

Early Biochemical Signals Arise from Low Affinity TCR–Ligand Reactions at the Cell–Cell Interface

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Summary

The kinetics of acid release by a mixture of T cells and antigen presenting cells were measured with a microphysiometer during a brief exposure to antigenic peptides. We find that some of the early biochemical events that lead to cellular proliferation cause a specific increase in the rate of acid release. The duration of this increase in acid release reflects the life-time of the peptide–MHC complexes. Peptides that form long-lived complexes produce a response that is stable for more than an hour. Serial TCR engagement is suggested by the observation that the amplitude of this stable response can be rapidly shifted up or down with additional agonist peptide or with antibodies that block T cell receptor binding. Cells briefly exposed to a peptide that forms short-lived peptide–MHC complexes produce a response that decays rapidly as peptide is washed away. A quantitative analysis of the kinetics of this decay in acidification demonstrates that intercellular TCR–ligand reactions are rapid, reversible, and of low apparent affinity with <20% of peptide–MHC ligand bound to a TCR at any one time. These results demonstrate that the fraction of peptide–MHC ligands bound to TCRs at the cell–cell interface is no higher than anticipated from the affinities observed in solution for isolated TCRs and ligands.

Many cellular recognition events involve specific interactions of receptor-ligand pairs at a cell-cell interface. It has been found that the binding affinities for these receptor-ligand pairs are often low when the molecules are isolated and studied in solution (1). However, intermembrane molecular events are approximately two-dimensional and involve additional adhesion molecules (2). Thus, the fraction of ligands bound to receptors at the cell-cell interface (i.e., the apparent affinity) might differ from that expected from the affinities measured in solution. To address this issue, we have studied the reactions of TCRs with their ligands at the cell-cell interface. The ligands are peptides bound to MHC proteins. The reactions of isolated TCRs and their ligands in solution are characterized with affinities in the range of 0.1–40 μM (3–7).

The rate of acid release, as measured with a microphysiometer, is a sensitive monitor of metabolic responses to extracellular ligands (8, 9). Addition of antigenic peptide to a mixture of T cells and APCs results in an increase in the acid release due to the interaction of new peptide–MHC complexes on the APCs with specific TCRs on the T cells (10). During initial peptide exposure, the rate of this increase is related to the formation of peptide–MHC com-

plexes and binding of these complexes to TCRs. If the cells are only briefly exposed to peptide and then washed to remove unbound material, new peptide–MHC complexes are no longer formed and changes in the response are directly related to the reactions of peptide–MHC complexes with TCRs at the cell–cell interface. We have used this latter feature to study the apparent affinities and kinetics for TCR–ligand reactions at the cell–cell interface.

Materials and Methods

Peptides. The peptides myelin basic protein (MBP) Ac(1–14), ASQKRPSPQRHGSKY, moth cytochrome *c* (MCC) 88–103, ANERADLIAYLKQATK, and analogues were synthesized with standard Fmoc chemistry, purified by reverse phase HPLC (Vydac C₁₈, acetonitrile/water) and characterized by mass spectrometry.

Cell Lines. The MCC-specific 5C.C7 T cells were derived from the 5C.C7 αβ-TCR transgenic mouse (11). Spleen cells, initially primed with 10 μM MCC and expanded for 8 d, were restimulated with irradiated (3,000 rad) B10.BR spleen cells and MCC. After 10 d of culture in the presence of IL-2, cells were sorted CD4⁺. The MBP-specific B10A.F2 T cell clone was obtained from lymph node cells harvested B10.A mice immunized subcutaneously at the base of the tail with 200 μg rat MBP Ac(1–11)

in a 50:50 emulsion of complete Freunds adjuvant *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). The cells were stimulated with 40 μ M MBP Ac(1-11) and irradiated (3,000 rad) B10.A spleen cells three times (8–10-d intervals) and then cloned by limiting dilution.

