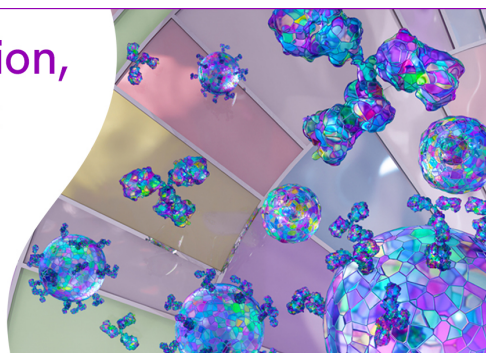


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# Supra-Agonist Peptides Enhance the Reactivation of Memory CTL Responses<sup>1</sup>

Fabiola Micheletti,\* Alessandro Canella,\* Simona Vertuani,\* Mauro Marastoni,<sup>†</sup> Lara Tosi,<sup>‡</sup> Stefano Volinia,<sup>‡</sup> Serena Traniello,\* and Riccardo Gavioli<sup>2\*</sup>

Single amino acid substitutions at TCR contacts may transform a natural peptide Ag in CTL ligands with partial agonist, antagonist, or null activity. We obtained peptide variants by changing nonanchor amino acid residues involved in MHC class I binding. These peptides were derived from a subdominant HLA-A2-presented, latent membrane protein 2-derived epitope expressed in EBV-infected cells and in EBV-associated tumors. We found that small structural changes produced ligands with vastly different activities. In particular, the variants that associated more stably to HLA-A2/molecules did not activate any CTL function, behaving as null ligands. Interestingly, T cell stimulations performed with the combination of null ligands and the natural epitope produced significantly higher specific CTL reactivation than reactivation of CTLs induced by the wild-type epitope alone. In addition, these particular variants activated memory CTL responses in the presence of concentrations of natural epitope that per se did not induce T cell responses. We show here that null ligands increased ZAP-70 tyrosine kinase activation induced by the natural epitope. Our results demonstrate for the first time that particular peptide variants, apparently behaving as null ligands, interact with the TCR, showing a supra-agonist activity. These variant peptides did not affect the effector T cell functions activated by the natural epitope. Supra-agonist peptides represent the counterpart of antagonists and may have important applications in the development of therapeutic peptides. *The Journal of Immunology*, 2000, 165: 4264–4271.

**T**he activation of CTL results from the recognition by the TCR of a processed immunogenic peptide, 8–10 aa long, bound to MHC class I molecules expressed at the surface of APCs (1). Crystallographic studies performed on purified MHC class I/peptide and TCR/MHC/peptide complexes have elucidated that the majority of peptide Ag residues are buried in the MHC groove, with only few side chains in the peptide center pointing out from it, and that the TCR binds MHC/peptide complexes in a diagonal manner and contacts peptide residues by the CDR3 domain (2–5).

TCR and/or peptide MHC have flexible binding surfaces that are stabilized upon binding (6, 7). The engagement of TCR by MHC-peptide complexes induces a cascade of intracellular signaling events that results in the activation of gene transcription, cell proliferation, and CTL effector functions.

It has been clearly demonstrated that a single TCR may cross-react with different ligands (altered peptide ligands). This phenomenon has been described for T cells of both CD4 and CD8 phenotypes, and it has been suggested to play a role in the positive and negative selection of thymocytes, the pathogenesis of autoimmune diseases, viral antagonism, and memory T cell maintenance (8–11); more importantly, it would be advantageous for the recognition pathogens, since an individual has far fewer lymphocytes than there are potential Ags (12). Recent data strongly suggest that TCR cross-reactivity may depend on the poor shape complementarity

between the Ag binding surfaces as well as on the conformational flexibility of TCR/MHC-peptide interactions (7).

Altered peptide ligands may display a wide range of stimulatory capabilities, ranging from the null response to full immunogenicity and, depending on the induced biological T cell response, have been classified as agonists/superagonists, partial agonists, antagonists, and null ligands (13). Distinct peptide-induced CTL responses have been ascribed to differences in the activation pattern of signal transduction events induced upon MHC/peptide recognition by the TCR, which, in turn, may depend on the affinity and off-rate kinetics of TCR with its ligand (14–18).

In previous studies (19), we analyzed peptide variants derived from the subdominant CLGGLLTMV (CLG) epitope, corresponding to an HLA-A2-presented nonamer derived from the latent membrane protein 2 (LMP2)<sup>3</sup> expressed in EBV-infected cells, including some EBV-associated tumors (20). The CLG natural epitope does not form stable HLA-A2/peptide complexes, and induces weak CTL responses. Since the CLG peptide may be regarded as a target of specific immunotherapies for the treatment of EBV-associated malignancies, we sought to increase its immunogenicity by defining CLG analogues forming stable complexes with HLA-A2 that are able to induce efficient CLG-specific CTL responses (19). The requirement for a prolonged stimulation to reach T cell commitment has long been recognized as essential for T cell activation by mitogens (21) and Ags (22). At the level of the T-APC synapse, a large number of TCRs can be serially engaged, triggered, and down-regulated by MHC/peptide complexes (23). Whereas the interaction between an individual TCR and its ligand occurs over a time frame of a few seconds, the interaction between a single T cell-APC pair has a time course of several hours. This long-lasting contact is necessary to ensure the sustained signaling that maintains gene transcription and promotes T cell cycle progression (24).

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<sup>3</sup> Abbreviation used in this paper: LMP2, latent membrane protein 2.

Among the analogues tested we identified a few peptides with superagonistic activity able to reactivate maximal CLG-specific CTL responses directed to the natural presented CLG epitope and a group of peptides that stably bound to HLA-A2 but did not induce any CTL activation, behaving as null ligands.

