

# Induction of T Cell Anergy by Low Numbers of Agonist Ligands<sup>1</sup>

Laura C. Korb, Saied Mirshahidi, Kasra Ramyar, Amir A. Sadighi Akha, and Scheherazade Sadegh-Nasseri<sup>2</sup>

Engagement of TCR by its ligand, the MHC/peptide complex, causes T cell activation. T cells respond positively to stimulation with agonists, and are inhibited by antagonist MHC/peptide ligands. Failure to induce proper conformational changes in the TCR or fast TCR/MHC dissociation are the leading models proposed to explain anergy induction by antagonist ligands. In this study, we demonstrate that presentation of between 1 and 10 complexes of agonist/MHC II by unfixed APC induces T cell anergy that persists up to 7 days and has characteristics similar to anergy induced by antagonist ligand or TCR occupancy without costimulation. Furthermore, anergy-inducing doses of hemagglutinin 306–318 peptide led to the engagement of less than 1000 TCR/CD3 complexes. Thus, engagement of a subthreshold number of TCR by either a low density of agonist/MHC or a 2–3 orders of magnitude higher density of antagonist/MHC causes anergy. Moreover, we show that anergy induced by low agonist concentrations is inhibited in the presence of IL-2 or cyclosporin A, suggesting involvement of the calcineurin signaling pathway. *The Journal of Immunology*, 1999, 162: 6401–6409.

T cell activation requires the engagement of TCR with the specific MHC/peptide ligand (signal 1) and CD28 on T cells with B7 on APCs (signal 2). Transmission of signal 1 in the absence of signal 2 leads to induction of anergy (1, 2). In the presence of costimulation, however, differences in signal 1 can also determine anergy or activation. Altered peptide ligands (APL)<sup>3</sup> have aided our understanding of the molecular events involved in transmission of signal 1 at the interface of T cells and APCs as well as early signal transduction processes (3, 4). APL are similar to the agonist peptide, except in one or more amino acid residues, generally those that contact the TCR, and can function as antagonists, or partial agonist ligands. Agonist peptides induce full T cell activation (both proliferation and cytokine production). Antagonist ligands are not capable of inducing T cell activation and prevent T cell activation in response to the agonist, and partial agonist peptides cause T cells to execute some, but not all, effector functions. Several groups have demonstrated that T cells stimulated with either antagonist or partial agonist peptides enter a state of anergy in which they are unresponsive to subsequent stimulation with the agonist peptide (5–9).

Currently, the conformational model and the kinetic discrimination model are the two leading theories to explain how APL and agonist peptides generate their different effects. The conformational model suggests that T cell activation depends on induction of a certain structure in the individual TCR that has been engaged.

Thus, APL induce T cell unresponsiveness by transmitting a negative signal due to their failure to induce a correct conformation in the TCR (10, 11). Alternatively, the kinetic discrimination model suggests that agonist peptides and APL engage TCR in a similar conformation, but that differences in T cell responses result from the shorter engagement time by APL/MHC complexes as compared with the agonist ligand (12, 13). Specifically, the rate of dissociation of the TCR from the MHC/peptide ligand determines anergy or activation. A derivative of this model is the avidity discrimination defined as the collective affinity between multiple TCR interacting with MHC/peptide complexes. Therefore, antagonist peptides may send a negative signal to the T cell because of failure to engage simultaneously a threshold number of TCRs as a result of fast dissociation of the TCR from MHC ligand.

T cell activation appears to be a continuous hierarchical process in which different effector functions are induced at different densities of Ag. For example, the ligand concentration needed to bring about early activation events, such as TCR down-regulation, and Ca<sup>2+</sup> influx is lower than that needed for later responses, such as proliferation and cytokine secretion (14–16). Activation of Th cell proliferation requires 100 or more MHC/peptide complexes (17–19). Activation of CD8 cells requires a similar number of class I/peptide complexes (20), although a much lower density of class I-peptide ligand for cytolytic killing has also been predicted (21). IFN- $\gamma$  production seems to require a lower concentration of MHC/peptide ligand than that needed for IL-2 production. Thus, selective activation of T cell effector functions can be achieved by either low agonist density or partial agonist peptides. It is unclear, however, whether both classes of peptides act through the same mechanism.

Since APL could potentially alter both the conformation and the kinetics of TCR interacting with ligand, differences in these models are best addressed in the absence of the multiple variables introduced by the use of APL. In this study, we investigate the outcome of T cell stimulation with a low density of agonist/MHC ligand. We find that T cells exposed to low agonist peptide concentrations, presented on fully competent live APCs, become anergic, as determined by their failure to mount a full response to

Department of Pathology and Graduate Program in Immunology, School of Medicine/Johns Hopkins University, Baltimore, MD 21205

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<sup>2</sup> Address correspondence and reprint requests to Dr. Scheherazade Sadegh-Nasseri, Department of Pathology, School of Medicine/Johns Hopkins University, 664E Ross Building, Baltimore, MD 21205. E-mail address: ssadegh@pathlan.path.jhu.edu

<sup>3</sup> Abbreviations used in this paper: APL, altered peptide ligand; CsA, cyclosporin A; HA, hemagglutinin.

a subsequent stimulatory dose of agonist peptide. These effects last up to 7 days and are quantitatively and qualitatively similar to those induced by an antagonist peptide. Furthermore, we find that the inhibitory agonist and antagonist peptide concentrations induce similar low levels of T cell activation, as assayed by proliferation, cytokine production, TCR engagement and down-regulation, and IL-2R $\alpha$  up-regulation. Furthermore, we show that anergy induced by low agonist concentrations is inhibited in the presence of IL-2 or cyclosporin A (CsA), suggesting involvement of the calcineurin pathway. The agonist concentrations that induce anergy are 10- to 100-fold lower than the concentrations necessary for induction of a detectable level of IL-2. In addition, we have determined the number of TCR engaged by the agonist peptide/DR1 and demonstrate that engagement of 100–1000 TCR induces anergy. Our data constitute that biological responses induced by fewer than 10 high affinity MHC/peptide complexes per APC are similar to those induced by several thousand-fold more antagonist/MHC per APC, indicating that quantitative and not qualitative differences in TCR ligand determine activation or anergy.

## Materials and Methods

### Cells and culture conditions

Clone 1 is a human CD4<sup>+</sup> Th1 clone specific for the peptide 306–318 of influenza hemagglutinin (HA) presented on HLA-DR1 (5), and HA1.7 is a human CD4<sup>+</sup> Th0 clone also specific for HA presented on HLA-DR1 (22). Although both clones have the same specificity, they were isolated at different times and from different individuals. EBV 1.24, a human HLA-DR1 (DRB1\*0101)-positive, EBV-immortalized, activated B cell line (EBV-B, or B cells), was used as APC. EBV-B cells constitutively express high surface levels of the costimulatory molecules B7.1 and B7.2, as determined by FACS staining with primary Abs BU63, anti-B7.2 (Calbiochem), and MAB104, anti-B7.1 (Immunotech, Westbrook, ME), followed by PE-conjugated secondary Abs (Sigma, St. Louis, MO).

