# Cytoskeletal Function in CD8- and T Cell Receptor-mediated Interaction of Cytotoxic T Lymphocytes with Class I Protein

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## Summary

Cloned allospecific cytolytic T lymphocytes (CTL) adhere to purified class I alloantigen immobilized on plastic and degranulate in response to it. Binding and degranulation are inhibited by drugs that impair cytoskeletal function. Cytochalasins D and E, which interfere with microfilament function, and colchicine, which disrupts microtubules, were used and gave qualitatively similar results. Concentrations of these drugs that inhibited degranulation in response to alloantigen did not inhibit response to immobilized anti-T cell receptor (TCR) antibody. Neither did they inhibit response when alloantigen was co-immobilized with an antibody against class I on the CTL to promote adhesion between the CTL and antigen-bearing surface. Thus, neither transmembrane signal generation via the TCR nor degranulation per se were prevented. Instead, the drugs act to prevent the initial adhesion to alloantigen. CTL binding to alloantigen depends in part on CD8-class I interaction, and adhesion via CD8 is "activated" by crosslinking the TCR with soluble anti-TCR antibody. This adhesion, too, is shown to be cytoskeleton dependent.

A variety of evidence indicates that cytoskeletal elements play important roles in T cell recognition and response to antigen-bearing cells. It has been known for some time that lysis of target cells by CTL is blocked by cytochalasins (1–6), which interfere with microfilament function, and by colchicine (4–8), which disrupts microtubules. More recently, selective reorganization of cytoskeletal proteins has been observed in both CTL and T helper cells upon interaction with antigen-bearing cells. Talin becomes localized inside the T cell in the region of cell-cell contact and the microtubule organizing center, Golgi apparatus, and granules undergo polar reorientation to the region of contact (9–14). These cytoskeletal changes are observed only when the T cell is in contact with a cell bearing antigen, and not when the T cell is nonspecifically bound to another cell. This dependence on specific antigen interaction suggests that these are triggered events, but the functional consequences of these cytoskeletal reorganizations are not known.

Cytochalasins prevent binding of CTL to target cells to form stable conjugates, but have little or no effect on the subsequent events leading to target cell lysis (1–6). CTL binding to targets is a complex process, with several CTL surface proteins implicated as having roles in adhesion, including the antigen-specific TCR, CD8, LFA-1/I-CAM, and CD2/LFA-3 (15, 16). To define better the role(s) of cytoskeletal elements in adhesion, we have examined the effects of cytochalasins and colchicine on CTL interacting with purified class I alloantigen.

Immobilized class I alloantigen is a sufficient ligand for binding of cloned CTL and triggering of the degranulation response (17, 18). Under conditions where class I protein is the only available ligand, binding and response depend upon both TCR binding to antigen and CD8 binding to nonpolymorphic regions of class I. Furthermore, it has been recently shown that binding of CD8 to class I is activated via the TCR, and that the CD8-class I interaction results in some additional signal(s) required to trigger degranulation when TCR crosslinking is minimal (19).

The results described in this report demonstrate that agents that perturb cytoskeletal function prevent binding to immobilized class I alloantigen and inhibit the degranulation response. In contrast, binding and degranulation in response to immobilized anti-TCR antibody are not inhibited by these agents in the same concentration range. Thus, neither TCR-mediated transmembrane signal generation nor degranulation per se are inhibited. Furthermore, it is shown that inhibition of the binding to alloantigen results, at least in part, from inhibition of the CD8 binding to class I that is activated via the TCR. These results have implications for the mechanism of TCR-activated, CD8-mediated adhesion of T cells.

## Materials and Methods

*Mice and Cell Lines.* C57BL/6 (H-$2^b$) mice were purchased from the Jackson Laboratory, Bar Harbor, ME. EL4 (H-$2^b$) C57BL/6 thymoma, P815 (H-$2^d$), a DBA/2 mastocytoma, and

RDM-4 (H-$2^k$), an AKR lymphoma, were maintained in tissue culture and passaged as ascites.

The $K^b$-specific clones 6, 11, and 13 were derived from peritoneal exudate cells of (B10.BR × B10.D2)$F_1$ (H-$2^k$ × H-$2^d$) mice 10–13 d after priming by intraperitoneal injection with 2 × $10^7$ EL4 cells. Cells were stimulated in vitro under limiting dilution conditions with irradiated B10.A(5R) (H-$2^{b/a}$) spleen cells and 10% rat Con A supernatants. Clones were maintained by weekly stimulation with C57BL/6 stimulator cells and human rIL-2 (10 U/ml) in culture medium consisting of RPMI 1640 supplemented with 20 mM Hepes, 2 mM L-glutamine, β-mercaptoethanol (5 × $10^{-5}$ M), penicillin-streptomycin (100 μg/ml), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10% heat-inactivated FCS.

*Monoclonal Antibodies.* The mAbs used were 11-4.1, anti-$K^k$ (20), M1/42, rat anti–mouse class I (21, 22), and F23.1, anti-Vβ8 TCR (23). 11-4.1 and F23.1 antibodies were purified by affinity chromatography using protein A–Sepharose and M1/42 by ammonium sulfate fractionation followed by DEAE-chromatography.

