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Alberto Paradela, Marina García-Peydró, Jesús Vázquez, Didier Rognan and José A. López de Castro

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# The Same Natural Ligand Is Involved in Allorecognition of Multiple HLA-B27 Subtypes by a Single T Cell Clone: Role of Peptide and the MHC Molecule in Alloreactivity<sup>1</sup>

Alberto Paradela,<sup>2\*</sup> Marina García-Peydró,<sup>2\*</sup> Jesús Vázquez,\* Didier Rognan,<sup>†</sup> and José A. López de Castro<sup>3\*</sup>

The human alloreactive CTL clone 27S69, raised against B\*2705, cross-reacts with B\*2702 and B\*2703, but not with B\*2701, B\*2704, B\*2706, or B\*2710. Its natural epitope was identified by electrospray/ion trap mass spectrometry, as the proteasome-derived RRFFPYV octamer. This is the first HLA-B27 ligand shown to be immunogenic in alloreactivity. The RRFFPYVYV nonamer, also found in the B\*2705-bound peptide pool, was recognized much less efficiently, demonstrating that an alloreactive CTL distinguishes between very similar natural ligands. Molecular modeling suggested that this was due to the different conformation of each peptide in complex with B\*2705. B\*2702- and B\*2703-RMA-S cells were lysed by CTL 27S69 when sensitized with the octamer, demonstrating that cross-reaction with these subtypes is through recognition of the same peptide as in B\*2705. B\*2704-, B\*2706-, and B\*2710-RMA-S cells were not sensitized for lysis, in spite of efficient binding of the octamer, indicating that polymorphism in these subtypes directly impairs allorecognition. B\*2701-RMA-S and -C1R cells were sensitized for lysis by the octamer, suggesting lack of the endogenous peptide epitope on this subtype. Absence of the octamer in the B\*2701-bound peptide pool further suggested that B\*2701 polymorphism impairs the generation of this peptide. *The Journal of Immunology*, 1998, 161: 5481–5490.

**T** cells recognize peptides presented by MHC proteins on the cell surface. The T cell repertoire of each individual is selected during ontogeny to become tolerant to self peptide/MHC complexes, and to react against foreign peptides presented by self MHC molecules. In allotransplantation, an extremely vigorous T cell response occurs, which is the basis for acute allograft rejection. This is due to activation of alloreactive T cells specific for the allogeneic MHC molecules.

The molecular basis for alloreactivity is poorly understood. MHC proteins constitutively present at the cell surface a large peptide repertoire, and the clonal diversity typical of alloreactive T cell responses is probably due to involvement of many MHC-bound peptides in allorecognition (1–4). However, alloreactive T cells may exhibit various degrees of peptide specificity (2), and a few might be peptide independent or recognize motifs common to many peptides (5–7).

Various major issues remain to be solved. One is the nature of the peptides involved in allorecognition. To our knowledge, only a natural HLA ligand recognized by a human alloreactive CTL has been

reported (8), and very few others have been identified in the mouse system (9–11). Therefore, the requirements for HLA-bound peptides to be immunogenic in alloreactive responses, such as affinity for MHC, expression level, and putative immunodominant character, have been insufficiently addressed. For instance, some of the peptides identified as allospecific epitopes were abundant and immunodominant (12, 13), but others were expressed at very low levels (8, 11).

A second issue is the molecular basis for the cross-reactions of alloreactive CTL. Self-restricted T cells can cross-react with peptides showing minimal homology to the nominal epitope (14–16). In one instance, an alloreactive CTL recognized two unrelated peptides in the context of two different allo-MHC molecules (17), and a third peptide in self-restricted fashion (18). In addition, a peptide isolated from a library that was recognized by an alloreactive T cell clone was different from the natural ligand recognized by that CTL (11, 19). In previous studies, alloreactive T cell clones raised against HLA-B27 (B\*2705) showed limited cross-reaction with non-B27 Ags, but cross-reacted frequently with other HLA-B27 subtypes (20). Whether these cross-reactions were due to recognition of the same or different peptides in the various contexts was not determined.

A third issue is the role of the structural features of the MHC molecule in allospecificity. Although probably only a limited subset of alloreactive T cells might be peptide independent (4, 7), x-ray diffraction studies show that about 75% of the surface area contacted by the TCR corresponds to the MHC molecule (21, 22). However, the actual contribution of MHC residues, relative to bound peptide, to the alloantigenic determinants, and to the affinity for allospecific TCR remains to be established.

In this work, we report the identification of a natural peptide ligand of HLA-B27 that is recognized by a human alloreactive CTL clone raised against B\*2705. We further establish the relationship between recognition of other HLA-B27 subtypes by this CTL clone and presentation of the peptide in the various contexts,

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and demonstrate the contribution of polymorphic HLA-B27 residues to the allospecific epitope. The same CTL clone distinguished between its peptide epitope and another closely related natural HLA-B27 ligand. The structure of the allospecific epitope and the molecular basis for this discrimination were analyzed by molecular modeling.

## Materials and Methods

### CTL 27S69

The anti-B\*2705 alloreactive CTL 27S69 clone and its culture conditions have been described (20). It was raised from donor SR (HLA-A3, 29; B7, 44; DR2, 7) by *in vitro* stimulation with LCL<sup>4</sup> R69 (HLA-A3, 24; B\*2705, 7; DR3, 5). Besides B\*2705, CTL 27S69 recognized B\*2702, B\*2703, and HLA-B61, but not B\*2701, B\*2704, or B\*2706, as established with HLA-typed LCL.

### HLA-B27 transfectant cell lines

Hmy2-C1R (C1R) is a human lymphoid cell line with low expression of its endogenous class I Ags. These cells and their transfectants expressing HLA-B27 subtypes were cultured in DMEM (Life Technologies, Paisley, U.K.) with 7.5% heat-inactivated FCS. T2 is a TAP-deficient human cell line of lymphoid origin (23). The B\*2705-T2 transfectant was a kind gift of Dr. David Yu (University of California, Los Angeles, CA). It was cultured in DMEM supplemented with 5% FCS. RMA-S is a TAP-deficient mutant cell line derived from the murine H-2<sup>b</sup> lymphoma RBL-5. These cells and their transfectants expressing HLA-B27 subtypes plus human  $\beta_2$ -microglobulin (6, 24–26) were cultured in RPMI 1640 supplemented with 10% FCS. When cultured at 26°C, T2 and RMA-S cells express class I molecules presumably devoid of peptides. These molecules are unstable at 37°C, but their surface expression at this temperature can be stabilized by exogenous peptide ligands.

### Isolation of HLA-B27-bound peptides

About  $1\text{--}1.5 \times 10^{10}$  B\*2705-C1R cells were lysed at 4°C in 20 mM Tris-HCl buffer, 150 mM NaCl, 1% Nonidet P-40, pH 7.5, containing the following protease inhibitors: 10  $\mu\text{g/ml}$  leupeptin, 2  $\mu\text{g/ml}$  pepstatin, 2.5  $\mu\text{g/ml}$  aprotinin (all from Boehringer Mannheim, Mannheim, Germany), 18.5  $\mu\text{g/ml}$  iodoacetamide, 1 mM EDTA, 2 mM PMSF, 258  $\mu\text{g/ml}$  1,10 phenanthroline (Sigma, St. Louis, MO), and 0.2% sodium azide. Cell lysates were centrifuged, and the supernatant was filtered, precleared through a CNBr-activated Sepharose 4B column (Pharmacia, Uppsala, Sweden), and subjected to affinity chromatography using the W6/32 mAb (IgG2a, specific for a monomorphic HLA-A, -B, and -C determinant). HLA-B27-bound peptides were eluted from the column with 0.1% TFA in water at room temperature, filtered through Centricon 3000 (Amicon, Beverly, MA), and concentrated to 500  $\mu\text{l}$  for fractionation by HPLC. This was conducted in a  $2 \times 150\text{-mm}$  Deltapak C18 (300Å) column (Waters, Milford, MA), at a flow rate of 100  $\mu\text{l/min}$ , as follows: isocratic conditions with buffer A (0.1% TFA in water) for 20 min, followed by a linear gradient of 0–44% buffer B (80% acetonitrile, 0.1% TFA in water) for 80 min, and a linear gradient of 44–100% buffer B for another 40 min. Peptide fractionation was simultaneously monitored at 210 and 280 nm, using a Waters 991 photodiode array detector. Fractions of 50  $\mu\text{l}$  were collected and stored at  $-20^\circ\text{C}$ .