EBV-B cells were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, nonessential amino acids (Sigma), and 1 mM sodium pyruvate (Sigma). T cells were grown in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 5% FCS (Life Technologies), 5% pooled human serum, 2 mM L-glutamine (Life Technologies), and 10 mM HEPES (Life Technologies). Every 7 days, T cell clones were maintained by restimulation of 10<sup>6</sup> T cells with 10<sup>6</sup> EBV-B cells (irradiated 10,000 rad), 10<sup>6</sup> human leukocytes (irradiated 10,000 rad), 1  $\mu$ M HA peptide, and 120 IU/ml human rIL-2 (Cetus, Norwalk, CT) in 2 ml of medium per well of a 24-well plate.

### Peptides

Peptides were synthesized, purified by HPLC, and analyzed by Peptide Express (Fort Collins, CO). HA<sub>306–318</sub>, PKYVKQNTLKLAT, is the full agonist peptide for both clones used in these experiments. ETEC, IYQIVVEKGGKKK, does not bind to DR1 (23), and YAK (AAYAAAAA KAAA) binds to DR1, but exhibits no agonist or antagonist properties (null peptide) for either clone (data not shown). N312Q, PKYVKQNTLKLAT, is an antagonist peptide of Clone 1 (5). The peptides were purified to apparent homogeneity of more than 95% by reverse-phase preparative HPLC, and their identities were confirmed by mass spectrometry. The concentration of the peptide stock solutions was determined by ninhydrin assay.

### Induction of T cell unresponsiveness

T cell anergy was induced by incubating 4  $\times$  10<sup>4</sup> T cells with 4  $\times$  10<sup>4</sup> irradiated (10,000 rad) EBV-B cells and variable concentrations of HA or N312Q, in 200  $\mu$ l in a 96-well round-bottom plate for 18–36 h at 37°C in 5% CO<sub>2</sub>. Alternatively, 2  $\times$  10<sup>6</sup> T cells and 2  $\times$  10<sup>6</sup> irradiated (10,000 rad) EBV-B cells were incubated with the indicated peptide concentrations for 18–36 h in a volume of 1 ml in a 48-well plate. At the end of this time, T cells were separated from B cells by density-gradient centrifugation over Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ), washed extensively, and counted. T cells were then stimulated with 0.1–10  $\mu$ M HA peptide or medium alone and freshly irradiated EBV-B cells. Persistence of anergy was examined by incubating separated T cells with fresh unpulsed B cells for 3, 5, 7, or 14 days. After these rest periods, peptide-pulsed B cells were added and T cells were assayed for proliferation. Proliferation or cytokine (IL-2 or IFN- $\gamma$ ) production was measured as indicated below.

In some assays, IL-2 (10 IU/ml) or CsA (1  $\mu$ g/ml) (Sandoz, Basel, Switzerland) was added during the initial phase. In these assays, cells were washed three times before the addition of the stimulatory dose of Ag. To exclude the possibility of errors in counting recovered cells, proliferation of T cells in response to IL-2 was measured.

### T cell proliferation assay

Seventy-two hours after the addition of the stimulatory dose of agonist peptide or peptide-pulsed APCs, each well was pulsed with 1  $\mu$ Ci [<sup>3</sup>H]thymidine (Amersham, Arlington Heights, IL). Cells were harvested and counted 14 h later using a beta counter (Packard Instruments, Meriden, CT). Each assay was done in triplicate.

### Cytokine assays

IL-2 release was measured using the IL-2-sensitive cell line HT-2. Culture supernatants were harvested 24 h after addition of a stimulatory dose of HA peptide, frozen, and then thawed once. Then 50  $\mu$ l of culture supernatant and 5  $\times$  10<sup>4</sup> HT-2 cells in RPMI supplemented with 10% FBS, 50  $\mu$ M 2-ME, and L-glutamine were combined in one well of a 96-well plate and incubated at 37°C at 5% CO<sub>2</sub> for 24 h. Each well was then pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine for an additional 14 h, harvested, and counted. All assays were performed in triplicate.

IFN- $\gamma$  production was measured in the culture supernatants harvested 24 or 48 h after addition of the stimulatory dose of agonist peptide by ELISA with a matched Ab pair, according to manufacturer's (PharMingen, San Diego, CA) suggested protocol. All assays were performed in duplicate.

### CD28 cross-linking

Wells of a 96-well plate were incubated for 3 h at 37°C with 50  $\mu$ l PBS without Ab or 10  $\mu$ g/ml goat anti-mouse IgG (Cappel), then washed three times in PBS. Clone 1 T cells were rendered unresponsive in either the absence or presence of 5  $\mu$ g/ml mouse anti-human CD28, as described (PharMingen). Proliferation to 1  $\mu$ M HA presented by EBV-B cells was assayed.

### Peptide quantification

Synthetic peptides were purchased from Molecular Resources (Colorado Springs, CO). Electrospray-mass spectrophotometry analysis of the HA peptide after synthesis as well as our own analytical HPLC analyses indicate that the peptide was greater than 97% pure and lacked any detectable contaminants with absorbance at 210 nm. Synthetic HA peptide (50 nmol) was iodinated with Iodo-Beads (Pierce, Rockford, IL). After iodination, the peptide was then purified by reverse-phase HPLC using a 3  $\mu$ m  $\times$  10-cm C-18 column (Thomson Instrument, Springfield, VA) with a 15-min 0–50% acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. Specific activity and recovery were determined by integration of area under the curve at 280 nm using serial dilutions of cold HA peptide as a reference. Specific activity of approximately 44 Ci/nmol was calculated.

Irradiated EBV-B cells (1  $\times$  10<sup>7</sup>/well) were pulsed for 18 h with the indicated doses of peptide under the conditions used for anergy induction. Quantitative analysis of complex formation was performed by a modification of a previously described method (17). Briefly, pulsed cells were washed three times in complete medium and then resuspended in 1 ml of 0.1  $\times$  PBS containing PMSF (10 mM) and aprotinin (1 mg/ml).  $\beta$ -Octyl glucopyranoside was then added to a final concentration of 1% and the cells were disrupted by mixing. After a low-speed centrifugation to remove insoluble debris, the lysate was precleared using Sepharose CL-4B. HA-DR1 complexes were immunoprecipitated using an excess of mAb L243-coupled CL-4B resin, which was then separated from the lysate by centrifugation over a 60% sucrose cushion. Precipitation efficiency was determined to be nearly 100% by a second immunoprecipitation of the cleared lysate. All manipulations (less than 2 h) were conducted on ice or at 4°C. Fractions were counted directly, for four minutes per sample, using a gamma counter. Precipitated counts were compared with a dilution series of the iodinated peptide alone.