Microphysiometry. Acid release was measured as described previously (10). Briefly, T cells rested from stimulation (7–12 d) were combined with APCs that had been cultured overnight in RPMI 1640 medium containing 5% fetal calf serum. APCs included Chinese hamster ovary (CHO) cells transfected (12) with I-E^k, murine L cells transfected (13) with I-A^k, CH27 B-cell lymphomas and B10.BR splenocytes. Cells ($3\text{--}6 \times 10^6$) mixed at a ratio of 20 T cells to 1 APC were collected by centrifugation and resuspended in 105 μ L medium which was then mixed with 35 μ L of melted low-temperature-melting agarose at 37°C. The agarose-cell mixture (10 μ L) was immediately spotted onto the membrane of a Cytosensor cell capsule (Molecular Devices, Sunnyvale, CA). After 10 min, the cell capsule was assembled and loaded in the microphysiometer chamber maintained at 37°C. The chamber was perfused (50 μ L/min) with low buffer RPMI 1640 medium containing 1 mM sodium phosphate, 1 mg/mL endotoxin-free bovine serum albumin (Calbiochem-Novabiochem, San Diego, CA) and no bicarbonate (pH 7.4). Extracellular acidification rates were determined with 20 s potentiometric rate measurements after a 58-s pump cycle and 10-s delay (90 s total cycle time). Maximal responses to antigenic peptide typically ranged from 150–200% of the initial basal acidification rates (60–100 μ V s⁻¹). Variations in maximal rates between different chambers during a single experiment were less than 10%.

Cell Proliferation. Proliferation of 5C.C7 T cells (3×10^4) incubated with irradiated (3,000 rad) B10.BR spleen cells (5×10^5) and peptide was measured as the uptake of [³H]thymidine (1 μ Ci/well). Maximum proliferation ($\sim 1 \mu$ M MCC) was $\sim 10^5$ cpm with a background $\sim 10^3$ cpm (no peptide). No counts above background were observed for incubations with MCC-99R, -99Q, or -99E at concentrations $\leq 50 \mu$ M. Proliferation of B10.AF2 T cells was measured using irradiated B10.A spleen cells.

Results and Discussion

Antigen activation of I-E^k restricted 5C.C7 T cells derived from the 5C.C7 $\alpha\beta$ -TCR transgenic mouse (11) was evaluated with a microphysiometer. Addition of MCC peptide to a mixture of 5C.C7 T cells and APCs results in an increase in the rate of acid production within minutes of peptide addition (Fig. 1 a). Inhibition of the response by amiloride (Fig. 1 a) demonstrates that the initial response involves the Na⁺/H⁺ antiport which is frequently activated by cellular stimuli (14). The MCC-99R, -99E, and -99Q peptides are single amino acid mutants modified at a 5C.C7 TCR contact (15). Each of these forms a stable complex with the I-E^k MHC protein (16). However, only the native MCC peptide causes the proliferation of 5C.C7 T cells (Materials and Methods) or an acidification response (Fig. 1). It has been demonstrated that anti-MHC antibodies can disrupt intracellular Ca²⁺ fluxes and T cell proliferation (4, 17). We observe that the I-E^k-specific 14-4-4S antibody (18), but not I-A^k-specific antibody, can block acid release by a mixture of 5C.C7 T cells and APCs that have previously responded to peptide; the acidification response is regained if antibody is washed out (data not shown). We also