### Mass spectrometry analysis and peptide sequencing

The peptide composition of individual HPLC fractions was determined by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. A calibrated Reflex instrument (Bruker-Franzen Analytik, Bremen, Germany) operating in the positive ion reflectron mode was used. Five-microliter aliquots of a given HPLC fraction were dried, resuspended in 2  $\mu\text{l}$  of 0.1% TFA in water:acetonitrile, 2:1, and mixed with 2  $\mu\text{l}$  of saturated  $\alpha$ -cyano-4-hydroxycinnamic acid matrix in 0.1% TFA in a water:acetonitrile ratio of 2:1. One microliter of the mixture was dried and subjected to analysis.

Peptide sequencing was conducted in a LCQ electrospray/ion trap mass spectrometer (Finnigan MAT, San Jose, CA), equipped with a microspray probe. HPLC fractions were dried down and resuspended in 5  $\mu\text{l}$  methanol/water (1:1) containing 0.1% formic acid. One microliter of this solution was used for analysis. Accurate peptide mass and charge of ionic species were determined by performing “Zoomscan” spectra. This is a high reso-

lution scanning method in which a precursor ion window is selected to incorporate several isotopomers. The charge states of individual product ions were determined at enhanced resolution by scanning across a limited mass/charge ( $m/z$ ) range. Collision energy and precursor ion resolution were optimized for each individual peptide to obtain the optimum fragmentation spectra. Putative peptide sequences were obtained by database comparison of the fragmentation spectra using the PEPSEARCH program (Bioworks package; Finnigan) in conjunction with the nr-BLAST database from the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MA), followed by manual assignment of expected fragments from the highest-score sequences. Sequence assignments were confirmed by comparing the fragmentation spectra with those of the corresponding synthetic peptides.

In one case, peptide sequencing was conducted by Edman degradation in an ABI 473A automated sequencer (Applied Biosystems, Foster City, CA). A single HPLC fraction of the B\*2705-bound peptide pool from  $10^{10}$  C1R transfectant cells, containing about 10 pmol of the major peptide species, was used.

### Peptide synthesis

Peptides were synthesized, using the standard F-moc or the t-BOC chemistries, and purified by HPLC. The correct composition and molecular mass of purified peptides were confirmed by amino acid analysis using a 6300 amino acid analyzer (Beckman, Palo Alto, CA), which also allowed their quantification, and by electrospray/ion trap mass spectrometry, respectively.

### Epitope stabilization assay

The quantitative epitope stabilization assay used was previously described (25). Briefly, RMA-S transfectants were incubated at 26°C for 24 h. They were then incubated 1 h at 26°C with  $10^{-4}$  to  $10^{-9}$  M peptides without FCS, transferred to 37°C, and collected for flow microcytometry analysis with the ME1 mAb (IgG1, specific for HLA-B27, B7, B22) after 4 h for B\*2705 and B\*2706, or after 2 h for all other HLA-B27 subtypes. Binding was expressed as the  $C_{50}$ , which is the molar concentration of the peptide at 50% of the maximum fluorescence obtained with that peptide in the concentration range used. Relative binding of multiple peptides was assessed as follows. First, the  $C_{50}$  of the reference peptide was calculated. Second, the concentration of each other peptide required to obtain the fluorescence value at the  $C_{50}$  of the reference peptide was found by interpolation. This was designated as  $EC_{50}$ , which is equal to the  $C_{50}$  for the reference peptide. Relative binding was expressed as the ratio between the  $EC_{50}$  values of the peptides compared. Peptides with  $EC_{50} \leq 5 \mu\text{M}$  were considered to bind with high affinity.  $EC_{50}$  values between 5 and 50  $\mu\text{M}$  were considered to reflect intermediate affinity.  $EC_{50} \geq 50 \mu\text{M}$  indicated low affinity.

### Peptide sensitization and other cytotoxicity assays

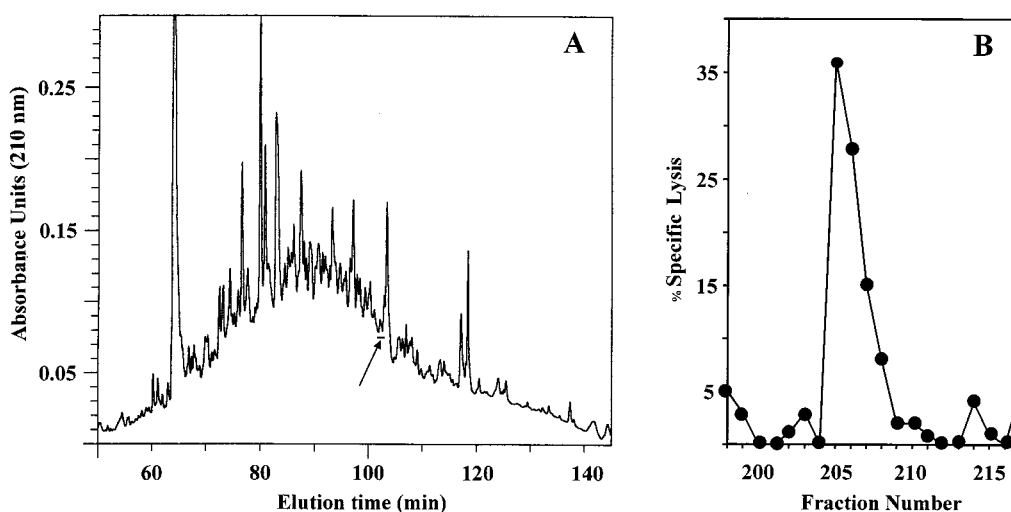
T2 or RMA-S transfectant cells were incubated for 24 h at 26°C in RPMI 1640 medium supplemented with 10% FCS, in the absence of peptide. Cells were then labeled for 90 min at 37°C with 50  $\mu\text{Ci}$  of  $^{51}\text{Cr}$ , washed (four times), resuspended in the same medium with 1% FCS, seeded in 96-well plates, and incubated for 30 min at room temperature with synthetic peptides or aliquots of particular HPLC fractions. Effector cells were then incubated with peptide-sensitized targets for 5 h at 37°C at given E:T ratios, and the supernatants were subjected to gamma counting. Percentage of specific  $^{51}\text{Cr}$  release was calculated as follows: (experimental lysis – spontaneous lysis)/(maximum release – spontaneous lysis)  $\times$  100.

A 4-h  $^{51}\text{Cr}$  release assay was used for other target cells, as described above, except that cells were not preincubated with exogenous peptide, unless otherwise specified, or at lower temperature.

