Sustained Signaling Leading to T Cell Activation Results from Prolonged T Cell Receptor Occupancy. Role of T Cell Actin Cytoskeleton

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Summary

Using antigen-specific T cell clones and peptide-pulsed antigen-presenting cells (APCs) we investigated the mechanisms that lead to sustained signaling, known to be required for activation of effector function. Four lines of evidence indicate that the T cell actin cytoskeleton plays a crucial role in T cell activation by antigen-pulsed APCs, but is not required when T cell receptor (TCR) is cross-linked by soluble antibodies. First, addition of antibodies to the major histocompatibility complex molecules recognized by the TCR aborts the ongoing intracellular calcium concentration ($[Ca^{2+}]_i$) increase in preformed T–APC conjugates, indicating that the sustained signaling requires the continuous occupancy of TCR. Second, time-lapse image recording shows that T lymphocytes conjugated to peptide-pulsed APCs undergo a sustained $[Ca^{2+}]_i$ increase, which is accompanied by the formation of a large and changing area of contact between the two opposing membranes. Third, drugs that disrupt the actin cytoskeleton, Cytochalasin D and and C2 *Clostridium botulinum* toxin induce a rapid block of $[Ca^{2+}]_i$ rise, coincident with a block of the cyclic changes in T cell shape. Finally, the addition of Cytochalasin D or of anti-MHC antibodies to preformed conjugates inhibits interferon γ production in an 1-antigen dose- and time-dependent fashion. These results identify T cell actin cytoskeleton as a major motor for sustaining signal transduction and possibly for driving TCR cross-linking and offer an explanation for how T cells equipped with low affinity TCR can be triggered by a small number of complexes on APCs.

It is well established that cross-linking of the TCR/CD3 complex with anti-CD3 antibodies (1), but not its monovalent ligation (2), results in the activation of signal transduction which includes early events, such as tyrosine phosphorylation and Ca^{2+} fluxes that occur within seconds (3), as well as late events, such as transcriptional activation of cytokine genes, that occur within hours (4). A characteristic feature of TCR signaling is that Ca^{2+} mobilization is sustained for at least 30 min (3, 5, 6) and that this prolonged signaling is required for induction of proliferation and cytokine production (7, 8).

The notion that TCR cross-linking is necessary for T cell activation is difficult to extrapolate to the physiological T cell–APC interaction. Indeed, the TCR has low affinity for peptide–MHC (9, 10) and high off rate (11). Yet as few as 100 peptide–MHC complexes displayed on the surface of APCs are sufficient to trigger T cells (12, 13). It is therefore difficult to envisage how low affinity receptors that readily dissociate from their ligand may be extensively cross-linked and may transduce a sustained signaling when they interact with a small number of peptide–MHC complexes (14, 15).

In this work we were interested to investigate in normal T cells interacting with peptide-pulsed APCs the mechanisms that sustain signaling and allow sensitive detection of a small number of ligands. We report that sustained signaling requires a prolonged TCR occupancy and that a functional actin cytoskeleton is required for prolonged signaling and for the activation of T cell effector function. We propose that via an actin cytoskeleton-dependent mechanism T cells can engage for a prolonged time a sufficient number of TCRs and in this way can respond to low number of cell-bound antigens.

Materials and Methods

Measurement of Intracellular Calcium Concentration ($[Ca^{2+}]_i$) and Conjugate Formation. $CD4^+$ T cells (clones KS-162 and KS-145, DR1104-restricted and specific for a tetanus toxin peptide $TT_{830-843}$ (16) were loaded with Indo-1 AM (Sigma Chemical Co., St. Louis, MO) as described (17). Cells were mixed at a 1:1 ratio with autologous EBV-B cells that had been pulsed 3 h at 37°C with $TT_{830-843}$, centrifuged 1 min at 1,500 rpm, incubated 1 min at 37°C, resuspended in the presence or absence of saturating concentrations of anti-DR antibody (L243; American Type Culture Collection [ATCC], Rockville, MD), and analyzed on a Coulter Elite Flow cytofluorimeter (Coulter Electronics Inc., Hialeah, FL) to detect Ca^{2+} fluxes and conjugate formation as described (17). Only

live (based on forward and side scatter [FSC] criteria) and Indo-1-loaded cells were included in the analysis. In this way, the unloaded APCs were only visible when conjugated with a loaded T cell. Because conjugates have a higher FCS than single T cells, we could estimate the percent of conjugates on the FSC histogram. In some experiments T cells were resuspended in the presence or absence of 10 μM Cytochalasin D (CD)[1] (Sigma Chemical Co.), a concentration that completely inhibits T cell spreading and motility (17) or C2 *Clostridium botulinum* toxin (1.5 μg C2I plus 3 μg C2II) (18). In parallel experiments, the cells were stimulated with cross-linked anti-CD3 (10 μg/ml TR66; 19) plus 20 μg/ml goat anti-mouse Ig (The Jackson Laboratory, Bar Harbor, ME).