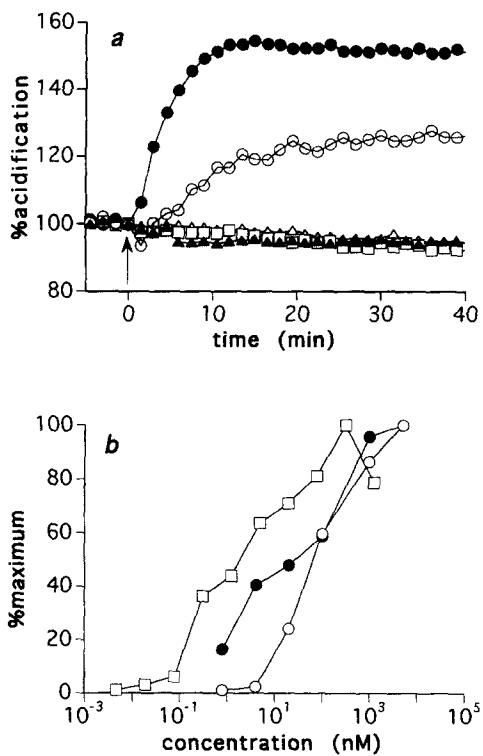


Figure 1. Antigenic peptide produces a specific increase in the rate of acid production for a mixture of T cells and APCs. (a) The initial acidification response of T cells from the 5C.C7 $\alpha\beta$ -TCR transgenic mouse to antigenic peptide (MCC) is partly blocked by amiloride, a Na⁺/H⁺ antiport inhibitor. Shown are the acidification rates for a mixture of 5C.C7 T cells and APCs (20:1) during continuous exposure to (●) 4 μ M MCC, (○) 4 μ M MCC + 5 μ M hexamethylene amiloride, (□) 5 μ M hexamethylene amiloride, (▲) 4 μ M MCC-99R peptide, and (△) media. Reagents were added at t = 0 min (arrow) and each data point corresponds to a 20 s acidification rate measurement recorded every 90 s. Acidification rates are normalized to the basal rates at t = 0 min (100%). CH27 B cell lymphomas were used as APCs. No increase in acidification was noted in response to the MCC-99Q or 99E peptides in multiple independent experiments (data not shown). (b) The proliferation (□) and microphysiometer (●, ○) dose responses of 5C.C7 T cells to MCC. Proliferation is measured as uptake of [³H]thymidine and microphysiometer response is measured as the % increase in acidification rate after 30 min of continuous exposure to peptide. Proliferation and % acidification are normalized to the corresponding maximum responses: (□) 1.55×10^5 cpm, (●) 121% and (○) 159%. B10.BR spleen cells were used as APCs for proliferation; (●) CHO cells transfected with I-E^k and (○) CH27 cells were used as APCs for the acidification dose responses. Acidification responses produced with spleen and CH27 cells are comparable (data not shown).

compared the proliferation and microphysiometer MCC dose responses of the 5C.C7 T cell line (Fig. 1 b). The proportional slopes of the acidification and proliferation dose-response plots suggest that the initial response is a quantitative measure of some early biochemical event related to cellular proliferation. The dose microphysiometer response is relatively insensitive to different APCs (Fig. 1 b).

A steady state of signals due to serial TCR engagement (19) is suggested by the sustained signaling of T cells after peptide exposure. Brief exposure (90 s) of a mixture of the 5C.C7 T cells and APCs to the MCC peptide produces a

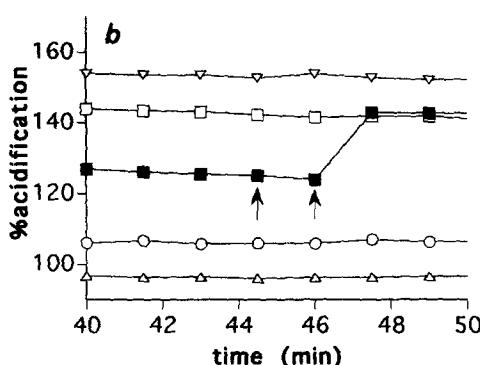
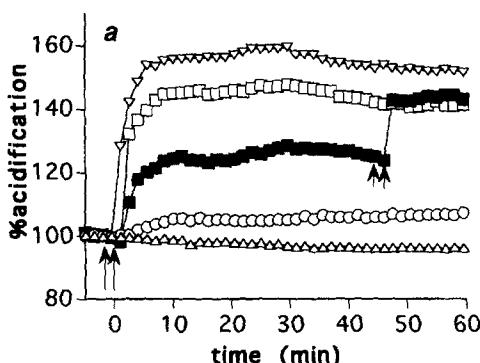


