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# A Patient-Derived Cytotoxic T-Lymphocyte Clone and Two Peptide-Dependent Monoclonal Antibodies Recognize HLA-B27-Peptide Complexes with Low Stringency for Peptide Sequences

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**HLA-B27 molecules expressed on the T2 mutant cell line do not have peptides. Such empty HLA-B27 molecules were not recognized by an HLA-B27-restricted cytotoxic T-lymphocyte (CTL) clone (auto-1) derived from synovial fluid. To test for peptide dependency of the clone, B27-T2 cells were incubated with a panel of 48 synthetic peptide nonamers in which P2 was arginine. Target cell lysis was induced by seven completely different peptides. This lack of stringency was compared with that of a peptide-dependent monoclonal antibody, B27.M2. Positive B27.M2 reactivity resulted when the B27-T2 cells were incubated with two peptides: RRKAMFEDI and RRMGPPVGHR, derived from *Chlamydia* HSP60 and human ribonucleoprotein, respectively. Because of the limited availability of CTL versus monoclonal antibody, the specificity of B27.M2 was studied in greater detail. The importance of the HLA-B27 heavy chain in antibody recognition of class I-peptide complexes was demonstrated by site-directed mutagenesis. The stringency of the peptide residues was tested by making analogs of each of the nine residues in RRKAMFEDI, creating a panel of 180 analogs. Although stringency was highest for the sixth position, as many as six different amino acids provided positive reactivity. These results indicate that immune recognition of HLA-B27–peptide complexes might have rather low stringency for the peptide sequences. In theory, then, pathogen-derived peptides which induce autoimmunity by generating autoreactive CTL might not share much sequence similarity with the responsible self peptides.**

The seronegative spondyloarthropathies, Reiter's syndrome and reactive arthritis, are closely associated with the major histocompatibility complex (MHC) class I molecule HLA-B27 and pathogenic bacteria, including the genera *Salmonella*, *Shigella*, *Yersinia*, and *Chlamydia*. The mechanisms responsible for this association remain undefined; however, a direct role for HLA-B27 has been postulated, including the possibility that autoreactive components such as serum antibodies and $CD8^+$ cytotoxic T lymphocytes (CTLs) recognize certain disease-specific MHC-peptide combinations (1, 17, 21). A role for bacteria in this process can be postulated either on the basis that these peptides are derived from bacterial antigens processed by the class I pathway or on the basis that the inflammatory response to infection induces presentation of cryptic endogenous peptides. Either of these possibilities could lead to the recognition of characteristic HLA-B27–peptide complexes by disease-related CTLs. The antigenic properties of MHC class I-peptide complexes have been studied with either CTL clones or monoclonal antibodies and mutant cell lines such as T2 and RMAS, which contain defects in the pathways for transport of peptides into the endoplasmic reticulum (24). Without the stability provided by bound peptides, T2 cells transfected with HLA-B27 express low levels of empty HLA-B27, which can bind exogenously added peptides of appropriate size and sequence (13, 33). These mutant cell lines transfected with human and mouse MHC class I have been used to demonstrate that certain monoclonal antibodies recognize MHC class I in a peptide-dependent manner, in that both peptide and MHC heavy chain appear to be involved in recognition (9, 23, 31). With several anti-H-2 monoclonal antibodies, the reactivity is influenced by changes in buried peptide residues. In other cases, multiple peptides of apparently unrelated sequences can lead to the same antibody specificity. Although antibodies and T-cell receptor probably interact differently with the MHC class I-peptide complex, similar observations have been made with several types of alloreactive CTL clones (2–4, 6, 8, 25, 26, 28).

We recently found that when synovial fluid T lymphocytes from patients with postinfectious spondyloarthropathies were cultured with stimulatory cells experimentally infected with arthritis-causing bacteria, HLA-B27-restricted CTL clones were generated. Some of these clones were cytotoxic to autologous monocytes and B-cell lines and to multiple $HLA\text{-}B27^+$ allogeneic B-cell lines but did not react with B27-T2 cells, which express empty B27 molecules, indicating that their reactivity was potentially peptide dependent (17). Because autoreactive CTLs and antibodies (21) with apparent peptide-dependent specificity have been found in spondyloarthropathy patients, understanding the factors that influence this type of recognition would have significant implications if extended to

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human disease-related HLA class I antigens such as the arthritis-predisposing HLA-B27.

