radioactivity will be referred to as target cells "lysed" or "nuclear damage." Spontaneous release of $^{51}$Cr equals the radioactivity released by target cells in the absence of effectors, and ranged from 15 to 18% and 30 to 38% for both total and soluble release in 6 and 18 h assays, respectively. Spontaneous release of $^{125}$IUdR, for up to 18 h, was <10% for both total and soluble release. In all instances, none of the reagents significantly altered the background release. Maximum release of $^{51}$Cr or $^{125}$IUdR equals the radioactivity released by targets exposed to 0.5 N NaOH. Results are expressed as the mean ± SEM of triplicate cultures, and are representative of two to four experiments with similar results.

### *TNF-$\alpha/\beta$, Bystander and Supernatant Transfer Assays*

*TNF-$\alpha/\beta$.* Radiolabeled ($^{51}$Cr or $^{125}$IUdR) target monolayers were exposed to rTNF-$\alpha$ or TNF-$\alpha/\beta$-containing supernatants at various concentrations (0.1 to 5000 U/ml) for up to 18 h, in the absence and presence of a metabolic inhibitor (AD, CHX, or EM) (21, 26, 27). After incubation, the percent specific release for both total and soluble radioactivity was determined as above.

*Bystander assays.* CD4+ T cells ($4 \times 10^5$/well) were assayed against the indicated radiolabeled target monolayer, in the absence and presence of the indicated stimulator cells ($4 \times 10^4$/well) added in suspension. In parallel cultures, lytic activity was assessed against $^{51}$Cr-labeled stimulator cells, in the presence of unlabeled target monolayers.

*Supernatant transfer.* CD4+ T cells ($4 \times 10^5$/well) were cultured without and with various unlabeled stimulator cells ($4 \times 10^4$/well) for 6 h in 200 $\mu$l of a 96-well, flat-bottomed microtiter plate (Flow Laboratories, McLean, VA). Additionally, targets were incubated in the absence of effectors. A total of 100 $\mu$l of cell-free supernatant fluid was collected from each well and mixed immediately with 100 $\mu$l of culture medium containing fresh, $^{51}$Cr or $^{125}$IUdR-labeled L929 monolayers. Assays were performed in the absence and presence of EM (5 $\mu$M), terminated after 18 h, and the percent specific release for total and soluble radioactivity determined as above.

## RESULTS

*CD4+ T cells can mediate detachment and lysis of MHC class II Ag-bearing targets.* In this study, we explored the hypothesis that T cell-induced target detachment is not restricted to CD8+ CTL and, therefore, can be expressed by other lymphocyte subpopulations, such as MHC class II-specific CD4+ T lymphocytes. To that end, we examined the ability of purified, alloreactive CD4+ T cells to mediate detachment using L cell lines transfected without (Ia$^-$) or with (I-A$^d$, I-A$^k$) MHC class II molecules.

Resting, nylon wool-purified T lymphocytes from CBA/J (H-$2^k$) mice (CD8+, 30 to 40%; CD4+, 60 to 70%) are enriched in CD4+ T cells by negative selection using anti-CD8 mAb+C (CD8+, ≤1%; CD4+, ≥90%). Both untreated and treated fractions are mixed in culture against irradiated DBA/2J (H-$2^d$) splenocytes. After the primary MLC, both fractions are isolated, analyzed by flow cytometry and used as effectors in the functional assays. The purity of the anti-CD8+C-treated population after the MLC is routinely ≥95% CD4+ and <1% CD8+. Table I (*top half*,

TABLE I

*Cell surface molecules involved in T cell-mediated detachment and lysis*

| Effector[a] | Treatment[b] | Percent Specific Release[c] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 H | | 6 H | | 18 H | |
| | | Detached | Lysed | Detached | Lysed | Detached | Lysed |
| CD4+ | None | NT[d] | | 39 ± 3 | 13 ± 2 | 82 ± 7 | 43 ± 4 |
| | Anti-CD8 | | | 43 ± 1 | 14 ± 2 | 88 ± 7 | 42 ± 3 |
| | Anti-CD4 | | | 6 ± 0 | 4 ± 1 | 35 ± 5 | 18 ± 3 |
| | Anti-I-A$^d$ | | | 15 ± 3 | 7 ± 0 | 56 ± 5 | 22 ± 2 |
| CTL | None | 60 ± 1 | 23 ± 0 | 79 ± 2 | 45 ± 2 | NT | |
| | Anti-CD8 | 23 ± 1 | 9 ± 1 | 49 ± 4 | 21 ± 2 | | |
| | Anti-CD4 | 58 ± 4 | 25 ± 2 | 75 ± 0 | 39 ± 1 | | |
| | Anti-I-A$^d$ | 57 ± 6 | 22 ± 3 | 87 ± 4 | 42 ± 1 | | |

[a] For generation of Ag-specific CD4+ T cells (H-2$^k$ anti-H-2$^d$), CD4+ T lymphocytes from CBA/J splenocytes were purified before the MLC and then stimulated in culture against irradiated DBA/2J splenocytes. Ag-specific CTL effectors (H-2$^d$ anti-H-2$^k$) were generated in culture by stimulation of DBA/2J splenocytes with irradiated CBA/J splenocytes.

[b] mAb = 10 μg/ml.

[c] Effectors were assayed against the transfected I-A$^d$ L cells for the indicated times, and then the percent specific release for detached and lysed targets determined. Percent CD4+ T cell activity against the transfected Ia$^-$ L cells after 6 h: 6 ± 0 (detached), 5 ± 0 (lysed); and 18 h: 16 ± 1 (detached), 8 ± 2 (lysed). Similar results were observed against the irrelevant I-A$^k$ L cells.