In the present investigation, we evaluated the capacity of CLG-derived null ligands to affect CTL responses when presented in combination with the natural ligand. We demonstrate that these peptides exhibit a supra-agonist activity, since they increase the reactivation of CLG-specific memory T cell responses and CLG-specific T cell proliferation. This behavior may be considered the opposite of that of antagonist peptides, which per se do not induce T cell activation but specifically inhibit T cell responses (25). Supra-agonist peptides may be useful for dissecting the highly sophisticated signal transduction pathways activated after TCR engagement by MHC/peptide complexes and may have important applications in immunotherapy.

## Materials and Methods

### Cell lines

The .174/T2 cell line (T2) was obtained by fusion of the peptide transporter mutant .174 LCL with the T cell line CEM (26). Cell lines were maintained in RPMI 1640 supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% heat-inactivated FCS (HyClone, Logan, UT). PHA-activated blasts were obtained by stimulation of PBLs with 1  $\mu$ g/ml purified PHA (Wellcome Diagnostics, Dartford, U.K.) for 3 days and were expanded in medium supplemented with IL-2 (Proleukin; Chiron, Milan, Italy) as described previously (27). T cell clones were obtained from CLG-specific CTL cultures by limiting dilution. Single cells were seeded in 96-well plates in 200  $\mu$ l of medium containing 10 U/ml human rIL-2 and  $10^5$  irradiated allogeneic PHA-activated PBLs as feeder. Growing cultures were transferred into 48-well plates and further expanded in IL-2-conditioned medium (27).

### Synthetic peptides

The CLGGLLTMV (CLG) peptide, corresponding to aa 426–434 of the EBV LMP2, and the relative analogues were synthesized by the solid phase method using a continuous flow instrument with on-line UV monitoring.

The stepwise syntheses were conducted by F-moc chemistry. The F-moc methylbenzhydrylamine resin (Novabiochem, Laufelfingen, Switzerland) was swelled in dimethylformamide and packed in the reaction column. F-moc amino acids were coupled in a 4-fold excess using diisopropylcarbodiimide in the presence of hydroxybenzotriazole. The F-moc group was cleaved with 20% piperidine-dimethylformamide solution. Protected peptides were cleaved from the resin by treatment with reagent B (88% trifluoroacetic acid, 5% H<sub>2</sub>O, and 7% Et<sub>3</sub>SiH), and the resulting products were collected by centrifugation. Crude deprotected peptides were purified by HPLC; purity was >98%. Structure verification was achieved by elemental and amino acid analyses and matrix-assisted laser desorption ionization-time of flight mass spectrometry (28). Peptide stocks were prepared in DMSO at a concentration of  $10^{-2}$  M, kept at  $-20^{\circ}\text{C}$ , and diluted in PBS before use.

### Detection of HLA-A2/peptide complex stability

Aliquots of  $5 \times 10^6$  T2 cells were cultured overnight in 2 ml of serum-free AIM-V medium (Life Technologies, Milan, Italy) containing  $10^{-4}$  M of the indicated peptides. Cells were then extensively washed, treated with mitomycin C (Sigma-Aldrich, Milan, Italy) to avoid cell proliferation, divided into aliquots, and maintained at  $37^{\circ}\text{C}$  for kinetic experiments in AIM-V medium containing 1  $\mu$ g/ml brefeldin A (Sigma-Aldrich) to block the egress of new class I molecules. Surface expression of HLA class I molecules was detected at different time points by indirect immunofluorescence using the mouse mAb W6-32, which recognizes HLA-A, -B, and -C molecules regardless of the associated peptide. The mean logarithm fluorescence intensity was measured with a FACS analyzer. The percent increase in HLA class I expression was calculated with respect to that in untreated T2 cells (29). Data are expressed as the half-life (hours) of HLA-A2/peptide complexes.

### Generation of CTL cultures

Monocyte-depleted PBLs from the EBV-seropositive donors MT (HLA-A2-B18), FR (HLA-A2, 24-B7, 35), and RG (HLA-A2-B8, 44) were plated

at  $1 \times 10^5$  cells/well in round-bottom microtiter plates. In some experiments populations of CD8<sup>+</sup>-enriched lymphocytes were used. CD8<sup>+</sup>-enriched lymphocytes were obtained by depleting CD4<sup>+</sup> T cells with two rounds of anti-CD4-coated magnetic beads (Dyna, Oslo, Norway). Aliquots of  $5 \times 10^6$  T2 cells were incubated overnight at  $26^{\circ}\text{C}$ , treated with mitomycin C, and then pulsed with peptides for 2 h at  $37^{\circ}\text{C}$ . After washing, peptide-pulsed T2 cells or a combination of peptide-pulsed T2 cells were added to PBLs always at a final responder:stimulator ratio of 20:1. All stimulations were performed in six replicates. The first stimulation was performed in AIM-V medium containing 10% FCS. A second stimulation was performed in the same conditions on day 7. Starting from day 8 the medium was supplemented with 10 U/ml rIL-2 (30). On day 14 T cell microcultures were split into two replicates and used as effectors in a standard 5-h <sup>51</sup>Cr release assay.

### Cytotoxicity tests

Cytotoxic activity was tested in standard 5-h <sup>51</sup>Cr release assay (31). PHA blasts were labeled with 0.1  $\mu$ Ci/ $10^6$  cells of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> for 90 min at  $37^{\circ}\text{C}$  and pulsed for 45 min with the indicated concentration of peptide at  $37^{\circ}\text{C}$ . Cells were then washed, and  $4 \times 10^3$  cells were used as targets of each CTL microculture.

The percent specific lysis was calculated as  $100 \times (\text{cpm sample} - \text{cpm medium}) / (\text{cpm Triton X-100} - \text{cpm medium})$ . The percent specific lysis of each CTL culture was determined by the mean lysis of the six replicates.

