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The Role of T Cell Antagonism and Original Antigenic Sin in Genetic Immunization[1]

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To counter highly mutable pathogens like HIV-1, a number of vaccines are being developed to deliver multiple mutant forms of viral Ags to provoke multivalent antiviral CTLs. However, it is uncertain whether such multiple mutant epitope vaccines will generate the diverse CTL responses desired or will instead create immune interference. To characterize the role of immune interference by mutant epitopes in this process, we have tested a "worst case" scenario in which the immunodominant epitope of OVA (SIINFEKL) and its in vitro TCR antagonist (SIINFEDL) have been used to genetically immunize C57BL/6 mice. We demonstrate here that sequential delivery of these mutant epitopes provokes original antigenic sin in CD8 T cells as demonstrated by attenuation of CTLs, intracellular IFN-γ production, and MHC I peptide-tetramer staining. By contrast, simultaneous exposure of the immune system to this agonist/antagonist pair not only fails to generate T cell antagonism in vivo, but also avoids original antigenic sin. These observations suggest that simultaneous immunization with vaccines containing mutant epitopes, even T cell antagonists, can indeed generate a diverse array of T cell responses and that at least some immune interference can be avoided by delivering mutant Ags to the immune system simultaneously. ***The Journal of Immunology,*** **2002, 169: 6779–6786.**

There is ample evidence for the protective role of vaccine-mediated CTLs in protection against a variety of infectious diseases. Although protection can be achieved against a number of pathogens by generating CTLs specific for a single epitope, control of viruses like HIV-1 is substantially more difficult, since mutable pathogens can mutate CTL epitopes to escape this limited immune responses (1, 2). One strategy to control as wide a spectrum of viral variants as possible is to deliver Ags from multiple viral variants or clades (3). Although this approach holds promise, it is not entirely clear whether stimulation of the immune system with multiple mutant epitopes will generate the desired multivalent immune responses or whether this approach will be limited by immune interference between these highly related Ags.

A variety of mechanisms could provoke immune interference of T cell responses after vaccination with mutant Ags. T cell antagonism and original antigenic sin (OAS)[3] are two such mechanisms, since both can be activated by exposure of the immune system to mutant epitopes or altered peptide ligands (APLs) (4). T cell antagonism occurs when an existing memory T cell is functionally inactivated by exposure to a point mutant of its cognate epitope presented on MHC I (reviewed in Ref. 5). A number of studies using the immunodominant OVA epitope SIINFEKL demonstrate that certain epitopes with single amino acid changes have the ability to antagonize T cell function by binding the TCR in the context of MHC I without inducing receptor activation (6). Given these observations, it is possible that immunization with a multiple mutant vaccine could generate T cell antagonism between mutant epitopes rather than multivalent CTL responses.

The dictum of OAS, discovered first in B cell biology, indicates that when an individual is exposed to an Ag that is similar to, but not identical to, an Ag to which he/she was previously exposed by either infection or immunization, the immune response to the second Ag will still be directed against the first Ag (7, 8). This phenomenon has subsequently been observed in T cells against lymphocytic choriomeningitis virus (LCMV) viral variants (4). In this case, exposure to first one and then a second LCMV strain generated CTLs specific to only the first strain. This observation suggests that vaccines delivering mutant epitopes, particularly at different times, have the potential to induce OAS to interfere with vaccine efficacy. Furthermore, these data suggest that a vaccine with limited epitopes may have the potential to prevent subsequent naive T cell responses against legitimate pathogens if their epitopes diverge from those of the original vaccine Ags.

In view of these observations, this study was performed to determine whether immune interference by T cell antagonism, OAS, or other mechanisms will limit the application of multiple mutant vaccines. To test this, we used an epitope system representing the "worst case" scenario for a vaccine in which an agonist epitope is combined with its own antagonist epitope. To do this, C57BL/6 (H2-K^b) mice were genetically immunized using a gene gun with minigenes encoding the H2-K^b-restricted immunodominant epitope of OVA ($OVA_{257–264}$ = SIINFEKL, OVA hereafter) and its point mutant antagonist (9) epitope SIINFEDL (OVANT hereafter). After immunization, Ag-specific memory and effector CTLs were analyzed by standard 4-h ^{51}Cr release assay, intracellular IFN-γ staining, and peptide-MHC-tetramer staining. Results presented here demonstrate that both OVA and OVANT are mutual in vitro antagonists and that sequential immunization with these epitopes in either order generates a novel T cell immune response which could be accommodated in the existing definition of OAS of CTLs. Furthermore, data presented here demonstrate that simultaneous immunization with this in vitro agonist/antagonist pair generates an in vivo T cell repertoire consisting of both OVA- and

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[3] Abbreviations used in this paper: OAS, original antigenic sin; APL, altered peptide ligand; LCMV, lymphocytic choriomeningitis virus.

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OVANT (mono-)- specific T cells without any sign of in vivo T cell antagonism or OAS of T cells even if the epitopes are expressed in the cytoplasm of the same cells. Since a number of persistent pathogens use antagonism and OAS to perpetuate in the host, the results presented in this study may help in the design and delivery of vaccines to effectively control these immunoevasive and mutable pathogens.