[d] NT, Not tested.

*first line*) illustrates the nonlytic and lytic responses of purified, alloreactive CD4+ T cells. We demonstrate that purified CD4+ T cells have the capacity to initiate detachment and lysis as separable events against the specific (I-A$^d$), but not irrelevant (Ia$^-$, I-A$^k$) target. Furthermore, detachment and lysis by purified CD4+ T cells proceeds at a dramatically slower rate than that of CTL effectors, as assessed using the same target (Table I: *top half, first line* vs *bottom half, first line*). Results similar to that seen with purified CD4+ T cells were also observed using unfractionated cultures (data not shown), suggesting that this particular MHC class II-mediated T cell function is not masked or suppressed by contaminating T cell subsets.

To determine if detachment by CD4+ T cells results from a mechanism or pathway similar to that of CD8+ CTL, we subsequently examined the role of specific effector and target cell surface molecules using mAb (Table I). In previous work (5), we demonstrated that CD8 plays an important role in CD8+ CTL-induced detachment and lysis. In an analogous fashion, we examine here the effects of anti-CD4 and show that this mAb specifically blocks the activity of CD4+ T cells, but not of CTL effectors (Table I). Conversely, anti-CD8 inhibits the activity of CTL effectors, but not CD4+ T cells. The observation that anti-CD8 has no effect on CD4+ T cells also precludes the possibility that any contaminating CD8+ T cells (which are <1% by flow cytometric analysis) contribute to this particular MHC class II-mediated T cell activity. In addition to anti-CD4, we show that anti-I-A$^d$ inhibits CD4+ T cell activity (Table I), suggesting that target cell-associated I-A$^d$ is requisite for the functional response. In contrast, anti-I-A$^d$ fails to block the activity of CTL effectors, which is anticipated because their function is generally MHC class I, but not class II specific (1, 2). Thus, detachment and lysis by CD4+ T cells require the participation of cell surface CD4 and I-A$^d$ molecules.

*Role of protein and RNA synthesis.* Earlier work established that CD8+ CTL-mediated detachment (5) and lysis (1–3) do not require de novo protein synthesis. Thus, we sought to determine whether the mechanism used by CD4+ T cells was similar to that of conventional CD8+ CTL. Although the functional result is similar, the time-course suggested that the mechanisms employed by CD4+ T cells and CD8+ CTL may be different. Furthermore, because CD4+ T lymphocytes are a prominent lymphokine-producing cell type (6, 7), we reasoned that protein synthetic capacity might be necessary for the synthesis of proteins associated with this activity. Therefore, we first examined the effects of CHX (28), and demonstrate that this drug strongly inhibits the activity of CD4+ T cells, but not of CTL effectors (Table II). CHX blocks protein synthesis in both effector and target populations by 90 to 95%, and does not significantly alter their viability or target cell expression of the I-A$^d$ Ag, as assessed by flow cytometry (mean channel fluorescence after 18 h ± CHX: control = 156 (2° Ab alone, 51); CHX = 161 (2° Ab alone, 44)). In contrast to CHX, AD has no effect in the assay at times ≤6 h (control: detached = 28 ± 5, lysed = 6 ± 1; AD: detached = 34 ± 2, lysed = 6 ± 0), which indicates that neither the effector nor target population requires the induction of new gene expression. Similarly, AD did not inhibit CD4+ T cell-mediated lysis of A20.1.11 (control = 28 ± 1; AD = 33 ± 1 at 20/1 E:T ratios) at times ≤6 h. Longer incubation in AD inhibited the various activities, but this effect is likely to be secondary to inhibition of protein synthesis.

*Protein synthesis is required by both effector and adherent target for detachment and its subsequent lysis.* Inasmuch as CHX is a reversible inhibitor of protein synthesis (28), it is uncertain whether the drug selectively alters the function of the CD4+ T cell, the target cell or both populations. Thus, to discern among these possibilities, we examined the effects of EM, an irreversible inhibitor of eukaryotic protein biosynthesis (28), on both CD4+ T cell and target responses (Table II). Additionally, CTL effectors are examined in parallel, and as with the previous experiments, the same transfected L cell line is used as the specific target for both effector groups. In contrast to that seen with CTL effectors, EM potently inhibits CD4+ T cell function (Table II). We show that EM exerts its effects on both the CD4+ T cell and the target, because pretreatment of either population inhibits the activity (Table II). Thus, these data not only strengthen the hypothesis that protein synthesis is required for CD4+ T cell-induced detachment and lysis, but also demonstrate that a requirement for protein synthesis resides within both the effector and target populations.

TABLE II

*Role of protein synthesis in T cell-mediated detachment and lysis*

| Effector[a] | Treatment[b] | Percent Specific Release[c] | | | |
|---|---|---|---|---|---|
| | | 6 H | | 18 H | |
| | | Detached | Lysed | Detached | Lysed |
| CD4+ | None | 27 ± 3 | 8 ± 1 | 73 ± 4 | 37 ± 2 |
| | CHX | 8 ± 1 | 3 ± 1 | 22 ± 2 | 11 ± 1 |
| | EM: | | | | |
| | in assay | 9 ± 1 | 5 ± 1 | 18 ± 2 | 7 ± 2 |
| | EC pretreat | 12 ± 1 | 7 ± 1 | 37 ± 2 | 20 ± 1 |
| | TC pretreat | 6 ± 0 | 2 ± 0 | 28 ± 3 | 10 ± 2 |
| CTL | None | 80 ± 1 | 53 ± 3 | 99 ± 1 | 78 ± 3 |
| | CHX | 90 ± 3 | 53 ± 1 | 103 ± 7 | 81 ± 7 |
| | EM: | | | | |
| | in assay | 80 ± 1 | 37 ± 1 | 88 ± 4 | 62 ± 6 |
| | EC pretreat | 77 ± 4 | 49 ± 2 | 95 ± 1 | 75 ± 2 |
| | TC pretreat | 77 ± 3 | 43 ± 3 | 85 ± 2 | 66 ± 2 |

[a] See Table I. CD4+ T cells, (H-2$^k$ anti-H-2$^d$); CTL effectors, (H-2$^d$ anti-H-2$^k$).

