Brief Definitive Report

Different Responses Are Elicited in Cytotoxic T Lymphocytes by Different Levels of T Cell Receptor Occupancy

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Summary

We have investigated the level of TCR occupancy required to elicit different biological responses in human CTL clones specific for an influenza matrix peptide. Specific cytotoxicity could be detected at extremely low peptide concentrations (10^{-12} to 10^{-15} M). However, IFN-γ production, responsiveness to IL-2 and Ca^{++} fluxes were observed only at peptide concentrations $>10^{-9}$ M, while autonomous proliferation required even higher peptide concentrations. In parallel experiments we measured TCR downregulation to estimate the number of TCRs triggered. We observed that at low peptide concentrations, where only cytotoxicity is triggered, TCR downregulation was hardly detectable. Conversely, induction of IFN-γ production and proliferation required triggering of at least 20–50% of TCRs.

Taken together these results indicate that a single CTL can graduate different biological responses as a function of antigen concentration and that killing of the specific target does not necessarily result in full activation.

It is well known that naive CD8^{+} T cells and mature CTL have different requirements for activation. On the one hand it has been shown that naive T cells require high antigen concentrations (1–3), professional APCs (4, 5) and T cell help (1, 6–8) in order to proliferate and mature into effector cells. On the other hand, mature CTL can kill any target cell displaying very low numbers of peptide-MHC complexes (9), even lower than that required to trigger Th cells (10, 11). These findings have generally been interpreted as evidence that the responsiveness of CTL to antigen is increased after activation and that CTL are far more sensitive than Th cells.

We have recently demonstrated that TCR downregulation can be used to measure the number of TCRs triggered at the level of T-APC interaction. Using this assay we observed that in Th clones activation to IFN-γ production requires that ~30% of the TCRs are triggered (12). Here we investigated the relationship between antigen concentration, TCR occupancy, and the induction of different biological responses in CTL. We found that in human CTL clones, cytotoxicity can be elicited by triggering a very small number of TCRs, in conditions where no other responses can be detected. At increasing levels of TCR occupancy, helper-dependent and -independent proliferation and IFN-γ production were observed as a function of the number of TCRs triggered. These results demonstrate that

different responses can be elicited in cytotoxic T lymphocytes by different levels of TCR occupancy.

Materials and Methods

T Cell Clones and Target Cells. Clones specific for the influenza matrix peptide 58-66 (M58-66, reference 13) were isolated from polyclonal cell lines derived from two different donors and maintained as described (14). The clones were used 10–30 d after restimulation. An HLA-A2[+] EBV-transformed B cell line (JY) was used as antigen presenting/target cell.

Cytotoxicity. Cytotoxicity was measured in a standard 4 h ^{51}Cr release assay using 5,000 target cells/well and different E:T ratios in the presence of different concentrations of M58-66.

TCR Downregulation and IFN-γ Production. JY cell were incubated 10 min with 1 μM BCECF-AM (2',7-bis-(carboxyethyl)-5(6')-carboxyfluorescein; Calbiochem, San Diego, CA) and washed four times. T cells and BCECF-loaded JY cells were incubated at 1:2 ratio in 200 μl RPMI-5% FCS medium in U bottom microplates in the presence of different peptide concentrations. The plates were centrifuged to allow conjugate formation and incubated 5 h at 37°C. Cells were resuspended, washed in PBS containing 0.5 mM EDTA to break the conjugates and stained with anti-CD3 (OKT3; American Type Culture Collection, Rockville, MD) followed by a phycoerythrin (PE)-labeled goat anti–mouse Ig (Southern Biotechnology Associates, Birmingham, AL). The CD3 fluorescence was analyzed on a FACScan (Becton-Dickinson, Mountain View, CA). EBV-B cells

were gated out using both FSC/SSC parameters and green BCECF fluorescence. Dead cells were excluded by propidium iodide staining. IFN-γ production was measured in the culture supernatant using an ELISA assay as described (14).