Fluorescence Imaging and $[Ca^{2+}]_i$ Analysis. T cells were loaded with 5 μg/ml FURA 2-AM (Molecular Probes, Inc., Eugene, OR) for 45 min at 37°C in 5% FCS Hepes-buffered RPMI medium and plated in 96-well flat-bottom microplates containing DR1101 L cell transfectants pulsed with 25 nM $TT_{830-843}$ and loaded with 0.5 μM 2',7'-bis-(carboxyethyl)-5(6')-carboxyfluorescein (BCECF-AM; Calbiochem-Novabiochem Corp., La Jolla, CA) for 30 min at 37°C. Fluorescence measurements were done on a Zeiss Axiovert microscope equipped with an Ion Imaging System (ImproVision, Coventry, UK). Cells were consecutively excited with 340 and 380 nm at 5-s intervals by means of a fast-spinning filter wheel. Both emissions were recorded with a CCD camera (model ISIS-2; Photonic Science, Millham, UK) through a 420-nm-long-pass optical filter. Using IonVision software, the 340:380 ratio was calculated for every time point. The BCECF-loaded cells did not change the exitation ratio during the whole experiment. The FURA-2-loaded T cells changed the ratio of the exitation at 340 and at 380 nm upon the $[Ca^{2+}]_i$ rise. The exitation intensity at any time point was rationed and the results displayed on a pseudocolor scale. Calculations of the ratios were done by the software on selected cells and exported in graph form. To enhance the visibility of the T cells conjugated to target cells, we changed the lower part of the pseudocolor scale (in which the APCs fall) into violet using the Adobe Photoshop program (Apple Computer Inc., Cupertino, CA).

Measurement of IFN-γ Production. 10^5 T cells and 10^5 APCs were mixed in 200 μl culture medium in 96-well U-bottom microplates. The stimulator cells for clone KS-162 were autologous EBV-B cells pulsed with high (50 μM) or low concentrations (25 nM) $TT_{830-843}$, for 3 h at 37°C. The stimulator cells for the alloreactive $CD8^+$ CTL clone KU10 were the U937 cell line (ATCC) either fresh or fixed for 30 s with 0.1% glutaraldehyde. The plates were centrifuged to allow conjugate formation and 10 μM CD or saturating concentrations of anti-DR antibody (L243) or anti-LFA-1 (HB 203; ATCC) plus anti-intercellular adhesion molecule ICAM-1 (RR1/1; provided by T. Springer, Department of Pathology, Harvard Medical School, Boston, MA), were added at different time points. In some experiments, T cells were stimulated with cross-linked anti-CD3 or with 100 nM PMA (Sigma Chemical Co.) plus 0.5 μg/ml ionomycin (Calbiochem Novabiochem Corp.). After 6 h of incubation, the supernatant was col-

[1] *Abbreviations used in this paper:* CD, Cytochalasin D; ICAM-1, intercellular adhesion molecule 1.