*Purification of Class I Antigens.* H-$2K^b$ was purified from TX-100 lysates of EL4 ($K^bD^b$) tumor cells by affinity chromatography on an M1/42 mAb column as previously described (22). The M1/42 mAb, a rat IgG that binds most murine class I proteins, does not bind H-$2D^b$ antigen under the conditions used for the affinity-chromatography (24). H-$2D^d$ was purified from TX-100 lysates of P815 ($K^dD^d$) tumor cells using an M1/42 mAb column as previously described (22, 24). H-2 preparations were quantitated by protein determination (25) and ELISA (24). For comparison, all H-$2K^b$ preparations were quantitated by ELISA relative to a single H-$2K^k$ preparation as the standard, using M1/42 mAb as the first antibody (24). Purity of class I protein preparations was assessed by SDS-PAGE, with protein visualized by staining with Coomassie Blue.

*Protein Immobilization on Plastic.* Purified H-2 eluted from mAb affinity columns was at concentrations of 25–150 μg/ml in 10 mM Tris buffer containing 0.5% deoxycholate and 0.15 M NaCl. Antigens were stored frozen in this buffer, and no losses in either serological activity or CTL stimulating activity have been noted over several months storage. For use, H-2 was diluted directly into Dulbecco's PBS, pH 7.4, to a concentration of 0.1–1 μg/ml. 0.1 ml of diluted antigen was then incubated for 1.5 h at 25°C in individual flat-bottomed microtiter wells (Falcon 3912 Flexible Assay Plate; Becton Dickinson, Oxnard, CA). After incubation for 1.5 h, the wells were washed four times with RPMI containing 2% FCS to remove unbound antigen and block unoccupied sites on the plastic. Characterization and quantitation of class I immobilization has been described in detail (24). mAbs were immobilized in plastic wells in the same way, with the exception that antibody preparations did not contain detergent. When antibody was co-immobilized with class I, a two-step procedure was used. mAb was first added to the well and incubated for 1.5 h at 25°C. Unbound antibody was then removed and class I protein was added and allowed to bind for 1.5 h. Wells were then washed and blocked with FCS as above. As previously described in detail (26), this procedure results in immobilization of both proteins over the concentration ranges described here.

*Assay of CTL Degranulation.* Release of serine esterase (SE)[1] activity in response to plate-bound proteins was determined by addition of $10^5$ CTL/well in 0.1 ml of RPMI medium buffered with 15 mM Hepes and containing 2% FCS. In cases where degranulation in response to target cells was measured, 1 × $10^5$ CTL and 2 × $10^5$ EL4 target cells were added to wells previously blocked by washing with 2% FCS. Plates were then briefly centrifuged and incubated at 37°C for varying times. After incubation, plates were again briefly centrifuged, 0.02-ml aliquots of supernatant were removed, and SE activity was determined as previously described (18, 27). SE substrate, *N*-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT), was purchased from Calbiochem-Behring-Corp., La Jolla, CA, and 5′5-dithiobis (2-nitrobenzoic acid) was purchased from Sigma Chemical Co. (St. Louis, MO).

Each experiment included CTL incubated in blocked wells in the absence of stimulus to determine the level of spontaneous SE release. Total cellular serine esterase activity was determined in each experiment by extracting CTL with 0.5% NP-40 and assaying activity in the extract (17, 26). Data are presented as percent specific esterase release calculated as: percent specific release = $100 \times [(E-S)/(T-S)]$, where $E$ is the experimental value, $S$ is the esterase activity spontaneously released by CTL in the absence of stimulation, and $T$ is the total esterase activity extractable with detergent. Spontaneous release ($S$) was <10% of stimulated release in all experiments. Measurement of total esterase activity in NP-40 extracts showed the target cells to have <1% of the activity present in the CTL.

All experiments had duplicate or triplicate wells for each condition being tested and these values varied by <10% in all cases. For the cloned CTL used in this study, antigen-stimulated degranulation normally results in 25–50% of the total SE present in the cell being released in 2–3 h (17–19). Longer incubation with antigen does not result in significantly more degranulation. All of the experiments examining effects of inhibitors on degranulation were repeated at least three times with essentially identical results, and the data shown are representative.

*Cytotoxicity Assay.* CTL lytic activity was determined in a 4-h $^{51}$Cr-release assay. Varying numbers of effector cells were incubated with 1 × $10^4$ $^{51}$Cr-labeled tumor cell targets in a final volume of 0.2 ml in V-bottomed wells. After 4 h at 37°C, 100 μl of the supernatants were harvested and radioactivity was determined. Spontaneous release (SR) from target cells was determined by incubation in the absence of CTL, and total releasable (TR) was determined by freeze thaw lysis. Percent specific cytotoxicity was calculated as: percent specific cytotoxicity = $100 \times [(\text{experimental release} - SR)/(TR - SR)]$. Cloned CTL used in these studies were periodically tested to insure that they retained specific cytolytic activity.

*Assay of CTL Binding.* CTL were labeled by incubating in PBS for 1 h at 37°C using 100 μCi $Na^{51}CrO_4$ per $10^7$ cells. Cells were then washed and resuspended at $10^6$/ml in RPMI medium containing 2.5% FCS. Binding was initiated by adding $10^5$ cells per well in 0.1 ml and centrifuging at 1,200 rpm for 1 min. Plates were then incubated for varying times at 37°C in a 5% $CO_2$ atmosphere. At the end of this time 0.1 ml of PBS was added, plates were placed on an ice/water bath for 10 min, and unbound cells were then removed by pipeting 10 times with a Pipetman (Gilson Co., Inc., Worthington, OH). Well bottoms were then cut off and radioactivity was determined. Spontaneous release of $^{51}$Cr was determined for cells incubated in parallel under identical conditions. Binding was calculated as percent cells bound = $100 \times [(\text{cpm bound})/(\text{total} - \text{spontaneous cpm})]$. Visual counting confirmed that this procedure provided an accurate measurement of bound cells. All of the experiments examining effects of inhibitors on binding were repeated at least three times with essentially identical results.