[b] Effectors were assayed against the transfected I-A$^d$ L cells, in the absence and presence of CHX (10 μM) or EM (3 μM). Additionally, EM-pretreated (3 μM) effector cells (EC) were tested against untreated target cells (TC) and vice versa.

[c] Reactions were terminated at the indicated times, and the percent specific release for detached and lysed targets determined. Percent CD4+ T cell activity against the transfected Ia$^-$ L cells after 6 h: 4 ± 1 (detached), 2 ± 1 (lysed); and 18 h: 13 ± 4 (detached), 5 ± 2 (lysed). EM alone did not significantly alter the background release.

EM blocks protein synthesis in pretreated effector cells and pretreated target cells by 90 to 98%, even up to 18 h in the subsequent absence of drug, and does not significantly alter their viability or target cell expression of the I-A$^d$ Ag, as assessed by flow cytometry (mean channel fluorescence: control = 156 (2° Ab alone, 51); EM pretreated = 162 (2° Ab alone, 48)). Moreover, EM pretreatment of CD4+ T cells, followed by an 18-h incubation in the absence of drug, did not alter effector cell surface expression of either the CD4 or CD3 molecule (mean channel fluorescence of CD4: control = 128 (2° Ab alone, 60); EM pretreated = 121 (2° Ab alone, 63); mean channel fluorescence of CD3: control = 106 (2° Ab alone, 52); EM pretreated = 106 (2° Ab alone, 52)). Similar results were also observed for both EM-pretreated effector and target populations following a 6-h incubation in the absence of drug. Thus, it is unlikely that inhibition of CD4+ T cell-mediated detachment and lysis by EM pretreatment (Table II) reflects modulation of either effector cell surface CD4 or CD3 or target cell surface I-A$^d$.

*Functional dissociation of detachment from lysis.* To elucidate the nature of the requirement for target cell protein synthesis, we compared the effects of EM on adherent targets (transfected I-A$^d$ L cells) to suspension targets (A20.1.11) for their susceptibility to CD4+ T cells (Table III). Although A20.1.11 is lytically more sensitive than L cells, EM reduces the activity against either target when the drug is present in the assay or after effector pretreatment (Table III). However, we demonstrate that EM pretreatment of the adherent, but not the suspension target inhibits CD4+ T cell activity, supporting the possibility that detachment, but not lysis requires target cell protein synthesis.

Inasmuch as the data on A20.1.11 (Table III) could also indicate that different targets (i.e., fibroblasts vs B lymphoma cells) react functionally different to the same pool of effectors, we examined the effects of EM on the I-A$^d$ L cells in both adherent and suspension states (Table III). Our findings indicate that similar to that observed with A20.1.11, EM inhibits lytic function against the suspension target when the drug is continuously present in the assay or after effector, but not target pretreatment. Because of the lower sensitivity of suspension L cells to lysis by CD4+ effectors, the experiment in Table III was carried out for 18 h. However, similar inhibition by EM on CD4+ effectors was observed in shorter (4 to 6 h) assays with A20.1.11 (data not shown). Although the suspension I-A$^d$ L cells are not as lytically sensitive as A20.1.11, the cytotoxic activity is Ag-specific. This is based on the observations that the lytic activity is strongly inhibited by anti-CD4 (>70%), anti-I-A$^d$ (>50%), but not anti-CD8 mAb. Moreover, these data substantiate

TABLE III

*Detachment, but not lysis requires target cell protein synthesis*

| Treatment[a] | Percent Specific Release | | | | | |
|---|---|---|---|---|---|---|
| | Adherent[b] | | Suspension[c] | | | |
| | I-A$^d$ L Cells | | A20.1.11 | | I-A$^d$ L Cells | |
| | Detached | Lysed | 10/1 | 3/1 | 100/1 | 30/1 |
| None | 64 ± 4 | 31 ± 3 | 61 ± 0 | 39 ± 2 | 35 ± 2 | 16 ± 1 |
| EM: | | | | | | |
| in assay | 19 ± 2 | 10 ± 2 | 16 ± 1 | 5 ± 1 | 6 ± 1 | 2 ± 1 |
| EC pretreat | 27 ± 1 | 16 ± 1 | 24 ± 1 | 12 ± 2 | 12 ± 1 | 6 ± 1 |
| TC pretreat | 23 ± 2 | 14 ± 2 | 58 ± 1 | 32 ± 1 | 31 ± 1 | 13 ± 1 |

[a] Effectors were assayed against their specific target, in the absence and presence of EM (5 μM). Additionally, EM-pretreated (5 μM) effectors were tested against untreated targets and vice versa.

[b] Transfected I-A$^d$ L cells were examined as an adherent target. Reactions were terminated after 18 h, and the percent specific release for detachment and lysis determined. (E:T ratio = 20/1.)