### Molecular modeling

The RRRFPYYV and RRRFPYYVY peptides in complex with B\*2705 were simulated by molecular dynamics, using the AMBER 5.0 package (27) with the AMBER95 force field (28). Initial coordinates for the Ag binding domain (residues 1–182) were taken from the crystal structure of HLA-B\*2705 (29) deposited in the Protein Data Bank. Both peptides were built by assembling two fragments (P1-P3, P4-P $\Omega$ ) generated from a crystal structure database of class I MHC-bound peptides. The P1-P3 part (RRF) was built from the B\*2705-bound ARAAAAAAA peptide (29). Rotameric states for the Arg1 and Phe3 side chains were assigned according to the structures of RGYVYQGL in complex with H-2K<sup>b</sup> (30), and of LLFGYPVYV in complex with HLA-A\*0201 (31). The C-terminal part (P4-P $\Omega$ ) has a less-defined conformation depending on its sequence and length (31). Thus, it was built from the structures of two class I MHC-bound peptides, an octamer and a nonamer, with Pro

<sup>4</sup> Abbreviations used in this paper: LCL, lymphoid cell line; TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight.



**FIGURE 1.** A, HPLC fractionation of the B\*2705-bound peptide pool from B\*2705-C1R cells. Only the peptide portion of the chromatogram is shown. The elution positions of fractions 205–208, which sensitized B\*2705-T2 cells for lysis by CTL 27S69, are indicated by an arrow. B, Lysis of B\*2705-T2 target cells sensitized with 8- $\mu$ l aliquots of HPLC fractions of the B\*2705-bound peptide pool by CTL 27S69. Specific lysis of the control B\*2705-C1R cells at the E:T ratio used (2:1) was 72%. Experimental conditions are described in *Materials and Methods*.

at P5. The FPYYV sequence (P4–P8) of the octamer was constructed from VPLRPMTY in complex with HLA-B\*3501 (32). For the nonamer, LLFGYPVYV in complex with HLA-A\*0201 (31) was used as a template to build the C-terminal FPYYVY sequence (P4–P9). Residue changes in P4 to P $\Omega$ -1 were introduced using the SYBYL modeling package (Tripos Associates, St. Louis, MO) without altering the dihedral angle of the side chains. The C-terminal side chain (Val8 or Tyr9) was orientated according to the two MHC-bound peptides described above (LLFGYPVYV, VPLRPMTY). The N- and C-terminal fragments were connected to define a *trans* peptide bond between P3 and P4. After adding all hydrogen atoms, both B\*2705-peptide complexes were energy minimized in vacuum, as previously described (33, 34). To sample the broadest conformation space in the bound state, the peptide was then annealed to 1000 K while maintaining the protein fixed, according to a described procedure (35). The MHC-peptide complex was then solvated in a shell of 1350 water molecules, minimized, and subjected to a 200-ps restrained molecular dynamics simulation, as previously reported (35).

## Results

### Identification of the peptide epitope recognized by CTL 27S69

The peptide dependency of CTL 27S69 was suggested by failure to lyse B\*2705-T2 cells. In a first attempt to determine the epitope recognized by this CTL, the B\*2705-bound peptide pool from about  $10^{10}$  C1R transfectant cells was fractionated by HPLC (Fig. 1A). Aliquots of individual fractions were incubated with B\*2705-T2 transfectants, and tested for lysis by the CTL clone. A peak of sensitizing activity spanning HPLC fractions 205–208 (Fig. 1B) suggested that CTL 27S69 recognized a peptide eluting in these fractions. Thus, their composition was determined by MALDI-TOF mass spectrometry. Fractions 205 and 206, which showed the highest sensitizing activity, contained at least 15 and 7 molecular species, respectively. The amino acid sequences of two of the four peptides that eluted in both of these fractions were determined by electrospray/ion trap mass spectrometry. The sequence of the RRFFPYVYV octamer (molecular mass: 1149 Da), a proteasome C5 subunit-derived peptide, was determined from its MS/MS spectrum (Fig. 2), as follows: matching of the observed pattern of product ions using the PEPSEARCH program in conjunction with the nr-BLAST database suggested the sequence mentioned above. Visual inspection confirmed that the data were consistent with this assignment (Fig. 2), and comparison with the product ion spectrum of the corresponding synthetic peptide

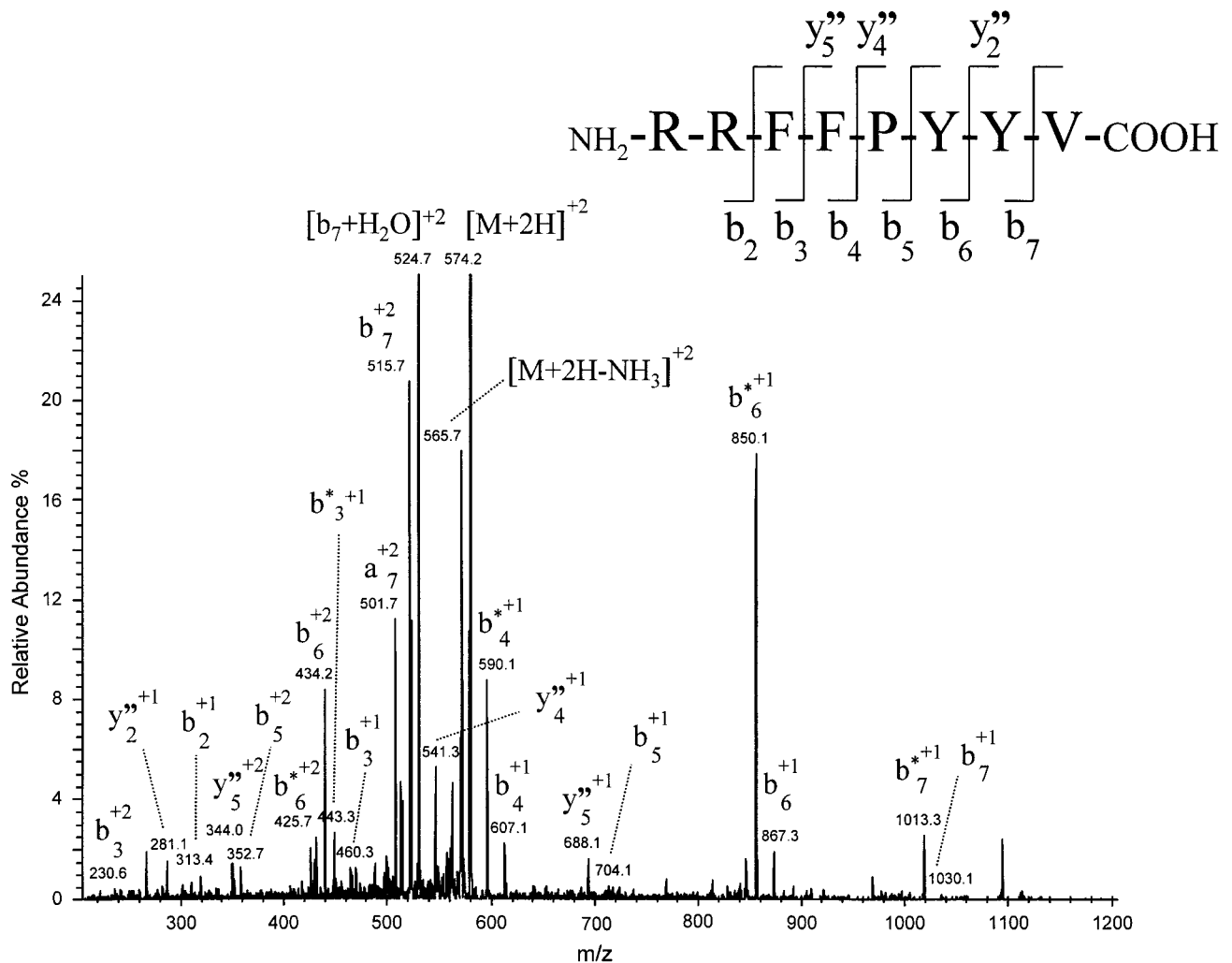
provided confirmation of the structure. A minor signal corresponding to the same peptide was detected in fraction 207. The second sequence (molecular mass: 1159 Da) was RRLPIFSRL, a peptide previously identified as a natural ligand of B\*2705 (36) and B\*2709 (37).