[Ca⁺⁺]i Measurement. CTL clones were loaded with Indo-1 AM (Sigma Chemical Co., St. Louis, MO) as described (14). Cells were mixed at a 1:2 ratio with JY cells that had been pulsed for 2 h at 37°C with different concentrations of M58-66. The cells were centrifuged 1 m at 1500 rpm, incubated 1 min at 37°C, resuspended, and analyzed on a Coulter Elite flow cytofluorimeter (Coulter Electronics Inc., Hialeah, FL) to detect [Ca⁺⁺], in T cell-APC conjugates. Only live, Indo-1 loaded and conjugated T cells were included in the analysis. The acquisition time was 4 min. The mean 405/525 fluorescence ratio of all the acquired events was represented in histogram form and plotted as function of peptide concentration.

Proliferation Assay. 5×10^4 CTL were mixed with 2×10^4 peptide pulsed, mitomycin-treated JY cells in 96-well flat-bottom microplates in 200 μl RPMI-10% FCS in the presence or absence of 30 U/ml human recombinant IL-2. After 48 h, the cultures were pulsed with 1 μCi [³H]thymidine and the radioactivity incorporated was measured after an additional 16 h by liquid scintillation.

Results

Cytotoxicity Is Efficient Over an Extremely Wide Range of Peptide Concentrations. HLA-A2–restricted CTL clones specific for the influenza matrix peptide 58-66 were used to study the relationship between antigen concentration and the induction of different biological responses. As evident from Fig. 1, detectable cytotoxicity could be elicited by extremely low peptide concentrations (10^{-15} M) and was comparable over a wide range of peptide concentrations (10^{-7} to 10^{-13} M).
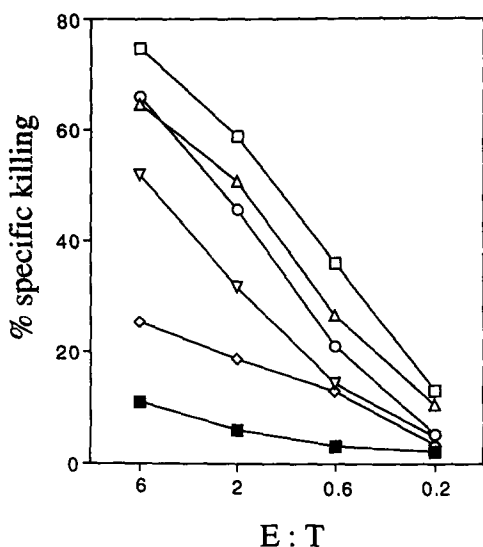

Figure 1. CTL can kill with comparable efficiency target cells in the presence of a very large range of peptide concentrations. Specific lysis of JY cells by clone CER43 in the absence (■) or in the presence of different concentrations of M58-66: 10^{-7} M (□); 10^{-9} M (△); 10^{-11} M (○); 10^{-13} M (▽); 10^{-15} M (◇)