### IL-2R $\alpha$ and CD3 expression

IL-2R $\alpha$  and CD3 expression were assayed by flow cytometry using a Becton Dickinson (Mountain View, CA) FACScan: 5  $\times$  10<sup>5</sup> Clone 1 T cells, 5  $\times$  10<sup>5</sup> EBV-B cells, and indicated concentrations of agonist, antagonist, or irrelevant peptide were incubated in one well of a 96-well round-bottom plate for 18 h. At this time, cells were either stained with anti-IL-2R $\alpha$  or anti-CD3 $\epsilon$  and analyzed by FACS, or 10  $\mu$ M HA peptide was added to each well for an additional 5 h before staining for CD3 expression or for an additional 24 h before staining for IL-2R $\alpha$  expression. Staining for CD3

expression was performed with mouse anti-human CD3 (OKT3; American Type Culture Collection, Manassas, VA) purified from ascites, followed by PE-conjugated goat anti-mouse IgG (Sigma). IL-2R was detected using mouse anti-human IL-2R $\alpha$  (T Cell Sciences, Cambridge, MA), followed by PE-conjugated goat anti-mouse IgG. Baseline expression was determined by incubating T and B cells without peptide during the initial incubation phase and/or the secondary activation phase. Maximal down-regulation of CD3 or up-regulation of IL-2R $\alpha$  was determined by incubating T cells and B cells without peptide during the initial phase, followed by stimulation with 10  $\mu$ M HA during the secondary incubation. T cells were gated by size and granularity.

#### Quantifying TCR down-regulation

Clone 1 T cells ( $10^6$ ), EBV-B cells ( $10^6$ ), and indicated concentrations of agonist or antagonist peptides were incubated in separate wells of a 24-well flat-bottom plate for 6 h. At this point, the cells were stained with saturating concentrations of (FITC-) conjugated anti-CD3 with known fluorescein: protein ratio (5:1), followed by PE-conjugated anti-CD4 (both from PharMingen). The specimens were examined by FACS analysis. The number of TCRs was estimated by determining the peak fluorescence channel for each sample and comparing with fluorescence of calibration particles with defined fluorescence intensities (Spherotech, Libertyville, IL).

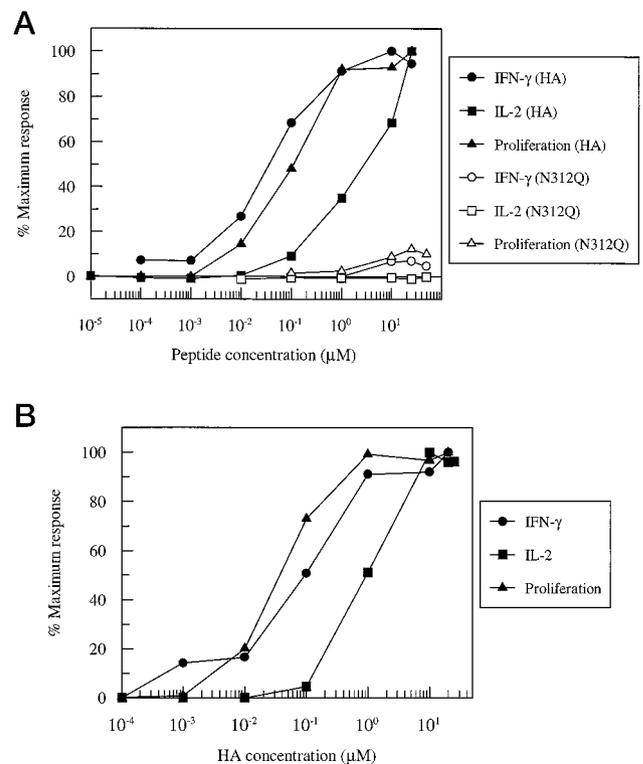
## Results

#### Cytokine production and proliferation of T cell clones in response to the agonist peptide HA<sub>306–318</sub>

Clone 1 and HA1.7 are T cell clones that are specific for the peptide 306–318 of influenza HA presented on the class II molecule HLA-DR1. N312Q is an analogue of HA, which contains a single amino acid substitution and is an antagonist of Clone 1 (5). Both clones proliferate and produce IL-2 and IFN- $\gamma$  upon stimulation with the agonist peptide. Clone 1 T cells showed extremely weak proliferation and IFN- $\gamma$  production and no detectable IL-2 production in response to the antagonist peptide N312Q concentrations up to 50  $\mu$ M (Fig. 1). Although both clones have the same peptide specificity and HLA restriction, they were isolated independently and use different TCR V $\alpha$ - and V $\beta$ -chains (22). In addition, peptides that have been shown to be antagonists of Clone 1 are not antagonists of HA1.7 (24).

#### Unresponsiveness induced by a low dose of agonist peptide

T cells were incubated with B cells in the presence of various doses of HA and, for Clone 1, of N312Q for 18 h. A second set of B cells, which were pulsed with 10  $\mu$ M HA (proliferation and IL-2) or 1  $\mu$ M HA (IFN- $\gamma$ ), was added to the culture, and T cell proliferation and IL-2 and IFN- $\gamma$  production were assayed (Fig. 2, A and B). The response of Clone 1 cells, which were untreated or pretreated with either 0.01  $\mu$ M HA, 25  $\mu$ M N312Q, or 0.01  $\mu$ M of the null peptide YAK to a range of stimulatory peptide concentrations, is also shown (Fig. 2C). When pretreated with a low concentration (0.01–0.001  $\mu$ M) of agonist peptide, both clones exhibited decreased proliferation and IFN- $\gamma$  production to the agonist ligand. Additionally, when pretreated with an inhibitory concentration of agonist peptide, neither clone produced any detectable IL-2 in response to the stimulatory dose of agonist peptide. The inhibitory effect of the agonist peptide occurs within a 2–3 log concentration range and is not observed at concentrations above 0.1  $\mu$ M or below 1 pM. Interestingly, the inhibitory effect of a low concentration of agonist is comparable with the effect of a high (25–50  $\mu$ M) concentration of N312Q. The irrelevant peptides ETEC and YAK had no inhibitory effect. Similar results were observed when T cells were separated from B cells after the initial step by centrifugation over Ficoll (as in Figs. 2D and 7). It has been shown that human T cells can present Ag in a tolerogenic fashion (5, 24, 25). However, high doses of peptide are necessary for this effect. In our system, T cells proliferate normally if either B or T cells are eliminated from the pretreatment step, and are