The material in HPLC fraction 208 contained two molecular species, one of which (1312 Da) was predominant in the MALDI-TOF spectrum. When subjected to Edman degradation, the RRFFPYVYV sequence, which corresponded to the predominant peptide in this fraction, was obtained. This peptide is a C-terminal extension of the proteasome-derived octamer in Fig. 2, and was previously identified as a natural ligand of B\*2701 (26), B\*2703, and B\*2705 (38).

The two peptides sequenced from fractions 205 and 206 were synthesized and tested for the capacity to sensitize B\*2705-T2 cells for lysis by CTL 27S69. In an initial experiment, the RRFFPYVYV octamer induced significant lysis even at 20 nM concentration, whereas RRLPIFSRL did not induce lysis at 200  $\mu$ M. This result indicates that the peptide epitope recognized by CTL 27S69 in the context of B\*2705 is the RRFFPYVYV octamer.

### CTL 27S69 distinguishes between closely related natural ligands

That HPLC fraction 208 sensitized poorly B\*2705-T2 transfectants for lysis (Fig. 1B) suggested that its predominant peptide RRFFPYVYV was inefficiently recognized by CTL 27S69. Thus, the capacity of this CTL clone to distinguish between this peptide and the RRFFPYVYV octamer was quantitatively analyzed. The octamer efficiently sensitized B\*2705-T2 (Fig. 3A) and B\*2705-RMA-S (Fig. 3B) cells for lysis: half-maximal lysis was obtained at  $1.8 \times 10^{-8}$  M and  $1.4 \times 10^{-7}$  M, respectively. This difference is consistent with the lower avidity of human CTL for murine, relative to human target cells. The nonamer was recognized about 100- or 1000-fold less efficiently on B\*2705-T2 and B\*2705-RMA-S targets, respectively. This was not due to lower binding of the nonamer: actually, this peptide bound better than the octamer to B\*2705-RMA-S cells (Fig. 3C). The nonamer was about four-fold more abundant than the octamer in the B\*2705-bound peptide pool, as estimated from the respective intensity peaks in the MALDI-TOF mass spectra of all of the HPLC fractions containing either peptide. This correlates with their relative binding to B\*2705 *in vitro*.



**FIGURE 2.** Sequencing of the RRFFPYYV peptide by electrospray/ion trap mass spectrometry. The MS/MS fragmentation spectrum of ion at  $m/z$  574.2 is shown. The y-axis represents relative abundance, expressed as a percentage of this ion peak, which was the major one in the spectrum. The assigned peptide sequence is indicated, detailing the observed backbone fragment ions according to the nomenclature of Roepstorff and Fohlman (57). Fragments labeled with asterisk come from ions of the same series after neutral loss of ammonia (17 Da). Ions of the “a” series are produced by neutral loss of carbon monoxide (28 Da) from ions of the “b” series. The precursor ion  $[M + 2H]^{+2}$ , and a related ion resulting from loss of ammonia are indicated. The high abundance of fragments with charge +2 is due to the presence of two basic residues at the N terminus.

Taken together, these results indicate that CTL 27S69 discriminates between the RRFFPYYV octamer and its C-terminally extended RRFFPYYVY nonamer, in a way unrelated to the relative binding or abundance of both peptides.

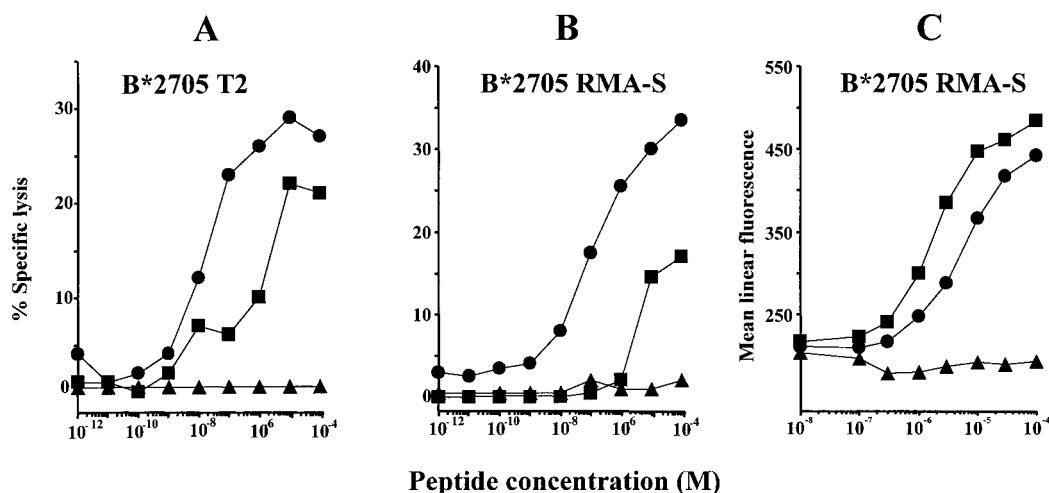
*CTL 27S69 cross-reacts with B\*2702 and B\*2703 through recognition of the same peptide as in B\*2705*

CTL 27S69 cross-reacts with B\*2702 and B\*2703 on LCL (20). Thus, binding of the RRFFPYYV octamer and the corresponding nonamer, and their recognition in the context of these two subtypes were tested. Both peptides bound to B\*2702 (Fig. 4A) and B\*2703 (Fig. 4B), with  $EC_{50}$  values in the range commonly found among natural ligands. Indeed, the nonamer was sequenced from the B\*2703-bound peptide pool (38). In addition, CTL 27S69 efficiently killed B\*2702-RMA-S targets sensitized with the octamer (half-maximal lysis at  $0.8 \times 10^{-7}$  M) and, about 70-fold molar less efficiently, with the nonamer (Fig. 4C). The octamer, but not the nonamer, was also recognized in the context of B\*2703 (half-maximal lysis at  $4.6 \times 10^{-7}$  M) (Fig. 4D).

These results indicate that the RRFFPYYV octamer binds to B\*2702 and B\*2703 with an affinity compatible with its binding in vivo to the two subtypes, and that cross-reaction of CTL 27S69 with B\*2702 and B\*2703 is through recognizing the same peptide as in B\*2705.

