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Upon encounter of a CTL with a target cell carrying foreign Ags, the TCR internalizes with its ligand, the peptide-MHC class I complex. However, it is unclear how this can happen mechanistically because MHC molecules are anchored to the target cell’s surface via a transmembrane domain. By using antigenic peptides and lipids that were fluorescently labeled, we found that CTLs promptly capture target cell membranes together with the antigenic peptide as well as various other surface proteins. This efficient and specific capture process requires sustained TCR signaling. Our observations indicate that this process allows efficient acquisition of the Ag by CTL, which may in turn regulate lymphocyte activation or elimination. The Journal of Immunology, 2001, 166: 3645–3649.

Upon physiological stimulation, receptors with tyrosine kinase activity (RTK)4 rapidly internalize together with their soluble ligands. The TCR clearly belongs to this family of receptors, and it too is internalized after stimulation (1). Intuitively, it seems unlikely that the TCR ligand, the peptide-MHC complex which is membrane-bound, could be efficiently transferred onto CTL. However, recently the absorption and internalization by T cells of MHC and costimulatory molecules derived from APC has been shown to occur during the recognition process (2, 3). The observation that CTL that recognized antigenic peptides subsequently became sensitive to fratricide killing (i.e., killing by T cells specific for the same Ag) (2, 3) suggested that the transfer probably involves the peptide in combination with the presenting MHC molecule. Here we examine the molecular processes involved in the capture of target cell molecules by CTL during the recognition process.

Materials and Methods

Cell lines and mice

The murine cell lines RMA and BALB/c 3T3 transfected with H-2D\(^b\) (3T3-D\(^b\)) were used as target cells. CTL lines specific for lymphocytic choriomeningitis virus gp33/H-2D\(^b\) were derived from the P14 TCR-transgenic mouse (4) or produced by standard peptide immunization procedure of C57BL/6 (B6) perforin-deficient (PKO) mice as described previously (5). CTL lines specific for the model peptide FITC-K9L presented by H-2D\(^b\) were also generated by peptide immunization.

Peptides

Synthesis of peptides has been described elsewhere (6). All peptides were HPLC-purified (>98%), and their identity was confirmed by mass spectrometry.

Functional assays

Cytotoxicity was measured in a classical 4- to 5-h (or 24-h when indicated) \(^{51}\)Cr release assay. For IFN-\(\gamma\) production, 100 \(\mu\)l were removed after 24 h from the same wells used for cytotoxicity assays and assayed for IFN-\(\gamma\) content by ELISA, as previously described, using R4-6A2 and biotinylated XMG1.2 (BD PharMingen, San Diego, CA) (7).

Flow cytometric analysis

Target cells were stained with the lipophilic dye PKH-26 (Sigma, St. Louis, MO) according to the manufacturer’s instruction, placed in U-bottom 96-well plates (100 \(\mu\)l with the indicated concentrations of peptides (0.2 \(\times\) 10\(^7\)cells/well in final volume of 200 \(\mu\)l), incubated for 1 h at 37°C, and then washed three to five times. After the last wash, cell pellets were resuspended with 100 \(\mu\)l of T cell suspension (0.1 \(\times\) 10\(^6\)cells/ml) centrifuged for 30 s at 1000 rpm to promote conjugate formation, and then left at 37°C for 1 h (or the indicated times). Conjugates were dissociated by washing cells twice in PBS containing 0.5 mM EDTA and were then stained with anti-CD8-Tricolor. In some experiments, T cells were treated with PP2 (10 \(\mu\)M; Sigma) or with blocking Abs either before or after conjugate formation.

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\(^6\)Abbreviations used in this paper: RTK, receptors with tyrosine kinase activity; PTK, protein tyrosine kinases; B6, C57BL/6; APL, altered peptide ligands; PKO, perforin-deficient; FSC, forward light scatter.

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Results and Discussion
To investigate how T cells acquire the antigenic peptides, we used two different fluorescently labeled antigenic peptides and measured their transfer onto specific CTL by flow cytometry. FITC-gp33 corresponded to the immunodominant epitope of the lymphocytic choriomeningitis virus (gp33, 33KAVYNFATC41) (8) to which a FITC moiety was attached on the ε amino group of Lys33, and Cys41 was replaced by Ile to avoid chemical reduction. The second peptide was the fluorescent probe FITC-K9L (FITC-KAIENAEAL) (6). Both peptides bind specifically and with a high affinity (Kd = 10–50 nM) to cells that express the H-2Db MHC class I molecule (data not shown). FITC-gp33 is recognized in cytotoxicity assays by P14 CTL specific for gp33 derived from transgenic mice expressing the P14 (Vα2, Vβ8.1) TCR (4), albeit more weakly than the original peptide (half-maximal lysis at 0.3 nM vs 1 pM, respectively; data not shown). FITC-K9L is recognized by CTL generated by standard peptide immunization.