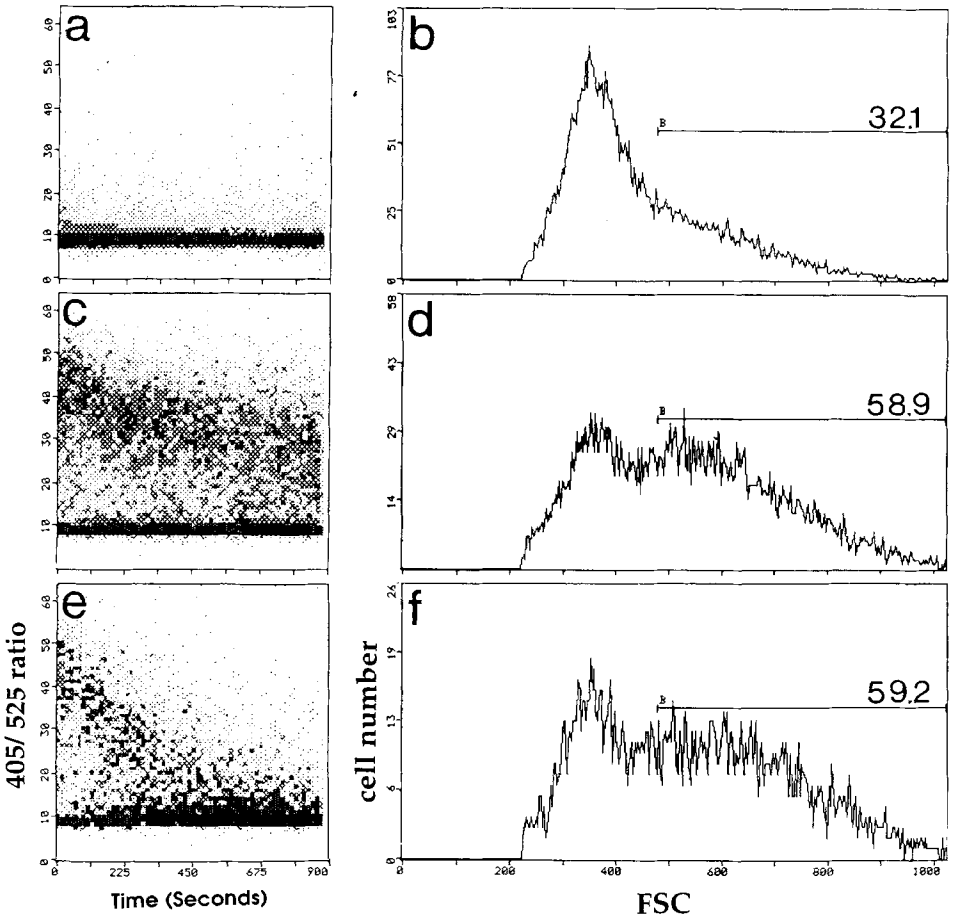

Figure 1. The sustained $[Ca^{2+}]_i$ increase in antigen-specific T-APC conjugates is blocked by addition of anti-DR antibody. Indo-1-loaded T cells were allowed to form conjugates with autologous APCs. (*a*, *c*, and *e*) Time course of $[Ca^{2+}]_i$ increase and (*b*, *d*, and *f*), percentage of T cells in conjugates with the APCs as assessed accumulatively over the entire recording time. (*a* and *b*) T cells conjugated to unpulsed APCs. (*c* and *d*) T cells conjugated with peptide-pulsed APCs (25 nM $TT_{830-843}$). (*e* and *f*) T cells conjugated with peptide-pulsed APCs to which saturating concentrations of anti-DR antibody were added 1 min after conjugate formation, i.e., time zero of recording. The number indicates the percentage of conjugates. Though some conjugates can be disrupted while passing through the sorter, note that the percentage of conjugates measured in the presence or absence of antibodies did not change.

lected and tested for the concentration of IFN-γ by a sandwich ELISA (20).

Results and Discussion

Sustained Signaling in T-APC Conjugates Is Blocked by the Addition of Anti-DR Antibodies. $[Ca^{2+}]_i$ increase is an early activation event detected within seconds after formation of specific conjugates and lasting for a time period of at least 30 min (5, 6). We were interested to investigate the mechanism of sustained signaling in T cells stimulated by the specific antigen. Antigen-specific, DR-restricted T cell clones were allowed to form conjugates with APCs pulsed with the specific peptide and $[Ca^{2+}]_i$ was then recorded by cytofluorimetric analysis. Fig. 1, *c* and *d* shows that, after conjugation with APCs pulsed with the specific peptide (25 nM $TT_{830-843}$), T cells undergo a rapid and prolonged $[Ca^{2+}]_i$ increase. Addition of saturating concentrations of anti-class II antibody after conjugate formation, at the beginning of recording, results, within 4–6 min, in a sharp inhibition of Ca^{2+}