*B\*2704, B\*2706, and B\*2710 bind, but do not present, the peptide epitope to CTL 27S69*

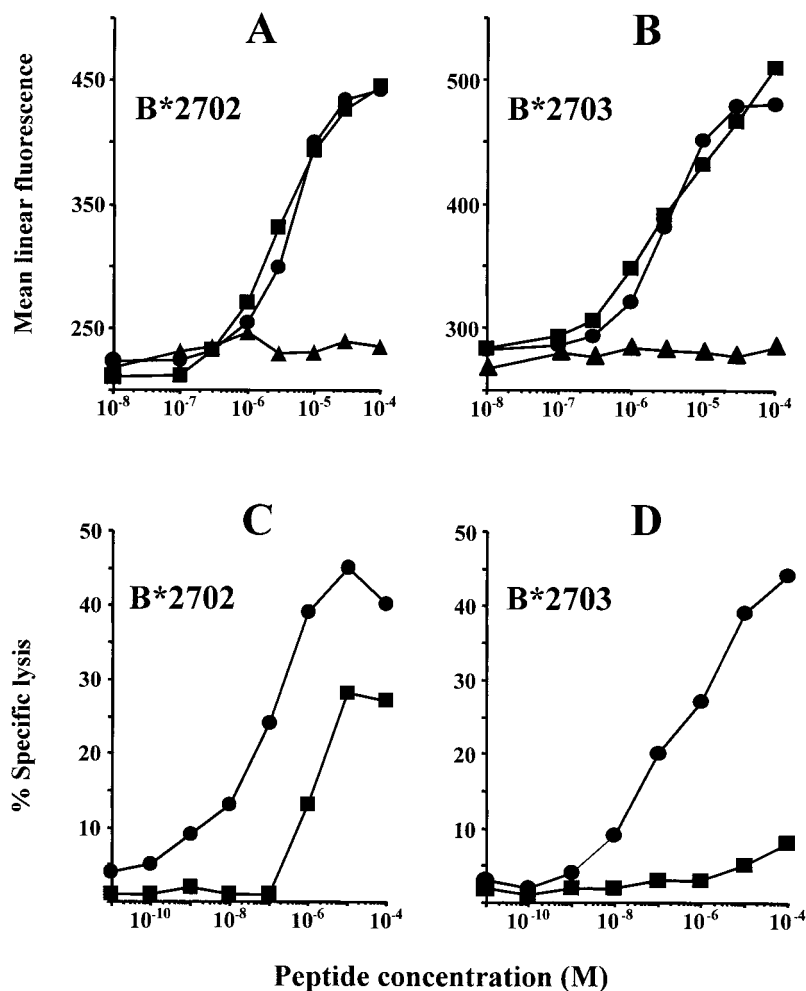
This CTL clone failed to lyse LCL expressing B\*2704 or B\*2706 (20), as well as B\*2710-C1R cells (data not shown). All three subtypes have Glu152, instead of Val152, which is present in all other HLA-B27 subtypes. This is the only change between B\*2710 and B\*2705. Binding to B\*2704-, B\*2706-, or B\*2710-RMA-S cells, and lysis of these transfectants in the presence of the peptide epitope were analyzed (Fig. 5). Both the RRFFPYYV octamer and the corresponding nonamer bound in vitro to B\*2704, B\*2706, and B\*2710 with  $EC_{50}$  values in the range of natural ligands (Fig. 5A–C). In addition, the RRFFPYYV octamer was identified and sequenced from a HPLC fraction of B\*2704-bound peptides,



**FIGURE 3.** A, Lysis of B\*2705-T2 target cells sensitized with the synthetic RRFPPYYV (●) and RRFPPYYVY (■) peptides by CTL 27S69. The B\*2705 natural ligand RRYQKSTEL (▲) was used as negative control. Half-maximal lysis was obtained at  $1.8 \times 10^{-8}$  M of the octamer. The same lysis required  $2.5 \times 10^{-6}$  M of the nonamer. Specific lysis of B\*2705-C1R cells at the E:T ratio used (0.5:1) was 49%. B, Lysis of B\*2705-RMA-S target cells sensitized with RRFPPYYV, RRFPPYYVY, or the negative control RRYQKSTEL by CTL 27S69. Half-maximal lysis was obtained at  $1.4 \times 10^{-7}$  M of the octamer. The same lysis required more than  $10^{-4}$  M of the nonamer. Specific lysis of B\*2705-C1R cells at the E:T ratio used (0.75:1) was 45%. C, Binding of RRFPPYYV, RRFPPYYVY, and KTGPIYKR as negative control to B\*2705 on RMA-S transfectant cells.  $EC_{50}$  was 2 and 8  $\mu$ M for the nonamer and the octamer, respectively. Data are means of two experiments. For experimental details, see *Materials and Methods*.

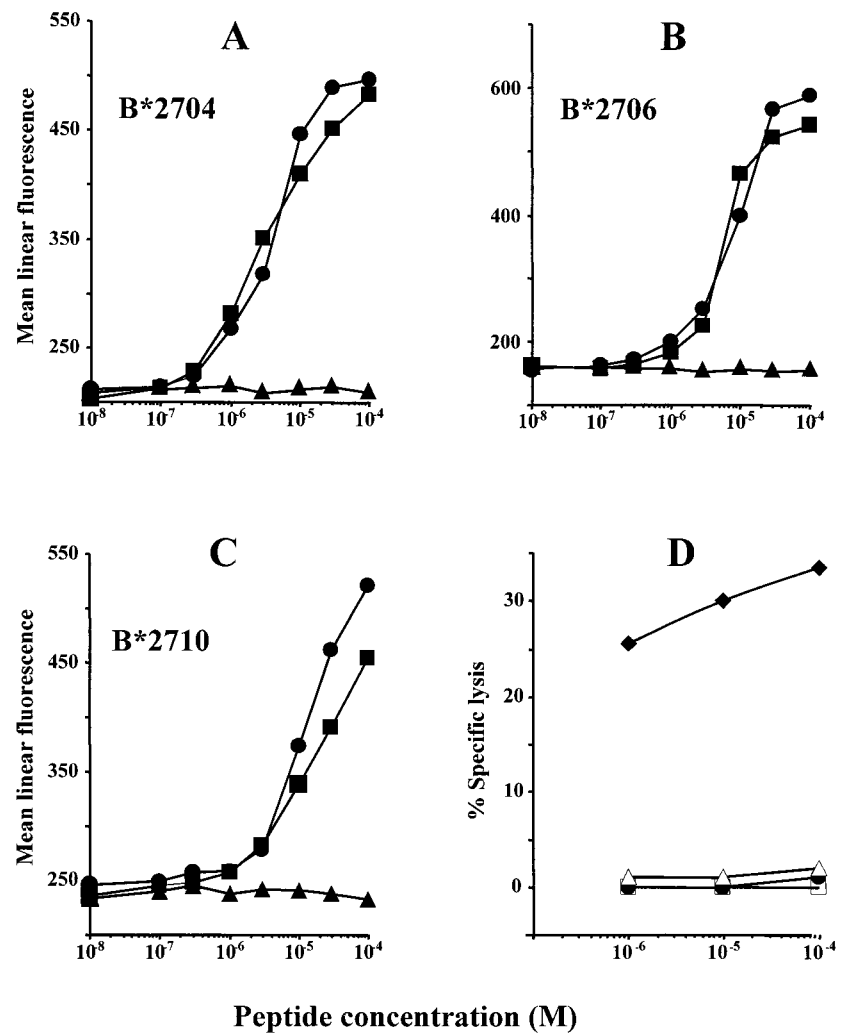
by electrospray/ion trap mass spectrometry (data not shown). In spite of the binding observed, CTL 27S69 failed to lyse B\*2704-, B\*2706-, or B\*2710-RMA-S cells even at the highest

peptide concentration used (Fig. 5D). The octamer also failed to sensitize C1R transfectants expressing these subtypes (data not shown).