Materials and Methods

Materials

Abs were purchased from BD PharMingen (San Diego, CA). Tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). T-Stim was purchased from Collaborative Biosciences (Bedford, MA). Endotoxin-free plasmid DNA purification columns were purchased from Qiagen (Chatsworth, CA). Peptides were synthesized by Research Genetics (Huntsville, AL). MHC I-SIINFEKL and MHC I-SIINFEDL tetramers were supplied by the National Institutes of Health Tetramer Core. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), or VWR Scientific (Houston, TX).

Mice

Female C57BL/6 (H-2^b) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). All mice were maintained in the American Association of Laboratory Animal Care-approved Center for Comparative Medicine at Baylor College of Medicine (Houston, TX).

Plasmids and genetic immunization

OVA was PCRed from a full-length OVA gene. OVANT was prepared by site-directed mutagenesis of a full-length OVA gene. Each construct was cloned in the pCMVi backbone. pCMVi was constructed from the pGL3 backbone from Promega (Madison, WI) to include the CMV promoter, a chimeric 5′ intron, and the human growth hormone polyadenylation sequence. Each epitope was cloned into the plasmid with the addition of a Kozak methionine (ACC ATG) before the epitope and a TAA stop after the last amino acid of the epitope. Four- to 6-wk-old mice were genetically immunized by gene gun transfection of the epidermis using the Helios biolistic device (Bio-Rad, Hercules, CA) using 250 psi of helium. A total of 2.5 μg of plasmid DNA was delivered per shot of 1.6-μm gold particles, which were prepared as per the device instructions. In most cases, two shots were delivered into the backs of the ears on each mouse at each immunization except where noted in the text. In all cases, mice were vaccinated in a single round of immunization without further boosts.

CTL assay

Four weeks after immunization, splenocytes were harvested and stimulated in vitro for 6 days with 1 μg of relevant peptide in RPMI 1640 medium containing 10% v/v FBS, 10 mM HEPES, 50 μM 2-ME, 10% v/v T-Stim (Collaborative Biomedical Products) as a source of IL-2 and antibiotic/antimycotic. On day 7, CTL activity of the cultured splenocytes was assayed by a 4-h ^{51}Cr release assay as detailed previously (10). Each experiment was done in triplicate and was repeated at least three times.

RMA target cells were labeled with 1 μM of the indicated peptide for 1 h at 37°C and excess peptide was removed by washing. Target cells were labeled with 100 μCi of ^{51}Cr (as sodium chromate) for 1 h in a 1-ml volume. After washing, 1×10^4 labeled target cells were added to effector cells at the indicated E:T ratios. Percent specific lysis was calculated as: ((cpm of sample − cpm of spontaneous release)/cpm of maximum release − cpm of spontaneous release)) × 100. Spontaneous release was defined as the mean cpm released from six replicates of 1×10^4 labeled cells incubated in medium alone. Maximum release was defined as the mean cpm released from six replicates of 1×10^4 labeled cells incubated in medium containing 1% SDS.

Tetramer staining

Four weeks after immunization, splenocytes from nonimmunized or immunized mice were harvested. Fresh lymphocytes or lymphocytes stimulated for 6 days with 1 μM peptide were used for tetramer staining. These stimulated cells were stained with 100 ng each of anti-CD3-PerCP (PerCP-conjugated hamster anti-mouse CD3ϵ (CD3ϵ chain) mAb) and anti-CD8-FITC (FITC-conjugated rat anti-mouse CD8a (Ly-2) mAb) or with isotype control of anti-CD8-FITC conjugate and PE-conjugated MHC I-SIINFEKL and MHC I-SIINFEDL tetramers for 30 min at 4°C in the dark in 100 μl of FACS buffer (PBS containing 2% FBS and 0.02% of sodium azide). Cells were then washed twice with FACS buffer and sample data were acquired and analyzed on a BD Biosciences FACScan instrument and analyzed using CellQuest software BD Biosciences, Mountain View, CA).

Intracellular IFN-γ staining

Fresh lymphocytes or lymphocytes stimulated for 6 days with peptides or cells were stimulated in vitro at 1×10^6 cells/ml for 4–6 h with 10 μM (OVA or OVANT) peptide in the presence of 5 μg/ml Golgi Plug (brefeldin A). These stimulated cells were then stained with 100 ng each of anti-CD3-PerCP (PerCP-conjugated hamster anti-mouse CD3ϵ (CD3ϵ chain) mAb) and anti-CD8-FITC (FITC-conjugated rat anti-mouse CD8a (Ly-2) mAb) or with isotype control of anti-CD8-FITC conjugate for 30 min at 4°C in the dark in 100 μl of FACS buffer (PBS containing 2% FBS and 0.02% of sodium azide). Cells were then washed twice with FACS buffer and intracellular IFN-γ staining was performed using a Cytofix/Cytoperm Plus kit from BD PharMingen according to the manufacturer's instruction. Briefly, CD3- and CD8-labeled cells were fixed and permeabilized for 20 min at 4°C in 250 μl of Cytofix/Cytoperm solution and then were washed twice with Perm/Wash solution. The permeabilized cells were then incubated with 100 ng of PE-conjugated anti-IFN-γ-labeled Ab for 30 min at 4°C. The cells were then washed twice with Perm/Wash solution and were resuspended in FACS buffer for further analysis.

Sample data were acquired and analyzed on a BD Biosciences FACScan instrument and analyzed using CellQuest software.