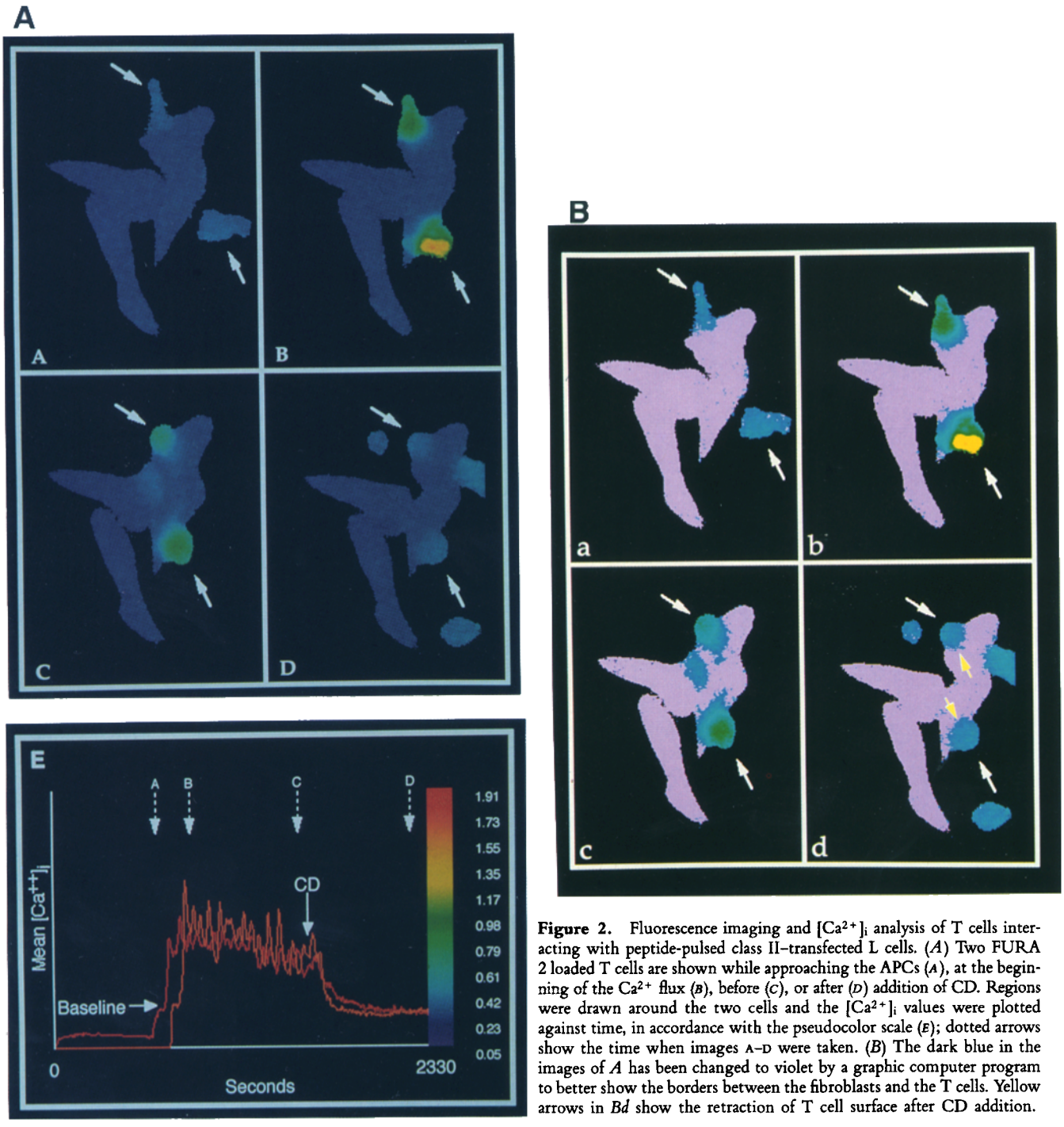

Figure 2. Fluorescence imaging and $[Ca^{2+}]_i$ analysis of T cells interacting with peptide-pulsed class II–transfected L cells. (*A*) Two FURA 2 loaded T cells are shown while approaching the APCs (*A*), at the beginning of the Ca^{2+} flux (*B*), before (*C*), or after (*D*) addition of CD. Regions were drawn around the two cells and the $[Ca^{2+}]_i$ values were plotted against time, in accordance with the pseudocolor scale (*E*); dotted arrows show the time when images A–D were taken. (*B*) The dark blue in the images of *A* has been changed to violet by a graphic computer program to better show the borders between the fibroblasts and the T cells. Yellow arrows in *Bd* show the retraction of T cell surface after CD addition.

mobilization (Fig. 1 *e*), without disrupting the conjugates (Fig. 1 *f*).

The possibility that anti-DR antibodies might interfere with T cell signaling by acting at the level of the responding T cell was excluded by the following experiments. First, whereas pretreatment of APCs with anti-DR antibodies results in a complete inhibition of $[Ca^{2+}]_i$ increase in DR-restricted T cell clones, pretreatment of the responding T cells did not have any effect. Second, anti DR antibodies do not inhibit $[Ca^{2+}]_i$ increase when added to conjugates between MHC class I–restricted cytotoxic T cells and target cells (data not shown). Third, an inhibitory effect of anti-class II antibody was observed in mouse class II–restricted T cells that do not express class II molecules (Valitutti, S., and J. Kirberg, unpublished observations).

The observation that an ongoing signaling can be aborted by the addition of anti-DR antibodies, indicates that the prolonged activation of the signal transduction machinery results from a long-lasting TCR occupancy. In principle, the anti-DR antibodies could interfere with a long-lasting occupancy of the TCR in two ways. First, they might disrupt by allosteric antagonism a substantial number of TCR/peptide–MHC complexes that otherwise would remain associated for prolonged time. Second, by saturating all unoccupied MHC class II molecules they should block the formation of new TCR/peptide–MHC complexes, as well as competitively impede the reassociation of complexes that have been transiently dissociated. We favor the second mechanism for the following reasons. The off rates of the TCR-ligand binding have been reported to be very fast (11) and, at the antigen concentration we used, the number of peptide–MHC complexes on APCs is very low (50–100 complexes/APC; Valitutti, S., and S. Müller, unpublished observations). In these conditions, a long-lasting TCR occupancy may require a sequential formation of new contacts between few peptide–MHC complexes and the previously engaged or additional TCRs, a process that is blocked by the binding of the antibodies to the MHC molecules.

A Functional Actin Cytoskeleton Is Required for Sustained Signaling in Antigen-stimulated T Lymphocytes. Indirect evidence suggests that the actin cytoskeleton may play a role in the process of T cell activation. T lymphocytes interacting with target cells undergo sequential changes of shape while fluxing Ca^{2+} (21). In addition, TCR triggering induces actin polymerization (22), an increase in the affinity of LFA-1 for its ligand (23), and a more stable association of LFA-1 with the cytoskeleton (24).