**FIGURE 4.** A, Binding of RRFPPYYV (●), RRFPPYYVY (■), and KTGPIYKR (▲) as negative control to B\*2702 on RMA-S cells.  $EC_{50}$  was 3 and 4  $\mu$ M for the nonamer and the octamer, respectively. B, Binding of the same peptides to B\*2703 on RMA-S cells.  $EC_{50}$  was 6 and 5  $\mu$ M for the nonamer and the octamer, respectively. Data for B\*2702 and B\*2703 are means of two experiments. C, Lysis of B\*2702-RMA-S cells sensitized with the RRFPPYYV and RRFPPYYVY peptides, by CTL 27S69. Half-maximal lysis was obtained at  $0.8 \times 10^{-7}$  M of the octamer. The same lysis required  $5.5 \times 10^{-6}$  M of the nonamer. Specific lysis of B\*2705-C1R cells at the E:T ratio used (0.9:1) was 76%. Data are means of three experiments. D, Lysis of B\*2703-RMA-S cells sensitized with the same peptides, by CTL 27S69. Half-maximal lysis was obtained at  $4.6 \times 10^{-7}$  M of the octamer. Specific lysis of B\*2705-C1R cells at the E:T ratio used (0.9:1) was 68%. Data are means of two experiments. For experimental details, see *Materials and Methods*.

**FIGURE 5.** *A*, Binding of RRFPPYYV (●), RRFPPYYVY (■), and KTGPIYKR (▲) as negative control to B\*2704 on RMA-S cells. EC<sub>50</sub> was 3 and 4 μM for the nonamer and the octamer, respectively. *B*, Binding of the same peptides to B\*2706 on RMA-S cells. EC<sub>50</sub> was 6 and 7 μM for the nonamer and the octamer, respectively. *C*, Binding of the same peptides to B\*2710 on RMA-S cells. EC<sub>50</sub> was 30 and 13 μM for the nonamer and the octamer, respectively. *D*, Lysis of RMA-S transfectants expressing B\*2705 (◆), B\*2704 (●), B\*2706 (△), and B\*2710 (□) sensitized with the synthetic RRFPPYYV octamer, by CTL 27S69. E:T ratio was 0.75:1, 1:1, 1.25:1, and 1:1, respectively. Specific lysis of the control B\*2705-C1R cells in these experiments was 45, 71, 71, and 58%, respectively. Data are means of two experiments. For experimental details, see *Materials and Methods*.



These results indicate that lack of cross-reaction of CTL 27S69 with B\*2704, B\*2706, and B\*2710 is not due to absence or inefficient binding of the corresponding peptide epitope, but to direct impairment of T cell recognition by structural features of these subtypes, including at least the Glu152 residue.

#### *B\*2701 presents exogenous, but not the endogenous peptide epitope to CTL 27S69*

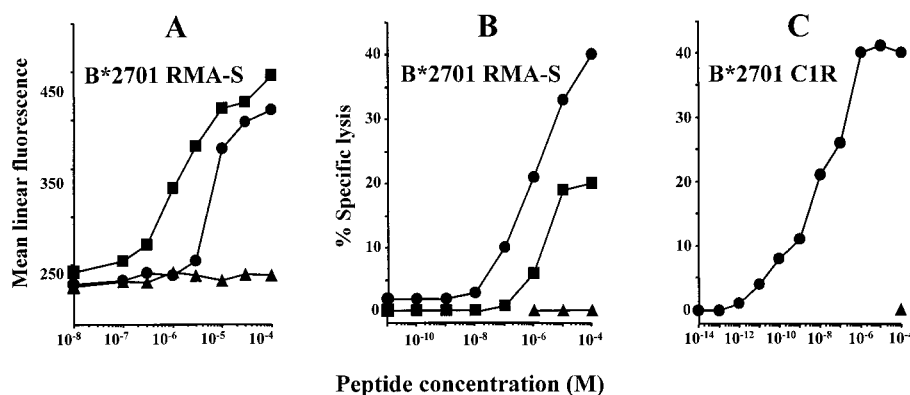
CTL 27S69 did not lyse B\*2701-positive LCL (20), or B\*2701-C1R transfectants. As in B\*2705, the nonamer, which is a natural B\*2701 ligand (26), bound better than the octamer to B\*2701-RMA-S cells (Fig. 6A). Although the EC<sub>50</sub> of the octamer was also in the range of natural ligands, it was not found in the corresponding HPLC fractions from the B\*2701-bound peptide pool, upon analysis by electrospray/ion trap mass spectrometry. Other peptides coeluting with the octamer in the B\*2705-bound peptide pool were also in the fractions analyzed from B\*2701 (data not shown). This suggests that the octamer is not bound *in vivo* to B\*2701. Thus, failure of CTL 27S69 to cross-react with B\*2701 LCL is probably due to absence of the peptide epitope on this subtype. In peptide sensitization assays, significant lysis of B\*2701-RMA-S cells was obtained with the octamer (half-maximal lysis at 10<sup>-6</sup> M) and, about 100-fold lower, with the nonamer (Fig. 6B). In addition, B\*2701-C1R cells were highly sensitized with the octamer (half-maximal lysis at 1.7 × 10<sup>-8</sup> M) (Fig. 6C). These re-

sults indicate that RRFPPYYV is efficiently recognized by CTL 27S69 in the context of B\*2701, further supporting that lack of cross-reaction with this subtype is due to lack of constitutive binding of the peptide to B\*2701 *in vivo*.

#### *Molecular modeling of the allospecific epitope recognized by CTL 27S69*

The molecular basis for the capacity of CTL 27S69 to distinguish between the RRFPPYYV and RRFPPYYVY ligands was addressed by molecular modeling of both peptides in complex with B\*2705. They were stabilized in the peptide-binding groove by numerous hydrogen bonds and nonpolar interactions. However, whereas hydrogen bonding was quantitatively similar in both complexes, the nonamer established significantly more nonpolar contacts (total of 72) than the octamer (total of 53), mainly because of additional interactions of Tyr9 in the F pocket. The total buried surface areas for the octamer and the nonamer bound to B\*2705 were 732 and 812 Å<sup>2</sup>, respectively. These results are consistent with the better binding of the nonamer to B\*2705 (Fig. 3).

The differential recognition of the octamer and nonamer by CTL 27S69 can be explained by the different conformations of both peptides in complex with B\*2705 (Fig. 7). Although the accessible surface area was only slightly higher for the octamer (539 Å<sup>2</sup>) than for the nonamer (506 Å<sup>2</sup>), the conformation of the main chain was different, especially in the central part of the sequence (P4-P5),



**FIGURE 6.** A, Binding of RRFFPYYV (●), RRFFPYYVY (■), and RRIKEIVKK (▲) as negative control to B\*2701 on RMA-S cells.  $EC_{50}$  was 1 and 7  $\mu$ M for the nonamer and the octamer, respectively. Data are means of two experiments. B, Lysis of B\*2701-RMA-S cells sensitized with RRFFPYYV and RRFFPYYVY, by CTL 27S69. Half-maximal lysis was obtained at  $1.2 \times 10^{-6}$  M of the octamer. The same lysis required more than  $10^{-4}$  M of the nonamer. The KRGILTLY peptide, a B\*2702 ligand that binds efficiently to B\*2701 (26), was used as negative control. Lysis of B\*2705-C1R cells at the E:T ratio used (0.9:1) was 74%. Data are means of three experiments. C, Lysis of B\*2701-C1R cells sensitized with RRFFPYYV or KRGILTLY by CTL 27S69. Half-maximal lysis was obtained at  $1.7 \times 10^{-8}$  M. Lysis of B\*2705-C1R cells at the E:T ratio used (2:1) was 56%. Data are means of three experiments. For experimental details, see *Materials and Methods*.

where it bulged out more prominently in the nonamer. Besides Arg1, which was partially accessible and similarly oriented in both peptides, three other side chains (Phe4, Pro5, and Tyr7) were accessible for the octamer, and four (Phe4, Pro5, Tyr7, and Val8) for the nonamer. Phe4 was rather similarly oriented in both cases. In contrast, Pro5 bulged out much more for the nonamer. In addition, a major qualitative difference occurred for Tyr7. In the nonamer, its side chain was located in a three-dimensional space not occupied by any atom of the bound octamer. Val8 was exposed for the nonamer. In the octamer, this residue is directed inward into the F pocket in the same way as Tyr9 in the nonamer, although the larger Tyr9 side chain gets deeper into this pocket.