Results and Discussion

Coimmunization with mutual in vitro antagonists does not result in in vivo antagonism

To determine whether T cell antagonism and OAS could interfere with immune responses to vaccines, we tested a defined epitope system in vivo by genetic immunization. The H-$2K^b$-restricted immunodominant peptide epitope of OVA ($Ova_{257–264}$ = SIINFE**K**L) was tested in combination with its point mutant SIINFE**D**L, which is a well-defined T cell antagonist of OVA-specific CD8 T cells (9) and carries a K → D point mutation at position 7, a primary TCR contact site. These epitopes were expressed as cytoplasmic peptides from minigene plasmids expressing either M-SIINFE**K**L or M-SIINFE**D**L. C57BL/6 H-2^b mice were genetically immunized with each plasmid once with a gene gun. Splenocytes harvested 4 wk later from both OVA and OVANT plasmid-immunized mice demonstrated potent epitope-specific CTL activity with little cross-reactivity (Fig. 1). The observed CTL response induced by minigenes occurred in the absence of delivery of any CD4 Th epitope. This is consistent with several reports demonstrating CD8 T cell responses in the absence of CD4 T cell help by the gene gun (Ref. 10 and the references therein). The antagonistic properties of the OVANT peptide were confirmed by the pre-pulse CTL lysis assay (9) and demonstrated that OVANT peptide does inhibit OVA-specific CTL activity in vitro (Fig. 1*A*). Moreover, OVA peptide reciprocally inhibited OVANT-specific CTLs in vitro (Fig. 1*D*). Although inhibition by OVA was detectable, this in vitro inhibition was substantially weaker than that mediated by OVANT.

To test whether similar immune interference effects occur in vivo, mice were immunized with both OVA and OVANT plasmids simultaneously. The two plasmids were either coprecipitated on the same gold particles (=TOGETHER) to ensure that both epitopes will be expressed by the same cell (11) or each plasmid was precipitated on separate gold particles (=SEPARATE) to ensure that only one epitope would be expressed by each host cell (11). Mice immunized with both plasmids produced robust CTL activity (Fig. 2), CD8/IFN-γ production (Fig. 3, *A–C*), and MHC I-tetramer-binding activity (Fig. 3, *D* and *E*) against both the OVA and OVANT epitopes. The specificity of CTL activity from the coimmunized mice depended entirely upon the peptide used for 6-day in vitro expansion of CTL effectors (Fig. 1), indicating that the splenocytes contained CTL precursors against both epitopes.