We therefore investigated the contribution of the actin cytoskeleton to TCR-coupled sustained signaling. We recorded morphology and $[Ca^{2+}]_i$ in single T cells interacting with peptide-pulsed, DR-transfected fibroblasts and tested in this system the effect of CD, a drug that interferes with actin cytoskeleton function (25, 26). In a typical experiment, T cells show high locomotion, i.e., they move on the plastic culture dish or on cell monolayers until they find a peptide-pulsed APC where they stop (Fig. 2 *A*, A and Fig. 2 *B*, B). This block of locomotion has been already observed (21) and may be related to the increase in affinity of LFA-1/ICAM-1 binding coupled to TCR signaling (23). After conjugation, T cells flux Ca^{2+} with a characteristic oscillatory pattern for a prolonged time (Fig. 2, *A*, B, C, and E) (5, 21). T cells anchored to the APCs and which have therefore stopped locomotion, undergo continuous changes in the area of contact with the APCs (compare Fig. 2 *B*, B and C). Upon addition of CD, within 2–3 min the level of $[Ca^{2+}]_i$ falls to baseline (Fig. 2 *A*, D and E), simultaneously with the arrest of motility and retraction of the area of interaction (Fig. 2 *B*, D). These effects were clearly observed in all T–APC conjugates examined.

By flow cytometry we quantified the effect of CD on Ca^{2+} mobilization in T cells that form conjugates with APCs pulsed with different concentrations of peptide. As shown in Fig. 3, *B* and *C*, CD added to preformed conjugates has an inhibitory effect on sustained $[Ca^{2+}]_i$ increase without reducing the number of conjugates. This result indicates that the inhibition of Ca^{2+} mobilization is not simply due to a loss of T cell–APC contact subsequent to cytoskeleton disruption. Furthermore, this inhibitory effect is more marked when APCs are pulsed with low peptide concentrations (Fig. 3 *C*). CD is not toxic for the signal transduction machinery of the T cell, since it does not inhibit, but in fact increases, $[Ca^{2+}]_i$ mobilization induced by cross-linked anti-CD3 antibodies (22, and Fig. 3 *A*).

To exclude the possibility that the inhibition by CD might be due to effects of the drug different from the well-known effect on cytoskeleton, we tested *C. botulinum* C2 toxin which affects the actin cytoskeleton with a different mechanism of action (18). Fig. 4 shows that this drug, like CD, blocks $[Ca^{2+}]_i$ mobilization induced by peptide-pulsed APCs, but affects neither the stability of preformed conjugates (Fig. 4 *B*) nor the signaling induced by anti-CD3 antibodies (Fig. 4 *A*). The time course of inhibition is slower (20–30 min), compatible with the time of intoxication (18).

Taken together, these results show that the disruption of the actin cytoskeleton affects neither the TCR-coupled signal transduction machinery nor the stability of T cell–APC conjugates, but inhibits the TCR triggering by the specific antigen. It is possible that the actin cytoskeleton could be required for the oligomerization of TCRs. However, we consider this possibility unlikely since we observed that CD inhibits signaling even when added 15 min after initiation of Ca^{2+} flux (Fig. 2), i.e., at a time when cross-linking and oligomerization should have occurred (27).

We propose that a functional actin cytoskeleton is used by T cells to form and maintain an area of interaction with the APC surface that favors the formation of new contact as well as the reassociation of TCR–ligand complexes that have been transiently dissociated.

T Cells Accumulate Triggering Signals Collected on the APC Surface. The above results indicate that the actin cytoskeleton plays an essential role in the antigen-specific activation of the TCR-coupled sustained signaling, particularly when the number of peptide–MHC complexes is low. As a consequence, a functional actin cytoskeleton should be important, espe-