In conclusion, molecular modeling provides an explanation for the moderate cross-reactivity between the octamer and the nonamer in complex with B\*2705. Conserved contacts with the TCR might be provided by the B\*2705 molecule, and by Arg1 and Phe4 in both peptides. The substantial differences in the conformation and/or accessibility of the Pro5, Tyr7, and Val8 side chains explain the weaker cross-reaction with the nonamer.

## Discussion

Molecular studies on alloreactivity are hampered by the difficulty of identifying the peptides involved in allospecific T cell epitopes. A natural ligand specifically recognized by an alloreactive CTL clone raised against B\*2705 was reported in this work. Aside from a peptide with an unclear relationship to the natural epitope (39), this is, to our knowledge, only the second naturally presented peptide identified as the epitope of an alloreactive CTL, and the first one from a known protein. It is also the first HLA-B27 ligand shown to be immunogenic in allospecific T cell responses. Its identification was possible through combining peptide sensitization of TAP-deficient target cells, and sequencing by electrospray/ion trap mass spectrometry. This is a powerful methodology, which adds to others, such as triple quadrupole (8, 40–42) and quadrupole/time of flight (43) electrospray mass spectrometry, as a suitable one for sequencing antigenic peptides eluted from class I MHC molecules.

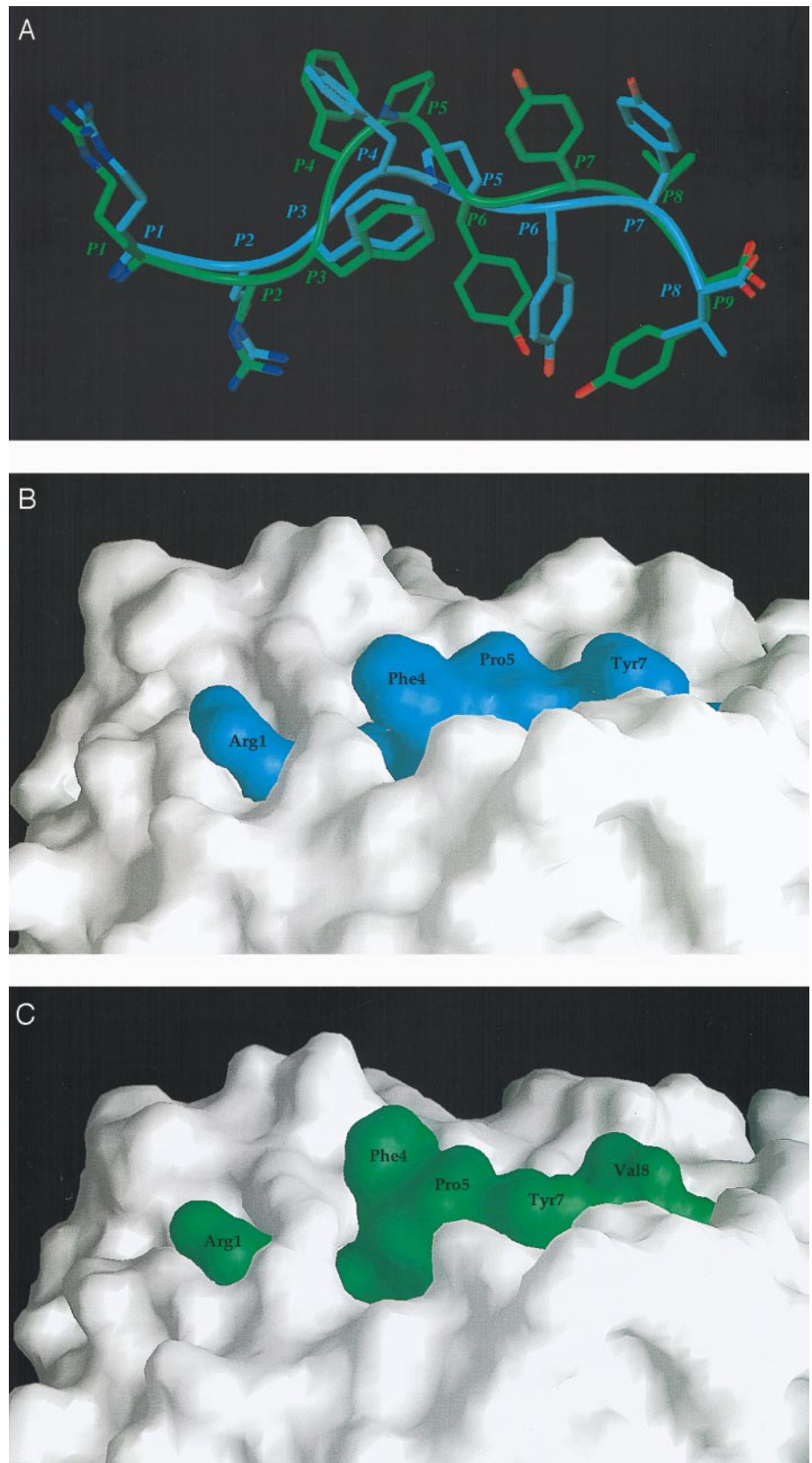
The RRFFPYYV octamer derives from the C5 subunit of the proteasome, which is abundant in the cell (44), and was recovered from the B\*2705-bound peptide pool in relatively substantial amounts. However, both abundant (9) and nonabundant (8, 11) peptides can stimulate alloreactive CTL. This epitope probably has

high affinity for the TCR of CTL 27S69, since this clone can efficiently lyse murine B\*2705-P815 (our unpublished results) and, in the presence of added peptide, RMA-S transfectant cells. Both TCR affinity and expression level of this peptide may contribute to its alloimmunogenicity. A minimum threshold of avidity is probably required to stimulate a given allo-CTL precursor. This threshold could be reached at low determinant density through high TCR affinity, or with high epitope expression in cases of lower affinity. Since serial TCR engagement by relatively few MHC-peptide complexes may be a mechanism of T cell triggering (45), non-abundant peptides may be allostimulatory. The requirements for alloimmunogenicity are important because they dictate the diversity of peptide epitopes in an allospecific T cell response. For instance, among the many class I-bound peptides involved in alloreactivity (1, 8), some can be immunodominant (13). The RRFFPYYV peptide was not recognized by three other anti-B\*2705 clones tested, including one from the same donor (data not shown), but we cannot rule out that it might be one of a relatively limited set of peptides driving the anti-B\*2705 response. This was suggested by restrictions in N+D $\beta$  and J $\alpha$  usage among B27-allospecific TCR (46).

Two aspects of this study provide novel insights into the molecular basis of T cell allorecognition. One is that another natural ligand closely related to the octameric epitope allowed us to assess the capacity of a CTL clone to distinguish between similar peptides in an allo-MHC context. The second aspect is the knowledge of the fine specificity of the CTL clone with HLA-B27 subtypes (20). This allowed us to analyze the relationship between expression and recognition of the peptide epitope in the context of different allo-MHC molecules, and therefore to assess the role of HLA polymorphism in allorecognition, aside from its effects on peptide binding.