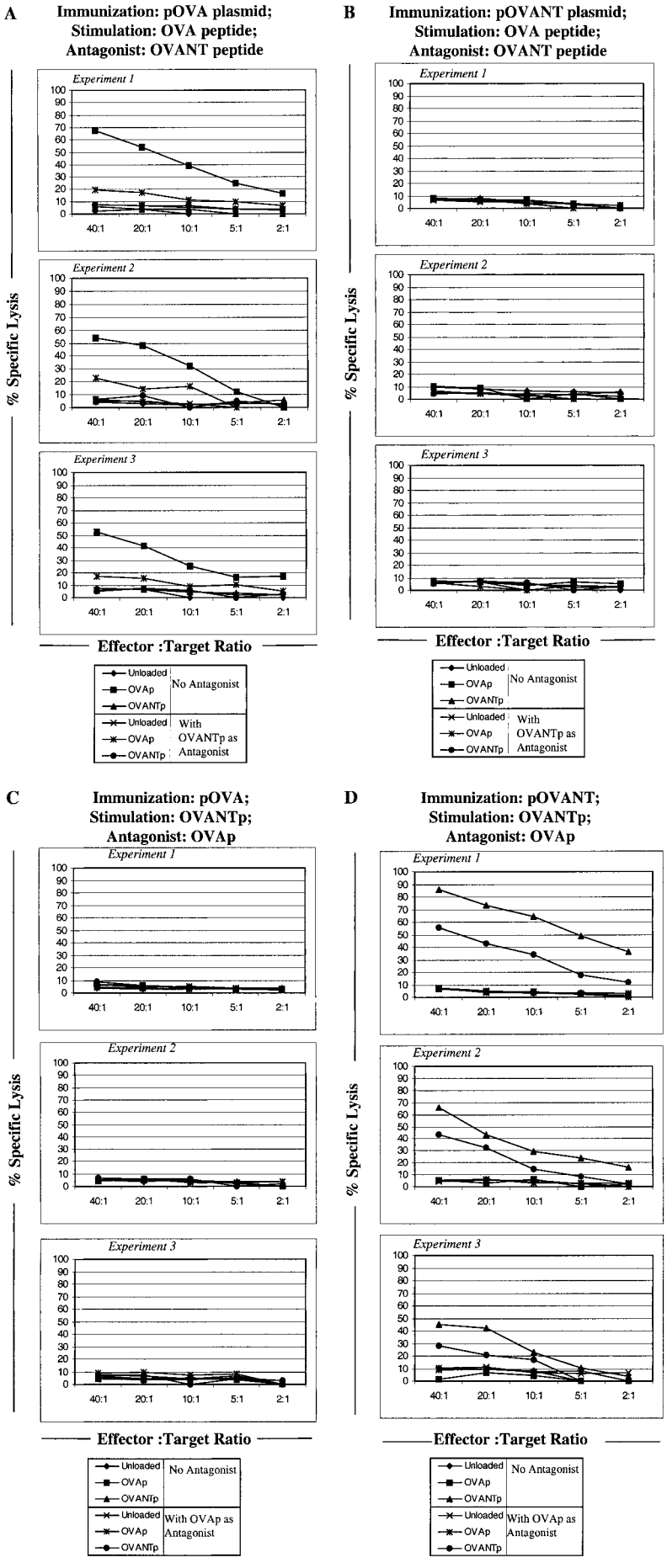

FIGURE 1. Immunization with T cell antagonists does not produce T cell antagonism in vivo. Six-week-old mice were genetically immunized with plasmid coding for OVA or OVANT by gene gun transfection of the epidermis using the Helios biolistic device (Bio-Rad) as in Ref. 19. Briefly, 2.5 μg of plasmid DNA was delivered per shot of 1.6-μm gold particles. Two shots were delivered into the backs of the ears on each mouse. In all cases, mice were vaccinated in a single round of immunization without further boosts. One month later, pooled splenocytes from two identically immunized mice were stimulated in vitro for 6 days with 1 μM OVA or OVANT peptide, as indicated, in RPMI 1640 medium containing 10% v/v FBS, 10 mM HEPES, 50 μM 2-ME, and 10% v/v T-Stim (Collaborative Biomedical Products) as a source of IL-2 and antibiotic/antimycotic. On day 7, OVA- and OVANT-specific CTL activity was measured by a standard 4-h ^{51}Cr release assay using either unlabeled target cells (1×10^4 RMA cells/well of a 96-well plate) or target cells pulsed with OVA or OVANT peptide. Antagonist assay was performed as described by Jameson et al. (9). For example, RMA cells were loaded with 1 μM OVA or OVANT peptide for 1 h at 37°C and after three washes they were resuspended in medium containing 1 μM OVANT or OVA peptide, respectively. One hundred microliters of target cells loaded with OVA and resuspended in OVANT peptide-containing medium was mixed with 100 μl of lymphocytes from OVA-immunized mice. CTL activity was measured by a ^{51}Cr release assay.