### TCR antagonism assay

Antagonism experiments were performed as described previously (11). Briefly, <sup>51</sup>Cr-labeled HLA-A2-positive PHA blasts were pulsed with the CLG peptide at concentrations ranging from  $10^{-6}$ – $10^{-12}$  M. The cells were then washed three times to remove the unbound peptide, seeded in 96-well V-bottom microtiter plates ( $4 \times 10^3$  cells/well), and treated for 1 h at  $37^{\circ}\text{C}$  with CLG analogues in a concentration range of  $10^{-4}$ – $10^{-8}$  M. Effector CLG-specific CTLs were then added to each well to give an E:T cell ratio of 10:1, and the percentage of specific lysis was detected as described above.

### T cell proliferation assay

T2 cells were incubated overnight at  $26^{\circ}\text{C}$ , treated with mitomycin C, and then pulsed with  $10^{-6}$  synthetic peptides for 2 h at  $37^{\circ}\text{C}$ . After extensive washing to remove the unbound peptide, 100  $\mu$ l of peptide-pulsed T2 cells ( $5 \times 10^4$  cells) or a combination of peptide-pulsed T2 cells ( $2.5 \times 10^4$  cells and  $2.5 \times 10^4$  cells) were added to round-bottom 96-well plates containing 100  $\mu$ l of CTLs ( $1 \times 10^5$  cells), with final responder:stimulator ratio of 2:1. After 3 days, T cell proliferation was determined by adding 1  $\mu$ Ci [<sup>3</sup>H]thymidine to each well for the last 18 h of culture. Cells were then harvested, and incorporated radioactivity was measured by scintillation counting (Top-Count, Packard, Downers Grove, IL). Results are expressed as the percent increase in proliferation calculated with respect to that of CTLs stimulated with untreated T2 cells.

### IFN- $\gamma$ production

CLG-specific CTLs ( $5 \times 10^4$ ) were incubated with  $5 \times 10^3$  T2 cells pre-pulsed with the indicated peptides as described for proliferation assay. After 5-h incubation, supernatants were collected and tested for the presence of IFN- $\gamma$  using the IFN- $\gamma$  ELISA kit in accordance with the manufacturer's instructions (human IFN- $\gamma$  deca kit; HyCult Biotechnology, Uden, The Netherlands). Data are expressed as picograms per milliliter of IFN- $\gamma$  produced.

### Tyrosine phosphorylation assays

CLG-specific CTLs ( $5\text{--}6 \times 10^6$ ) were added to  $2.5\text{--}3 \times 10^6$  T2 cells, pulsed or not with  $10^{-5}$  M peptide, in Eppendorf tubes; the cells were pelleted by centrifugation; and the tubes were placed at  $37^{\circ}\text{C}$  for 10 min. At the end of the incubation period, the tubes were transferred to ice. Cells were resuspended in ice-cold TBS, repelleted, and lysed for 15 min with ice-cold lysing buffer containing 1% Nonidet P-40, 137 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM PMSF, 1 mM sodium orthovanadate, 0.1 mM DTT, 25  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml pepstatin A (Sigma-Aldrich). Cell debris were removed by centrifugation, and the protein concentration of clear supernatants was determined using the bicinchoninic acid protocol (Pierce, Rockford, IL).

Tyrosine-phosphorylated proteins and ZAP-70 were immunoprecipitated from cell lysates containing equal amounts of proteins with 4G10 mAb (Upstate Biotechnology, Lake Placid, NY) or anti-human ZAP-70 polyclonal Ab (Upstate Biotechnology), respectively. After 3 h protein

A-Sepharose was added for 1 h at 4°C. Precipitated proteins were solubilized in sample buffer, resolved by SDS-PAGE under reducing conditions, and transferred to nitrocellulose membrane (Amersham International, Aylesbury, U.K.). The blots were probed with anti-human ZAP-70 mAb (Upstate Biotechnology) or 4G10 mAb, followed by peroxidase anti-mouse (Roche, Indianapolis, IN), and were revealed by chemiluminescence (ECL; Amersham).

## Results

### *CLG-derived peptide variants generate different T cell responses*

The CLGGLLTMV (CLG) peptide derives from the EBV LMP2, aa 426–434. CLG-derived analogues were obtained by single or combined amino acid substitutions at positions 1, 3, and 4 that represent secondary anchors for HLA-A2 binding. The amino acids tyrosine (Y), alanine (A), and serine (S) were selected because they have been demonstrated as preferred nonanchor residues at these positions (32–34). All peptides were previously tested for their capacity to associate with HLA-A2 molecules, to induce reactivation of CLG-specific CTLs, to sensitize target cells to lysis by CLG-specific CTLs, and to inhibit CTL cytotoxicity induced by the CLG peptide (19). A summary of representative peptide activities is reported in Table I. All variants showed an increased capacity to form stable HLA-A2 peptide complexes, confirming that we substituted nonanchor amino acids with residues that favor the interaction with HLA-A2 molecules. Among the CLG variants we identified a peptide (3A) that induced higher CTL reactivation compared with the natural ligand and that sensitized target cells to lysis, here classified as superagonist; a weak agonist (3Y) that induced memory CTL reactivation and target cell lysis less efficiently than the CLG peptide; a partial agonist (1Y-3A) that was able to reactivate CLG-specific CTLs, but failed to sensitize target cells to lysis; and two null ligands (4S and 3Y-4S) that, in contrast with the fact that they produced stable HLA-A2/peptide complexes, failed to activate CTLs. The peptides did not show any antagonistic activity at any of the concentrations tested ( $10^{-4}$ – $10^{-8}$  M).

### *4S and 3Y-4S peptides increase CLG-specific CTL reactivation induced by the natural epitope*

The stability of MHC/peptide complexes plays an important role in determining CTL responsiveness (35–38). We have previously demonstrated that the CLG peptide that does not produce stable HLA-A2/CLG complexes induces subdominant CTL responses, while the 3A variant that produces stable HLA-A2/peptide complexes induces strong reactivation of CLG-specific memory CTLs (19). Surprisingly, the 4S and 3Y-4S peptides, which produce much more stable HLA-A2/peptide complexes compared with the CLG and 3A variants, did not activate any CTL function, behaving as null ligands.