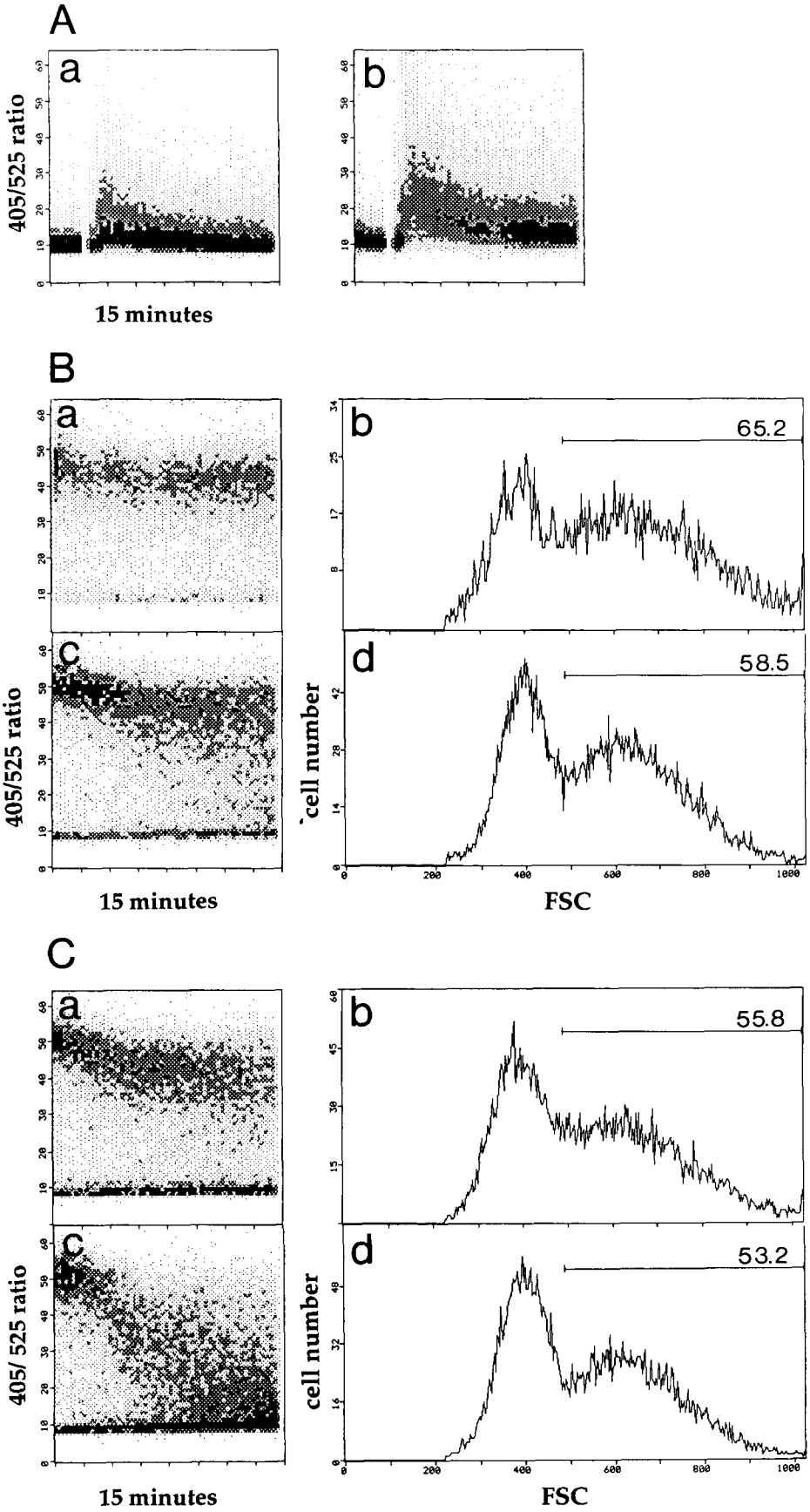

Figure 3. The sustained $[Ca^{2+}]_i$ increase in preformed antigen-specific conjugates is inhibited by CD. (*A*) Time course of $[Ca^{2+}]_i$ increase in T cells stimulated by anti-CD3. T cells were untreated (*a*) or preincubated for 10 min with CD (*b*). (*B*) Time course of T cell $[Ca^{2+}]_i$ rise and percentage of conjugates between T cells and EBV-B cells pulsed with 50 μM $TT_{830-843}$. The cells were untreated (*a* and *b*) or treated with CD at 37°C 1 min after conjugate formation, i.e., time zero of recording (*c* and *d*). (*C*) As in *B*, using EBV-B cells pulsed with 25 nM $TT_{830-843}$. The vehicle of the drug (DMSO 0.1%) did not affect the number of conjugates and $[Ca^{2+}]_i$ increase (not shown).