RMA cells coated with increasing concentrations of either FITC-gp33 or FITC-K9L were thoroughly washed to remove any free peptide and were then incubated with either P14- or K9L-specific CTL. After dissociation of the conjugates, the amount of free peptide and were then incubated with either P14- or K9L-specific CTL. After dissociation of the conjugates, the amount of free peptide and were then incubated with either P14- or K9L-specific CTL. After dissociation of the conjugates, the amount of free peptide and were then incubated with either P14- or K9L-

RMA cells pulsed with 1 μM of FITC-gp33 (top) or FITC-K9L (bottom) were mixed with P14 CTLs (left panels) or K9L CTLs (right panels). After incubation at 37°C for 1 h, conjugates were dissociated and stained with anti-CD8-Tricolor and then analyzed by flow cytometry. The analysis was performed on gated CD8+ live T cells as identified by FSC parameter. The dark line shows green fluorescence intensity on CTLs exposed to peptide-coated RMA, as compared with noncoated ones (thin line). B. Dose-response curves of FITC-gp33 (●) or FITC-K9L (○) transfer from RMA cells to P14 CTLs (left) or K9L CTLs (right). C. 3T3-Dβ cells biotinylated on their cell surface were pulsed (lane 2) or not (lane 1) with 1 μM gp33 and were then incubated with P14 for 30 min at 37°C. P14 CTLs were separated from 3T3-Dβ cells, lysed in N-octylglucoside, and the lysates were analyzed by Western blot analysis revealed by streptavidin-HRP and chemiluminescence. Lanes 3 and 4 correspond to small amounts of lysates from biotinylated 3T3-Dβ and P14, respectively.

FIGURE 1. Specific capture of target cell-derived antigenic peptides by T cells. A. RMA cells pulsed with 1 μM of FITC-gp33 (top) or FITC-K9L (bottom) were mixed with P14 CTLs (left panels) or K9L CTLs (right panels). After incubation at 37°C for 1 h, conjugates were dissociated and stained with anti-CD8-Tricolor and then analyzed by flow cytometry. The analysis was performed on gated CD8+ live T cells as identified by FSC parameter. The dark line shows green fluorescence intensity on CTLs exposed to peptide-coated RMA, as compared with noncoated ones (thin line). B. Dose-response curves of FITC-gp33 (●) or FITC-K9L (○) transfer from RMA cells to P14 CTLs (left) or K9L CTLs (right). C. 3T3-Dβ cells biotinylated on their cell surface were pulsed (lane 2) or not (lane 1) with 1 μM gp33 and were then incubated with P14 for 30 min at 37°C. P14 CTLs were separated from 3T3-Dβ cells, lysed in N-octylglucoside, and the lysates were analyzed by Western blot analysis revealed by streptavidin-HRP and chemiluminescence. Lanes 3 and 4 correspond to small amounts of lysates from biotinylated 3T3-Dβ and P14, respectively.

likely that the major band detected around 45 kDa corresponds to MHC class I molecules, the precise identity of this and other proteins remains to be determined.
How might the passage onto T cells of so many different molecules from the plasma membrane of the target cell be explained? We investigated the possibility that membrane fragments could convey these proteins by analyzing the transfer to T cells of a fluorescent lipid (PKH26) initially incorporated into the target cell membranes. As shown in Fig. 2A, P14 CTL acquired PKH26 when incubated with RMA cells pulsed with 1 μM gp33, but not with 1 μM of the control peptide K9L or RMA cells left unpulsed. Reciprocally, significant PKH26 transfer onto K9L-specific CTL occurred only when K9L, but not gp33, was used to coat the target cells. The amount of lipid transferred depended on the dose of peptide used in both cases (Fig. 2B). The transfer observed specifically concerns membranes because when RMA cells were stained with CFSE, a stable internal protein dye, no transfer of fluorescence from RMA cells to P14 CTL was detected (data not shown).

When we incubated RMA cells that were both pulsed with FITC-gp33 and labeled with PKH26 with P14 CTL, we observed a parallel capture of antigenic peptide and lipid by T cells (data not shown). To a rough approximation based on levels of fluorescence intensity per cell, we estimate that after 1 h CTLs acquire 5–10% of the amount of lipid and peptide initially present on a target cell. Capture of both peptide and lipid was partially inhibited by anti-P14 TCR Abs (anti-Vβ2 and anti-Vβ8.1), but not by anti-Vβ6 or anti-TCR Cβ control mAb (data not shown). After P14 CTLs were exposed to gp33-pulsed RMA cells, we also observed fratricide killing by fresh P14 CTLs (data not shown). These observations suggest that specific MHC-TCR interactions are directly involved in the capture process.