To determine whether null ligands may affect CTL functions induced by the natural CLG peptide, we initiated a series of experiments using combinations of null ligands along with the natural epitope. We evaluated first the effect of such combinations on the reactivation of memory T cell responses. PBLs from the HLA-A2-positive EBV-seropositive donors MT, FR, and RG were stimulated in microassay with the mutant T2 cell line pulsed with  $10^{-6}$  M of the natural CLG epitope, the 4S and 3Y-4S variants, or the unrelated GILGFVFTL (GIL). The GIL peptide derives from the influenza virus matrix Ag and has been previously shown to have high affinity for HLA-A2 (19, 39). In parallel, we performed CTL stimulations using the combination of T2 pulsed with the natural ligand plus T2 pulsed with the peptide variants. CTL cultures were tested after two consecutive stimulations against HLA-A2 single-matched PHA blasts treated or not with  $10^{-7}$  M CLG peptide. Fig. 1A shows a mean of data obtained from three separate experiments. As previously observed, the CLG peptide induced weak CTL responses, whereas the 4S and 3Y-4S peptide variants did not induce any CLG-specific cultures. Interestingly, the combination of 4S and 3Y-4S peptides with the natural epitope produced significantly higher CLG-specific CTL reactivity compared with CLG-stimulated PBLs. Similar results were obtained by CTL stimulation performed with T2 cells pulsed first with the CLG peptide and subsequently treated with 4S or 3Y-4S peptides (not shown). Stimulations performed with the combination of GIL and CLG peptides induced CLG-specific cultures with an efficiency comparable to that of stimulations performed with the CLG alone.

To determine the specificity of 4S and 3Y-4S peptides on the reactivation of CLG-specific CTL responses, we performed stimulations in the same conditions as those described above, evaluating the effects of CLG analogues on CTL responses specific for the CLG-unrelated YLLEMLWRL (YLL) epitope. YLL is a subdominant epitope that derives from LMP1 of EBV, aa 125–133. The 4S and 3Y-4S peptides did not increase YLL-specific CTL responses, demonstrating that the CLG analogues exert their effect on CLG-specific CTL precursors (Fig. 1B). None of the CTL cultures lysed untreated PHA blasts. As already demonstrated (11, 19, 38), CTL cultures obtained by peptide stimulation efficiently recognize epitopes endogenously presented by EBV-infected cells (data not shown).

To control whether the effect of 4S and 3Y-4S peptides was dependent on the presence of CD4<sup>+</sup> cells, we performed CTL stimulations using CD4-depleted T cells. In these conditions we also observed that variant peptides increase CTL reactivation induced by the natural epitope (not shown).

These experiments indicate that 4S and 3Y-4S ligands contribute to the reactivation of CLG-specific memory CTL precursors when presented in combination with the natural weak epitope.

Table I. Sequence and activity of peptides derived from the CLG epitope

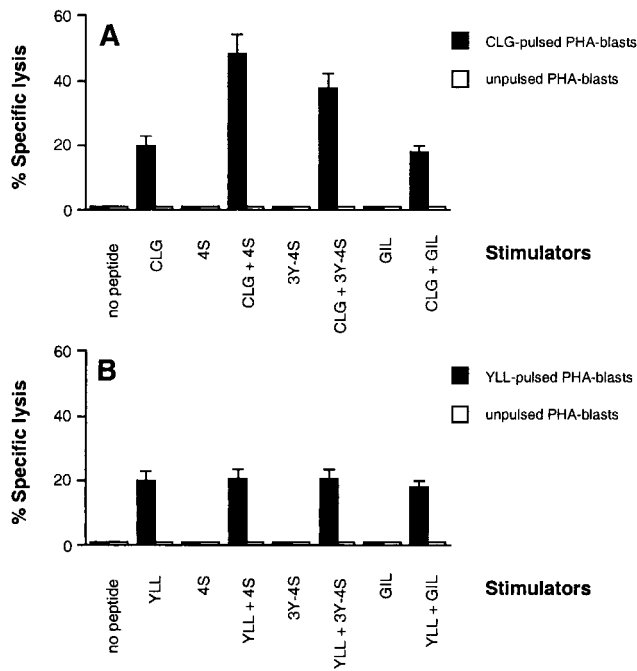
Code	Sequence	HLA-A2/Peptide Stability <sup>a</sup>	CTL Stimulation <sup>b</sup>	Target Cell Sensitization <sup>c</sup> (M)	Antagonist Activity <sup>d</sup>	Peptide Activity
CLG	CLGGLLTMV	12 ± 3	34 ± 10	10 <sup>-9</sup>		Natural agonist
3A	CLAGLLTMV	18 ± 4	50 ± 15	10 <sup>-9</sup>	0	Superagonist
3Y	CLYGLLTMV	24 ± 1	30 ± 5	10 <sup>-8</sup>	0	Weak agonist
1Y-3A	YLAGLLTMV	20 ± 1	60 ± 13	10 <sup>-5</sup>	0	Partial agonist
4S	CLGSLLTMV	29 ± 3	1 ± 5	>10 <sup>-4</sup>	0	Null ligand
3Y-4S	CLYSLLTMV	27 ± 2	1 ± 5	>10 <sup>-4</sup>	0	Null ligand

<sup>a</sup> Half life of HLA-A2/peptide complexes (hours ± SD).

<sup>b</sup> Percent CLG-specific lysis ± SD of CTL cultures obtained by stimulation with the indicated peptides.

<sup>c</sup> Peptide concentration to obtain half-maximal lysis of PHA blasts by CLG-specific CTL cultures.

<sup>d</sup> Percent inhibition of CLG-pulsed target cell lysis by the indicated peptides (see *Materials and Methods*). Mean of five different experiments.