[c] A20.1.11 and transfected I-A$^d$ L cells were examined as suspension targets. Monolayers of I-A$^d$ L cells were grown on non-tissue culture-treated dishes and then were collected in suspension by pipetting without the use of trypsin and/or EDTA. Reactions were terminated after 18 h, and lytic activity against each target determined at two E:T ratios.

the observations made with A20.1.11 and strengthen the hypothesis that CD4+ T cell-mediated detachment, but not lysis requires target cell protein synthesis. Thus, L cells in the adherent, but not the suspension state require protein synthetic capacity for lysis. This observation along with the fact that detachment temporally precedes lysis and requires protein synthesis implies that lysis of the adherent target cell by these primary CD4+ effectors can only be accomplished after the target has detached from the substratum.

*T cell-induced detachment and lysis are associated with nuclear damage.* As a final issue of comparison between CD4+ T cells and CTL effectors, we examined them for their capacity to induce nuclear damage of the transfected I-A$^d$ fibroblasts (Fig. 1). Earlier work from our laboratory (29), and that of others (30, 31), have established that during CD8+ CTL-mediated lysis, nuclear damage to the target cell usually precedes or occurs concomitantly with cell death. Furthermore, others (10, 14) have shown that in addition to conventional MHC class I-specific/restricted CTL cells, MHC class II-specific/restricted CTL can also efficiently mediate target nuclear destruction.

Target monolayers were radiolabeled with either $^{125}$IUdR or $^{51}$Cr, before their interaction with CTL or CD4+ T effectors, to assess for nuclear damage and detachment/lysis. We show that for CTL effectors, detachment and lysis occur concomitantly with changes in nuclear activity (Fig. 1, $^{51}$Cr:$^{125}$IUdR ratio). Thus, for soluble $^{51}$Cr:soluble $^{125}$IUdR release, a ratio of 1.8 is obtained, suggesting a close association between target cytolysis and the induction of nuclear damage (Fig. 1*A*). More importantly, we show that, as with CTL effectors, CD4+ T cells can also effect nuclear damage, yielding a similar ratio of soluble $^{51}$Cr:soluble $^{125}$IUdR release (Fig. 1*B*, ratio = 1.7). Thus, during Ag-specific attack, detachment and lysis by alloreactive CTL or CD4+ T effectors is associated with nuclear destruction, suggesting a common lymphocyte-mediated mechanism that is in accord with earlier studies (10, 14). The observation that $^{51}$Cr release precedes $^{125}$IUdR release substantiates earlier work (31) on the differential sensitivity of different targets, including fibroblasts, to nuclear attack by CD8+ CTL. Also, for both CTL and CD4+ T effectors, the total release of $^{125}$IUdR approximates the total release of $^{51}$Cr, implying that neither effector population reacts with a selective subset of target cells.

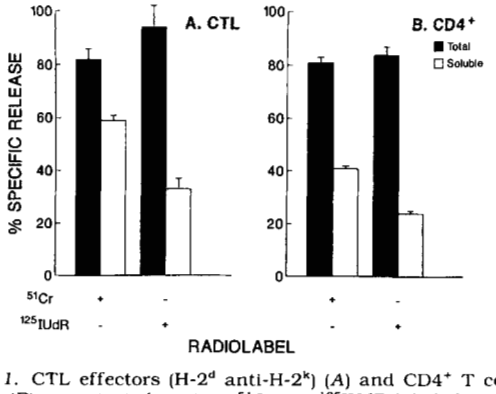


*Figure 1.* CTL effectors (H-2$^d$ anti-H-2$^k$) (*A*) and CD4+ T cells (H-2$^k$ anti-H-2$^d$) (*B*) were tested against $^{51}$Cr- or $^{125}$IUdR-labeled I-A$^d$ L cells. Assays were terminated after 18 h, and the percent specific release for both total and soluble radioactivity determined. CTL effectors caused 82 and 57%, soluble $^{51}$Cr and soluble $^{125}$IUdR release, respectively, against L929 cells.

*Role of soluble mediators in CD4+ T cell-mediated detachment and lysis.* The previous experiments demonstrate that CD4+ T cell-mediated detachment and lysis require effector protein synthesis. Although the actual role of protein synthesis remains uncertain, it may be required for the production of intracellular proteins having effector function. To examine the potential effector role of CD4+ T cell-derived lymphokines as mediators of detachment as well as obligatory components for lysis, we first performed bystander experiments, which assess the ability of third party targets to respond to soluble products secreted from Ag-specific, E:T interactions. We compared L929, a prototype target for TNF-$\alpha/\beta$-sensitivity (26, 32), to the transfected Ia$^-$ L cells for their susceptibility to serve as bystander targets (Table IV).

We show that Ag-specific stimulation of CD4+ T cells with A20.1.11 or transfected I-A$^d$ L cells results in high levels of bystander activity against L929, revealing the involvement of soluble factors as effector components. Although L929 cells are lytically sensitive, such soluble mediators appear unable to induce a distinct detachment event, since the levels for target cell detachment and lysis are approximately the same. Much lower levels of activity are achieved using the transfected Ia$^-$ L cells as stimulator cells (Table IV), indicating a requirement for an Ag-specific effector-stimulator cell interaction to induce the bystander effect. In parallel cultures, lytic activity is assessed against the stimulator cells (Table IV). As can be seen, high levels of lytic activity are expressed against A20.1.11, whereas lower levels are detectable against the transfected I-A$^d$ L cells. Thus, although the transfected I-A$^d$ L cells, when used as a suspension target, display weak sensitivity to the lytic mechanism (Tables III and IV), they appear to be an effective stimulator of bystander lysis (Table IV). Importantly, however, we show that under similar incubation conditions, the transfected Ia$^-$ L cells appear to be resistant to bystander activity (Table IV). Taken together, these data support the hypothesis that CD4+ T cells can mediate detachment and lysis through soluble factor-dependent and -independent pathways.

