T cell unresponsiveness *in vitro* can be due to activation *in vivo*

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**Abstract**

During investigations into the behaviour and fate of ovalbumin (OVA)-specific CD8⁺ T cells in a TCR transgenic system, cytotoxic T lymphocyte (CTL) activity (as measured by classical *in vitro* stimulation followed by ⁵¹Cr-release assay) was found to be reduced after OVA peptide administration. This paradoxically occurred even during the peak of activation and expansion of these T cells. The reduced responsiveness occurred for both classical CTL assays and *in vitro* proliferation assays, and would apparently be consistent with induction of anergic or suppressor T cells. Instead, we provide evidence that *in vivo* peptide treatment generated activated killers which consequently killed the stimulator cells during *in vitro* culture, thus resulting in the unresponsive phenotype. In co-culture experiments, the proliferation and classical CTL activity were severely reduced when naive OVA-specific CD8⁺ T cells (OT-I) cells were co-cultured with cells from OVA peptide-treated mice. Moreover, cells obtained after peptide injection did not require *in vitro* stimulation to be able to kill target cells. Therefore, activation of killers *in vivo* should be considered as one pathway whereby unresponsiveness is found in assays requiring *in vitro* stimulation.

One approach toward treatment or prevention of autoimmune disease is to induce tolerance in autoreactive cells by administration of soluble antigen, usually by the mucosal or i.v. route (1–6). For example, peptide injection of the immunodominant epitope of myelin basic protein has been used to prevent experimental autoimmune encephalitis (1) and soluble glutamic acid decarboxylase antigen has been used to prevent spontaneous diabetes in the non-obese diabetic mouse (7,8). Tolerance of CD8 T cells by i.v. injection of a peptide from lymphocytic choriomeningitis virus also prevented diabetes in a virally induced autoimmune model (9).

There is consensus that in the periphery, soluble peptide usually leads to T cell tolerance while peptide in Freund’s adjuvant usually leads to T cell priming (10–15). After administration of soluble peptide, clonal expansion of reactive T cells is followed by both peripheral deletion (antigen induced cell death) and induction of unresponsiveness in remaining cells (2,11,13).

During studies on tolerance of anti-ovalbumin (OVA) cytotoxic T lymphocytes (CTL) in TCR transgenic mice (designated OT-I; 16), we found that i.v. injection of soluble OVA peptide (OVAp) led to clonal activation, expansion by day 2 and deletion by day 7 after peptide administration. On days 2 and 7, no CTL activity could be shown after the standard *in vitro* stimulation for 5 days. We show that what initially appeared to be an unresponsive phenotype was the consequence of activation of killer cells *in vivo* by soluble peptide, and that these activated cells killed stimulator cells and reduced the *in vitro* proliferation and cytotoxicity responses.

We have shown that i.v. OVAp administration results in the activation and proliferation of OT-I cells (17). Two days post OVAp administration OT-I cells in the spleen and lymph nodes exhibited elevated levels of the activation marker CD44. Furthermore, there was an increase in the absolute number of OT-I cells in mice treated with OVAp. The peptide-induced proliferation of OT-I cells in the periphery was followed by a decrease in the absolute number of OT-I cells by day 7. The spleens of mice treated with 2 nmol OVAp exhibited a decrease in absolute numbers from 164% on day 2 to 62% of control on day 7 (P < 0.001); likewise in the lymph node, the absolute numbers decreased from 267% on day 2 to 43% of control on day 7 (P < 0.001).

The cytotoxic activity of splenic OT-I cells was also analyzed 2 and 7 days after i.v. administration of OVAp. Microtitre
A with in vitro stimulation