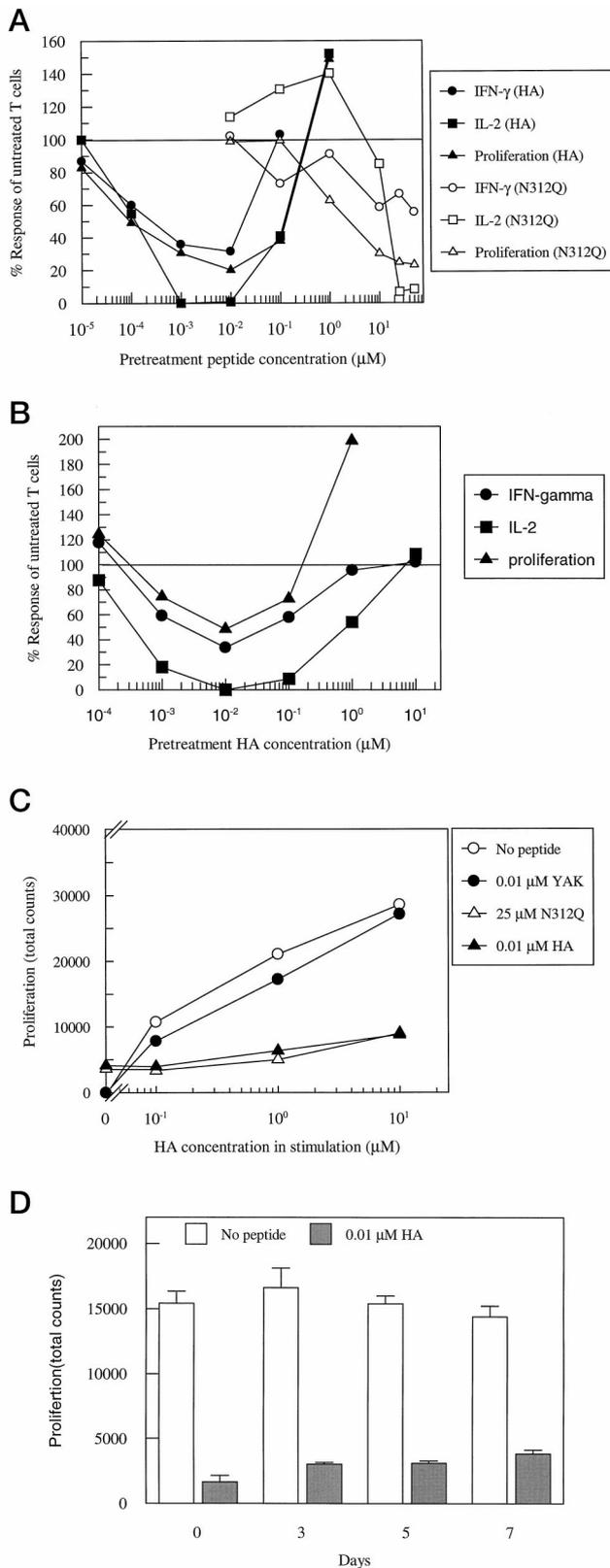
**FIGURE 1.** Cytokine production and proliferation of T cell clones in response to the agonist peptide HA<sub>306–318</sub>. IFN- $\gamma$  production (circles), IL-2 production (squares), and proliferation (triangles) dose-response curves of Clone 1 (A) and HA1.7 (B) T cells to agonist peptide HA (filled symbols) or N312Q (open symbols) presented by EBV-B cells. Maximum response is the highest response observed when cells were stimulated with HA peptide. For Clone 1, total counts for maximum proliferation are 96,601, total counts for maximum IL-2 production are 46,738 for HT-2 indicator cells, and maximum IFN- $\gamma$  production is 24.8 ng/ml. For HA1.7, maximum proliferation is 69,197 total counts, maximum IL-2 production is 79,066 total counts of HT-2 indicator cells, and maximum IFN- $\gamma$  production is 19.6 ng/ml.

added instead at the stimulation step, indicating that B cell presentation of peptide/DR1 is necessary for inducing unresponsiveness (data not shown).

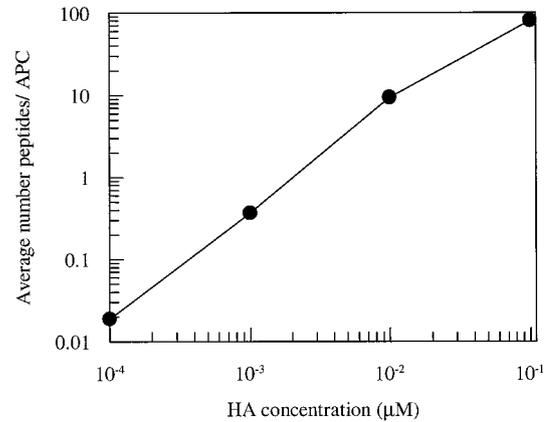
T cell anergy can result when TCR ligation occurs in the absence of CD28 engagement. Although EBV-B cells express high levels of B7.1 and B7.2 and can fully activate T cell clones tested (Fig. 1, A and B), to ensure effective engagement of CD28 and delivery of signal 2 during the initial phase, we examined the effect of CD28 cross-linking during the exposure of T cells to a low concentration of agonist peptide. Wells of a 96-well plate were either left uncoated or coated with cross-linked anti-human CD28. Clone 1 T cells were then incubated with EBV-B cells without peptide or 0.01  $\mu$ M HA. Fresh B cells pulsed with 1  $\mu$ M HA were added to wells, 18 h later, and proliferation to this challenge was measured. Unresponsiveness was induced even in the presence of cross-linked anti-CD28. The effectiveness of this treatment in providing signal 2 was confirmed by its enhancement of T cell proliferation in response to anti-CD3 as signal 1 (data not shown).

#### T cell unresponsiveness lasts up to 7 days

Persistence of anergy was examined by incubating T cells that had been anergized by 0.01  $\mu$ M HA-pulsed B cells with fresh unpulsed B cells for 3, 5, 7, or 14 days. After these rest periods, peptide-pulsed B cells were added and T cells were assayed for proliferation. As shown in Fig. 2D, cells remained anergic up to 7 days of



**FIGURE 2.** Exposure of T cells to a low concentration of agonist peptide renders cells less responsive to a stimulatory Ag concentration. Clone 1 (A) or HA1.7 (B) T cells were incubated with irradiated EBV-B cells and various concentrations of HA (filled symbols) or N312Q (open symbols) for 24 h. Fresh irradiated B cells and a stimulatory concentration of HA peptide ( $10 \mu\text{M}$  in proliferation and IL-2 assays and  $1 \mu\text{M}$  in IFN- $\gamma$  assays) were then added to cultures, and proliferation (triangles), IL-2 production (squares), and IFN- $\gamma$  production (circles) were measured. Results are shown as the percentage of the response of T cells pretreated in the absence



**FIGURE 3.** The concentration of HA peptide that induces T cell anergy results in the formation of less than 10 HA-DR1 complexes per APC. Irradiated EBV-B cells were pulsed for 18 h with the indicated doses of  $^{125}\text{I}$ -labeled HA peptide. DR1/peptide complexes were immunoprecipitated from detergent-solubilized cells, as described in *Materials and Methods*, and counted directly using a gamma counter. Precipitated counts were compared with a dilution series of the  $^{125}\text{I}$ -labeled HA.

rest period before the peptide challenge. However, cells recovered from anergy if rested for another 7 days (data not shown).

#### Quantification of DR1-HA complexes that induce anergy

We used an  $^{125}\text{I}$ -labeled HA peptide to determine the number of DR1/HA complexes formed per APC in our pretreatment step to estimate the number of complexes that induce T cell anergy. Our previous work (26) had shown that iodination of HA peptide does not alter its DR1-binding affinity, as the iodinated HA peptide could compete equally with the unmodified peptide in peptide competition assays. EBV-B cells were incubated with various concentrations of iodinated HA under the same conditions used in the pretreatment step of our assay. Cells were then washed extensively and solubilized with the detergent  $\beta$ -Octyl glucopyranoside. DR1 molecules were specifically immunoprecipitated, and the number of bound peptides was determined by direct gamma counting (Fig. 3). A second immunoprecipitation did not yield counts above the background level, indicating that nearly all DR1 molecules were immunoprecipitated. Assuming an equal distribution of complexes, we found that approximately 10 HA-DR1 complexes were formed per APC when cells were incubated with  $0.01 \mu\text{M}$  HA and that a nearly linear relationship existed between the peptide concentration and the number of complexes formed in the range measured. Thus, at an HA concentration of  $0.1 \mu\text{M}$ , 100 complexes of peptide/MHC were formed per APC, and at  $1 \text{ nM}$ , the average number of HA-DR1 per APC was one. Notably, even a single agonist-MHC II complex is sufficient to induce a measurable effect in specific T cells.

of peptide. C, Clone 1 T cells were pretreated as in A and B in the absence of peptide ( $\circ$ ), with  $0.01 \mu\text{M}$  YAK ( $\bullet$ ), with  $25 \mu\text{M}$  N312Q ( $\Delta$ ), or with  $0.01 \mu\text{M}$  HA ( $\blacktriangle$ ), and proliferation to a range of HA concentrations was assayed. D, Clone 1 T cells were cultured with irradiated B cells and no peptide or  $0.01 \mu\text{M}$  HA; 18 h later, T cells were separated from B cells over a Ficoll gradient, washed, and rested with fresh irradiated B cells for the time indicated. A second batch of freshly irradiated but peptide-pulsed B cells was added after 0, 3, 5, or 7 days, and proliferation was measured 3 days later by [ $^3\text{H}$ ]thymidine.