*Capacity of rTNF-$\alpha$ to induce detachment and lysis.* The preceding experiments implicate a functional role for soluble factors in the bystander, but not the Ag-specific response (Table IV). Next, we assayed directly the sensitivity of the different target populations to rTNF-$\alpha$. Our observations confirmed those in the bystander assay, and showed that only L929 (Table V), but not the transfected L cells, whether Ia$^-$ or I-A$^d$, are responsive (data not shown). In fact, no detachment or lytic activity was detectable against either transfected L cell population. Similar results were obtained with TNF-$\alpha$ + TNF-$\beta$-containing supernatants (data not shown), derived from a lymphotoxin-secreting T cell line (F128) or clone (F128.G5) (21).

Furthermore, as revealed by the kinetic and dose-response experiments (Table V), rTNF-$\alpha$-mediated lysis of L929 occurs concomitantly with, rather than separable from, detachment. Moreover, in further contrast to CTL or CD4+ effectors (Fig. 1), rTNF-$\alpha$-mediated lysis is not accompanied by nuclear disintegration (Table V, soluble $^{125}$IUdR). However, rTNF-$\alpha$ does stimulate the rapid release of $^{125}$IUdR-labeled L929 cells from substrate (Table

TABLE IV

*Ability of CD4+ T cells to mediate detachment and lysis of bystander targets*

| Target Monolayer | Stimulator Cells | CD4[a] (−/+) | Percent Specific Release | | |
|---|---|---|---|---|---|
| | | | Adherent[b] | | Suspension[c] |
| | | | Detached | Lysed | |
| L929 | None | + | 14 ± 1 | 14 ± 1 | |
| | A20 | − | −2 ± 2 | −2 ± 1 | |
| | | + | 54 ± 3 | 52 ± 5 | 73 ± 2 |
| | Ia− | − | 3 ± 1 | 3 ± 1 | |
| | | + | 21 ± 2 | 21 ± 2 | −8 ± 2 |
| | I-A$^d$ | − | 7 ± 2 | 7 ± 1 | |
| | | + | 60 ± 3 | 59 ± 3 | 14 ± 1 |
| Ia− | None | + | 14 ± 1 | 11 ± 2 | |
| | A20 | − | −2 ± 1 | −3 ± 1 | |
| | | + | 21 ± 2 | 15 ± 1 | 72 ± 2 |
| | Ia− | − | 0 ± 1 | 0 ± 1 | |
| | | + | 13 ± 3 | 6 ± 3 | −6 ± 1 |
| | I-A$^d$ | − | −3 ± 0 | −3 ± 1 | |
| | | + | 11 ± 2 | 5 ± 1 | 13 ± 1 |
| I-A$^d$ | None | + | 67 ± 4 | 39 ± 2 | |

[a] CD4+ T cells (H-2$^k$ anti-H-2$^d$) were assayed against the indicated target monolayer, in the absence and presence of the indicated stimulator cells added in suspension. Furthermore, the effect of each stimulator population alone on the target monolayer was also determined.

[b] Assays were terminated after 18 h, and the percent specific release for detached and lysed targets determined.

[c] In parallel cultures, lytic activity was assessed against $^{51}$Cr-labeled stimulator cells, in the presence of unlabeled target monolayers.

TABLE V

*Ability of rTNF-α to induce detachment and lysis*

| Time (H) | TNF-α[a] (U/ml) | Percent Specific Release[b] | | | |
|---|---|---|---|---|---|
| | | $^{51}$Cr | | $^{125}$IUdR | |
| | | Detached | Lysed | Detached | Lysed |
| 4 | 1 | 1 ± 1 | 1 ± 1 | 5 ± 2 | 2 ± 2 |
| | 100 | 4 ± 1 | 4 ± 1 | 14 ± 1 | 2 ± 1 |
| 10 | 1 | 16 ± 3 | 14 ± 1 | 47 ± 2 | 2 ± 1 |
| | 100 | 36 ± 1 | 30 ± 1 | 69 ± 1 | 5 ± 1 |
| 18 | 1 | 61 ± 3 | 51 ± 5 | 79 ± 2 | 7 ± 1 |
| | 100 | 77 ± 4 | 67 ± 5 | 84 ± 7 | 7 ± 1 |

[a] L929 cells were radiolabeled with $^{51}$Cr or $^{125}$IUdR and then tested for their susceptibility to murine rTNF-α at different concentrations.

[b] Reactions were performed in the presence of EM (5 μM). Assays were terminated at the indicated times, and the percent specific release for detachment and lysis/nuclear damage determined. Similar results were observed at both higher (1000 U/ml) and lower (0.1 U/ml) rTNF-α concentrations. Furthermore, similar results were obtained in the absence of EM, although the overall activity was less. In contrast to L929, rTNF-α had no detectable effect on the transfected Ia− or I-A$^d$ L cells.

V; up to a 10-h incubation, total $^{125}$IUdR leads total $^{51}$Cr). Inasmuch as a 1.5 hr pulse was used for isotope labeling, the target cells in the S through M phase of the cell cycle are enriched in $^{125}$IUdR. The rapid detachment of this $^{125}$IUdR-labeled L929 fraction in response to rTNF-α is consistent with earlier work (32), revealing that cells in the $G_2$ through M phase of the cell cycle are more sensitive to TNF-α.

*Ability of EM to modulate Ag-specific-mediated lysis.* Our data suggest that although CD4+ T cells can produce cytotoxic lymphokines following Ag-specific stimulation, target cytolysis may result from a soluble-factor (TNF-α/β)-independent pathway (Table IV). Alternatively, it remains possible that such targets become susceptible to soluble lytic agents only after appropriate "sensitization" (cell surface or internal) incurred by a functional E:T interaction.