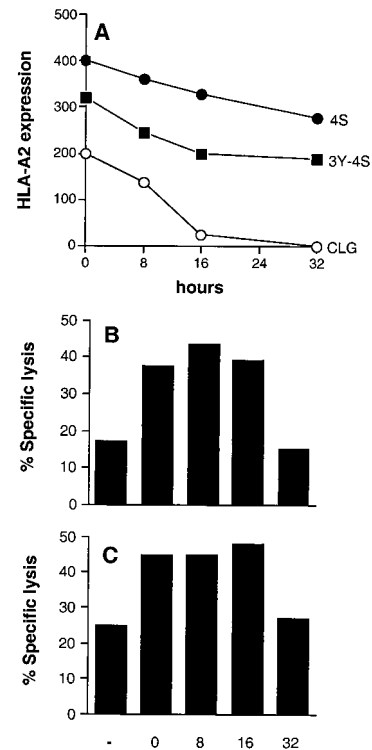
**FIGURE 1.** 4S and 3Y-4S peptides increase CLG-specific CTL reactivation induced by the natural epitope. Freshly isolated PBLs derived from the HLA-A2-positive, EBV-seropositive MT, FR, and RG donors were stimulated with T2 cells pulsed with  $10^{-6}$  M of the indicated peptides at a final responder:stimulator ratio of 20:1. CTL cultures obtained after two consecutive stimulations were tested in cytotoxicity assays against HLA-A2-positive PHA blasts, pulsed or not with  $10^{-7}$  M CLG (A) or YLL peptide (B). The mean  $\pm$  SD percent specific lysis of three separate experiments is shown.

#### 4S and 3Y-4S peptides increase CLG-specific CTL reactivation only in the presence of the natural epitope

PBLs isolated from the EBV-seropositive donors FR and RG were first stimulated with CLG-pulsed T2 cells, then, at different time points, further stimulated with T2 cells pulsed with 4S (Fig. 2B) or 3Y-4S (Fig. 2C) peptides. After 14 days, CTL cultures were tested against HLA-A2 single-matched PHA blasts treated or not with  $10^{-7}$  M CLG peptide. None of the CTL cultures killed untreated PHA blasts (data not shown). As already observed, the stimulation performed with the CLG peptide induced weak CTL responses. A high increase in CLG-specific CTL reactivation was observed in CTL cultures to which were added the 4S and 3Y-4S variants. This effect was detectable in cultures restimulated with the CLG analogues at 0, 8, and 16 h, whereas it completely disappeared at 32 h. It should be noted that at 32 h the CLG-pulsed T2 cells did not express the CLG epitope (Fig. 2A). This demonstrates that 4S and 3Y-4S peptides exert their effects only in the presence of the related natural epitope.

#### 4S and 3Y-4S peptides induce CTL activation in the presence of suboptimal concentrations of natural epitope

We then tested the stimulatory capacities of peptide variants in combination with different concentrations of CLG peptide. PBLs from the EBV-seropositive donor FR were stimulated with the T2 cell line pulsed with concentrations of natural CLG epitope ranging from  $10^{-6}$ – $10^{-12}$  M. In parallel, we performed stimulations using the combination of T2 cells pulsed with the indicated concentrations of natural ligand plus T2 pulsed with  $10^{-6}$  M 4S peptide. CTL cultures were tested for specificity after two consecutive stimulations against HLA-A2 single-matched PHA blasts treated

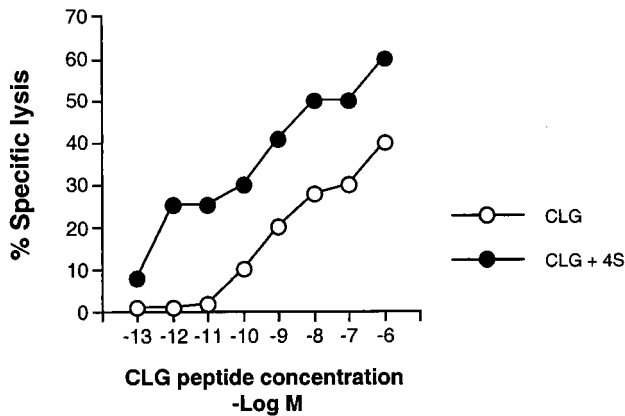


**FIGURE 2.** 4S and 3Y-4S peptides increase CLG-specific CTL reactivation only in the presence of the natural epitope. A, T2 cells were preincubated overnight at 37°C in serum-free medium containing  $10^{-4}$  M of the indicated peptides. Cells were washed, treated with mitomycin C to avoid cell proliferation, divided into aliquots, and maintained at 37°C for kinetic experiments. At the indicated time points, the surface expression of HLA class I molecules was detected by indirect immunofluorescence using the W6-32 mAb. Results are expressed as the mean fluorescence intensity. B and C, Freshly isolated PBLs isolated from the EBV-seropositive donors FR and RG were first stimulated with CLG-pulsed T2 cells, then, at different time points, further stimulated with T2 cells pulsed with 4S (B) or 3Y-4S (C) peptides. Control cultures (–) were stimulated only with CLG-pulsed T2 cells at time zero. CTL cultures were tested in cytotoxicity assays against HLA-A2 single-matched PHA blasts treated or not with  $10^{-7}$  M CLG peptide. One representative experiment is shown.

or not with  $10^{-7}$  M CLG peptide. As shown in Fig. 3, stimulation by the 4S peptide increases CLG-specific CTL reactivation induced by the CLG peptide; it is noteworthy that CTLs can be reactivated at CLG concentrations that per se are unable to induce CTL responses. None of the cultures lysed untreated PHA blasts (data not shown). Similar results were obtained with the 3Y-4S peptide (data not shown).