Figure 2. A sustained T-cell acidification response is maintained by steady state triggering of TCRs. (a) Shown are the acidification rates of a mixture of 5C.C7 T cells and APCs in response to (○) 1 nM, (■) 10 nM, (□) 100 nM, (▽) 1 μ M MCC peptide and (△) media added for 90 s (first two arrows). At $t = 45$ min, the chamber that had been exposed to 10 nM peptide (■) was perfused with 100 nM MCC peptide for another 90 s (second two arrows). (b) An expanded view of data from a showing the increase in acidification during the second 90-s exposure to peptide. CHO cells transfected with I-E k were used as APCs.

rapid increase in acid production that remains at a stable plateau for more than an hour (Fig. 2 a). After 45 min, an additional pulse (90 s) of a higher concentration of MCC peptide causes a rapid (90–180 s) increase to a higher plateau (Fig. 2 a). The plateau attained after the second pulse (100 nM MCC) is the same as that attained by a single pulse of 100 nM MCC peptide (Fig. 2 a). Evidently the T cell response to peptide is not decreased by prior stimulation during the first hour of stimulation. The increase after the second pulse stabilizes 1–2 min after peptide has been washed out (Fig. 2 b). This duration is independent of the peptide concentration used in the second pulse (data not shown). Control experiments in which pH is monitored during introduction and removal of peptides show that peptide diffusion through the microphysiometer chamber is fast. The chamber can be completely saturated with or rinsed free of peptide in less than 20 s (data not shown). The time required to reach a new plateau after excess peptide is completely removed, about 1–2 min, is thus likely to be an upper limit for the time constants for the TCR-ligand binding and dissociation reactions.

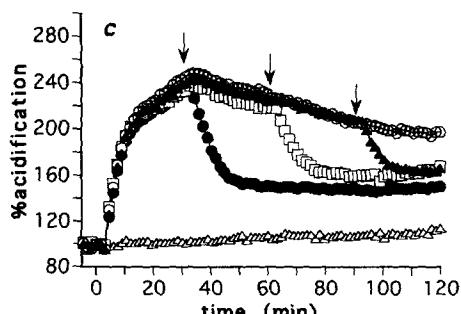
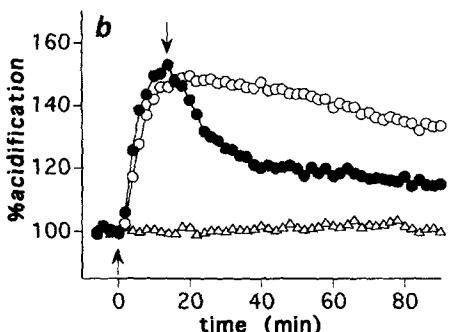
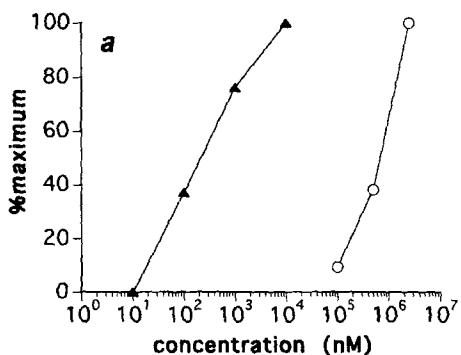