In some experiments, SE release and binding were examined for

[1] *Abbreviations used in this paper:* CD, cytochalasin D; CE, cytochalasin E; SE, serine esterase.

the same cells. In this case, at the end of the 37°C incubation, 0.02-ml aliquots were removed from wells for esterase activity determination, and the wells were then treated as above to determine binding. Direct comparison of unlabeled and $^{51}Cr$-labeled cells showed that degranulation in response to antigen or anti-TCR antibody was not affected by the $^{51}Cr$ labeling (data not shown).

*Inhibitors of Cytoskeletal Function.* Colchicine and cytochalasins D (CD) and E (CE) were purchased from Sigma Chemical Co. Colchicine was dissolved directly in assay medium for use. Cytochalasins were dissolved in DMSO at a concentration of 2 mM, and diluted into medium to the desired final concentration. Controls done in all experiments included addition of DMSO to the same final concentration present when cytochalasin was added. Over the concentration range used (up to 1% final concentration), DMSO had no significant effects on degranulation, binding or cytolysis. Cytochalasins and colchicine had no effect on measured SE activity when they were added directly to the enzyme assay (data not shown). Thus, effects on SE release are not due to direct inhibition of the activity of the enzyme following its release by exocytosis.

## Results

*Inhibition of Target Cell–stimulated Responses.* Cytochalasins and colchicine inhibit lysis of target cells by CTL populations (1–8) and cloned CTL lines (28). This was confirmed to be the case for cloned, H-2K$^b$ alloantigen–specific CTL lines used in this study. CD inhibited lysis of EL4 tumor targets by C11 with an $IC_{50}$ (concentration required for 50% inhibition) of 3.3 $\mu$M (Fig. 1 *A*) and CE with an $IC_{50}$ of 0.25 $\mu$M (Fig. 1 *B*). Colchicine was also inhibitory, with an $IC_{50}$ of 1 mM (data not shown). These values are in good agreement with those previously reported for inhibition of CTL-mediated target lysis. Visual inspection confirmed that inhibitory concentrations of these agents blocked conjugate formation with the target cells.

Cloned CTL are stimulated by target cells to undergo degranulation which results in release of granular contents, including SE activity, into the medium (27). This response is antigen-specific and dependent on the TCR, and can be easily quantitated by measuring the released SE activity. SE release by C11 in response to EL4 target cells was inhibited by CD and CE with essentially the same dose responses as for target cell lysis (Fig. 1, *A* and *B*).

*Inhibition of Responses to Immobilized Alloantigen or Anti-TCR Antibody.* Cloned CTL are also stimulated to degranulate upon interaction with anti-TCR antibodies (18, 29) or affinity-purified class I alloantigen immobilized on plastic surfaces (18). Immobilized F23.1 anti-TCR antibody and K$^b$ alloantigen stimulated very comparable levels of degranulation by C11 (Fig. 2). However, cytochalasins and colchicine had very different effects on responses to these two stimuli. Response to the K$^b$ alloantigen was effectively inhibited by CD and colchicine (Fig. 2), with $IC_{50}$s in the same range as those found for inhibition for target cell lysis. In contrast, CD had little or no effect on the response to anti-TCR at concentrations up to 20 $\mu$M. Colchicine also had little or no effect on the response to F23.1 at levels that effectively inhibited response to K$^b$, although it did at least partially inhibit response to F23.1 at higher concentrations (Fig. 2 *B*). The $IC_{50}$ for inhibition by colchicine was somewhat variable in different experiments; it ranged from 1 to 4 mM for inhibition of the response to immobilized F23.1 mAb. In all cases, however, the $IC_{50}$ for inhibition of response to K$^b$ was 5–10-fold lower than for F23.1 mAb. Similarly, CE inhibited the response to anti-TCR, but only at concentrations fivefold higher than required to cause comparable inhibition of the response to K$^b$ (data not shown). In all of these experiments, K$^b$ was immobilized at a density that stimulates optimally (18), and further increasing K$^b$ density had no effect. Thus, for both colchicine and CE, response to K$^b$ was inhibited in the same dose range as target cell lysis, while response to anti-TCR mAb was only partially affected and only at much higher concentrations.

These results suggested that cytochalasins and colchicine did not significantly affect the degranulation response per se,

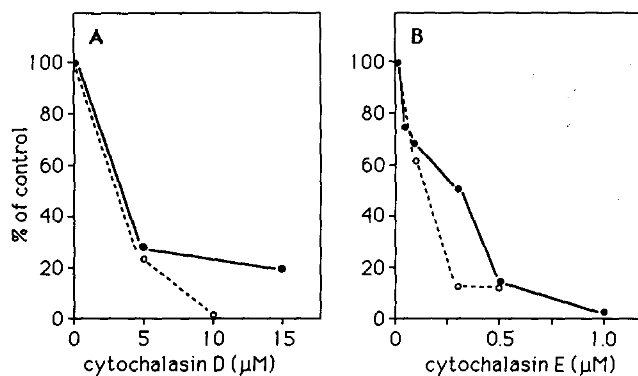


**Figure 1.** Cytochalasins inhibit target cell cytolysis and degranulation by CTL. Clone 11 lysis of $^{51}Cr$-labeled EL4 target cells and degranulation in response to EL4 targets were assayed as described in Materials and Methods. Results are expressed as percent of the control response in the absence of cytochalasin. Control cytolysis at an E/T of 1:1 was 52%, and responses were compared based on lytic units of activity determined from lysis at three different E/T ratios. SE release was measured after a 3-h incubation at 37°C. DMSO had no significant effects on lytic activity or degranulation. Cytolysis (●); degranulation (O).