*Similar levels of TCR down-regulation and IL-2R up-regulation by inhibitory concentrations of antagonist or agonist peptides*

Upon activation with Ag, T cells down-regulate their surface expression of TCR (27, 28) and up-regulate their surface expression of the IL-2R. We compared the effect of the low concentration of agonist peptide with the inhibitory dose of antagonist peptide on TCR and IL-2R expression on Clone 1. T cells and EBV-B cells were incubated either in the absence of peptide or with 0.01  $\mu$ M HA or 25  $\mu$ M N312Q for 18 h. T cells were then evaluated for CD3 and IL-2R $\alpha$  expression by flow cytometry. We found that 0.01  $\mu$ M HA and 25  $\mu$ M N312Q caused similar shifts in the level of CD3 expression, indicating similar down-regulation in the level of TCR by either ligand (Fig. 4A). A similar increase in the level of IL-2R was also observed by both ligands at the above concentrations (Fig. 4B). Interestingly, it appears that stimulation with these two different peptides caused the same low levels of activation.

*Quantification of TCR engagement*

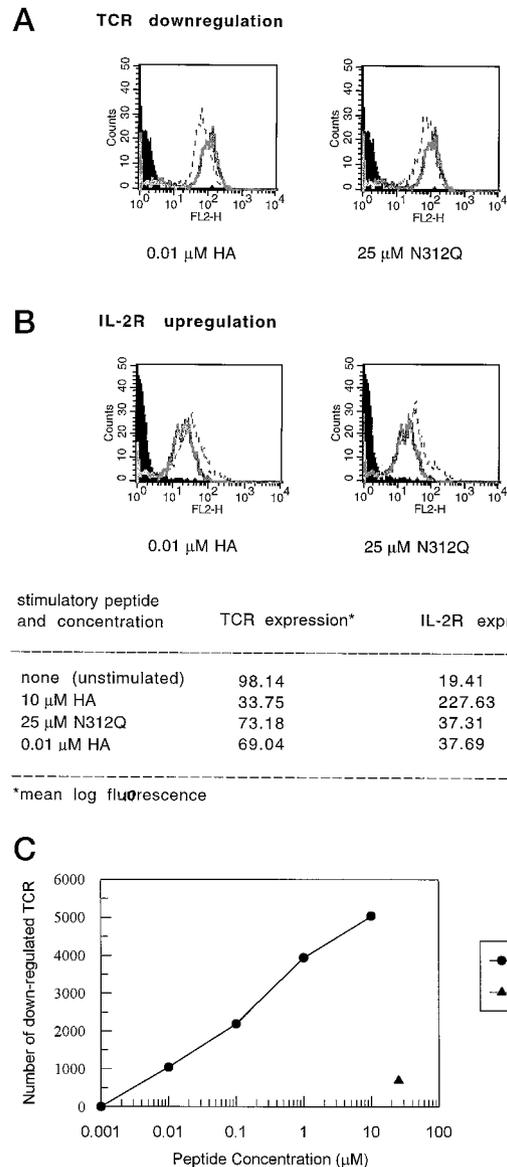
TCR down-regulation is shown to be an indication of TCR engagement. To quantify the number of TCR engaged by the low numbers of HA/DR1 complexes, Clone 1 T cells were incubated with EBV-B cells, pulsed with 0, 0.001, 0.01, 0.1, 1, or 10  $\mu$ M HA or 25  $\mu$ M N312Q for 6 h (maximal TCR down-regulation). TCR-CD3 complexes were enumerated by direct staining with FITC-labeled anti-CD3 $\epsilon$  Ab, with known numbers of FITC per molecule, followed by FACS analysis. The number of TCR engaged at each peptide concentration was estimated by subtracting the fluorescence signal on stimulated T cells from that of resting T cell. The mean channel fluorescence was compared with the fluorescence of calibration beads for estimation of the number of TCR stained. This showed a basal expression of 8594 on each resting T cell. Cells exposed to 0.001, 0.01, 0.1, 1, or 10  $\mu$ M HA had 0, 1093, 2186, 3934, and 5027 receptors down-regulated, respectively. Incubation with 25  $\mu$ M of N312Q led to the down-regulation of 687 TCR/CD3 complexes.

*T cells rendered unresponsive show normal TCR down-regulation and IL-2R up-regulation in response to a stimulatory dose of the agonist peptide*

We also examined whether Clone 1 T cells that had been pretreated with a low dose of agonist or with an inhibitory dose of N312Q exhibited these signs of activation upon subsequent stimulation with a high dose of agonist peptide (Fig. 5). All groups of T cells showed equal levels of CD3 and IL-2R expression after stimulation with 10  $\mu$ M HA, regardless of prior exposure to inhibitory peptide. Thus, pretreatment apparently did not affect the ability of the TCR to be engaged by a second dose of agonist peptide nor did pretreatment prevent IL-2R up-regulation. The same phenotype is observed when anergy is induced by signal 1 in the absence of signal 2 (29).

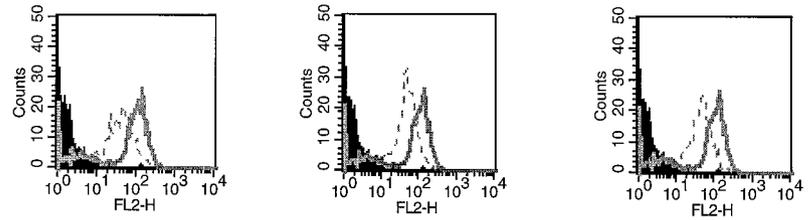
*T cells rendered unresponsive proliferate normally to exogenous IL-2 and to a stimulatory dose of agonist in the presence of exogenous IL-2*

In light of the data that T cells exposed to low agonist concentrations retained the ability to up-regulate IL-2R expression, we next examined whether these T cells are also able to proliferate in response to exogenous IL-2. Clone 1 T cells were pretreated with B cells in the absence of peptide or in the presence of 0.01  $\mu$ M HA. T cells were then stimulated with either 10 IU/ml IL-2 or 1  $\mu$ M HA, or both, and assayed for proliferation. Both groups of T cells proliferated equally well in response to the IL-2 or the peptide plus IL-2, but not to the peptide alone (Fig. 6). These results indicate