Interestingly, mAb 53.6.7.2 (or its Fab9), which binds to CD8α, markedly inhibited the transfer of both PKH26 (Fig. 3A) and FITC-gp33 (data not shown). Because this mAb stabilizes the membrane, it acts very early in the TCR signaling cascade (13). At 10 μM, a nontoxic concentration that blocks T cell activation completely (data not shown), PP2 fully inhibited the acquisition of PKH26 by P14 CTL induced by gp33 (Fig. 3A). Even though we cannot exclude an effect of PP2 on endocytosis, this result, together with the Ab-blocking experiments presented above, strongly suggests a role for TCR signaling in the capture process.

Interestingly, the transfer of RMA cell membranes to P14 CTL was already detectable after a 5-min incubation and reached its maximum as early as after 30 min, with 50% of the transfer being achieved at 12 min (Fig. 3B). The fact that the increase of PKH26 staining on P14 with time was progressive and followed a monomodal profile (data not shown) suggests that multiple transfers of small amounts of target cell plasma membrane occurred during CTL-target cell recognition rather than a single event involving the capture of a large piece of membrane.

As shown in Fig. 3C, PP2 inhibited the transfer of PKH26 to T cells even when added after conjugation, with the extent of inhibition diminishing progressively with time and indicating that a sustained TCR signaling was required to maintain the capture process. A similar time-dependence was observed with anti-CD8 mAb (data not shown). These kinetics are remarkably comparable to those for inhibition of T cell function when similar approaches are used (13, 14). Finally, these data strongly support our view that small fragments of membrane are progressively captured by CTL in a manner that can be interrupted with treatments affecting proximal events of TCR signaling.

Because PKH26 transfer required sustained TCR signaling and this signaling can be differentially affected by altered peptide ligands (APL) (15, 16), we next compared the ability of 16 APL to trigger cytotoxicity, cytokine production, and capture of PKH26. The 16 peptides all bound to H-2D<sup>b</sup> with affinities comparable to the natural peptide (i.e., around 1 nM) (data not shown).
FIGURE 4. Induction of target cell membrane capture by altered peptide ligands. RMA cells pulsed with the indicated concentrations of peptides were tested for their ability to activate P14 cytotoxicity in a 51Cr release assay (upper row), target cell membrane capture by flow cytometry (middle row), and IFN-γ production by ELISA. The names of the APL used to obtain the data shown here are indicated on the top row, and those of additional APL with similar behavior are indicated at the bottom of each column.

FIGURE 5. Target cell membrane capture by perforin-deficient CTL cytotoxicity. A, RMA cells pulsed with the indicated concentrations of gp33 were challenged with P14 CTLs (○) or gp33-specific CTLs generated in PKO B6 mice (○) for 1 h at 37°C in a 4-h (left panel) or 24-h (right panel) 51Cr release assay. In control samples using K9L-pulsed RMA cells, no killing was observed after short or long incubation (data not shown). B, Capture of target cell membranes was analyzed by flow cytometry after incubating the two CTL populations for 60 min with PKH26-labeled gp33-pulsed RMA cells.

for the half-maximal capture to be reached in PKO vs P14 CTLs relates to the avidity of both CTL lines for the Ag (Fig. 5A). Finally, the kinetics of membrane capture was also the same when PKO or P14 CTLs were used (data not shown). Therefore, these observations rule out that this capture process requires the involvement of perforin’s cytolytic activity and indicate that the capture process is not a consequence of perforin-mediated damages caused to target cells. The possibility that CTLs scavenge membrane fragments nonspecifically was ruled out by this fact that K9L-specific CTLs did not capture any PKH26 when co-incubated with P14 CTLs in the presence of PKH26-labeled RMA cells pulsed with gp33 (data not shown). The tight correlation between short-term (perforin-dependent) cytotoxicity and membrane capture indicate that either these two processes are independent but have similar requirements or that they are intimately linked. In light of our data, membrane capture is independent of perforin release and could correspond to an initial “love bite” preceding CTL’s kiss of death. Further studies are required to explore the nature of the correlative link we observed between these two events.

One point to consider is the role(s) that this capture phenomenon might play in regulating CTL activity. By providing prolonged engagement of the TCR, membrane fragments captured from Ag-bearing target cells could in the first place favor T cell activation. However, whether soluble or membrane-bound ligands of RTK could participate in signaling once inside the cell is still a matter of debate (17, 18). In turn, in the case of important antigenic load, those activated CTL will inherit enough antigenic material to become sensitized to killing by other CTLs carrying TCRs of the same specificity as their own. This fratricide killing was proposed previously to explain the phenomenon of lymphocyte “exhaustion” (2, 3, 19). Our data not only support this notion, but also provide a mechanistic explanation for this phenomenon.

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