Although self-restricted CTL are sensitive to subtle peptide changes, the capacity of alloreactive CTL to distinguish between related ligands could be limited by a more prominent contribution of MHC residues to the allospecific determinant. Cross-reaction of CTL 27S69 with the RRFFPYYVY nonamer might be due, on the basis of modeling, to conservation of a portion of the epitope involving the MHC molecule and the peptidic Arg1 and Phe4. However, conformational and accessibility differences in the P5-P8 region explain the better recognition of the octamer. Thus, the





**FIGURE 7.** A, Overlay of the B\*2705-bound conformations of RRFFPYYV and RRFPPYYVY. Backbone atoms of the bound peptide are displayed as ribbon tubes (cyan, RRFFPYYV; green, RRFPPYYVY). Peptide side chains are represented by sticks with the following color coding (blue, nitrogen; red, oxygen; cyan, carbon atom of RRFFPYYV; green, carbon atom of RRFPPYYVY). C $\alpha$  atoms of the peptides are labeled from the N terminus (P1) to the C terminus (P8 or P9). The B\*2705 protein is not displayed. The figure has been prepared using the MOLMOL program (58). B, Side view, from the  $\alpha$ 2-helix toward the  $\alpha$ 1-helix, of B\*2705 (white) in complex with RRFFPYYV (cyan). A TCR- $\alpha\beta$  would bind diagonally across the binding groove (22) at the top of the figure. Both protein and peptide are represented by molecular surfaces, computed and displayed by the GRASP program (59). Only TCR-accessible peptide positions are labeled. C, Side view of B\*2705 (white) in complex with RRFPPYYVY (green).

different alloantigenicity of these highly similar ligands illustrates the critical role of peptide conformation in alloreactivity.

CTL cross-reacting with other MHC Ags do not necessarily recognize the same peptide in the various contexts. For example, the murine alloreactive CTL clone 2C recognized three unrelated pep-

tides in the context of L<sup>d</sup>, K<sup>bm3</sup>, and, in self-restricted fashion, K<sup>b</sup>, respectively (9, 17, 18). Although CTL cross-reactions with HLA-B27 subtypes were assumed to reflect similarities among subtype-bound peptide repertoires (20, 47), recognition of the same peptide by CTL 27S69 on B\*2705, B\*2702, and B\*2703 provides the first

formal demonstration of this assumption. It also indicates that the changes between B\*2702 or B\*2703 with B\*2705 (Y→H59 in B\*2703; D→N77, T→I80, and L→A81 in B\*2702) are not critical for this epitope. Presentation of the same viral peptide to B\*2705-restricted CTL by B\*2702 has been shown for EBV-specific T cells (48, 49). However, 7 of 10 B\*2705-restricted CTL clones failed to recognize the viral epitope in the context of B\*2702 (49). Thus, although not for CTL 27S69, polymorphic B\*2702 residues can alter some T cell epitopes without impairing binding of the corresponding peptide.

From the results with B\*2702 and B\*2703, it should not be concluded that, upon expression of the relevant peptide, the structure of the alloantigen is not critical for allorecognition. Indeed, in spite of good binding, the RRFFPYV octamer was not recognized in the B\*2704, B\*2706, or B\*2710 context. Direct impairment of TCR binding by the E152 residue in these three subtypes is strongly supported by the fact that this is the only change between B\*2710 and B\*2705. We have reported recently that, in spite of little cross-reaction of anti-B\*2705 CTL with B\*2710, both subtypes bind *in vivo* similar peptide repertoires. Molecular modeling further suggested that the E152 change in B\*2710 did not alter the conformation of bound peptides, but directly impaired TCR interaction (35). CTL 27S69 now provides the first example of an anti-B27 alloreactive CTL clone that fails to recognize its peptide epitope across the V→E152 change. Therefore, besides the peptide, the structure of HLA-B27 directly and critically contributes to the allospecific epitope. The contribution of the MHC molecule to allorecognition is likely to severely limit cross-reactivity between class I alloantigens that bind common peptides (50).

Cross-reaction of CTL 27S69 with B\*2701 only in the presence of exogenous peptide strongly suggests that the octamer epitope is not endogenously presented by this subtype, in spite of significant binding *in vitro*. This was supported by failure to detect the octamer in the B\*2701-bound peptide pool by mass spectrometry. Since B27-bound peptides were isolated from C1R transfectants both for B\*2701 and other subtypes, it is extremely unlikely that the octamer is not generated in B\*2701-C1R transfectants due to a defect of these cells, independently of B\*2701. Since the octamer bound similarly *in vitro* to B\*2701 and B\*2705 (EC<sub>50</sub> 7 and 8 μM, respectively), it also seems unlikely that failure to bind *in vivo* could be due to disadvantageous competition with other ligands for binding to B\*2701, relative to B\*2705. A possibility that we favor is that the octamer might be generated by trimming of the HLA-B27-bound nonamer, rather than by proteasome-mediated cleavage, and that in B\*2701 such trimming is impaired. This is suggested by a report that MHC class I molecules influence the precise structure of endogenous ligands (51). Since the RRFFPYVYV nonamer is naturally presented by B\*2701 (26), lack of cross-reaction with B\*2701 indicates that the avidity of CTL 27S69 for the constitutive B\*2701 + nonamer complex is insufficient for lysis.

In conclusion, identification of a peptide epitope involved in HLA-B27 alloreactivity allowed us to establish that T cell allorecognition is very dependent both on the precise structure of the peptide, as to discriminate between closely related natural ligands, and on the structure of the MHC molecule. This may either influence expression of the peptide at the cell surface, or directly impair TCR binding.

The findings reported in this work have implications for the pathogenetic role of HLA-B27 in ankylosing spondylitis and other spondyloarthropathies. Among other possible mechanisms, the arthritogenic peptide hypothesis proposes that peptide(s) presented by HLA-B27 would be recognized by autoreactive CTL activated upon external challenge, such as a bacterial infection (52). This

hypothesis must explain that multiple subtypes, such as B\*2705, B\*2702, and B\*2704, are associated with ankylosing spondylitis (53), whereas B\*2706 and B\*2709 are less or not associated with this disease (54, 55). Presumably, disease-associated subtypes should present some common peptides to CTL. Our results demonstrate that B\*2705 and B\*2702 present the same peptide to an alloreactive CTL, as they do to some EBV-restricted CTL clones (49). A potential problem arises from the fact that B\*2704, although it binds some of the same ligands as B\*2705 and B\*2702 (25, 56), cannot present those that have been tested to the same CTL, as shown in this work and for EBV epitopes (48, 49). Cross-reaction of some anti-B\*2705 alloreactive CTL clones with B\*2704 or with B\*2704 plus B\*2702, respectively (20), suggests that, in spite of residue 152, some peptides might be presented by all three B\*2705, B\*2702, and B\*2704 subtypes to the same CTL. However, this awaits molecular identification of the corresponding peptide epitopes. There are at least two possibilities that make compatible the peptide-presenting properties of these three subtypes with their association to spondyloarthropathy. One is that putative arthritogenic peptides may be different for different subtypes. A second possibility, that might seem more likely, is that the same peptide is recognized in the context of different subtypes by arthritogenic CTL.

Although, obviously, alloreactive CTL are not related to B27-mediated spondyloarthropathy, their recognition of a same peptide in the context of different subtypes illustrates the extent to which HLA-B27 subtypes may interchangeably act as restriction elements for given peptides. This feature would be critical for a putative pathogenetic role of HLA-B27 as a peptide-presenting molecule.

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