**FIGURE 4.** Inhibitory agonist and antagonist peptide concentrations cause similar levels of TCR down-regulation and IL-2R up-regulation. Clone 1 T cells and irradiated B cells were incubated with no peptide, 0.01 mM HA, or 25 mM N312Q for 18 (A) or 24 (B) h. Cells were then washed and stained with mAbs against CD3 (A) or IL-2R (B), followed by a PE-labeled goat anti-mouse IgG, and surface protein expression levels were determined by flow-cytometric analysis. Solid lines show surface receptor expression of cells mock stimulated in the absence of peptide. Dashed lines show surface receptor expression level of cells stimulated with indicated peptide concentrations. Solid histograms show staining with an isotype-matched negative control Ab. Mean log fluorescence of TCR and IL-2R expression on T cells stimulated with various peptide concentrations is also given. C, The amount of TCR engaged after exposure to different concentrations of HA (●) or 25  $\mu$ M of N312Q (▲). T cell down-regulation was quantified by incubating Clone 1 T cells and EBV-B cells without peptide or in the presence of 0.001, 0.01, 0.1, 1, or 10  $\mu$ M HA or 25  $\mu$ M N312Q for 6 h. The number of TCR-CD3 complexes was estimated by comparing the anti-CD3 staining with the standard curve of calibration particles with defined fluorescence intensities. A basal expression of 8594 TCR on each T cell was determined. Cells exposed to 0.001, 0.01, 0.1, 1, or 10  $\mu$ M HA had 0, 1093, 2186, 3934, and 5027 receptors down-regulated, respectively. Incubation with 25  $\mu$ M of N312Q led to the down-regulation of 687 TCR/CD3 complexes.

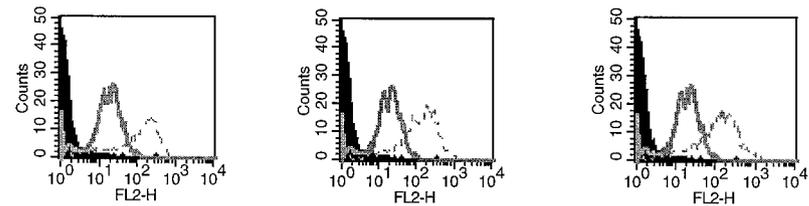
### A. TCR downregulation



pretreatment: no peptide

0.01  $\mu\text{M}$  HA25  $\mu\text{M}$  N312Q

### B. IL-2R upregulation



pretreatment: no peptide

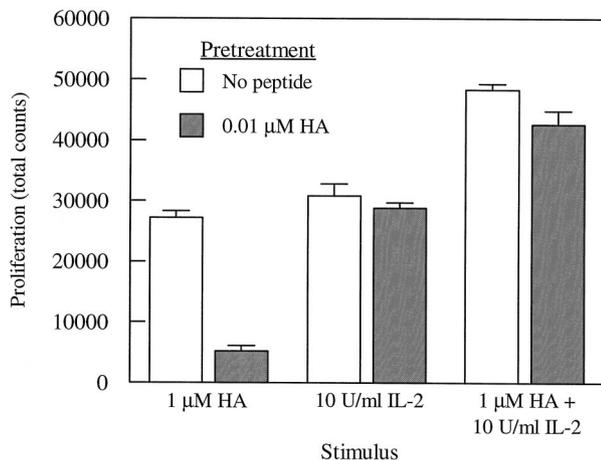
0.01  $\mu\text{M}$  HA25  $\mu\text{M}$  N312Q

**FIGURE 5.** Peptide-induced anergy does not change TCR down-regulation or IL-2R up-regulation in response to 10  $\mu\text{M}$  HA. Clone 1 T cells were incubated for 24 h with irradiated B cells and indicated peptide concentrations. Fresh B cells and 10  $\mu\text{M}$  HA or no peptide were then added to cultures. Five (A) or twenty-four (B) hours later, cells were washed and stained with mAbs against CD3 (A) or IL-2R (B), followed by a PE-labeled goat anti-mouse IgG, and surface protein expression levels were determined by flow-cytometric analysis. Solid lines show surface receptor expression of cells pretreated and mock stimulated in the absence of peptide. Dashed lines show surface receptor expression level of cells pretreated with indicated peptide concentration and stimulated with 10  $\mu\text{M}$  HA. Solid histograms show staining with an isotype-matched negative control Ab. Mean log fluorescence of TCR and IL-2R expression under different pretreatment and stimulation conditions is also given.

pretreatment	stimulation	TCR expression*	IL-2R expression*
no peptide	no peptide	94.92	19.51
no peptide	10 $\mu\text{M}$ HA	42.59	172.30
0.01 $\mu\text{M}$ HA	10 $\mu\text{M}$ HA	48.38	160.77
25 $\mu\text{M}$ N312Q	10 $\mu\text{M}$ HA	44.25	160.25

\*mean log fluorescence

that unresponsiveness was not due to T cell deletion. Instead, it may be due to a failure of IL-2 production, as proliferation was seen not only in response to IL-2 alone, but also to peptide in the



**FIGURE 6.** Clone 1 T cells pretreated with a low concentration of agonist peptide proliferate as effectively as untreated cells upon stimulation with exogenous IL-2 or with Ag plus IL-2. Clone 1 T cells were incubated together with B cells and either 0.01  $\mu\text{M}$  HA or no peptide; 18 h later, T cells were stimulated with either fresh irradiated B cells + 1  $\mu\text{M}$  HA, 10 IU/ml human IL-2, or fresh irradiated B cells + 1  $\mu\text{M}$  HA + 10 IU/ml human IL-2, and proliferation was measured by [ $^3\text{H}$ ]thymidine uptake.

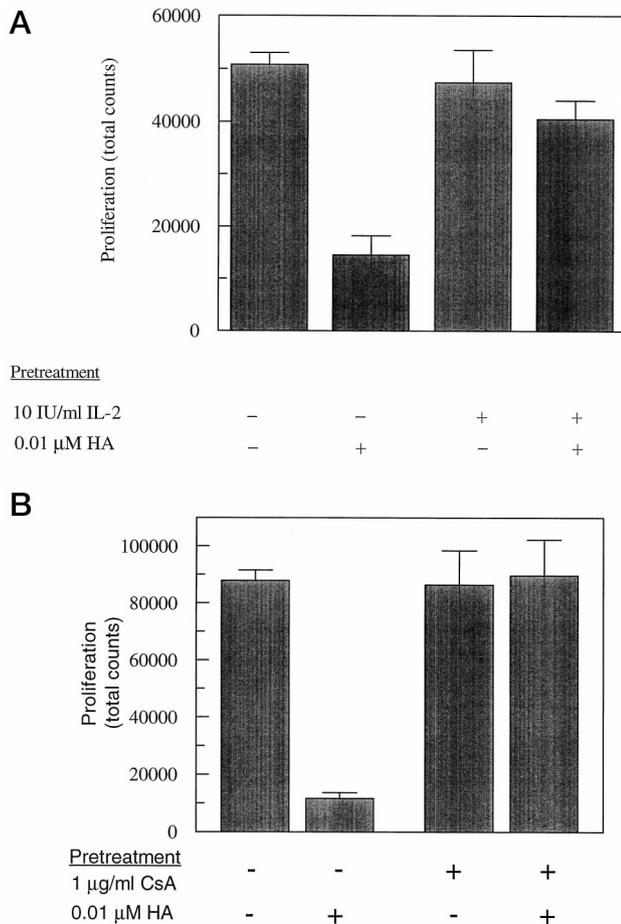
presence of IL-2. The proliferation of all groups of T cells in response to peptide plus IL-2 was greater than that induced by IL-2 alone, indicating that the T cells were responding to the peptide in addition to the IL-2.