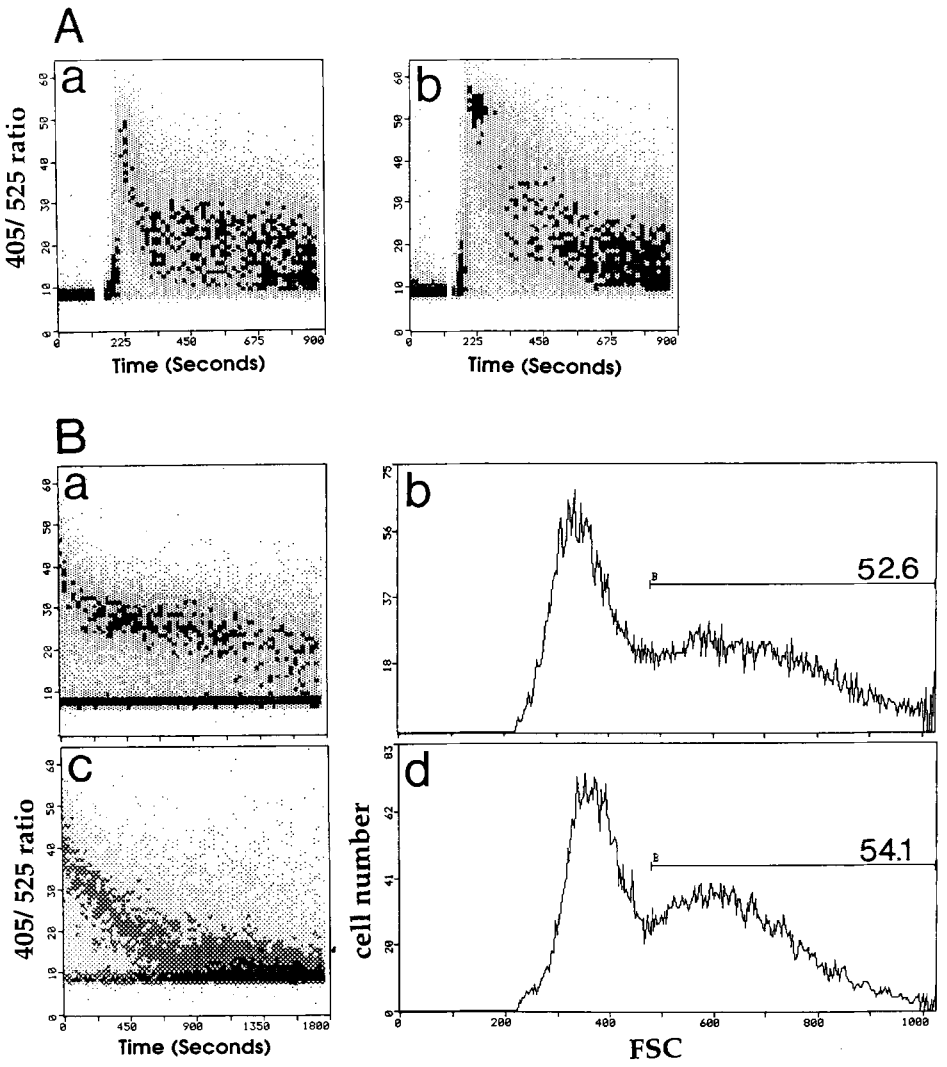

Figure 4. Effect of *C. botulinum* C2 toxin on T cell responses to peptide-pulsed APCs. The experiments were performed as in Fig. 3. (*A*) Response to cross-linked anti-CD3 in T cells untreated (*a*) or preincubated for 30 min with C2 toxin (1.5 μg C2I plus 3 μg C2II) (*b*). (*B*) $[Ca^{2+}]_i$ and the percentage of conjugates in T cells forming specific conjugates. The T cells were either untreated (*a* and *b*) or preincubated for 15 min with C2 toxin (1.5 μg CI plus 3 μg CII) (*c* and *d*).

cially when antigen is present at low (physiological) concentrations, to allow the T cell to accumulate and integrate trigger events required for activation of effector function.

We therefore used IFN-γ production as a readout for T cell activation and tested the effect of disrupting the cytoskeleton or preventing antigen recognition at different times after conjugate formation. As shown in Fig. 5 *a*, CD does not inhibit IFN-γ production when T cells are stimulated by cross-linked anti-CD3 antibodies or PMA plus ionomycin, demonstrating that CD is not toxic to the biological response of T cells. Interestingly, when T cells were conjugated by centrifugation with APCs pulsed with low peptide concentrations (25 nM, inducing 50–60% of the maximal IFN-γ response), CD had a marked and time-dependent inhibitory effect. Addition of CD 10 min after conjugate formation resulted in a 90% inhibition, whereas addition at later time points resulted in only a partial inhibition of IFN-γ production (Fig. 5 *b*). However, when T cells were stimulated by APCs pulsed with high peptide concentrations (50 μM), CD had little inhibitory effect. A similar time-dependent inhibition was observed in a cytotoxic alloreactive T cell clone stimulated by fixed as well as fresh target cells (Fig. 5 *c*), indicating that the integrity of the T cell cytoskeleton and not of the APC cytoskeleton is required for T cell activation. Finally, a time and antigen dose-dependent inhibition was observed when antibodies to DR molecules or to LFA-1 and ICAM-1 were added to preformed conjugates (Fig. 5, *d* and *e*).

These results suggest that at low antigen concentrations, T cells use the cytoskeleton and adhesion molecules to accumulate activation signals up to a threshold required for the activation of the effector function.

Concluding Remarks

We have shown that the sustained signaling that leads to T cell activation requires a prolonged TCR occupancy and a functional T cell actin cytoskeleton.