IFN-γ Production, Unlike Cytotoxicity, Requires High TCR Occupancy. We have previously demonstrated that in T helper clones the activation of IFN-γ production requires triggering of a substantial fraction of TCRs as measured by TCR/CD3 downregulation (12). We therefore conjugated CTLs with JY cells at 0.5 E:T ratio and measured at different peptide concentration cytotoxicity, IFN-γ production and the level of TCR occupancy, as detected by TCR downregulation. As shown in Fig. 2, IFN-γ production was detected only when target cells were pulsed with peptide concentrations $>10^{-9}$ M (Fig. 2 B), that correspond to the concentrations where a substantial fraction of TCRs was downregulated (Fig. 2 A). At lower peptide concentrations (10^{-15} to 10^{-10} M) TCR downregulation was not significantly different from that induced by unpulsed cells. Yet, in this range of antigen concentrations, the target cells were efficiently killed (Figs. 1 and 2 C). These results indi-
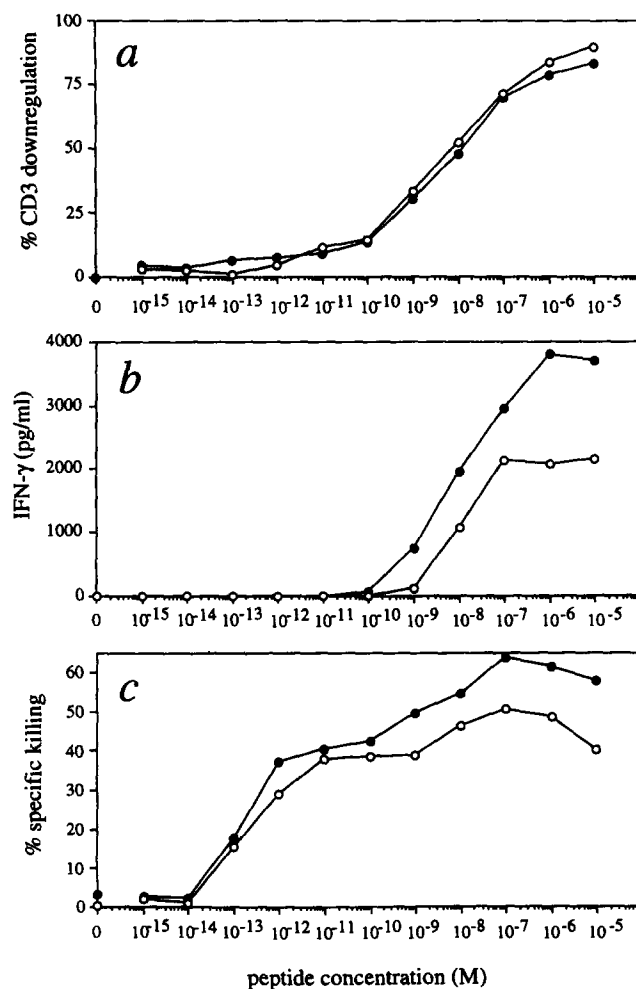

Figure 2. IFN-γ production by CTL clones requires a much higher antigen concentration and TCR occupancy than cytotoxicity. CTL clones CER43 (○) and MC2 (●) were conjugated at E:T = 0.5 with JY cells in the presence of different concentrations of M58-66. (a) CD3 downregulation. (b) IFN-γ production. (c) cytotoxicity. Comparable results were obtained with five CTL clones from two different donors

cate that in CTL, as in Th cells (12), IFN-γ production requires a substantial level of TCR occupancy, whereas cytotoxicity can be elicited by triggering only very few TCRs.