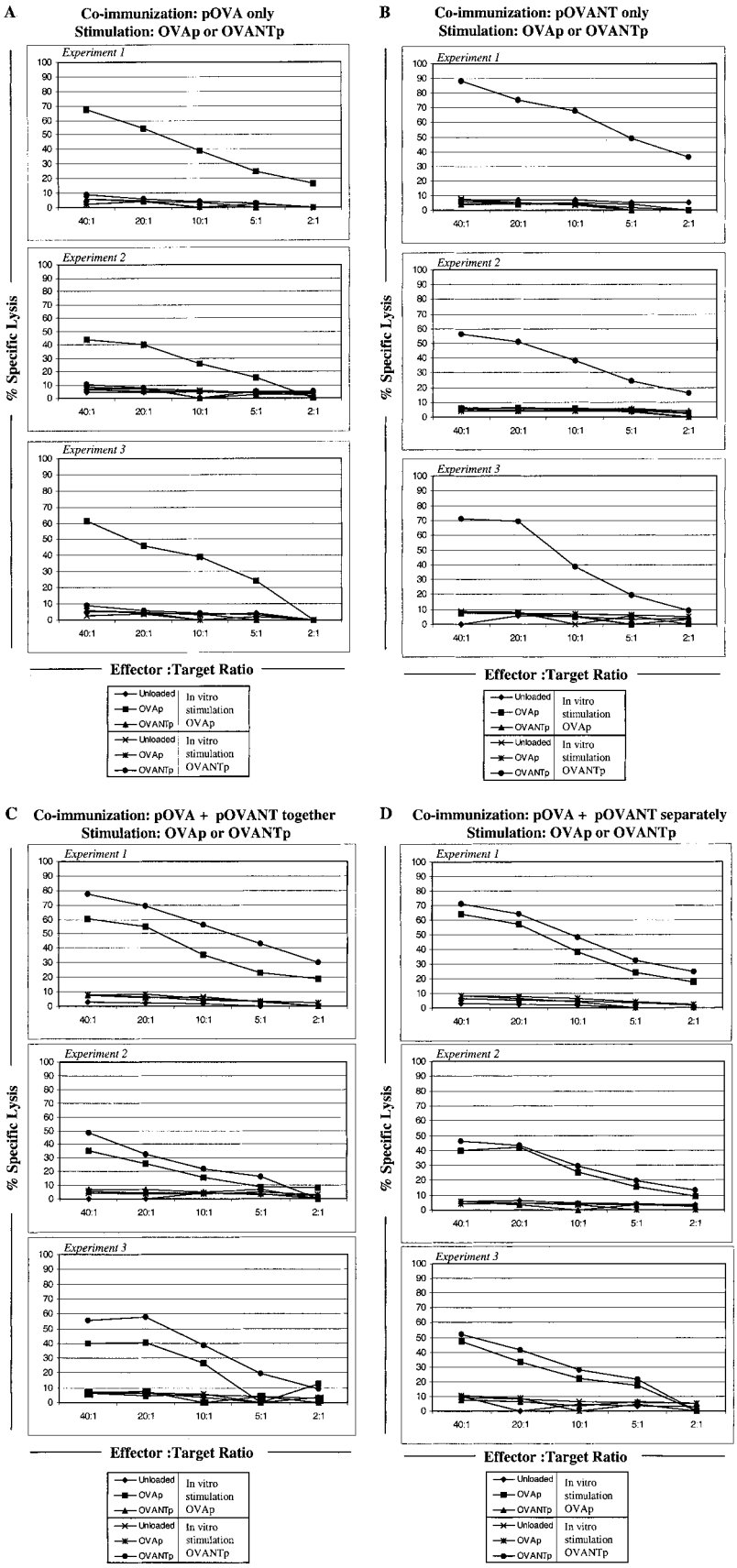

FIGURE 2. Simultaneous immunization generated combined OVA- and OVANT-specific CTLs with no overt T cell antagonism in vivo. For coimmunization, the plasmids (pOVA plus pOVANT) were precipitated TOGETHER on the same gold particle or on SEPARATE gold particles that were mixed and delivered at the same site of the ear. Briefly, 2.5 μg of each plasmid was delivered in coimmunization. In mice receiving only pOVA or pOVANT alone, 2.5 μg of plasmid pGL was added to balance the total DNA to 5 μg. One month after immunization, pooled splenocytes from two identically immunized mice were stimulated in vitro with 1 μM OVA or OVANT peptide for 6 days, and the CTL activity was measured on day 7 using 1×10^4 peptide-unloaded and OVA or OVANT peptide-loaded RMA cells as targets. Data are shown from each experiment comparing the indicated variables. All of the experiments were done in triplicate. Each CTL curve was generated from pooled splenocytes from two mice. Splenocytes were pooled to provide sufficient cells to allow parallel CD8/IFN-γ and tetramer analysis on the same samples.

T cells from OVA-immunized mice were slightly cross-reactive against the OVANT-MHC I tetramer, while OVANT-specific T cells cross-reacted slightly with the OVA-tetramer (Fig. 3, *C* and *D*). Partial cross-reactivity of the T cells for the alternate epitope was expected given that a T cell antagonist must bind TCR to evoke its inhibitory effects (9). Although there was some low level