These results are compatible with the following model of T cell–APC interaction (28). T cells use their intrinsic motility to form random conjugates with other cell types. Upon initial TCR triggering by engagement of a minimum number of TCRs with the cognate peptide–MHC complex, the adhesion increases (23) and T cells polarize towards the APCs (27). These conjugates, however, are not static since T cells

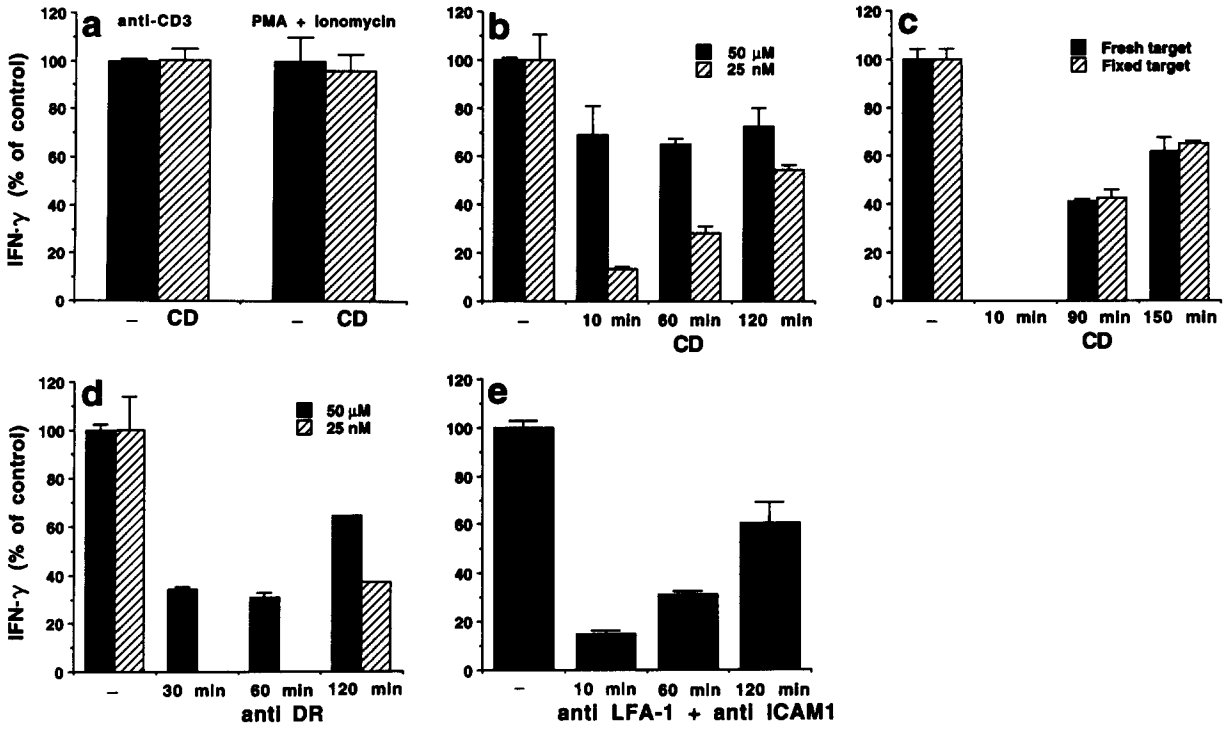

Figure 5. IFN-γ production by antigen-stimulated T cells is inhibited by CD and by antibodies to DR and adhesion molecules in an antigen dose- and time-dependent fashion. (*a*) IFN-γ production by KS-162 T cell clone stimulated with cross-linked anti CD3 or ionomycin plus PMA in the absence (*black bars*) or in the presence (*shaded bars*) of CD; (*b*) IFN-γ production by KS-162 stimulated with EBV-B cells pulsed with 50 μM (*black bars*) or 25 nM (*shaded bars*) $TT_{830-843}$ in the absence or presence of CD added at different times after conjugation. (*c*) IFN-γ production by an alloreactive CTL clone (KU10) stimulated with fresh (*black bars*) or fixed (*shaded bars*) target cells in the presence of CD added at different times. (*d*) IFN-γ production by KS-162 T cell clone stimulated with 50 μM (*black bars*) or 25 nM (*shaded bars*) $TT_{830-843}$ in the absence or presence of anti-DR L243 antibody. (*e*) IFN-γ production by the KU10 CTL clone in the absence or presence of a mixture of anti LFA-1 plus anti-ICAM-1 antibodies added at different times after conjugation.

continually change the area of contact with the APC surface. This could allow the T cell to scan the APC surface and gather new peptide–MHC complexes as well as reengage complexes that may have been spontaneously dissociated. As a consequence, a small number of peptide–MHC complexes can engage and trigger over time a much higher number of TCR/CD3 complexes than those engaged at any time point.

This model can explain how T cells can be triggered by a very small number of ligands (12, 13). We have shown that at low antigen concentration T cells integrate, for a time period of at least 2 h, the signals collected on the APC surface. Since the peptide–MHC complexes are stable and can be presented for a long time on the surface of the APC (29), even a very low number of such complexes may be sufficient to engage, in several rounds of ligation, a relatively high number of TCRs, thus triggering T lymphocyte responses.

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