#### 4S and 3Y-4S peptides increase CLG-specific CTL proliferation induced by the natural epitope

We next investigated the capacity of CLG-derived ligands to stimulate the proliferation of CLG-specific T cell cultures. T2 cells were treated with  $10^{-6}$  M CLG, 4S, 3Y-4S, and GIL peptides and used as stimulators of CLG-specific cultures obtained from different donors. Mitomycin-treated stimulators were added to  $1 \times 10^5$  CTLs in a responder:stimulator ratio of 2:1. After 3 days of coculture in serum-free medium, CTL proliferation was determined by adding [ $^3$ H]thymidine for the last 18 h. Fig. 4 shows the mean of three experiments performed with CLG-specific CTL cultures obtained from different donors. The CLG peptide induced a low level of proliferation, while 4S, 3Y-4S, and GIL peptides did not significantly enhance T cell proliferation compared with CTLs



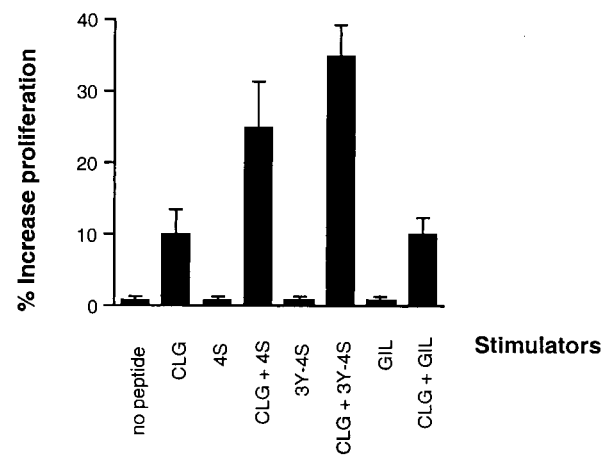
**FIGURE 3.** 4S and 3Y-4S peptides enable CTL activation at suboptimal concentrations of natural epitope. Freshly isolated PBLs derived from the HLA-A2-positive, EBV-seropositive donor FR were stimulated with T2 cells pulsed with the indicated concentrations of CLG peptides (○) or with combinations of T2 cells treated with  $10^{-6}$  M 4S peptide plus T2 treated with the indicated concentrations of CLG peptide (●) at a final responder:stimulator ratio of 20:1. CTL cultures, obtained after two consecutive stimulations, were tested in cytotoxicity assays against HLA-A2-positive PHA blasts pulsed with  $10^{-7}$  M CLG peptide. One representative experiment is shown.

stimulated with untreated T2. Stimulations performed with the combination of CLG-pulsed T2 cells and T2 cells pulsed with 4S or 3Y-4S peptides greatly enhanced CTL proliferation compared with stimulations performed with CLG-pulsed T2 cells. No effect was observed after stimulation with the combination of GIL and CLG peptides.

#### 4S and 3Y-4S peptides do not affect the effector functions of CLG-specific CTL cultures

The 4S and 3Y-4S peptides were not able to sensitize target cells to lysis (Table I). To establish whether the combination of CLG analogues and natural epitope could affect the recognition of CLG-pulsed target cells, we tested the cytotoxic activity of CLG-specific cultures and CTL clones against PHA blasts pulsed with different concentrations of CLG peptide in the presence of unlabeled PHA blasts pulsed with  $10^{-6}$  or  $10^{-8}$  M 4S peptide. As shown in Fig. 5, PHA blasts pulsed with the wild-type peptide were lysed at comparable levels independently of the presence of PHA blasts treated with the 4S ligand. Similar data were obtained with the 3Y-4S peptide (not shown).

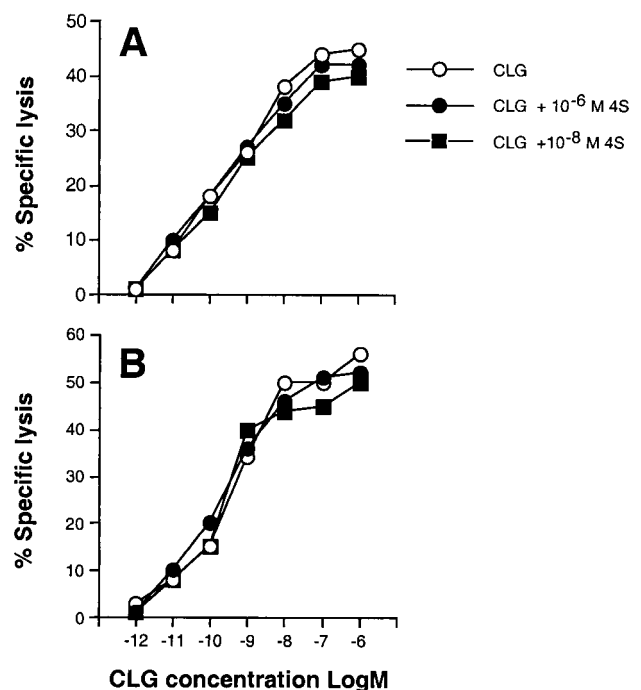
We next evaluated the IFN- $\gamma$  release induced by the combination of peptide variants and natural epitope. T2 cells were treated with  $10^{-6}$  M CLG, 4S, 3Y-4S, and GIL peptides and used as stimulators of CLG-specific cultures and CLG-specific CTL clones. The stimulators were added to  $5 \times 10^4$  CTLs to give a final responder:stimulator ratio of 10:1. After 5 h of coculture in serum-free medium, IFN- $\gamma$  release was determined by ELISA. Fig. 6 shows the mean of data obtained by using three different CLG-specific CTL cultures. The CLG peptide stimulated IFN- $\gamma$  release, while the 4S, 3Y-4S, and GIL peptides did not induce any significant release compared with CTL stimulated with untreated T2. Stimulations performed with the combination of CLG-pulsed T2 cells and T2 pulsed with 4S, 3Y-4S, or GIL peptides did not enhance IFN- $\gamma$  release compared with stimulations performed with CLG-pulsed T2 cells. Similar results were obtained with CLG-specific CTL clones (data not shown).