[Ca^{++}]$_i$ Increase Is Proportional to the Level of TCR Occupancy. Activation of Th cells to IFN-γ production is accompanied by a strong and sustained [Ca^{++}]$_i$ increase (14). We therefore investigated the level and time course of [Ca^{++}]$_i$ increase in CTL conjugated with peptide pulsed JY cells. Fig. 3 *A* shows that a raise in [Ca^{++}]$_i$ could be detected only in the high range of peptide concentrations (>10^{-9} M), with a kinetics parallel to that of TCR downregulation and IFN-γ production. On the contrary, at lower peptide concentrations, where only specific cytotox-
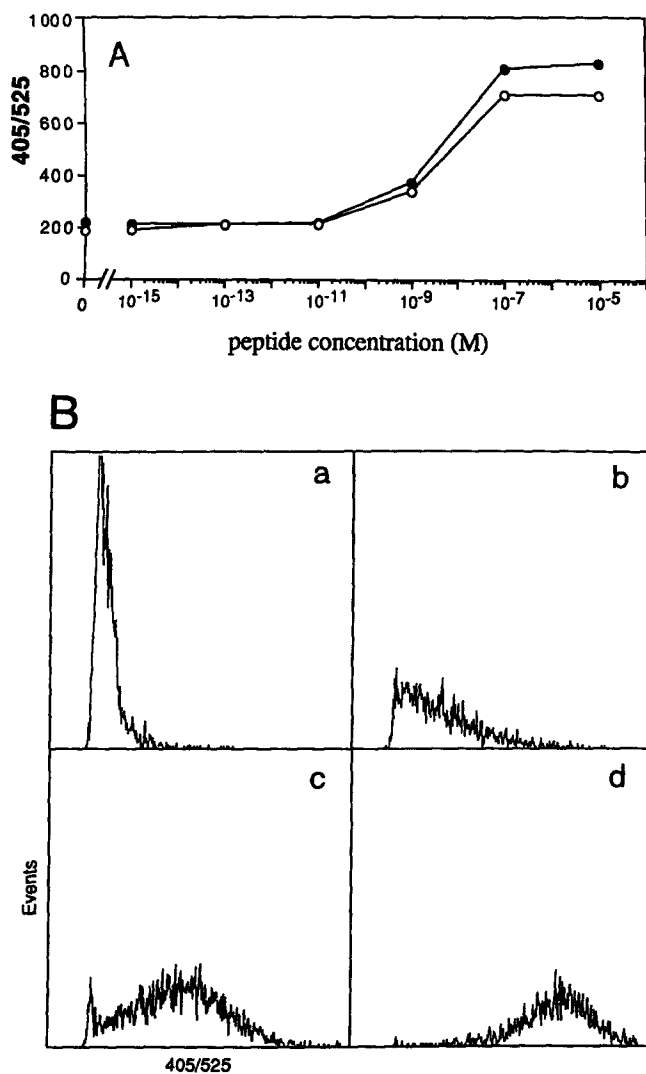
icity was observed, no [Ca^{++}]$_i$ rise could be measured. It is interesting to note that decreasing peptide concentrations led to a decrease in the mean [Ca^{++}]$_i$ of all T cells rather than in a decrease in the percent of fluxing T cells at any given time (Fig. 3 *B*). These results indicate that the level of TCR stimulation that is sufficient to trigger cytotoxicity is not sufficient to induce a detectable [Ca^{++}]$_i$ increase, not even transiently.

Helper-dependent and -independent Proliferation as a Function of TCR Occupancy. In different experimental systems it has been shown that CTL stimulated by specific antigen can either proliferate autonomously or increase their responsiveness to exogenous IL-2. We asked whether these different responses could reflect different levels of TCR occupancy.

CTL clones were cultured with APC pulsed with different peptide concentrations and proliferation was measured in the presence or absence of exogenous IL-2. As shown in Fig. 4, CTL stimulated by very high peptide concentrations (10^{-8} M) proliferate autonomously, indicating that they are able to produce their own growth factors, while at intermediate concentrations (10^{-10}–10^{-9} M), only a proliferative response to exogenous IL-2 was measured. A marked

Figure 3. [Ca^{++}]$_i$ increase in CTL-target conjugates can be measured only at relatively high peptide concentrations. CTL clones were loaded with Indo-1 and conjugated at E:T = 0.5 with JY cells that were either unpulsed or pulsed with different concentrations of peptide. (*A*) Mean level of [Ca^{++}]$_i$ as a function of peptide concentration in clones CER43 (●) and MC2 (○). (*B*) Distribution of [Ca^{++}]$_i$ levels in clone MC2 conjugated with EBV-B cells unpulsed (*Ba*) or pulsed with 1 nM (*Bb*), 10 nM (*Bc*), or 100 nM peptide (*Bd*).
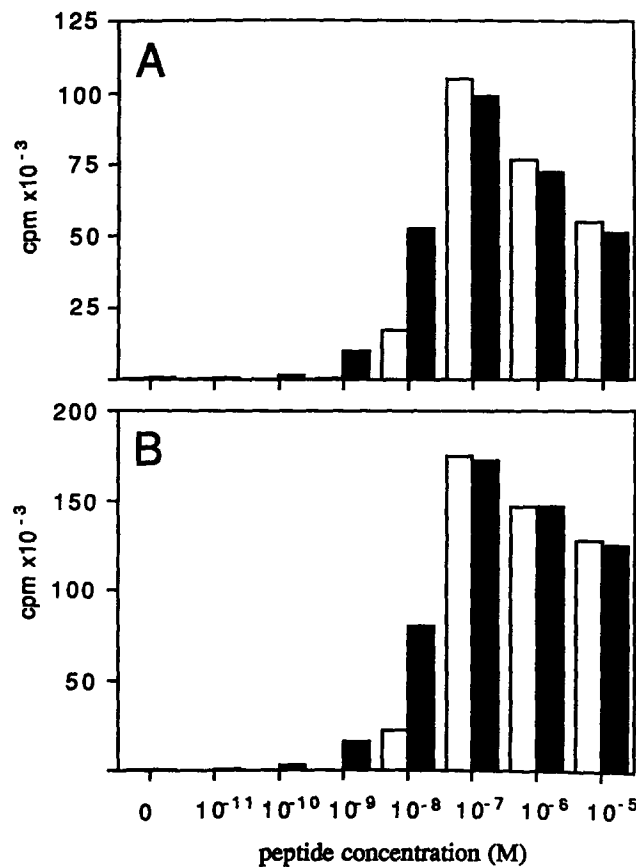