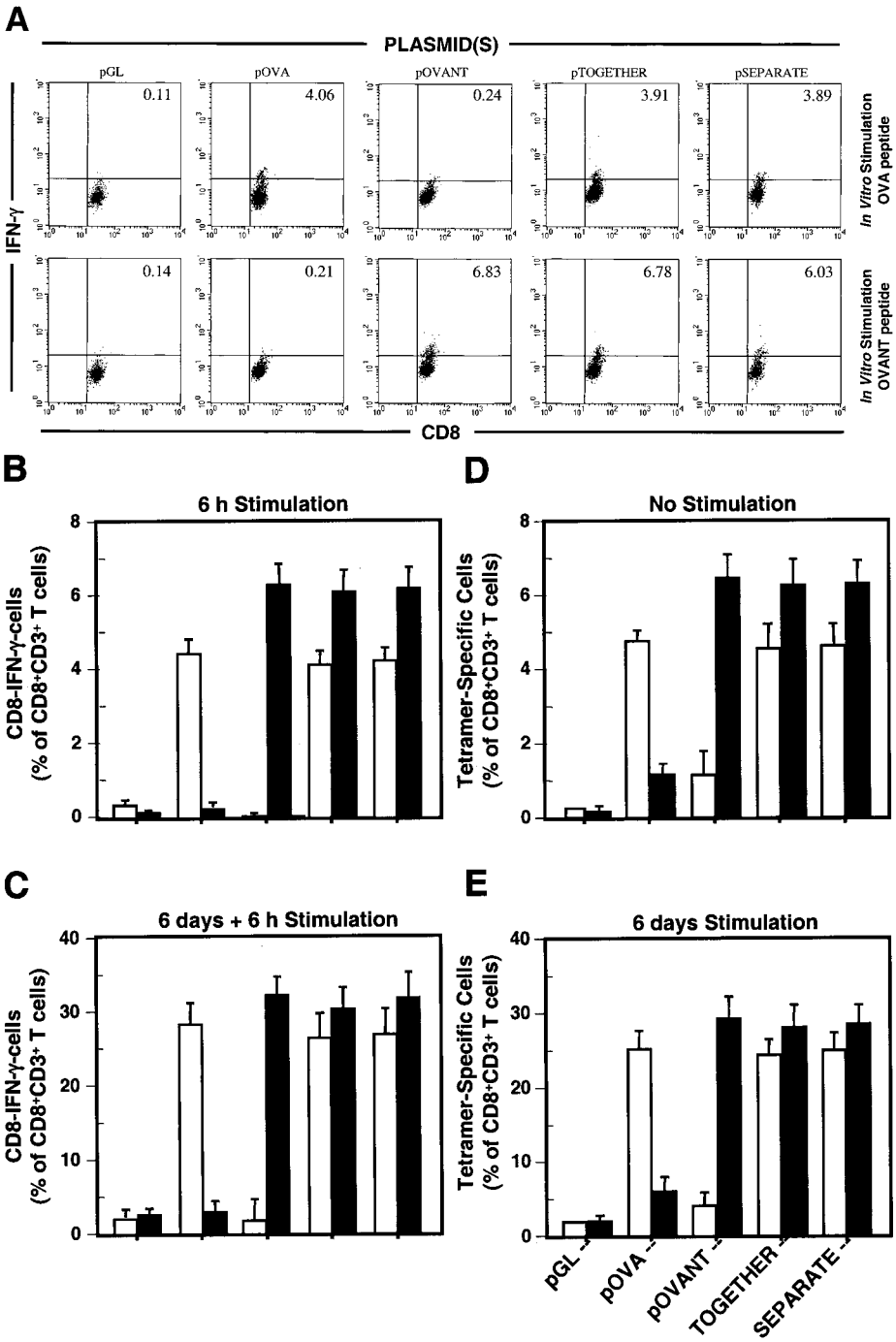

FIGURE 3. Quantitation of OVA- and OVANT-specific CD8 T cells in mice coimmunized with pOVA and pOVANT. C57BL/6 mice were coimmunized with plasmids coding for OVA or OVANT as described in Fig. 1*B*. Negative control mice were immunized with plasmid pGL encoding luciferase. One month later, pooled splenocytes that were freshly isolated (6-h stimulation) or expanded for 6 days (6 days plus 6-h stimulation) were assayed for OVA- and OVANT–specific CD8 T cells by flow cytometry. *A–C,* Quantitation of IFN-γ-producing OVA- and OVANT-specific CD8 T cells in mice coimmunized with pOVA and pOVANT. Intracellular IFN-γ staining was performed using a Cytofix/Cytoperm kit (BD PharMingen). Briefly, 1×10^6 freshly isolated (*A* and *B*) or cultured lymphocytes (*C*) were stimulated at 37°C for 6 h with 10 μM OVA or OVANT peptide in the presence of 5 μg/ml brefeldin A. For cultured splenocytes, cells were expanded for 6 days with 1 μM OVA peptide and then were stimulated for 6 h with 10 μM OVA or 10 μM OVANT peptide just before assay. Cells expanded for 6 days with 1 μM OVANT peptide were also stimulated for 6 h with 10 μM OVANT or 10 μM OVA peptide. After a 6-h stimulation, cells were then washed and stained with anti-CD3-PerCP- and CD8-FITC-conjugated Abs for 30 min at 4°C in the dark. Cells were then washed, fixed, and permeabilized before staining with anti-IFN-γ Ab or its isotype control. CD3/CD8 double-positive cells ($5–10 \times 10^4$/sample) were analyzed for intracellular IFN-γ production on a FACScan using CellQuest software. Dot plot shown in Fig. 2*A* represents raw FACS data (of gated CD3/CD8 double-positive T cells) and bar graphs shown in Fig. 2, *B* and *C*, are mean ± SD from three independent experiments. □, Splenocytes stimulated with OVA peptide; ■, splenocytes stimulated with OVANT peptide. *D* and *E,* Peptide-MHC I-tetramer staining of OVA- and OVANT-specific CD8 cells in mice coimmunized with pOVA and pOVANT. For tetramer staining, fresh (*D*) or 6-day cultured splenocytes (*E*; 1×10^6/sample) were stained with anti-CD3-PerCP, CD8-FITC-conjugated Abs, and PE-conjugated OVA-MHC I-tetramer or OVANT-MHC I-tetramer (both from the National Institutes of Health Tetramer Core) for 30 min at room temperature. On a FACScan, CD3/CD8 double-positive cells ($5–10 \times 10^4$/sample) were analyzed for the presence of tetramer-positive cells using CellQuest software. All of the experiments were repeated at least three times. Data shown are the mean ± SD from six mice from two different immunizations. □, Splenocytes tested with OVA peptide-MHC I tetramer; ■, splenocytes tested with OVANT peptide MHC I tetramer.