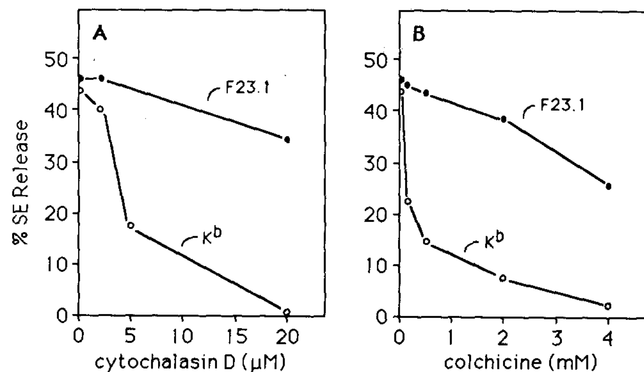


**Figure 2.** Effects of CD and colchicine on degranulation in response to immobilized anti-TCR mAb and class I alloantigen. F23.1 anti-TCR mAb was immobilized at 1 $\mu$g/well and K$^b$ at 0.1 $\mu$g/well, levels that stimulate maximally, as described in Materials and Methods. SE release by C11 was determined after incubation for 2.5 h at 37°C.

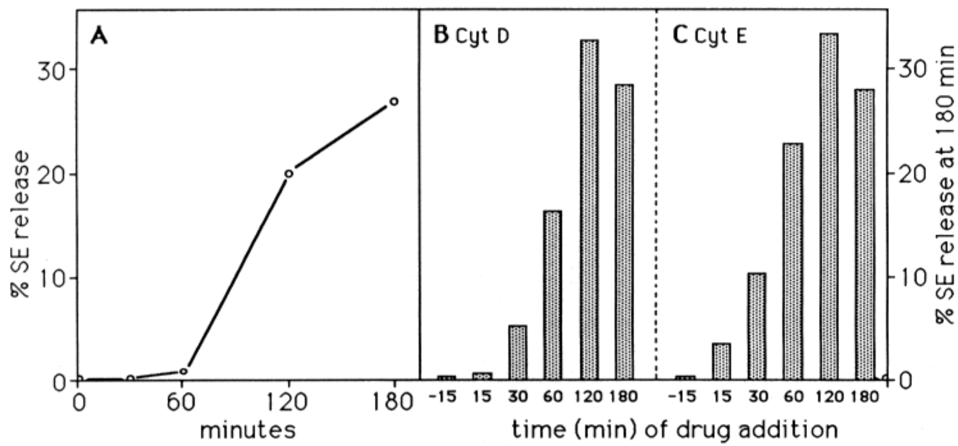


**Figure 3.** Cytochalasins prevent degranulation only when present early in the response. SE release by C11 in response to $K^b$ immobilized at 0.1 μg/well was determined as described in Materials and Methods. (*A*) Kinetics of degranulation. SE release was determined after the indicated time of incubation at 37°C. (*B*) Effect of CD on degranulation. CD (20 μM) was added to wells at the indicated time (time 0 = time of addition of C11 cells). SE release in all cases was measured 180 min after addition of cells to the wells. (*C*) CE (1 μM) was added at the indicated time and SE release measured 180 min after addition of cells to the wells.