We examined whether the addition of EM (in the absence or presence of rTNF-α) to the Ag-specific reaction several hours after culture initiation would now exert a positive effect, similar to that normally seen against L929, and now render I-A$^d$ L cells sensitive to either endogenous or exogenous lytic agents, such as TNF. In this experiment (Table VI), EM is added either at the beginning of the assay or 6 h after initiation, when it has little inhibitory effect on detachment. The reaction is then allowed to proceed for at least an additional 15 h to maximize the impact of EM, with or without rTNF-α. Our data indicate that the addition of EM 6 h after assay initiation does not enhance lytic sensitivity, but rather still partially inhibits the response (Table VI; 34% vs 16%). Furthermore, rTNF-α alone or combined with EM, also fails to augment lytic sensitivity. In contrast, we show that EM, whether added at either the beginning of the assay or 6 h post-initiation, can potentiate rTNF-α-mediated lysis of L929 to the same extent (Table VI). Similarly, EM, when added at T (6) to bystander assays (using A20.1.11 or transfected I-A$^d$ L cells as stimulator cells), augments lysis of L929 (data not shown). Taken collectively, these data reveal that CD4+ T cells do not have the capacity to convert the phenotype of TNF resistant L cells into a TNF sensitive one, further demonstrating that detachment and lysis against those targets results from a TNF-α/β-independent pathway.

*Direct evidence for TNF-α/β-dependent and -independent pathways.* A direct role of TNF-α/β in Ag-specific and bystander-induced detachment/lysis by CD4+ T cells was assessed using anti-murine TNF-α/β mAb (21). We also explored the role of IFN-γ in both processes using anti-murine IFN-γ mAb (22), because this cytokine has been shown to possess a variety of immunoregulatory properties, including its ability to up-regulate TNF-α/β receptors (33). Our data indicate that each mAb alone or in combination fails to block detachment and lysis against the Ag-specific target (Table VII). In contrast, each mAb alone partially inhibits bystander activity, albeit anti-IFN-γ is less effective. Furthermore, the combination of both mAb leads to a slightly further, yet reproducible reduction in bystander activity (Table VII). Thus, the observations that these mAb can affect bystander, but not Ag-specific activity further demonstrate that

TABLE VI

*Effect of CD4+ T cell-I-A$^d$ L cell interaction on target cell susceptibility to TNF*

| Time (H) | TNF[a] (−/+) | EM[b] (−/+) | Percent Specific Release[b] T (0) Detached | T (0) Lysed | T (6) Detached | T (6) Lysed |
|---|---|---|---|---|---|---|
| 6 | − | − | 25 ± 2 | 9 ± 1 | | |
| | − | + | 8 ± 2 | 1 ± 1 | NT[c] | NT |
| 21 | − | − | 68 ± 2 | 34 ± 1 | | |
| | − | + | 12 ± 4 | −2 ± 3 | 53 ± 1 | 16 ± 1 |
| | + | − | 69 ± 5 | 34 ± 3 | | |
| | + | + | 21 ± 2 | 1 ± 2 | 63 ± 3 | 21 ± 2 |
| 21 | +[d] | + | 92 ± 4 | 79 ± 1 | 87 ± 6 | 73 ± 7 |

[a] CD4+ T cells (H-2$^k$ anti-H-2$^d$) were assayed against the I-A$^d$ L cells in the absence or continuous presence of rTNF-$\alpha$ (1000 U/ml), alone or in combination with EM (5 $\mu$M).

[b] EM was added either at the beginning of the assay (T(0)) or 6-h post-initiation (T(6)). Assays were terminated after a total incubation time of 6 or 21 h, and the percent specific release for detached and lysed targets determined. Percent CD4+ T cell activity against the transfected Ia$^-$ L cells after 6 h: 6 ± 1 (detached), 3 ± 1 (lysed); and 21 h: 19 ± 1 (detached), 13 ± 2 (lysed).

[c] NT, Not tested.

[d] In parallel and under identical culture conditions, L929 cells were exposed to rTNF-$\alpha$ (1000 U/ml) and EM (5 $\mu$M).

TABLE VII

*Effects of anti-TNF-$\alpha/\beta$ and anti-IFN-$\gamma$ on CD4+ T cell-induced detachment and lysis of Ag-specific and bystander targets*

| Treatment[a] | Percent Specific Release[b] I-A$^d$ (Ag-Specific) Detached | I-A$^d$ (Ag-Specific) Lysed | L929 (Bystander) Detached | L929 (Bystander) Lysed |
|---|---|---|---|---|
| None | 94 ± 2 | 46 ± 2 | 63 ± 2 | 62 ± 2 |
| Control Ig | 105 ± 5 | 52 ± 4 | 66 ± 2 | 58 ± 2 |
| Anti-TNF-$\alpha/\beta$ | 92 ± 2 | 46 ± 3 | 38 ± 1 | 37 ± 2 |
| Anti-IFN-$\gamma$ | 89 ± 2 | 46 ± 1 | 54 ± 2 | 51 ± 2 |
| Anti-TNF-$\alpha/\beta$ + anti-IFN-$\gamma$ | 97 ± 4 | 50 ± 4 | 31 ± 0 | 30 ± 1 |

[a] Anti-TNF-$\alpha/\beta$ (50 $\mu$g/ml); anti-IFN-$\gamma$ (50 $\mu$g/ml); control Ig (100 $\mu$g/ml).