Figure 3. Short-lived peptide-MHC complexes produce a transient increase in T cell acidification. (a) The increase in acid release is proportional to the log of peptide concentration (see also Fig. 1 b). Shown are acidification dose responses for B10A.F2 T cells exposed to (○) MBP Ac(1-14) and (▲) Ac(1-14)4A normalized to the % acidification after 30 min exposure to the at maximum peptide concentration. Half-maximal proliferation for MBP Ac(1-14) and Ac(1-14)4A is obtained at peptide concentrations of 10 μ M and 1 nM, respectively. (b) The acidification rates of a mixture of B10A.F2 T cells and APCs briefly exposed to (○) 10 μ M MBP Ac(1-14)4A, (●) 100 μ M MBP Ac(1-14), and (△) media. Peptide was added at $t = 0$ min (first arrow); at $t = 15$ min (second arrow) peptide addition was stopped and the chamber was continuously perfused with media. (c) The long-lived T cell response to MBP Ac(1-14)4A is sustained by active TCR ligation. Mixtures of B10A.F2 T cells and APCs were exposed to (●, □, ▲, ○) 1 μ M MBP Ac(1-14)4A or (△) media for 30 min. 10 μ g/mL of the I-A k -specific 10-2.16 antibody was added (●) immediately, (□) 30 min, and (▲) 60 min after peptide exposure (first, second, and third arrows, respectively). Identical results were also obtained with 1 and 100 μ g/mL 10-2.16 antibody (data not shown). L cells transfected with I-A k were used as APCs.

The kinetics of T cell acidification responses to short-lived peptide–MHC complexes provide further insight into TCR–ligand interactions at the cell–cell interface. The NH₂-terminal MBP peptide is a murine autoantigenic peptide that forms short-lived complexes with the I-A^a and I-A^k MHC proteins (20, 21). The peptide in which the lysine at position-4 is substituted with alanine, MBP Ac(1-14)4A, dissociates from detergent-soluble (21) or cell surface I-A^k (K. Tate and P. P. Jones, unpublished results) with $t_{1/2} = 30$ min. Presumably dissociation of the native MBP Ac(1-14) peptide from I-A^k is much faster (i.e., $t_{1/2} = \sim 1$ min) since binding of the native peptide to I-A^k is not detected with these methods (21). Half-maximal proliferation of the MBP-specific T cell clone B10A.F2 with MBP Ac(1-14) and Ac(1-14)4A is obtained at peptide concentrations of 10 μM and 1 nM, respectively. These large differences in proliferation dose response are at least partly explained by differences in the peptide affinities for the MHC protein I-A^k. It is notable that the half-maximal acidification responses for these two peptides also differ by about four orders of magnitude (Fig. 3 a). This further demonstrates the similarity of proliferation and acidification dose responses.

A 10-min exposure of a mixture of the B10A.F2 T cells and APCs to the MBP Ac(1-14)4A peptide produces a rapid increase in acid production that slowly decays as the peptide is washed out (Fig. 3 b). The response to the native MBP Ac(1-14) peptide decays even more rapidly as the peptide is washed out (Fig. 3 b). In contrast, the response of 5C.C7 T cells briefly exposed to MCC is sustained despite continual perfusion of the cells with media (Fig. 2). These differences are consistent with the observation that MCC forms a long-lived peptide–MHC complex (16) and the MBP peptides form short-lived peptide–MHC complexes (20, 21). Evidently, the interaction of peptide–MHC ligand with its specific TCR at the cell–cell interface does not significantly affect the rate of peptide loss. Peptide and MHC side-chains recognized by the TCR are exposed at the membrane distal face of the MHC protein (22). Presumably the TCR binds to this surface forming a complex in which the peptide is sandwiched between the MHC and TCR protein surfaces. Since dissociation of peptide from this TCR–ligand complex is likely to be slow relative to dissociation from free MHC, we assume that peptide dissociates primarily from MHC not bound to a TCR (Fig. 4). Thus, we attribute the decay in acidification observed for the MBP peptides (Fig. 3 b) to rapid dissociation of the peptide–MHC ligand from the TCR, followed by subsequent dissociation of the peptide from the MHC.