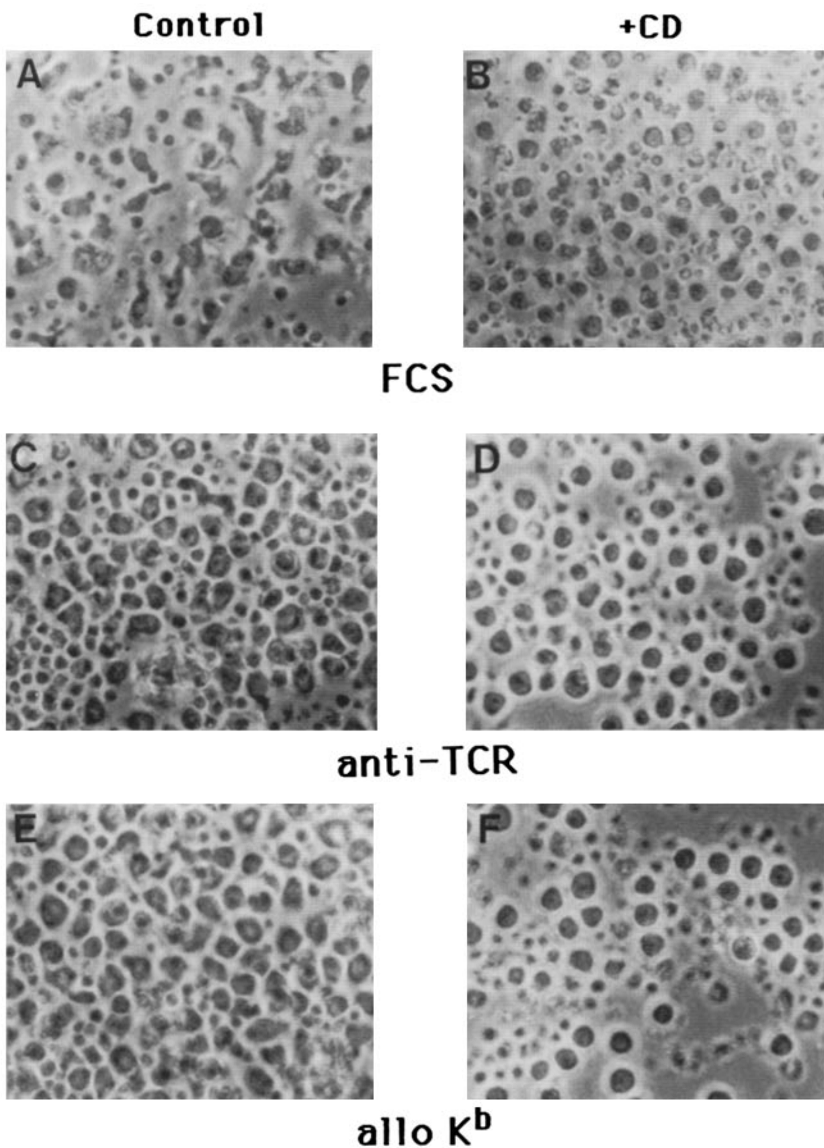


**Figure 4.** Effects of CD on Clone 11 morphology. C11 cells were placed in wells that were coated with (*A* and *B*) FCS, (*C* and *D*) F23.1 anti-TCR mAb immobilized at 1 μg/well or (*E* and *F*) $K^b$ immobilized at 0.1 μg/well. Plates were briefly centrifuged at room temperature to initiate contact and incubated 60 min at 37°C. Cells were then photographed using an inverted microscope. CD (20 μM) was added to the cells 15 min before addition of the cells to wells in panels *B*, *D*, and *F*.

but rather earlier events that lead to antigen-specific activation. The time course of degranulation in response to immobilized alloantigen displays a distinct lag phase of 30–60 min, during which little or no SE release is detectable. After this, release occurs over the next 2 h (Fig. 3 *A*). To determine the point at which interference with cytoskeletal function affects response, inhibitors were added at various times and the extent of degranulation then determined at the end of 3 h. Addition of CD or CE to the CTL 15 min before they are placed in contact with alloantigen results in complete inhibition of SE release over 3 h, and addition 15 min after exposure to antigen still effectively inhibits subsequent response (Fig. 3, *B* and *C*). In contrast, when cytochalasin addition is delayed until 30 or 60 min after exposure to antigen, substantial response is detected at the end of the 3-h period. Thus, addition during the latter portion of the lag phase, at a time when degranulation is not yet detectable, does not effectively prevent subsequent serine esterase release. Essentially, the same results have been obtained in experiments using colchicine at a concentration of 2 mM (data not shown). These results further support the conclusion that intact microfilament and microtubule functions are critical for early events leading to activation, but not for degranulation per se.

*Effects of Cytoskeletal Perturbants on CTL Morphology.* CTL undergo rapid flattening and spreading when centrifuged onto the coated surfaces of the microtiter plate wells used in these experiments, and many cells appear motile. This occurs irrespective of whether a stimulus is present; cells on wells coated with FCS do not appear different than those on wells having immobilized F23.1 or $K^b$ when examined immediately after centrifugation. CD inhibits spreading, and the cells are rounded in its presence. After incubation at 37°C in FCS-coated wells, cells remain spread and many appear motile as evidenced by the presence of pseudopods (Fig. 4 *A*). In contrast, cells on F23.1- or $K^b$-coated wells remain spread but motile-appearing cells are no longer present (Fig. 4, *C* and *E*; this is consistently and reproducibly seen, and in blinded experiments, wells having an immobilized stimulus can be readily distinguished on this basis). In all cases, cells in the presence of CD retain a rounded morphology throughout the incubation period (Fig. 4, *B*, *D*, and *F*).

Visual inspection of cells at varying CD concentrations indicates that the dose response for the morphological effects is very comparable to that for inhibition of degranulation in response to $K^b$. However, the morphological effects are clearly dissociated from inhibition of degranulation in the case of stimulation by anti-TCR antibody. With this stimulus, CD causes the cells to remain rounded up under the same conditions where degranulation is occurring, and flattening and spreading are prevented over the entire time course of the response (Fig. 4 *D* and data not shown). CE had essentially the same effects on CTL morphology as those described for CD. In contrast, colchicine did not prevent flattening and spreading at concentrations which inhibited degranulation in response to alloantigen (data not shown).

*Response to Alloantigen Is not Inhibited when Additional Surface Interactions Are Provided by Co-immobilized Antibody.* We have previously shown that co-immobilizing a suboptimal level of alloantigen together with an antibody directed against a surface protein on the CTL can dramatically augment the degranulation response obtained (26). This can occur using antibodies against any of a variety of CTL proteins, including class I, Thy-1, CD8, and CD45. For all of these, the immobilized antibody alone stimulates no response, but in its presence a response is obtained at a surface density of alloantigen that alone is insufficient to stimulate. It was also found that the lag period in alloantigen-stimulated degranulation was greatly reduced or eliminated when augmenting antibody was present. This suggested the possibility that augmentation with co-immobilized antibody might bypass the cytoskeletal-dependent events that appear to be critical for response to antigen.