In the present study, we screened a panel of peptides derived from eukaryotic, bacterial, and viral proteins by using B27-T2 cells to identify peptides that could induce positive reactivity of B27-T2 cells with patient-derived CTL clones. One of the CTL clones, designated auto-1, recognized HLA-B27 in the context of several peptides of different sequences. We used two peptide-dependent monoclonal anti-HLA-B27 antibodies, B27.M2 and Ye-2 (13, 33), to investigate in greater detail this ap-parent lack of stringency in HLA-B27 immune recognition. Because of limited cell numbers, detailed analysis of specificity was not possible with CTLs; however, analysis of the reactivity of the antibodies by using single-amino-acid substitutions of peptides and the HLA-B27 heavy chain indicated that the lack of peptide stringency was caused by multiple factors. These principles are important in the search for disease-related peptides.

## MATERIALS AND METHODS

**Cell lines.** The cell lines T2 and T2 stably transfected with the B*2705 gene (abbreviated B27-T2) were kindly provided by P. Cresswell, Yale University, New Haven, Conn. Mouse L cells stably transfected with both B*2705 and human $\beta_2$-microglobulin (abbreviated as B27-$\beta_2$m-L) were kindly provided by J. Taurog, University of Texas, Dallas. They were maintained as described previously (13).

**Antibodies.** Three monoclonal antibodies with relative specificity for HLA-B27 were used: ME1, B27.M2, and Ye-2 (7, 10, 15); the first is immunoglobulin G, and the other two are immunoglobulin M. All antibodies except Ye-2 were purchased from the American Type Culture Collection, Rockville, Md.

**Generation of CTL clones.** Generation of the CTL clones used in this study and their testing by the standard $^{51}$Cr release assay were reported previously (12, 17). The clones previously termed P6.2.36, P6.3.68, and P1.3.65 are designated auto-1, auto-2, and yer, respectively, in this paper. The participation of HLA-B27 and CD8 in CTL activity was verified by the inhibitory effect of anti-HLA-B27 and anti-CD8 antibodies, respectively (17).

**Peptide synthesis and loading onto cultured cells.** Two methods of peptide synthesis were used. The first was the standard F-moc chemical synthesis. These peptides were purified by high-performance liquid chromatography. In the other method, peptides were synthesized in 1-mg quantities on polyethylene rods (pin method) by Chiron Mimotopes, Clayton, Australia. The peptides were supplied in free form with free amino and carboxyl termini.

To load a peptide onto HLA-B27 for antibody analysis, approximately $3 \times 10^5$ to $5 \times 10^5$ cells were cultured at 37°C in 5% $CO_2$ for 14 to 18 h in a well of a 96-well plate containing 200 μl of Dulbecco's minimum essential medium supplemented with either 0.1% bovine serum albumin (BSA) or 10% fetal calf serum. For the evaluation of the peptide specificity of HLA-B27-restricted CTL, B27-T2 cells were first labeled with $^{51}$Cr as described previously (17), washed, and seeded into 96-well flat-bottom microtiter plates at 5,000 cells per well. These B27-T2 target cells were then cultured with the different peptides at a final concentration of 400 μg/ml for 1 h before the effector T cells were added.

**Flow microfluorometry analysis.** Cells were harvested and washed twice with Hanks' balanced salt solution containing 1% BSA and 0.2% sodium azide and incubated at 4°C for 60 min with saturating amounts of monoclonal antibodies in the form of culture supernatants. After being washed, the cells were incubated with *R*-phycoerythrin-conjugated F(ab′)$_2$ fragment of goat anti-mouse immunoglobulin G or M (Jackson Immunoresearch Labs, West Grove, Pa.) at 4°C for another 60 min. The cells were then washed and fixed with 2% paraformaldehyde in phosphate-buffered saline, and 2,500 cells per sample were analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) and the version B FACScan research software. Viable cells were gated by their light-scattering characteristics. The data were presented in two different forms: as a histogram on a log scale of fluorescence channels and as mean fluorescence intensity on a linear scale. Histogram data were generated by analysis of 5,000 viable cells, using an EPICS elite flow cytometer (Coulter Cytometry, Hialeah, Fla.).