To confirm that acid release observed after peptide addition reflects active TCR triggering, the I-A^k-specific 10-2.16 antibody (23) was added to previously activated T cells at different times after removal of peptide. The rapid decreases in acid production after each addition of antibody (Fig. 3 c) demonstrates that the sustained response to the MBP Ac(1-14)4A peptide is due to the continued presence of peptide–MHC complexes. In addition, the residual acidification rate observed upon completion of the antibody

blockade is independent of when antibody was added (Fig. 3 c). Thus, the gradual decrease in acid production seen after peptide removal (Fig. 3, b and c) is most likely related to the loss of peptide as it dissociates from MHC protein. We also added MBP Ac(1-14)4A peptide for 2 h (as compared to the 30 min in Fig. 3 c) to determine whether these observations are sensitive to the duration of T cell activation. The rate of decrease in acid production as peptide was washed away after the prolonged peptide exposure was identical to that observed in Fig. 3 c (data not shown).

The decrease in acid production after MBP peptide removal can be related to the rate of dissociation of peptide from MHC at the cell–cell interface. We observe that the increase in acid release from activated T cells is proportional to the logarithm of peptide concentration over a 100-fold concentration range (Figs. 1 b and 3 a). The MBP Ac(1-14)4A concentration used for the experiments described in Fig. 3, b and c is in the log-linear range of this dose response. We assume that the observed logarithmic dependence of acidification on peptide concentration implies a logarithmic dependence on the amount of peptide–MHC ligand bound by TCRs. For MBP Ac(1-14)4A concentrations $10 \text{ nM} < [P] < 10 \mu\text{M}$, the acidification (A) follows the empirical relation $A = B + C \log([P]/P^0)$ where B and C = empirical constants, $[P]$ = peptide concentration (nM) and $P^0 = 1 \text{ nM}$. Note that A is defined as the acidification rate normalized to the maximum observed acidification. The slope of the dose response for MBP Ac(1-14)4A gives $C = 34\%$ (Fig. 3 a). We assume that the concentration of peptide–MHC, $[PM]$, is proportional to added peptide, i.e., $[PM] = \alpha [P]$. The concentration of peptide–MHC at time t as peptide dissociates from MHC is $[PM]_t = PM_i e^{-kt}$ where k = peptide dissociation rate con-

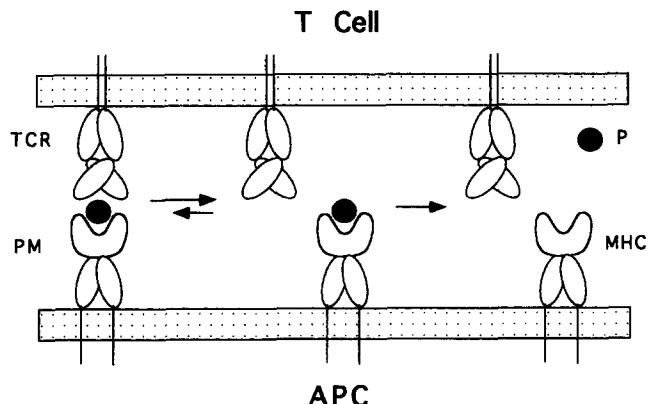


Figure 4. TCR–ligand interactions at the cell–cell interface. The TCR–ligand complex (TCR–PM) initiates biochemical signals that cause increased acid release. Dissociation of the trimolecular complex gives free TCR and peptide–MHC ligand (PM) which subsequently dissociates to give free peptide (P) and MHC. Biochemical signals leading to increased acid release terminate after TCR–ligand dissociation. If peptide dissociation from PM is fast relative to dissociation from TCR–PM, the rate at which peptide is lost from the T cell–APC interface is approximately the intrinsic peptide–MHC dissociation rate multiplied by the fraction of free peptide–MHC ligands: i.e., $k_{\text{obs}} = k_{\text{off}} (PM)/(PM + TCR-PM)$.

stant, PM_i = initial peptide–MHC concentration. Thus, the acidification during peptide dissociation is $A = D + C \log(e^{-kt})$ where $D = B + C \log(PM_i/\alpha P^0)$. Therefore, the decay in acidification during peptide dissociation should be linear with respect to time as observed for MBP Ac(1–14)4A in Fig. 3, b and c. From six different experiments with MBP Ac(1–14)4A we calculate an average slope of $0.38 \pm 0.08\% \text{ min}^{-1}$ which gives peptide dissociation rates with $t_{1/2} = 27 \pm 6 \text{ min}$.