It was found that degranulation in response to $K^b$ co-immobilized with anti-$K^k$ mAb (specific for class I on the C11 CTL) showed the same CD insensitivity as did the response to immobilized anti-TCR. In contrast, the response to the same level of immobilized $K^b$ alone was effectively inhibited (Fig. 5). Additional experiments confirmed the decreased lag period (26) and lack of CD inhibition in response to alloantigen augmented with co-immobilized antibody (not shown). Thus, it appears that the TCR can effectively bind alloantigen and signal for response in the presence of CD, provided that cytoskeletal-dependent events are bypassed by increasing surface interactions with an augmenting antibody.

*Inhibition of CTL Binding to Alloantigen.* The results described above strongly suggested that the inhibition of degranulation in response to alloantigen by cytochalasins and colchicine resulted from a requirement for cytoskeletal function in order to obtain sufficient surface interaction and TCR occupancy to initiate signaling for response. The avidity of

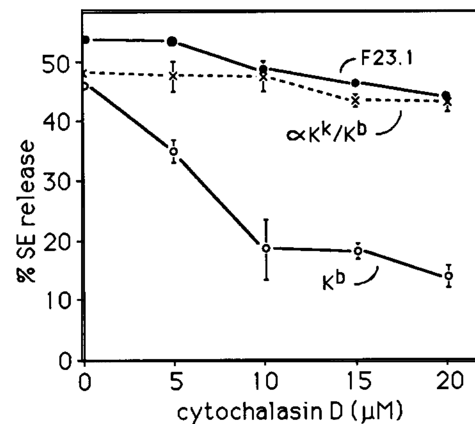


**Figure 5.** Co-immobilized antibody that binds class I on the CTL eliminates CD inhibition of the response to alloantigen. SE response by C11 was determined after incubation for 3 h at 37°C. CD was added to cells 15 min before adding cells to wells and initiating contact. Proteins immobilized on wells were: F23.1 (1 μg/ml) (●); $K^b$ (0.2 μg/well) (O); 11-4.1 mAb (anti-$K^k$, 0.9 μg/well) co-immobilized with $K^b$ (0.2 μg/well) (X). 11-4.1 and $K^b$ were co-immobilized in a two-step procedure as described in Materials and Methods. No response was obtained with cells in wells having just immobilized 11-4.1 (not shown). Error bars indicate standard deviations of triplicate samples.

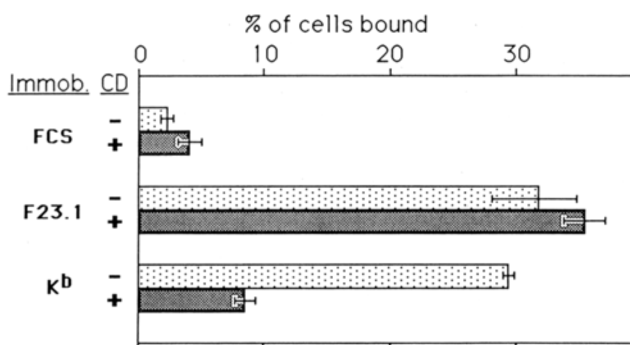


**Figure 6.** CD inhibits CTL binding to alloantigen but not to immobilized F23.1 anti-TCR mAb. $^{51}$Cr-labeled C11 cells were placed in wells having the indicated immobilized protein in the absence (−) or presence (+) of CD at 15 μM. F23.1 was immobilized at 1 μg/well and $K^b$ at 0.2 μg/well. Binding was measured after a 150-min incubation at 37°C as described in Materials and Methods. Total incorporation was 21,853 cpm/$10^5$ cells and spontaneous release after 150 min was 1,708 cpm/$10^5$ cells. CD had no effect on the level of spontaneous release. Error bars indicate standard deviations of triplicate samples.

surface interaction can be directly determined by measuring TCR-dependent CTL binding to alloantigen-bearing surfaces by either visual counting or by using $^{51}$Cr-labeled CTL. Cloned CTL bind specifically to alloantigen but not to irrelevant class I proteins (17, 19). Using the assay described in Materials and Methods, 20–40% of the cells in the well are found to be bound. This is consistent with observations

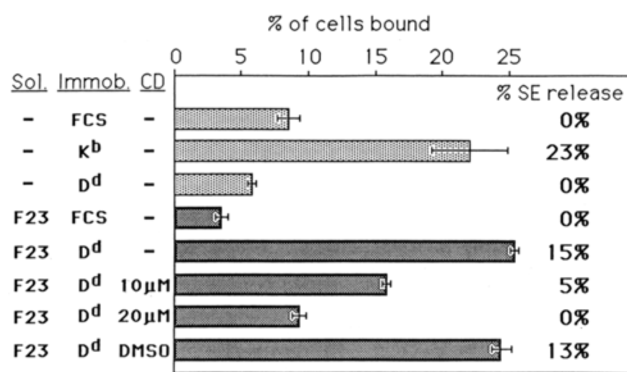


**Figure 7.** CD inhibits CD8-mediated CTL binding to class I triggered by soluble anti-TCR mAb. Wells were prepared having the indicated immobilized $K^b$ alloantigen (0.2 μg/well) or nonantigenic $D^d$ (0.2 μg/well). Where used, soluble F23.1 mAb was present at 0.025 μg/well and was added to the cell suspension immediately before placing the cells into wells and initiating contact by centrifugation. When used, CD or DMSO were added to the cell suspension 15 min before placing the cells into wells. DMSO was present at a final concentration of 1%, corresponding to the level of DMSO present when 20 μM CD was added. After incubation for 180 min at 37°C, aliquots were removed for determination of SE release, and cell binding in the same wells was then measured. Total $^{51}$Cr incorporation was 14,643 cpm/$10^5$ cells and spontaneous release was 589 cpm/$10^5$. Spontaneous release was not significantly different in the presence of CD or DMSO. All measurements were done for duplicate wells and error bars indicate the range for the duplicates.

of others that only a fraction of cells in a clonal population form conjugates with targets (28, 30).