**Plasmids used for mutation of the HLA-B27 gene.** Plasmids used for site-directed mutagenesis were provided by J. Taurog. By using an *Avr*II site, a genomic DNA fragment containing the HLA-B27 gene was subcloned into two fragments. The 5′ 2.2-kb fragment contained the exons for the leader sequence and the α1 and α2 domains. This was inserted into the *Eco*RI-*Xba*I sites of M13mp18. The plasmid was termed mB27mϕ-RA. The 3′ 2.3-kb fragment containing exons 4 to 7 was inserted into the *Xba*I-*Pst*I sites of pUC19. The plasmid was termed pB27mϕ-AP (29).

**Site-directed mutagenesis procedure.** The mutations produced in the B*2705 gene product were designated R62E, K70Q, R79G, and R83G to indicate a change in the numbered codon from the first to the second amino acid (e.g., in R62E, R62 was changed to E62). Reagents for oligonucleotide-directed in vitro mutagenesis were purchased from Amersham, Arlington Heights, Ill. The procedures followed the manufacturer's protocols as well as those of Taurog and El-Zaatari (29). DNA from mB27mϕ-RA was used as the template for mutagenesis. The oligonucleotides for mutants R62E, K70Q, R79G, and R83G were 5′CAGATCTGTGTCTCCTCGTCCCAATA3′, 5′AGTCTGTGCCTGGGCCTTGCA3′, 5′AGCAGGGTCCCCAGGTCCT3′, and 5′GTTGTAGTACCCGAGCAGGGT3′, respectively. The successfully generated mutants were selected by sequencing, using the protocol and reagents provided by the manufacturer (United States Biochemical Corp., Cleveland, Ohio). The sequencing primer, designed to sequence exon 2, was 5′GACCCGGGCCGTACGTG3′. The mutated fragment was excised from M13 with the *Eco*RI and *Hin*cII sites and ligated to the *Eco*RI-*Sma*I fragment of pB27mϕ-AP. The correctness of the sequencing was again confirmed before the mutated gene was transfected with calcium phosphate into the L cells for expression. During such sequencing, we found that in the particular mB27mϕ-RA clone used, the sequence encoding L82 was GTC and not CTC as in the parent genomic DNA. Hence, L82 in our parent HLA-B27 was V82 in this particular clone. This was a conservative change and did not affect the antibody reactivities. As a control for our designed mutations, we also ligated this fragment to pB27mϕ-AP. This spontaneous mutant was designated mutant L82V. This change was not observed in the parent genomic DNA before subclones were generated. The cell line transfected with that clone was designated the parent.

TABLE 1. Effect of peptides derived from *Yersinia* HSP60 on the susceptibility of B27-T2 cells to lysis by CTL clones[a]

| *Yersinia* peptide | Sequence | % Lysis with CTL clone: | | |
|---|---|---|---|---|
| | | auto-1 | auto-2 | Yer |
| 1 | DRGIDKAVI | 87.9 | 0 | 0 |
| 2 | DRGYLSPYF | 0 | 1.2 | 4.6 |
| 3 | IREMLPVLE | 0 | 5.4 | 0 |
| 4 | MRGIVKVAA | 0 | 1.9 | 0 |
| 5 | DRRKAMLQD | 0 | 4 | 0 |
| 6 | RRKAMLQDI | 0 | 0 | 0 |
| 7 | KRVVINKDT | 13 | 0 | 0 |
| 9 | GRVTQIRQQ | 0 | 0 | 2.6 |
| 10 | IRQQIEEAT | 0 | 0 | 0.3 |
| 11 | TRAAVEEGV | 0 | 0 | 0 |
| 12 | IRAASAITA | 45 | 0 | 45 |
| 13 | LRAMESPLR | 0 | 0 | 0 |
| 14 | LRQIVVNAG | 0 | 0 | 4.4 |
| 236 | ARIKMLRGV | 6.1 | ND[b] | 4.4 |
| 237 | LRGVNILAD | 5.3 | ND | 4.6 |
| 238 | GRNVVLDKS | 45.8 | ND | 5.1 |
| 239 | AREIELEKD | 6.5 | ND | 3.4 |
| 240 | TRSALQYAA | 4.3 | ND | 0 |
| 241 | PRDDKGADM | 1.3 | ND | 4.3 |
| 242 | GRRSLSLVV | 1.2 | ND | 12.9 |

[a] Effector/target ratio, 1:1.
[b] ND, not done.