Figure 4. Autonomous CTL proliferation at peptide concentrations higher than those eliciting IL-2-dependent proliferation. Proliferative response of CER43 (*A*) and MC2 (*B*) cultured with mitomycin-C-treated JY cells pulsed with different concentrations of peptide in the presence (*black bars*) or absence (*open bars*) of 30 U/ml IL-2.

upregulation of CD25 paralleled the induction of IL-2 responsiveness (data not shown).

Discussion

Our results demonstrate that CTL can give different biological responses as a function of the concentration of antigen offered and consequently of the number of TCR engaged. Target cells pulsed with extremely low peptide concentrations (10^{-15} to 10^{-10} M) can efficiently trigger cytotoxicity, which occurs in the absence of measurable TCR downregulation and Ca^{++} fluxes. This result is compatible with the notion that cytotoxicity can be elicited by as few as 1–10 peptide-MHC complexes (9), that may trigger only a few TCRs during the transient interaction of a CTL with the target cell.

The fact that the induction of cytotoxicity requires such a low level of TCR occupancy is perhaps not surprising if we consider that, unlike T cell activation, cytotoxicity is a cytosolic event that may be localized to a very small cellular domain. It is possible that even a single peptide-MHC complex by rapidly triggering several TCRs may transduce a local signal (including a small $[Ca^{++}]_i$ elevation) sufficient to trigger the delivery of the lethal hit.

The capacity to deliver the lethal hit in response to triggering of very few TCRs appears to be a peculiar characteristic of effector CTL. It is interesting that cytotoxicity by $CD4^+$ class II restricted T cell clones is elicited only at concentrations of antigen that induce high TCR occupancy and IFN-γ production (data not shown).

Target cells pulsed with higher peptide concentrations ($>10^{-9}$ M) trigger a substantial fraction of TCRs, as detected by TCR downregulation. This higher level of TCR occupancy results, in addition to cytotoxicity, in the induction of proliferation and cytokine production. It is interesting that the level required for the induction of IL-2 responsiveness (mediated by IL-2 receptor upregulation) is lower than that required for the induction of autonomous proliferation.

The observation that a spectrum of biological responses can be elicited as a function of the amount of antigen may help to explain some aspects of the cytotoxic response. In the induction phase, clonal expansion will be driven by antigen displayed on professional APC. These cells may not only display high levels of complexes but in addition possess adhesion and costimulatory molecules that facilitate TCR engagement resulting in the high TCR occupancy required for the induction of proliferation (4). It is possible that when the occupancy is not sufficient to induce an autonomous response, the CTL may still be activated to proliferate if exogenous IL-2 is provided by other cells (8). In the effector phase, cytotoxicity requires a very low level of TCR occupancy and can be readily triggered by non professional APCs even if they display few complexes or lack accessory molecules. The fact that this interaction is transient and involves only a limited number of TCRs allows the CTL to spare TCRs and be able to kill many target cells. Even in this case, however, the level of TCR occupancy may be insufficient and fail to induce cytokine production leading to clonal exhaustion (15).

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