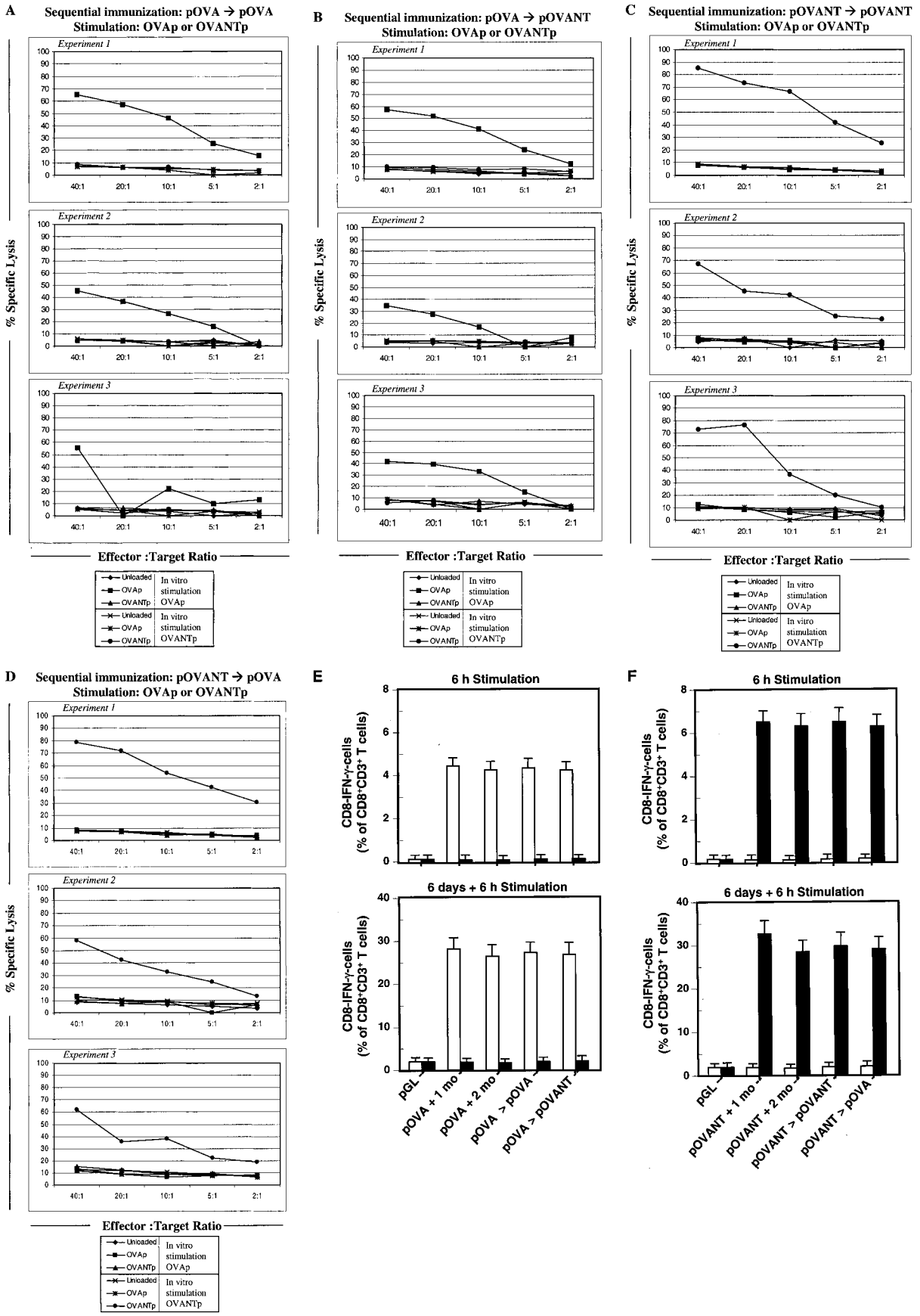

FIGURE 4. Sequential immunization with pOVA and pOVANT plasmids in either order induces OAS of CTLs. *A–D*, OVA- and OVANT-specific CTL activity in mice sequentially immunized with pOVA and pOVANT in either order. Mice were primed with either pOVA or pOVANT as described in Fig. 1*A*. One month later, pOVA-primed mice were boosted with either pOVA (pOVA→pOVA) or pOVANT (pOVA→pOVANT) plasmid. Similarly, pOVANT-primed mice were boosted with either pOVANT (pOVANT→pOVANT) or pOVA (pOVANT→pOVA) plasmid. A total of 5μg of plasmid (*Figure legend continues*)

cross-binding of TCR by the two epitopes on MHC I, this did not translate into the production of functionally cross-reactive T cells, as 1) splenocytes from mice immunized with only OVA and stimulated with OVANT peptide failed to generate OVANT-specific CTLs (Fig. 1*C*) or CD8/IFN-γ production (Fig. 3) and 2) stimulation of splenocytes from OVANT-immunized mice with OVA peptide failed to produce OVA-specific CTLs (Fig. 1*B*) or CD8/IFN-γ production (Fig. 3).

Therefore, although both OVA and OVANT inhibit each other's T cells to varying degrees in vitro, they fail to induce immune interference in vivo when they are presented to the immune system at the same time or even on the same cell. These results suggest, but do not formerly prove, that T cell antagonism or epitope competition may largely be an in vitro phenomenon where high levels of an inhibitor peptide can attenuate agonist-specific T cell responses. By contrast, too little inhibitor peptide may be produced by vaccines in vivo to effectively antagonize CD8 T cells (5). This model is consistent with observations of in vivo MHC II-restricted T cell antagonism in which antagonism can be avoided by decreasing the ratio of antagonist to agonist (12).

Sequential immunization with agonist/antagonist pair results in OAS of CTLs

In vivo immune interference was not observed after coimmunization. In contrast, sequential exposure of the immune system to mutant epitopes or APLs can reveal OAS in which pre-existing CD8 T cell responses against one epitope reduces subsequent CD8 responses against a point mutant of the same epitope (4). Given this observation, we tested whether the OVA/OVANT system would generate OAS when these APLs were delivered sequentially rather than simultaneously. C57BL/6 mice were immunized by gene gun with either the OVA or OVANT plasmid. One month later, mice were reimmunized with the same epitope or with the counter epitope and their splenocytes were assayed 4 wk later for CTL and tetramer binding. Splenocytes from mice immunized twice with OVA plasmid produced OVA-specific CTLs, CD8/IFN-γ production, and OVA tetramer binding, but negligible OVANT-specific responses (Fig. 4 and data not shown). Mice immunized with OVA plasmid and then boosted with OVANT plasmid showed high OVA-specific CD8 responses, but no OVANT-specific CD8 responses. Reciprocal immunizations demonstrated that OVANT plasmid priming ablated subsequent OVA-specific CD8 responses (Fig. 4).

These data demonstrate that sequential delivery of the OVA and OVANT APLs generates a very potent form of OAS. These results are consistent with the observation of OAS in LCMV (4), but differ from that model in that immune responses against the first epitope are not boosted by a second immunization with the mutant epitope. Furthermore, unlike the LCMV model, OVA- and OVANT-specific CD8 T cells did not functionally cross-react against each other's epitopes. These differences between our observations and those of Klenerman et al. (16) are likely due to the fact that the OVA and OVANT APLs are reciprocal antagonists or competitive inhibitors, whereas the LCMV APLs may be partial agonists. Therefore, in the OVA/OVANT system, boosting of primary responses by the counter epitope likely does not occur because these epitopes reciprocally antagonize or inhibit each other's T cells.