## RESULTS

**Analysis of HLA-B27-restricted CTL clones for recognition of HLA-B27–peptide complexes.** Three HLA-B27-restricted CTL clones from synovial fluid of patients with reactive arthritis were tested. One of these clones, yer, was cytolytic only to HLA-B27 target cells infected in vitro with *Yersinia enterocolitica*. The other two clones, auto-1 and auto-2, were cytolytic to autologous monocytes and all HLA-B27 cell lines except B27-T2 (17). To identify a peptide which might induce the B27-T2 cells to become susceptible to killing by the auto-1 CTL clone, we synthesized 48 nonamers by the pin method. These peptides were derived from the sequences of the HSP60 protein of *Y. enterocolitica*, *Mycobacterium tuberculosis*, and humans. To provide the anchor residue for HLA-B27, the sequences of all these peptides carried arginine at P2. Peptides were used at a concentration of 400 μg/ml to sensitize B27-T2 cells and tested with the auto-1 clone at an effector/target ratio of 1:1. More than 30% lysis was observed for seven peptides

TABLE 2. Effect of peptides derived from human HSP60 on susceptibility of B27-T2 cells to the CTL clone auto-1[a]

| Human peptide | Sequence | % Lysis with CTL auto-1 |
|---|---|---|
| 207 | LRLPTVFRQ | 3.8 |
| 208 | FRQMRPVSR | 0 |
| 209 | SRVLAPHLT | 0 |
| 210 | TRATAKDVK | 1.6 |
| 211 | ARALMLQGV | 1.2 |
| 212 | GRTVIIEQS | 1.9 |
| 213 | ARSIAKEGF | 5.2 |
| 214 | IRRGVMLAV | 48.9 |
| 215 | GRKGVITVK | 3.7 |
| 216 | DRGYISPYF | 6.6 |
| 217 | NRLKVGLQV | 0 |
| 218 | NRKNQLKDM | 0 |
| 219 | KRIQEIIEQ | 39.9 |
| 220 | ERLAKLSDG | 5.5 |
| 221 | DRVTDALNA | 3.5 |
| 222 | TRAAVEEGI | 3.8 |
| 223 | LRCIPALDS | 4.5 |
| 224 | KRTLKIPAM | 60.7 |
| 225 | VRTALLDAA | 7.1 |

[a] Effector/target ratio, 1:1.

(Tables 1 to 3). The same seven peptides induced B27-T2 cells to become susceptible at an effector/target ratio of 0.5:1. Because cell availability was limited, the auto-2 clone was tested with only 26 peptides. More than 30% lysis was observed only with the peptide Inf (SRYWAIRTR) (Table 3). The yer clone was tested with 20 peptides derived from the *Yersinia* HSP60. Only *Yersinia* HSP60-derived peptide IRAASAITA induced susceptibility to lysis (Table 1).

In summary, at least seven different peptides sensitized B27-T2 cells to induce >30% lysis by the auto-1 clone at an effector/target ratio of 1:1. When the sequences of the seven peptides were aligned, the only common feature was that P2 was arginine (see Table 5).

**Positive B27.M2 antibody reactivity induced in B27-T2 cells by two peptides (Ch2 and Endo14) with sequences RRKAMFEDI and RRMGPPVGGHR, respectively.** To compare the degree of stringency of the CTL clones with that of the peptide dependent anti-HLA-B27 monoclonal antibody B27.M2, we synthesized a panel of 28 peptides according to the putative HLA-B27-binding motif (Table 4). Fifteen of the peptides were derived from eukaryotic proteins (20); 10 were from the *Chlamydia* HSP60. The peptides were incubated with B27-T2 cells and tested for reactivity with the B27.M2 antibody. At a peptide concentration of 1 mg/ml, reactivity with the B27.M2 antibody was highly positive with only two peptides: Ch2 and Endo14, with sequences of RRKAMFEDI and RRMGPPVG GHR, respectively (Fig. 1a; Table 5). The effects of these two peptides were also tested at 0.125, 0.25, 0.5, and 1.0 mg of peptide per ml. For Ch2, these are equivalent to 43.7, 87.3, 436, and 872 μM, respectively. Near plateau antibody reactivity was observed with the two highest concentrations (Fig. 1b). For Ch2, we also tested the variability of the experimental conditions by culturing cells in triplicate with 1.0 mg of the peptide per ml. The highest and lowest fluorescence intensities in the three samples were within 5% of the average value.