#### IL-2 inhibits induction of unresponsiveness

Previous studies have indicated that APL and fixed APCs induce unresponsiveness because of their failure to stimulate IL-2 production and that IL-2 inhibits the induction of unresponsiveness (30). To determine whether anergy induced by a low density of agonist ligand is also reversible in the presence of IL-2, we included exogenous IL-2 during the initial phase of our assay. T cells were separated from the dead B cells and washed to remove residual IL-2, and T cell proliferation in response to stimulation with 1  $\mu\text{M}$  HA was assayed. T cells exposed to a low agonist concentration in the presence of IL-2 responded to Ag to the same degree as T cells that were cultured in the absence of peptide (Fig. 7A), indicating that IL-2 prevents the induction of anergy by the low concentration of agonist.

#### CsA prevents induction of unresponsiveness

CsA has been shown to bind cyclophilin and inhibit activation of calcineurin, a calcium- and calmodulin-dependent phosphatase, resulting in inhibition of dephosphorylation of cytoplasmic nuclear factors of activation in T cells, NF-AT, necessary for transcription of IL-2 gene (31-34). Previous studies of anergy induced by APL (6) or by chemically fixed APCs (35) showed that anergy was not



**FIGURE 7.** IL-2 and CsA prevent the induction of T cell anergy by low agonist peptide concentration. *A*, Clone 1 T cells were cultured with irradiated B cells and no peptide or 0.01  $\mu\text{M}$  HA in the absence or presence of 10 IU/ml IL-2; 18 h later, T cells were separated from B cells over a Ficoll gradient, washed, and stimulated with fresh, irradiated B cells and 1  $\mu\text{M}$  HA. Proliferation was measured 3 days later by [ $^3\text{H}$ ]thymidine. *B*, T cells were cultured with irradiated B cells and no peptide or 0.01  $\mu\text{M}$  HA in the absence of drug or in the presence of 1  $\mu\text{g}/\text{ml}$  CsA. Eighteen hours later, T cells were separated from B cells, and proliferation was assayed as in *A*.

induced in the presence of CsA. Thus, we tested whether unresponsiveness induced by a low dose of agonist was sensitive to CsA. Clone 1 T cells and irradiated EBV-B cells were incubated in the absence of peptide or with 0.01  $\mu\text{M}$  HA peptide in the presence or absence of 10  $\mu\text{g}/\text{ml}$  of CsA. EBV-B cells were removed, T cells were washed extensively, and proliferation in response to 1  $\mu\text{M}$  HA was assayed. As shown in Fig. 7*B*, CsA prevented induction of T cell unresponsiveness, suggesting that induction of unresponsiveness involves the calcineurin pathway.

## Discussion

*T cell unresponsiveness can be induced by a low density of agonist or a high density of antagonist MHC/peptide presented by live APCs*

The findings described in this work address the relationship between the number of MHC/peptide complexes on APCs and differential signaling transduced in T cells. We find that low concentrations of agonist peptide presented on live APCs can induce T cell anergy in CD4<sup>+</sup> T cell clones. Similar effects are induced by 10,000-fold higher concentration of antagonist peptide. This treat-

ment inhibits expression of late activation events such as proliferation and production of IL-2 or IFN- $\gamma$ , but does not affect TCR down-regulation, an early activation event. IL-2R $\alpha$  expression is also unaffected by pretreatment of T cells with a low agonist or inhibitory antagonist peptide concentration, suggesting that not all signaling pathways in the T cell are impaired. We estimate that approximately 1–10 agonist MHC/peptide complexes per APC induce T cell anergy, and that similar effects are observed in response to three orders of magnitude higher concentrations of the antagonist peptide.

We demonstrate that anergy induction is not likely to be due to failure of APCs to provide an adequate costimulatory signal, as the EBV-B cells used as APCs express high levels of B7.1 and B7.2, as determined by flow-cytometric analysis. Moreover, cross-linking of CD28 with plate-bound anti-human CD28 during the pretreatment of cells with low agonist concentration does not prevent induction of anergy (data not shown). T cells exposed to low concentrations of agonist peptide retain the ability to proliferate in response to exogenous IL-2, ruling out the possibility of cell loss or death. Moreover, anergy induction is not due to T cell Ag presentation, as reported earlier. In those studies, the concentration of peptide necessary for induction of unresponsiveness in T cells was several orders of magnitude higher than that necessary for T cell activation. In our system, T cell unresponsiveness is induced by extremely low doses and is dependent on presentation by B cells.

We have used T cell clones specific for HA<sub>306–318</sub> peptide bound to DR1 because of the stability of HA/DR1 complexes. We have measured a dissociation  $t_{1/2}$  of 140 h (6 days!) at 37°C for  $^{125}\text{I}$ -labeled HA/DR1 complexes (26). Such remarkable stability minimizes errors in underestimating the number of  $^{125}\text{I}$ -labeled peptide/MHC complexes due to dissociation during the process. Thus, a more accurate estimation of the number of complexes necessary for induction of T cell responses can be achieved. The only other report in literature regarding a measurable effect induced by a single peptide/MHC complex comes from Sykulev et al. (21), that contrary to our report, observed cytolytic killing of target cells expresses as few as a single peptide/class I MHC ligand. This difference might be due to different activation requirements for CD8 versus CD4 T cells.

We can dismiss the possibility that unresponsiveness is induced by contaminating antagonist peptides found in the agonist peptide preparation in several ways. First, our peptides are purified to apparent homogeneity of >95% by reverse-phase preparative HPLC, and their identities were confirmed by MALDI mass spectrometry. Second, during the course of these experiments, we have used several independent batches of peptides that all have consistently induced unresponsiveness at the same doses of agonist peptide. Finally, the possibility of a contaminant peptide that induces anergy only at extremely low but not at higher doses of peptide seems implausible.

### *Agonist ligands provide tools for discrimination among different models of T cell activation*

Use of APL for T cell stimulation has provided a means to study mechanisms of TCR engagement and the signals transduced. Several models have been proposed that rely on qualitative or quantitative differences between APL and the agonist ligands. By using agonist ligand at a low density on APCs, we have circumvented these differences. In this study, because of the use of the agonist peptide, the conformation or quality of the TCR ligand remains the same. Additionally, the kinetics of an individual MHC/peptide-TCR interaction remains unchanged. Thus, we can evaluate the concepts of conformational or kinetic models in engagement of TCRs. Our findings suggest that transduction of signals that cause

T cell unresponsiveness does not occur through qualitative differences at the level of individual MHC/peptide-TCR interactions. Rather, we propose that anergy is the result of a collective or two-dimensional decrease in affinity or so-called avidity of the T cell for the APC. Our results show that induction of a specific, unique TCR conformation, as has been proposed as a mechanism of anergy induction by antagonist ligands (8, 11, 36), is not necessary for induction of T cell anergy. Furthermore, our data do not support models of kinetic discrimination at the level of single TCR-ligand interactions for the same reason that anergy can be induced by the agonist ligand. However, we cannot rule out the possibility that engagement of TCR by a high density of high affinity ligand might induce structural differences that would not be induced by a low density of ligand. Multimerization of TCR either in random clusters (37) on the surface of T cells or as orientation-specific multimers (38) or ordered oligomers (39) represents alternative models for T cell activation in response to formation of specific TCR arrays. The likelihood of formation of sufficient numbers of correctly oriented multimers is much increased if many TCRs are engaged by their ligands.