In the presence of CD, binding of C11 to $K^b$ alloantigen was reduced to almost background levels, while binding to immobilized F23.1 anti-TCR antibody was unaffected (Fig. 6). $^{51}$Cr labeling of CTL does not affect stimulated degranulation (unpublished results) and SE release can therefore be determined for the same cells as those assayed for binding. In the experiment shown in Fig. 6, CD inhibited SE release in response to $K^b$ by 88%, but did not inhibit the response to immobilized F23.1 mAb. Thus, neither degranulation nor tight binding of the CTL to allogeneic class I can occur when microfilament function is disrupted.

*Inhibition of CD8-dependent Binding of CTL to Class I Conserved Determinants.* Degranulation in response to alloantigen depends on both TCR-antigen binding and CD8 binding to nonpolymorphic class I determinants (18). More recently, it was shown that CD8-mediated binding of CTL to class I could only be detected after stimulation via the TCR (19). Thus, CTL do not bind to class I (other than the $K^b$ alloantigen) when unstimulated, but bind effectively when stimulated by addition of soluble F23.1 anti-TCR mAb. Furthermore, neither soluble anti-TCR mAb alone nor nonantigenic class I alone will stimulate degranulation. Degranulation does occur, however, when CTL are treated with soluble anti-TCR and then allowed to bind to class I. Thus, it appears that CD8 binding is activated via the TCR and that the resulting CD8–class I interaction provides a signal required for response when TCR crosslinking is minimal (19). The levels of binding and degranulation obtained in response to soluble anti-TCR plus class I are very comparable to those obtained when alloantigen is the stimulus (19).

The fact that TCR-antigen binding and signaling were not inhibited by CD when an augmenting antibody was co-immobilized on the surface suggested that the early cytoskeletal-dependent events seen when alloantigen is the only ligand might involve binding and/or signaling via CD8. Consistent with this possibility, the kinetics of degranulation in response to both immobilized alloantigen (Fig. 3) and to soluble anti-TCR plus immobilized irrelevant class I (data not shown) displayed significant lag periods, while little or no lag is seen for responses to immobilized anti-TCR or to alloantigen co-immobilized with an augmenting antibody (26).

CD was found to inhibit CD8-dependent binding of C11 to irrelevant class I (Fig. 7). In the experiment shown, ~22% of the cells bound to $K^b$ while no binding to $D^d$ above background occurred in the absence of soluble anti-TCR mAb. In the presence of soluble anti-TCR mAb 25% of the cells bound to $D^d$. CD effectively inhibited this binding, while DMSO had no effect at the same final concentration as used for CD addition. As expected, degranulation occurred in response to the combination of soluble anti-TCR mAb and $D^d$, but not to either stimulus alone. CD inhibited the degranulation in parallel with inhibition of binding (Fig. 7). Colchicine similarity inhibited binding and degranulation in response to soluble anti-TCR mAb and irrelevant class I protein in the 0.5–5 mM concentration range (data not shown).

## Discussion

Degranulation in response to target cells is inhibited both by cytochalasins and by colchicine. Similarly, both inhibit degranulation in response to immobilized class I alloantigen, and do so with dose responses comparable to those found for target-stimulated responses. However, at these same concentrations neither cytochalasins nor colchicine effectively inhibited degranulation when immobilized anti-TCR mAb was used as the stimulus. This suggested that neither generation of transmembrane signals nor granule exocytosis per se was affected. Receptor-stimulated degranulation in other cell types is similarly not prevented by cytochalasins, an example being IgE receptor–mediated degranulation (31, 32).

Colchicine can significantly inhibit degranulation in response to immobilized anti-TCR mAb at high concentrations. The Golgi apparatus, microtubule organizing center, and granules of CTL undergo reorientation to the region of contact when CTL bind to target cells (9–14). It may be that microtubule-dependent movement of granules is needed for their translocation to the site of fusion with the plasma membrane, and that colchicine interferes with this. At low doses (<1 mM; Fig. 2), the effective inhibition of response to alloantigen but lack of inhibition of response to anti-TCR mAb strongly suggest that microtubule-dependent events are also required before initiation of degranulation when alloantigen is the stimulus. The differential dose responses may reflect differences in the stability of the microtubule structures involved in antigen interaction versus granule exocytosis.

Inhibition of response to antigen but not to anti-TCR mAb (Fig. 2) suggested, in the case of antigen, that early cytoskeletal events are necessary to achieve sufficient receptor occupancy for signaling to occur. This was further supported by experiments examining the kinetics of degranulation and the time of addition required for effective inhibition. Degranulation in response to immobilized anti-TCR mAb is linear from the time of initial contact with the stimulus. In contrast, a distinct lag of 30–60 min occurs when antigen is the stimulus, and during this time little or no SE release can be detected. Addition of cytochalasins or colchicine early in the lag phase prevents response, but addition late in the lag phase, at a time when degranulation cannot yet be detected, fails to prevent subsequent degranulation (Fig. 3). These results strongly suggest that after initial contact with the antigen-bearing surface, cytoskeletal dependent events must occur that lead to sufficient receptor occupancy for signaling and response to occur.