All of the peptides except Ch4, Ch7, Ch10, and the control induced stable expression of HLA-B27, as demonstrated by their ability to induce B27-T2 cells to become reactive with the ME1 antibody. ME1 reactivity is influenced only by the increased expression of HLA-B27 caused by the presence of binding peptides but not by the peptide sequences (13, 33). We compared the stabilizing activity of Ch2 and Endo14 by using a range of concentrations of several endogenous peptides and

TABLE 3. Effect of peptides on the susceptibility of B27-T2 cells to CTL clones[a]

| Peptide | Sequence | % Lysis with CTL: | |
|---|---|---|---|
| | | auto-1 | auto-2 |
| *M. tuberculosis* | | | |
| 1 | HRIEDAVRN | 0 | 0 |
| 2 | RRGLERGLN | 23.7 | 0 |
| 3 | GRNVVLEKK | 0 | 0 |
| 4 | RRKAMLQDM | 2.3 | 0 |
| 5 | ARRGLERGL | 2.7 | 0 |
| 6 | LRNVAAGAN | 0 | 0 |
| 8 | ERLAKLAGG | 0.4 | 0 |
| 9 | VREGLRNVA | 2.3 | 0 |
| 10 | GRNVVLEKK | 1.1 | 0 |
| Listed in Table 4 | | | |
| Endo1 | RRIKEIVKK | 0 | 6.2 |
| Endo2 | RRVKEVVKK | 1.1 | 4.4 |
| Endo15 | RRYQKSTEL | 0.6 | 4.5 |
| Inf | SRYWAIRIR | 41.7 | 38.4 |

[a] Effector/target ratio, 1:1.

TABLE 4. Peptides screened for their ability to induce positive antibody reactivity with B27-T2 cells

| Peptide designation | Parent protein[a] | Sequence |
|---|---|---|
| Ch1 | *Chlamydia* HSP60 | KRGIDKAAK |
| Ch2 | *Chlamydia* HSP60 | RRKAMFEDI |
| Ch3 | *Chlamydia* HSP60 | ARKKIQKGV |
| Ch4[b] | *Chlamydia* HSP60 | GRHVVIDKS |
| Ch5 | *Chlamydia* HSP60 | LRNVTAGANPM |
| Ch6 | *Chlamydia* HSP60 | ARCESIKKQI |
| Ch7[b] | *Chlamydia* HSP60 | NRGYLSSYF |
| Ch8 | *Chlamydia* HSP60 | NRIRGGFRV |
| Ch9 | *Chlamydia* HSP60 | IRGGFRVCA |
| Ch10[b] | *Chlamydia* HSP60 | FRVCAVKAPG |
| Endo1 | HSP89 alpha | RRIKEIVKK |
| Endo2 | HSP89 beta | RRVKEVVKK |
| Endo3 | Elongation factor 2 | RRWLPAGDA |
| Endo4 | ATP-dependent helicase | RRSKEITVR |
| Endo5 | Yeast ribosomal protein | GRIDKPILK |
| Endo6 | Rat 60S ribosomal protein L28 | FRYNGLIHR |
| Endo7 | Unidentified | KRFEGLTQR |
| Endo8 | Unidentified | RRISGVDRY |
| Endo9 | Breast basic conserved protein | ARLFGIRAK |
| Endo10 | Rat ribosomal protein L36 | GRLTKHTKF |
| Endo11 | Immediate-early response gene product | RRFGDKLNF |
| Endo12 | Cytochrome P-450 | TRYPILAGH |
| Endo13 | Cattle C-kinase substrate | KRFSFKKSF |
| Endo14 | Ribonucleoprotein L | RRMGPPVGGHR |
| Endo15 | Histone H3 | RRYQKSTEL |
| Inf | Influenza virus nucleoprotein | SRWAIRTR |
| HIV | HIV[c] gp120 | RIQRGSGRAFVTIGK |
| Control[b] | HLA-B27 | AQTDREDLR |

[a] Ch1 to Ch10 peptides were derived from the sequence of *Chlamydia* HSP60 (5). Endo1 to Endo15 were derived from sequences of peptides eluted from HLA-B27 (24). Unless otherwise noted, proteins are from human sources.