Although the exact mechanism of OAS for T cells remains unclear, at least three models have been proposed (13): 1) During OAS, a weakly cross-reactive second epitope reactivates the larger number of memory CTLs against the first epitope more effectively than it activates the small number of naive T cells specific for the second epitope. 2) OAS is due to killing or deactivation of cells presenting the second Ag by primary CTLs that cross-react against the second Ag. 3) OAS occurs because CTLs responding to the variant epitope experience "a form of T cell antagonism" where CTL clones could react suboptimally to the variant epitope and become partially activated, leading to anergy.

In OAS with the OVA/OVANT system, models 1 and 2 appear unlikely, since these mechanisms both require cross-recognition and cross-activation of the original T cells by the second Ag which does not appear to occur between OVA and OVANT. However, it is possible that more subtle interactions that are not represented by our in vitro assays may occur in vivo to invoke these models. Model 3 cannot be ruled out, but seems unlikely since T cell antagonism in vivo in the MHC II system required the antagonist to be present in higher amounts than the agonist (12). In contrast, after genetic immunization with a gene gun, 95% of expressed transgene product was lost within 5 days (14). Therefore, the amount of second Ag produced upon second immunization should be in marked excess over the residual potentially antagonistic epitope remaining from the first immunization. However, it is conceivable that the first Ag could be sequestered in high concentrations on dendritic cells in the draining lymph nodes that could overcome the Ag ratio requirement for in vivo antagonism.

Although our data cannot unequivocally exclude models 1 and 2, we propose an alternative model to account for OAS for the OVA/OVANT system. We hypothesize that the large population of pre-existing memory T cells may cross-bind the APLs on MHC I dendritic cells in the draining lymph nodes, thereby blocking access to the APCs by low frequency naive T cells. Complete blockade of all MHC I molecules would not be required for OAS. Rather, memory T cells would need only to block sufficient numbers of MHC I to prevent the naive T cells from triggering the ~8000 TCRs needed for T cell activation (15). The "masking" model predicts that any cross-reactive APLs will generate OAS. OAS involving full T cell antagonists will ablate subsequent responses and not boost primary responses. By contrast, OAS by

DNA was delivered at both priming and boosting. One month after boosting, pooled splenocytes from two identically immunized mice were stimulated in vitro for 6 days with 1 μM OVA or OVANT peptide. *E,* Mice primed with pOVA and boosted with pOVANT failed to show a pOVANT-specific CD8-IFN-γ response. Mice were immunized with plasmid pGL or pOVA. One month later, pOVA-immunized mice were boosted with either pOVA or pOVANT. One month postboosting, pooled splenocytes from two identically immunized mice were stimulated in vitro for 6 days with 1 μM OVA or OVANT peptide. Both freshly isolated (6-h stimulation) and cultured (6 days plus 6-h stimulation) splenocytes were analyzed for OVA- and OVANT-specific CD8-IFN-γ-positive T cells as described in Fig. 2. All of the experiments were repeated at least three times. Data shown are the mean ± SD from six mice from two separate immunizations. □, Splenocytes stimulated with OVA peptide; ■, splenocytes stimulated with OVANT peptide. *F,* Mice primed with pOVANT and boosted with pOVA did not show OVA-specific CD8-IFN-γ-positive cells. Mice were immunized with plasmid pGL or pOVA. One month later, pOVANT-immunized mice were boosted with either pOVANT or pOVA. One month postboosting, pooled splenocytes from two identically immunized mice were stimulated in vitro for 6 days with 1 μM OVA or OVANT peptide. Both freshly isolated (*top panel*) and cultured (*bottom panel*) splenocytes were analyzed for OVA- and OVANT-specific CD8-IFN-γ-positive T cells as described in Fig. 2. All of the experiments were repeated at least three times. Data shown are the mean ± SD from six mice from two separate immunizations. □, Splenocytes stimulated with OVA peptide; ■, splenocytes stimulated with OVANT peptide.

partial T cell agonists/antagonists may only partially attenuate subsequent responses and will likely partially boost primary responses of T cells against the first mutant epitope (4).

For mutable, immunoevasive pathogens like HIV-1, there might be a selective advantage for viruses bearing antagonistic APLs over other APLs, since antagonistic peptides cannot only escape pre-existing CTLs, but also inactivate pre-existing CTLs (at least in vitro) (16, 17). Our data favoring a masking model suggest that antagonist APLs may also activate OAS to suppress new CTLs against variant viruses. Although continuous large-scale production of divergent APLs by HIV-1 may eventually overcome OAS, even transient reductions or delays in the production of new CTLs may confer significant selective advantages to mutable viruses over the course of latent infection.

This work also has practical implications for the delivery of multimutant vaccines. Sequential immunization with APLs generates the extreme case of immune interference by activating OAS. By contrast, simultaneous delivery of the same problematic epitopes avoids both overt antagonism and OAS in vivo. These data, along with data demonstrating the ability to provoke multivalent T cell responses by simultaneous delivery of 64 Th epitopes (18) or a 32-plasmid genetic vaccine (19), suggest that the immune system can indeed respond effectively to multiple APLs, even if they include T cell antagonists. Simultaneous APL delivery contrasts with the sequential APL exposure produced by annual vaccines like influenza (7, 8) and during on-going infections by mutating pathogens like HIV-1 (16, 17), in which OAS and T cell antagonism are observed. Problematic natural immune interference mechanisms can be readily avoided by simultaneous delivery of multigene, multimutant vaccines.

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