As in the case of immobilized anti-TCR, a requirement for early cytoskeletal events is also bypassed when an antibody directed against class I protein on the CTL is co-immobilized on the antigen bearing surface (Fig. 5). Here too, the lag phase is eliminated (26) and cytochalasins and colchicine do not inhibit. The co-immobilized anti–class I mAb might simply serve to increase surface interactions and thus promote a higher level of TCR occupancy. Alternatively, antibody binding to the CTL class I protein might deliver some additional signal. In any case, the response remains completely dependent on the presence of antigen, thus allowing the conclusion that cytochalasins and colchicine do not directly interfere, at the level of the individual molecular interactions, with antigen binding by the TCR.

Cytochalasins and colchicine, at least partially, block CTL-target conjugate formation (1–7, 28). It appeared likely that the early events involved in stimulation by antigen might result from a dependence on cytoskeletal function for tight adhesion to the antigen-bearing surface. This was confirmed by the finding that cytochalasins and colchicine inhibited CTL binding to alloantigen. This binding is mediated by both TCR-antigen and CD8–class I interactions, and recent results have shown that CD8 is "activated" to bind class I nonpolymorphic determinants upon minimal crosslinking of the TCR (19). Furthermore, the CD8–class I interaction provides some additional signal(s) needed to activate degranulation. Both cytochalasins and colchicine inhibited the TCR-triggered, CD8-dependent binding and degranulation in the same dose range required for inhibition of binding and response to alloantigen (Fig. 7).

A variety of evidence indicates that initial CTL contact with alloantigen also results in TCR-mediated activation of CD8, which then binds to class I (18 and unpublished data). Only when this has occurred are the CTL tightly adhered to the antigen-bearing surface (as defined by the binding assay used here) and only then does the degranulation response occur. Our results strongly suggest that the cytoskeletal dependence for binding and response to antigen, as defined by sensitivity to agents that interfere with microfilament and microtubule function, results from a requirement for cytoskeletal function for CD8 binding to class I protein. Results obtained using pharmacological agents as inhibitors must be interpreted with some caution, as most of these can have multiple actions. Similar results using CD and CE give considerable confidence that disruption of microfilament function is the likely basis for their effects on binding and response. Whether this is due to a direct involvement of microfilament function in these processes or alternatively to microfilament disruption causing changes in the intracellular environment (e.g., altered cyclic nucleotide levels) remains to be determined. Disruption of the cytoskeleton might interfere with generation of the TCR-derived signal to "activate" CD8. However, we do know that signaling via the TCR can occur, since signal generation for triggering degranulation in response to immobilized anti-TCR mAb, or antigen co-immobilized with antibody, is not inhibited by cytochalasins or colchicine.

T cells undergo cytoskeletal reorganization upon stimulation (9–14), and these may be directly involved in CTL adhesion to antigen bearing surfaces. In the absence of an understanding of the mechanism of CD8 "activation" and binding, one can only speculate as to how cytoskeletal function(s) might be involved. Activated binding might involve cytoskeletal-dependent, directed redistribution of CD8 to the region of surface contact. This would appear unlikely, however, since soluble anti-TCR mAb would presumably not provide a polar signal. Alternatively, activated binding might involve interaction of CD8 with cytoskeletal elements that could, for example, result in formation of CD8 microclusters. C3bi receptors form microclusters upon treatment of polymor-

phonuclear leukocytes with PMA, and there is good correlation between the extent of microclustering and the capacity of the cells to bind C3bi-coated particles (33). Clustering of receptors might enhance the avidity of interaction by lowering the apparent off rate.

Another possibility for a role of the cytoskeleton is suggested by the morphological observations that cytochalasins prevent flattening and spreading of the CTL on the antigen-bearing surface (Fig. 4). CTL interactions with target cells normally involve contact between large areas of the cell surfaces (10) and binding might involve a process of zipping-up the two surfaces, with CD8 and class I providing the zipper. If so, limiting the area of surface contact by interfering with cytoskeletal-dependent deformation of the cell might result in formation of too few CD8–class I bonds to yield high avidity binding or adequate signal generation for a response. A requirement for highly multivalent interaction over a large surface area is supported by the findings that effective CTL interaction with alloantigen on artificial membranes critically depends on (*a*) the alloantigen surface density being in the range of that found on normal cells, a requirement that results primary from the density dependence for effective CD8–class I interaction (18 and Kane, K.P., A.M. O'Rourke, and M.F. Mescher, manuscript in preparation), and on (*b*) the size of the artificial membrane, and thus the area available for surface contact with the CTL (17, 34).

The binding of CD8 to class I appears to be the cytoskeletal-dependent component of the CTL response when class I is the only ligand on the antigen-bearing surface. It is therefore very likely to contribute to the cytoskeletal dependence of CTL interaction with targets, but it may not be the only component of this more complex set of surface interactions that depends on cytoskeletal function. Several observations suggest that LFA-1 binding to its ligand(s) may be another. A variety of evidence indicates that LFA-1 binding to ligands on the target cell can make an important contribution to CTL-target conjugate formation (15, 16). Furthermore, Dustin and Springer (35) and van Kooyk et al. (36) have recently presented evidence that LFA-1–mediated binding of T cells is activated via the TCR. Finally, LFA-1–mediated cell–cell interactions are induced by PMA treatment in many cell types, and these interactions are sensitive to inhibition by cytochalasins (15, 37, 38). It therefore appears likely that both CD8/class I and LFA-1/I-CAM interactions represent TCR-activated, cytoskeletal-dependent adhesion systems important in CTL lysis of target cells.

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