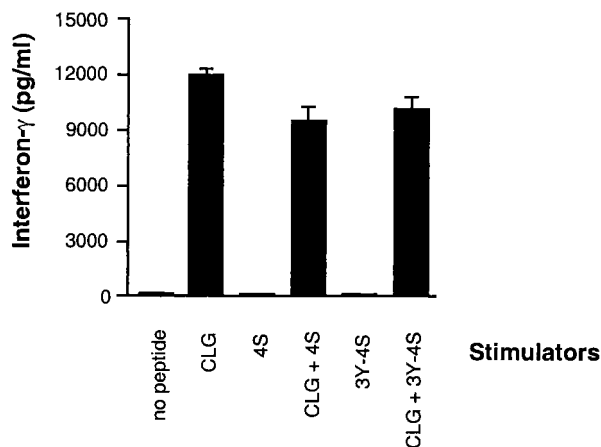
**FIGURE 4.** 4S and 3Y-4S peptides increase CLG-specific CTL proliferation induced by the natural epitope. T2 pulsed or not with  $10^{-6}$  M of the indicated peptides were mitomycin C treated and used as stimulators of CLG-specific cultures. Stimulators were added to  $1 \times 10^5$  CTLs in a final responder:stimulator ratio of 2:1. After 3 days of coculture in serum-free medium, CTL proliferation was determined by adding [ $^3$ H]thymidine for the last 18 h. Results are expressed as the percent increase in [ $^3$ H]thymidine incorporation calculated with respect to that by CTLs stimulated with untreated T2 cells. The mean  $\pm$  SD of three separate experiments is shown.

#### 4S and 3Y-4S peptides increase ZAP-70 activation induced by the natural epitope

Differential T cell activation by variant TCR ligands correlates with distinct patterns of TCR-associated protein phosphorylation.



**FIGURE 5.** Recognition of the CLG epitope is not affected by 4S and 3Y-4S peptides. A CLG-specific culture from donor RG (A) and the CLG-specific CTL clone MT16 (B) were tested for the ability to lyse HLA-A2-matched PHA blasts pulsed with different concentrations of CLG peptide in the absence or the presence of unlabeled PHA blasts pulsed with the indicated concentration of 4S peptide. The percent specific lysis recorded at a 20:1 (A) or 5:1 (B) E:T cell ratio is shown.



**FIGURE 6.** IFN- $\gamma$  production by CLG-specific CTLs is not affected by 4S and 3Y-4S peptides. T2 cells were pulsed with  $10^{-6}$  M of the indicated peptides and used as stimulators of CLG-specific cultures. The final responder:stimulator ratio was 10:1. IFN- $\gamma$  production was evaluated after 5 h of incubation at 37°C. The mean  $\pm$  SD of three separate experiments are shown.

In particular, high levels of ZAP-70 activation are induced by superagonist peptides (17). We then examined recruitment and activation of ZAP-70 in CLG-specific cultures activated with T2 cells untreated or treated with  $10^{-6}$  M of the natural CLG epitope or the 4S peptide. In parallel, we performed CTL stimulations using the combination of T2 pulsed with the natural ligand plus T2 pulsed with the 4S variant. The different cell lysates containing an equal amount of proteins were divided into two aliquots and immunoprecipitated with either anti-phosphotyrosine or anti-ZAP-70 Abs (Fig. 7).

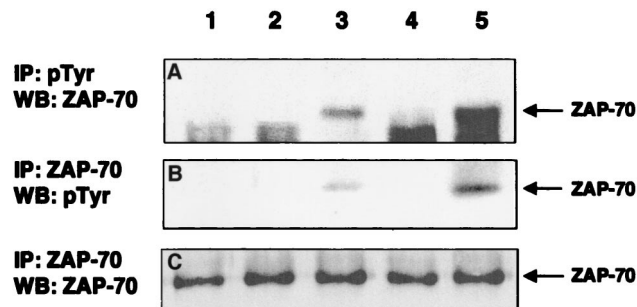
Phosphotyrosine immunoprecipitates obtained from the different CTL lysates were blotted with anti-ZAP-70 mAb. As shown in Fig. 7A, we were able to detect expression of ZAP-70 in immunoprecipitates obtained from CLG-stimulated CTLs, but not from 4S-stimulated CTLs. Stronger expression of ZAP-70 was detected after CTL activation with the combination of CLG and 4S peptides.

We immunoprecipitated ZAP-70 from the same CTL lysates and analyzed the levels of tyrosine phosphorylation with the 4G10 mAb. The combination of 4S and CLG peptide induced higher phosphorylation of ZAP-70 than that obtained with CLG alone, whereas the 4S peptide did not induce any detectable ZAP-70 phosphorylation (Fig. 7B). As shown in Fig. 7C, all CTL lysates contained similar amounts of ZAP-70. Similar results were obtained with the 3Y-4S peptide (data not shown).

These experiments demonstrate that 4S and 3Y-4S peptides, when presented in combination with the natural epitope, contribute to the activation of CLG-specific T cell precursors by increasing recruitment and phosphorylation of ZAP-70 protein kinase.

## Discussion

In the present report, we describe peptides that per se do not induce any CTL activity, but in combination with the natural epitope efficiently stimulate specific memory CTL responses. These peptides did not affect the activation of T cell effector functions. Peptide ligands were obtained by changing nonanchor amino acids of the natural HLA-A2-presented CLG epitope (40). The differential recognition of these analogues by CTLs may be due to conformational changes affecting residues pointing out toward TCR or, alternatively, affecting the orientation of neighboring MHC side chains.