The rate MBP Ac(1–14)4A peptide loss can be related to the apparent TCR–ligand affinity. We assume that the rate at which peptide dissociates from the TCR–ligand complex is slow relative to dissociation from free peptide–MHC. Thus, the observed rate of peptide loss from the T cell–APC interface is approximately the intrinsic peptide–MHC dissociation rate multiplied by the fraction of free peptide–MHC ligands (Fig. 4). Since the $t_{1/2}$ of dissociation for MBP Ac(1–14)4A from I–A^k in the absence of T cells is 30 min (21) (K. Tate and P.P. Jones, unpublished results), we infer that no more than 20% of the peptide–MHC complexes are TCR bound at any one time. Higher fractional receptor occupancies might have been anticipated given that TCR–ligand affinities measured in solution are about 0.1–40 μM (3–7) and the large number of TCRs at the cell–cell interface corresponds to an effective concentration of $\sim 30 \mu\text{M}$ (24).

Because of the rapid loss of the native MBP(1–14) peptide, the acidification response is below the log-linear range within $\sim 10 \text{ min}$ (Fig. 3 b). From the initial acidification slopes ($\sim 10 \text{ min}$) during loss of the native MBP Ac(1–14) peptide, we estimate rates that correspond to a peptide dissociation $t_{1/2} = 1\text{--}2 \text{ min}$. However, the rate of acidification decrease upon antibody addition (Fig. 3 b) also corresponds to an apparent TCR–ligand dissociation $t_{1/2}$ of 1–2 min. In addition, the changes in steady state signaling (Fig. 2) are also limited by some process with $t_{1/2}$ of 1–2 min. These observations demonstrate that some event other than pep-

tide dissociation with $t_{1/2}$ of 1–2 min is associated with changes in TCR ligation and acid release. It is possible that this is an intracellular biochemical process that mediates the triggering of a TCR and acid release. The rate limiting event could also be dissociation of the TCR–ligand complex. In either case, dissociation of the peptide–MHC ligand from the TCR at the interface of living cells occurs at a rate in which $t_{1/2} \leq 1\text{--}2 \text{ min}$. We note that Valitutti et al. have shown that TCR epitopes present on T cells are lost during T cell activation by a process with a lifetime on the order of 1–2 min (19).

Although TCR–ligand affinities measured for isolated molecules in solution are low (3–7), one might have expected enhanced apparent affinities at the cell–cell interface. Membrane bound ligands and receptors have restricted degrees of freedom as compared to those in solution and TCR–ligand reactions are associated with additional coreceptor and adhesion molecules (1). The local concentration of TCRs is also expected to be large since they accumulate as dense clusters at the cell–cell interface (25). However, the cell–cell interface is a dynamic environment due to cytoskeletal movements during T cell activation (26). Since proteins involved in T cell signaling are associated with the cytoskeleton (27), it is likely that cytoskeletal motions will affect the TCR–ligand binding and dissociation reactions. Despite these complexities, our analysis of T-cell triggering indicates that these reactions can be described simply in terms of rapid, reversible bimolecular reactions. We estimate that only a fraction (<20%) of peptide–MHC ligands are bound to a TCR at any one time. Thus, in those cases where T cells are activated by 50–200 ligands (28), only a small number of ligands (i.e., 10) may be actively engaged at any moment. These results support the emerging view that specific cellular recognition can be mediated by a small number of weak, transient receptor–ligand interactions at the cell–cell interface.

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