[b] Ag-specific CD4+ T cell activity (H-2$^k$ anti-H-2$^d$) was determined against the I-A$^d$ L cells. Bystander activity against L929 was induced by CD4+ T cells stimulated by A20.1.11. Assays were performed in the absence and presence of mAb, terminated after 18 h, and the percent specific release for detached and lysed targets determined. Percent CD4+ T cell activity against the transfected Ia$^-$ L cells: 18 ± 1 (detached), 12 ± 0 (lysed); and L929 (without A20.1.11): 11 ± 2 (detached), 9 ± 2 (lysed). A20.1.11 alone against L929: 0 ± 1 (detached), 0 ± 1 (lysed).

CD4+ T cells can mediate their effector function through both TNF-$\alpha/\beta$- (and IFN-$\gamma$)-dependent and independent pathways.

Direct evidence for the production of cytotoxic lymphokines by CD4+ T cells is provided by supernatant transfer experiments. Here, we isolated supernatants from CD4+ T cells stimulated for 6 h by their specific target (A20.1.11) and tested them immediately for their capacity to lyse L929 (Table VIII). Similar to that seen in the bystander (Tables IV and VII) and rTNF-$\alpha$ assays (Table V), we show that such supernatants contain a soluble cytotoxic activity which can efficiently lyse L929 cells without inducing a distinct detachment event. In contrast to L929, A20.1.11 and the transfected L cells (Ia$^-$, I-A$^d$) failed to respond to the coculture supernatants. Table VIII also demonstrates that anti-TNF-$\alpha/\beta$ mAb, but not the control Ig, inhibits almost all of the activity in the coculture supernatants, confirming that CD4+ T cells secrete TNF-$\alpha/\beta$ in response to Ag-specific recognition. Interestingly, more anti-TNF-$\alpha/\beta$ inhibition is observed here than in the bystander assays (Table VII), which may, in part, reflect the accessibility of the mAb for TNF-$\alpha/\beta$ in the local microenvironment. Hence, the mAb may be more accessible to TNF-$\alpha/\beta$ contained freely in the supernatant (Table VIII) than of TNF-$\alpha/\beta$ secreted between closely juxtaposed cells (Table VII). Nevertheless, CD4+ T cells can produce soluble effector molecules, including TNF-$\alpha/\beta$, after Ag-specific stimulation; however, the exact nature of the target response reflects its inherent susceptibility to a particular effector mechanism.

## DISCUSSION

Recently, we identified and began the functional characterization of a unique lymphocyte function, reflecting the ability of CD8+ T cells to stimulate adherent targets to detach from their substratum as an early consequence of the E:T interaction (4, 5). Under physiologic conditions, CD8+ T cell-induced detachment is a distinct and rapid prelytic event, although through experimental manipulation, detachment can occur in the absence of lytic expression, demonstrating that both activities are functionally separable (4, 5).

In this report, we demonstrate that CD4+ T cells, as with CD8+ CTL, can mediate target detachment. Although the functional outcome by both lymphoid subsets is similar, evidence is provided to indicate that the effector pathways are different. Here, we show that protein synthesis is essential for the CD4+, but not the CD8+ pathway, with the metabolic requirement residing in both the effector and target populations (Tables II and III).

Interestingly, we demonstrate that the adherent target cell requirement for protein synthesis is likely for detachment, and not lysis (Table III). We show that pretreatment of targets with EM reduces their ability to lose adherence, but not their apparent sensitivity to the lytic event. On the surface, this observation appears inconsistent with our findings that reveal that CHX or EM blocks the subsequent lysis of adherent targets (Tables II and III at 18 h). This paradox could suggest that for an adherent L cell target to be lysed, it must first be dislodged from substrate. If so, then a protein synthesis-dependent loss of adherence step may necessarily precede a protein synthesis-independent lytic event. The role of target cell protein synthesis is uncertain, but may reflect the turnover of unique cell surface "adhesion" molecules, distinct from I-A$^d$, required for efficient recognition and conjugation leading to detachment, but not lysis. Alternatively, target cell protein synthesis may be required for intracellular events or pathways, which underlie an "active"

TABLE VIII

*Inhibition of CD4+ T cell-target cell coculture supernatant activity by anti-TNF-α/β*

| Soluble Factor[a] | Percent Specific Release[b] | | | | | |
|---|---|---|---|---|---|---|
| | None | | Control Ig | | Anti-TNF-α/β | |
| | Detached | Lysed | Detached | Lysed | Detached | Lysed |
| rTNF-α (U/ml) | | | | | | |
| 10 | 69 ± 2 | 65 ± 2 | 75 ± 1 | 72 ± 1 | 5 ± 1 | 4 ± 1 |
| 100 | 74 ± 1 | 73 ± 1 | 78 ± 3 | 75 ± 2 | 9 ± 1 | 6 ± 2 |
| 1000 | 73 ± 3 | 70 ± 3 | 80 ± 2 | 69 ± 2 | 28 ± 1 | 27 ± 1 |
| Supernatants | | | | | | |
| CD4 alone | 8 ± 1 | 7 ± 1 | 7 ± 1 | 5 ± 2 | 1 ± 1 | 0 ± 1 |
| A20 alone | 1 ± 1 | 0 ± 1 | NT[c] | NT | NT | NT |
| CD4/A20 | 70 ± 3 | 66 ± 5 | 68 ± 3 | 63 ± 2 | 3 ± 1 | 1 ± 1 |

[a] $^{51}$Cr-labeled L929 cells were exposed to either rTNF-α or to CD4+ T cell/A20.1.11 coculture supernatants.

[b] Reactions were performed in the presence of EM (5 μM), alone (none), or combined with anti-TNF-α/β (50 μg/ml) or a control Ig (50 μg/ml). Assays were terminated after 18 h, and the percent specific release for detached and lysed targets determined.

[c] NT, Not tested.

target response to CD4+ T cell attack. Thus, the slower kinetics observed with CD4+ T cells, in part, may reflect the requirement of protein synthesis by both partners to maximally elicit the cellular response.