B without in vitro stimulation

C without in vitro stimulation: increased no. of effectors

Fig. 1. (A) Reduced CTL activity of splenic cells from OT-I mice treated 2 days previously with OVAp. OT-I mice bearing Vα2Vβ5 TCR specific for the OVA257–264 peptide (OVAp, sequence SIINFEKL) were bred and housed at the WEHI animal facilities. OVAp and control Kβ-binding insulin peptide8–15 (INSp, sequence GSHLVEAL) were dissolved in PBS and 2 or 20 nmol (~2 or 20 µg respectively) injected i.v. as a single dose. Between 2 and 7 days after injection spleens were harvested for analysis. For microtitre culture CTL analysis 5×10⁴ splenocytes from peptide (OVAp or INSp) treated OT-I mice were serially diluted 3-fold in 96-well U-bottom microtitre plates. Then, 5×10⁴ irradiated (200 Gy) OVA-bearing (EG7; 18) stimulator cells were then added to each well in a final volume of 200 µl of RF10 (RPMI 1640, 10% FCS, 2 mM L-glutamate and 5×10⁻⁵ M 2-mercaptoethanol) and incubated at 37°C, 5% CO₂. After 5 days culture a 4⁵¹Cr-release assay was performed. Control (EL4) or OVA-bearing (EG7) targets (10⁵) were labelled with 100 µCi ⁵¹Cr for 90 min at 37°C. Then, 100 µl of medium was removed from microtitre cultures and the cellular pellets resuspended before the addition of 10⁴ target cells. After 4 h incubation at 37°C, 5% CO₂, 100 µl supernatants were harvested and γ counted for 1 min. The percentage specific lysis was determined using the following formula: % specific lysis = (c.p.m. test release – c.p.m. spontaneous release)/(c.p.m. maximum release – c.p.m. spontaneous release). (B) Cells from OT-I mice treated 2 days previously with OVAp exhibit OVA-specific CTL activity without the need for prior in vitro stimulation. Cells (5×10⁴) from mice treated with OVAp or INSp were combined with 5×10⁴ INSp or B6 cells (starting E:T ratio of 10) respectively and placed immediately into a ⁵¹Cr-release assay. (C) Cells from OT-I mice treated 2 days previously with OVAp exhibit OVA-specific CTL activity without the need for prior in vitro stimulation at higher E:T ratios. Method as stated in (B).

culture for CTL priming was performed using several dilutions of effectors (OT-I spleen cells) with a constant number of OVA-bearing stimulators (EG7; 18). The effector:target (E:T) ratios represent the ratio of the OT-I cells originally placed in microtitre culture to the constant number of ⁵¹Cr-labelled targets cells added 5 days after stimulation in vitro. CTL activity was consistently shown to be dramatically reduced in the OVAp-treated mice, at both 2 and 20 nmol doses and on days 2 and 7; results for day 2 are shown in Fig. 1(A).

OT-I cells from mice treated 2 or 7 days previously with OVAp displayed a similar reduction in CTL activity. This was despite the fact that 2 days after OVAp there was activation and an increase in the absolute number of OT-I cells in the periphery while 7 days after OVAp there was a decrease.
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Fig. 2. (A) OT-I cells from OVAp-treated mice inhibit the proliferation of naive OT-I cells to OVAp. CD8+ splenocytes (10^4) from mice treated with INSp or OVAp were serially diluted (2-fold) in 96-well U-bottom microtitre plates. CD8+ splenocytes from a naive OT-I mouse were then added at 10^3/well. The number of CD8+ cells in spleen samples was determined by staining with FITC-conjugated anti-CD8 (Caltag, San Francisco, CA) and analyzed with a FACScan and Lysys II software (Becton Dickinson, San Francisco, CA). Peptide was added to a final concentration of 10 ng/ml in a final volume of 200 µl of RF10 and incubated at 37°C in 5% CO2. On day 3 of incubation the cells were pulsed with 0.25 µCi [3H]thymidine for 8 h. Samples were counted on a TopCount microplate scintillation counter (Packard, Meridan, CT). Shown are the proliferative responses to OVAp. Proliferation to the negative control INSp was 200 c.p.m. and for the sake of clarity is not shown. (B) Exogenous IL-2 can overcome inhibition of proliferation by OT-I cells from OVAp-treated mice. Cells were combined as stated previously with the addition of recombinant human IL-2 (Cetus, Emeryville, CA) to a final concentration of 25 U/ml in the culture medium. Shown are the proliferative responses to OVAp. Proliferation to the control INSp was 200 c.p.m. and for the sake of clarity is not shown.

One explanation for this apparent anomaly was that perhaps despite showing signs of activation and clonal expansion at day 2, OT-I cells were in the process of being deleted (hence reduction in absolute numbers seen at day 7) and therefore unable to elicit cytotoxic activity. Perhaps OT-I cells were rendered unresponsive in agreement with conclusions drawn by others using soluble peptide and CTL systems (11,15). Alternatively, the cells may have already been activated killers (supported by CD44 expression) when placed into the 5 day stimulation culture and this somehow interfered with further CTL induction, e.g. by killing stimulator cells.