**FIGURE 7.** The 4S peptide in combination with the natural epitope increases recruitment and activation of ZAP-70 kinase in CLG-specific CTLs. Equal amounts of proteins from cell lysates derived from unstimulated CLG-specific CTLs (lane 1) or CLG-specific CTLs stimulated with unpulsed (lane 2),  $10^{-6}$  M CLG-pulsed (lane 3), or  $10^{-6}$  M 4S-pulsed (lane 4) T2 cells or with the combination of  $10^{-6}$  M CLG-pulsed and  $10^{-6}$  M 4S-pulsed T2 cells (lane 5) were divided in two aliquots and used for immunoprecipitation assays. ZAP-70 expression was evaluated from phosphotyrosine immunoprecipitates by anti-ZAP-70 immunoblotting (A), and ZAP-70 phosphorylation was tested from anti-ZAP-70 immunoprecipitates by anti-phosphotyrosine 4G10 mAb (B). All aliquots contained similar amounts of ZAP-70 (C).

Our study originated from the observation that the CLG natural epitope induced weak CTL responses, perhaps due to its poor capacity to form stable HLA-A2 complexes. This result is in line with a large body of data showing that immunodominant CTL responses are directed to epitopes presented for a long time at the cell surface of APC (36, 38, 41–44).

The 4S and 3Y-4S peptides produced much more stable HLA-A2/peptide complexes compared with the CLG epitope. This increased stability could render these analogues capable of efficient CTL stimulation (38). However, functional assays performed with 4S and 3Y-4S peptides demonstrated that they do not activate any CTL function, behaving as null ligands. Since these modified CLG-derived peptides should have maintained the residues necessary for TCR contact, we reasoned that the CLG-specific TCR should still be capable of recognizing them by using alternative contacts or else adapting to rather similar ligands. Indeed, we have shown that these peptides, when used in combination with the natural ligand, interact with the CLG-specific TCR, since 1) the synergistic effect of these particular combinations on the reactivation of memory CTLs was not observed with unrelated ligands such as the GIL peptide, which forms stable HLA-peptide complexes at levels comparable to 4S and 3Y-4S peptides (19); and 2) the CLG-derived variants did not affect the reactivation of CLG-unrelated CTL responses directed to subdominant epitopes.

These apparently null-behaving ligands have been defined supra-agonists, since they increase specific T cell responses only in the presence of the agonist peptide. We have also demonstrated that the combination of supra-agonists and natural epitope induced activation and phosphorylation of ZAP-70 in CLG-specific cultures at levels much higher than those induced by the natural ligand, while stimulation with the null ligand alone did not induce ZAP-70 activation. In our system it appears that activation of ZAP-70 is a key proximal signaling molecule whose activity determines the extent of memory T cell reactivation and proliferation, while the extent of its activation does not correlate with the CTL effector functions. Indeed, high levels of ZAP-70 activation have been detected after T cell stimulation with all ligands showing selective superagonistic activity on memory CTL reactivation (A. Canella and R. Gavioli, unpublished observations).

Together, the data presented in this study suggest that supra-agonists are capable of low-affinity interactions with the TCR. These interactions can be detected by functional and biochemical assays where these particular variants and natural epitope are simultaneously presented to CTL. We propose two alternative models to explain this synergistic effect. In the first model we assume that the supra-agonist may induce conformational changes at the TCR level, so that the CLG peptide may bind with a lower energy barrier. The supra-agonist, by favoring interaction of the CLG epitope with its TCR, could determine an increase in intracellular signaling and higher T cell activation. It has recently been demonstrated that the low affinity of the TCR-peptide-MHC interaction is a consequence of highly unfavorable entropic changes associated with the conformational adjustments and reduction of flexibility required for binding (6, 7). In our case, it may be that the conformational flexibility of CLG-specific TCR in its unbound state is compatible with some nonproductive interactions with the supra-agonist. These may induce conformational rearrangements that result in a more favorable TCR conformation for binding to the natural epitope and higher kinetics of activation, in the end producing greater CTL activation. A similar mechanism, based on receptor conformational changes, has recently been described for low affinity peptides that bind to MHC class II molecules, which after association induce a receptive conformation until a stably binding Ag is encountered (45).

Alternatively, the supra-agonist, after interaction with the specific TCR, may induce an as yet undefined signal(s) that per se is not sufficient to trigger any CTL function but, in combination with signals induced by the natural ligand, determines an increase in CTL activation. In favor of this hypothesis, although we cannot completely exclude the first model, we have observed that supra-agonists induce partial hypophosphorylation of  $\zeta$ -chains (A. Canella, unpublished observations). It remains to be seen how this partial phosphorylation affects the downstream pathways associated with T cell activation. Further studies are needed to characterize the biochemical events induced by superagonists and their impact on CTL activation.

It should be noted that the ligands described here do not increase the effector functions of CLG-specific CTLs. This behavior is in line with results obtained with antagonist peptides that were shown to block defined subsets of T cell functions (46) and further confirms our previous findings showing that the reactivation of memory CTL precursors and triggering of the cytotoxic function have different, as yet undefined, requirements (11).

In conclusion, we have defined peptides apparently behaving as null ligands that, in combination with the natural epitope, induce efficient CTL stimulation directed to the wild-type epitope. These peptides, here defined as supra-agonists, may represent the counterpart of TCR antagonists, since they act on CTL functions in the presence of the agonist with the final effect of increasing, and not blocking, specific CTL activities. It remains to be seen whether such variant peptides exist in vivo, and if these play a role in thymic positive selection and lymphocyte differentiation (47), in memory T cell maintenance (11), or in determining the immunodominance of CTL responses to a given epitope.

This class of peptide variants may be useful for a better understanding of the TCR/peptide interactions and TCR-mediated signaling, complex phenomena that are finely regulated depending on the quality of ligands. Furthermore, peptides with the characteristics described in this paper may be useful for therapeutic applications in EBV malignancies expressing the CLG epitope. Since the natural ligand induces weak CTL responses that are unable to control in vivo tumor development, we have found peptides that are able to selectively induce stronger CTL reactivation and to con-

tribute to CTL activation even in the presence of low, nonproductive concentrations of natural ligand. These CLG-derived peptides have the advantage of forming stable HLA-A2 complexes that are not recognized by CLG-specific CTLs, a factor that would increase the safety and efficacy of a peptide-based CTL immunotherapy.

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