Because CD4+ T cells can mediate their effector function via the release of lymphokines (6, 7), it is possible that the requirement for protein synthesis is for the production of relevant intracellular components. However, based on bystander, supernatant transfer and anti-TNF-α/β mAb inhibition experiments, our data do not support a role for soluble factors, notably TNF-α/β and possibly proteases, in detachment and as the sole mediators of lysis by CD4+ T cells. Nevertheless, it is possible that effector protein synthesis is required for the production of other endogenous cytokines or lytic agents, perhaps novel or metastable in solution and yet unidentified. This contention is in accord with Ju et al. (15), who demonstrated that CD4+ T cell clones can mediate target cytolysis through a TNF-α/β-independent pathway, suggesting the involvement of a unique array of effector molecules. Moreover, similar to our findings for detachment and lysis, Ju et al. (15) showed that protein synthesis was obligatory for both TNF-α/β-dependent and -independent lytic pathways. Alternatively, detachment and lysis by CD4+ T cells may proceed by a mechanism independent of soluble factors, involving direct cell-cell interaction as proposed by others (14). The protein synthetic requirement for CD4+ T cells in this capacity, therefore, may be associated with the turnover of a unique triggering molecule and/or an intracellular metabolic pathway leading to detachment and lysis.

The lack of requirement for RNA synthesis in our system, however, suggests that transcripts encoding for detachment/lytic-specific proteins already pre-exisit within the effector or the target; therefore, the induction of new mRNA is not essential for the manifestation of these short term lymphocytes activities. Ju et al. (15) reported that AD can inhibit CD4+ T cell-mediated lysis (as determined in a 5-h assay). The reasons for this difference are unclear, but may reflect the clonal nature and/or activation status of the different effector populations at the time of testing (i.e., primary MLC-derived CD4+ T cells isolated 5 days after stimulation as described here vs CD4+ clones used 10 to 21 days after stimulation as described by Ju et al. (15)).

The role of soluble factors as putative mediators of detachment and lysis by CD4+ T cells was also explored and, overall, the data demonstrate that: 1) detachment is initiated by direct cell-cell interaction, independent of TNF-α/β; 2) lysis can be mediated by either TNF-α/β-dependent or -independent pathways; and 3) the nature of that lytic response reflects target susceptibility to a particular effector mechanism. Thus, these findings are in accord with both types of proposed models, supporting the contention that bystander (8–11) as well as contact-dependent (12–15) pathways are operational during CD4+ T cell-mediated lysis. The inability of TNF-α/β to lyse a more extensive array of targets, including the L cell lines examined here, could reflect inherent characteristics of the targets, such as an absence of specific cell surface receptors (34) and/or the failure to transduce a postbinding lytic signal (35). Direct TNF-α binding assays reveal that there are no cell surface receptors for TNF-α on the transfected I-A$^d$ L cells, whereas L929 cells contain >1000 receptors/cell (K.C.F. Sheehan and S.I. Abrams, unpublished observations). Nevertheless, it is the basic finding that TNF-α/β-resistant targets are sensitive to CD4+ T cell attack that unmasks and illustrates the multi-functional potential of CD4+ T cells.

Furthermore, our data reveal an important and fundamental distinction between T cells and rTNF-α, in regard to their ability to induce DNA fragmentation. We (29) and others have shown that CD8+ CTL (30, 31) or lytic CD4+ T cell clones (10, 14) have a unique capacity to induce target nuclear damage before or in association with alterations in cytoplasmic homeostasis. Here, we demonstrate that with allospecific CTL or CD4+ T effectors, but not rTNF-α or TNF-α/β supernatants, lysis of susceptible targets is accompanied by nuclear dissolution (Fig. 1; Table V). Thus, these data reveal functional similarities (between CD8+ CTL and CD4+ T cells) as well as differences (vs Ab + C (Refs. 4 and 29) and cytotoxic lymphokines) among the various effector systems. The inability of TNF-α/β to induce nuclear disintegration of L929 (Table V) is in accord with Laster et al. (27), but not Schmid et al. (10). The exact reasons for this discrepancy are uncertain, but based upon the findings of Laster et al. (27) using a variety of cell types, these differences may reflect intrinsic properties of the responding targets.

The failure of TNF-α/β, as with Ab + C (4), to initiate detachment as a separable, prelytic event, further illustrates basic differences among the various effector path-

ways and strengthens the concept that detachment is a distinct and potentially relevant in vivo phenomenon. The ability of $CD8^+$ CTL or $CD4^+$ T cells to mediate detachment may account for a unique form of immune damage that contributes to both normal and pathologic responses. Such an interaction between Ag-specific T lymphocytes and the vascular endothelium, for example, may contribute to tissue injury through nonlytic disruption of tissue architecture. This immunologic reaction, therefore, may underlie a mechanism for coagulation necrosis that is an important pathway of allograft rejection (36). Moreover, such a nonlytic response resulting from the interaction between T cells and/or their products with adherent target cells introduces the possibility that the immune system, at least via T cells, may play a role in promoting tumor metastases. This hypothesis is consistent with the concept of "immune enhancement of tumor growth" (37, 38), and supported by our earlier observations revealing that targets released by Ag-specific $CD8^+$ CTL in the absence of extracellular $Ca^{2+}$ are not programed to die and will form colonies with equivalent plating efficiencies as the untreated controls (4). Thus, the capacity of $CD8^+$ CTL or $CD4^+$ T cells to stimulate detachment in vivo may constitute a novel form of immune damage, perhaps underlying inflammation, allograft rejection, and tumor metastases.

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