To test for these hypotheses, OT-I cells from OVAp-treated mice were mixed with spleen cells from naive OT-I mice (treated with control non-cognate INSp) and co-cultured with EG7 stimulators. If cells from OVAp-treated mice were unable to induce CTL because they were in the process of being deleted, the CTL activity of the cells from naive OT-I mice would be unaffected. On the other hand, if the latter hypothesis was correct and OT-I cells from OVAp-treated mice could kill the EG7 stimulators during culture, the observed CTL activity of cells from naive mice would be reduced due to the lack of available stimulators. Naive OT-I cells co-cultured with cells from mice treated 2 days prior with OVAp exhibited reduced CTL activity compared to OT-I cells from naive mice co-cultured with irrelevant C57Bl/6 (B6) spleen cells, supporting our latter hypothesis (results similar to Fig. 1A).

To determine whether OVAp-stimulated OT-I cells became functional killers in vivo, cells were harvested from peptide-treated mice and examined directly for CTL activity (i.e. without in vitro stimulation). As shown in Fig. 1(B), cells exhibited OVA-specific CTL activity without the need for in vitro stimulation. This was relatively low in mixing experiments ranging from 13 to 25% specific lysis for an E:T ratio of 10. With an E:T ratio as high as 50, however, specific lysis of 85% could be observed (Fig. 1C).

To ascertain if activated OT-I cells from OVAp-treated mice could similarly inhibit proliferation, these cells were cultured with cells from a naive OT-I mouse. The number of naive OT-I cells was kept constant for every well. The proliferation of naive OT-I cells to OVAp was dramatically inhibited by co-culturing with cells from OVAp-treated mice when the latter was added at 2.5- to 10-fold excess (Fig. 2A). In contrast co-culture of naive cells with cells from INSp-treated mice had no effect or increased the level of incorporation (when in 5- to 10-fold excess, presumably due to the increase in OT-I cells added). Thymidine incorporation by cells from OVAp-treated mice was 100-fold less than that from INSp-treated mice when there was a 10-fold excess of peptide treated cells. Figure 2(A) shows the co-culture results of one set of mice; this was representative of the seven sets performed. Anergic T cells have also been shown to inhibit proliferation in co-culture experiments and that this phenomenon was reversible by the addition of exogenous IL-2 (14,19). We found that inhibition of proliferation by activated OT-I cells could also be overcome by the addition of IL-2 (Fig. 2B). This is not surprising as co-stimulation by antigen-presenting cells (APC) (notably via B7:CD28 pathway) greatly increases production of IL-2 by T cells, so addition of exogenous IL-2 would overcome that deficiency when there are reduced APC (20,21).
Our explanation for the apparent reduced T cell activity is that OVAp activates the OT-I cells in vivo (consistent with CD44 expression and proliferation) and killers are generated that are capable of killing the stimulator cells during in vitro culture. Two lines of evidence support this. Firstly, the unresponsive phenotype in either proliferation or cytotoxicity assays persisted when cells from OVAp-treated mice were co-cultured with cells from naive OT-I mice. This suggests an active process by the cells from OVAp-treated mice. Secondly, OT-I cells taken directly ex vivo from OVAp-treated mice could lyse cognate target cells (EG7) without in vitro culture. EG7 cells, albeit irradiated, are also used in our stimulation cultures for CTL assays. Two reports provide supportive evidence for this latter finding. Kyburz et al. (11) found that ex vivo isolated spleen cells from peptide injected mice exhibited cytotoxic activity, although there was no discussion as to how this may be related to the lack of CTL activity after in vitro stimulation. Similarly, Mamalaki et al. (13) found that after peptide injection, cognate T cells were able to kill targets in the absence of any in vitro stimulation. Our findings in conjunction with these reports and others showing that CTL can inhibit APC function (22,23) suggest that activated CTL can result in unresponsive in vitro phenotypes. How general this in vitro phenomenon may remain moot as CD4+ T cell clones have been shown to proliferate in the presence of clones which have been rendered unresponsive by peptide treatment (24).

We advise, therefore, that caution should be exercised over the interpretation of in vitro data where there is in vitro stimulation (as for standard proliferation and CTL assays) and where there is any opportunity for CTL to have already been generated. The effect we describe may not be limited to CTL, as other activated T cells may have noxious effects on APC (e.g. via cytokine). We argue that our findings can provide an alternative interpretation of in vitro assays that indicate anergy/suppression/unresponsiveness.

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