By increasing the number of ligands on a cell surface, the avidity of the T cell-APC interaction will be increased. This alone could determine anergy or activation. One may consider the avidity model as a subset of the kinetic discrimination model because the extent of TCR engagement is dictated not only by the dissociation of individual TCR/MHC/peptide ternary complexes, but also by the number of interactions that occur. Nevertheless, the avidity model may be applicable if interactions between TCR and MHC/peptide ligand show 1:1 stoichiometry.

We demonstrate that Th cells respond to fewer than 10 ligands per APC, as determined by assessment of down-regulation of the surface expression of TCR. Viola et al. have estimated serial engagement of 8000 TCR as a prerequisite for transduction of activation signals (19). Our experiments (Fig. 4C) determining the number of TCR engaged by I-10 complexes of peptide/DR1 suggest that engagement of fewer than 1000 TCR per T cell has a negative effect on the T cell signaling machinery.

#### *Induction of unresponsiveness affects IL-2 production*

Anergy is thought to be the consequence of T cell stimulation that fails to induce IL-2 production (29, 30). Chemically modified APCs prompt T cell anergy by signaling through the TCR, but failing to provide costimulation (1), resulting in undetectable IL-2 production. Additionally, partial agonist and antagonist peptides cause anergy by failing to activate IL-2 production. In all of these cases, anergy does not result in the presence of exogenous IL-2. Relatively high concentrations of Ag are necessary to induce IL-2 production in T cells (15, 16), and we now show that stimulation with a peptide concentration that is insufficient to result in detectable IL-2 can also induce unresponsiveness. Our findings that IL-2 or CsA prevents induction of anergy in response to a low density of agonist MHC/peptide ligand are consistent with a role for IL-2 in regulating T cell anergy.

Although we have not yet determined the signaling pathways involved in this anergy induction, we show that it is inhibited by CsA, providing evidence for the involvement of the calcineurin pathway. In addition, we can postulate that only some signaling pathways are affected by pretreatment with low agonist concentrations because the energized T cells retain the ability to increase surface expression of the IL-2R upon subsequent stimulation.

#### *Similarities between antagonist and agonist peptides in TCR reactivity*

The remarkable phenotypic similarities between anergy prompted by a low density of agonist ligand and those induced by partial agonist or antagonist ligands (5, 6, 8) suggest the possibility of shared operating mechanisms. In particular, 10–50  $\mu\text{M}$  N312Q and 0.01  $\mu\text{M}$  or lower doses of HA, the concentrations of antagonist, and agonist peptide that induce anergy in Clone 1, cause similar levels of TCR down-regulation and IL-2R up-regulation, and also induce only slightly detectable T cell activation, as measured by proliferation and IFN- $\gamma$  production. The peptide concentrations that induce anergy do not induce any detectable IL-2.

Antagonist peptides are reported to function in two distinct ways. N312Q, the antagonist of Clone 1 (5), appears to work differently from antagonists identified in the hemoglobin system (6). In the latter case, anergy can only be induced when antagonist peptide is presented to T cells in the absence of the agonist peptide. In the former case, however, the antagonist MHC/peptide complex functions closer to the pharmacologic definition of a receptor antagonist, i.e., a high dose of antagonist ligand competes with the agonist ligand present at subthreshold quantities for TCR engagement. For example, N312Q can prevent T cell activation when present on the same APCs as agonist peptide in vast molar excess (40). However, our experiments reveal that this antagonist peptide can also induce anergy if presented to T cells in the absence of agonist peptide. Thus, combined with its ability to trigger some activation signals, N312Q can justifiably be reclassified as a partial agonist, suggesting that both classes of antagonists may ultimately use the same mechanism to induce anergy. Furthermore, since the dissociation of antagonist ligands from TCR is several orders of magnitude faster than the agonist ligands (13, 41), a corresponding larger number of such ligands is necessary to transduce a negative signal to the T cells. Our data suggest that antagonist peptides at high doses are similar to agonist peptides at low doses in offering a low stimulus to the T cell.

Several groups have found that stimulation of T cells with APL causes altered phosphorylation of TCR- $\zeta$  and lack of ZAP-70 activation. In one report, similar phosphorylation patterns were observed in primary thymocytes after stimulation with APL and low concentrations of agonist peptide, while high concentrations of the agonist peptide caused the expected complete phosphorylation of TCR- $\zeta$  and ZAP-70 (42). In other reports, low concentrations of agonist peptide were shown not to cause this altered phosphorylation pattern (3, 4); however, concentrations of peptides used in those studies were not shown to induce anergy. Although we have not examined TCR- $\zeta$  or ZAP-70 phosphorylation in our system, it is reasonable to imagine that a weak signal could be generated as a result of many incomplete or a very few complete signaling events.

#### *Similar requirements for anergy induction and thymic positive selection*

Our results provide another similarity between the requirements for thymic positive selection and those for anergy induction. The concentration of peptide ligand necessary to induce positive selection is inversely correlated with its affinity for the TCR (43). Both positive selection and anergy can be induced *in vitro* by either APL or low concentrations of agonist peptide (44). Thus, the agonist peptide can cause either positive or negative selection of a T cell, depending upon its abundance.

#### *Significance of low dose anergy *in vivo**

Induction of T cell unresponsiveness by a low concentration of MHC/peptide ligand is a reasonable mechanism for maintaining

peripheral tolerance in vivo. Self Ags encountered in the periphery may not only be present at sufficient concentrations to induce T cell activation, but may also induce T cell unresponsiveness, thus preventing response to transient increases in Ag concentration.

The tolerogenic effect of 1–10 MHC/peptide complexes versus the necessity of recognition of more than 100 complexes for activation of T cell effector functions leaves a safety zone for the immune system in deciding between induction and maintenance of self-tolerance or mounting an autoimmune response. A disruption in this balance could result in autoimmune disease.

### Conclusions

In conclusion, our data indicate that T cell unresponsiveness can be induced in vitro by a low concentration of agonist peptide. We propose that the undetermined mechanism by which low dose anergy is induced involves the avidity of the T cell-APC interaction if a 1:1 stoichiometric relation exists between TCR and MHC/peptide, or alternatively, if subthreshold numbers of TCR are serially engaged by as few as 1–10 MHC/peptide complexes. Our data suggest that the phenomena of TCR antagonism and partial agonism are not unique to altered peptide ligands, but rather that these ligands are capable of providing only a very low stimulus to the T cell.

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