[b] Peptides that did not stabilize HLA-B27 as defined by a greater-than-twofold increase in ME1 staining intensity over B27-T2 with no peptide as measured by flow cytometry.

[c] HIV, human immunodeficiency virus.

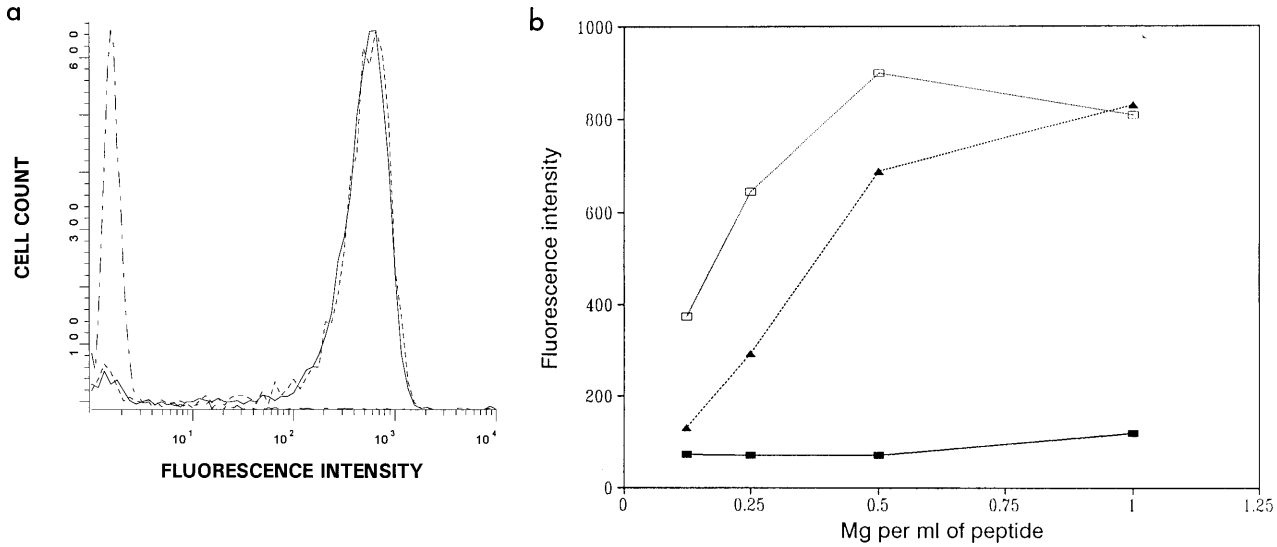


FIG. 1. (a) Histograms of flow cytometry analysis of B27-T2 cells and B27.M2 antibody conducted with Ch2 (solid line) and Endo14 (dashed line) peptides or without (broken line) preincubation of cells with peptide. (b) Reactivity of B27-T2 cells with the B27.M2 antibody after incubation with peptides Ch2 (RRKAMFEDI), Endo14 (RRMGPPVGGHR), and, as control, Endo1 (RRIKEIVKK). Cells were incubated with peptides over a range of concentrations before being tested with the B27.M2 antibody. Symbols: □, B27-T2 cells plus peptide Endo14; ▲, B27-T2 cells plus peptide Ch2; ■, B27-T2 cells plus peptide Endo1.

the ME1 antibody. Endo1 and Endo2 showed at least a 10-fold-greater ability than did Ch2. To ensure that the increases in antibody binding were dependent on HLA-B27, we incubated peptides with the T2 cells that had not been transfected with the HLA-B27 gene. No reactivity was observed with the ME1 or the B27.M2 antibody.

**Effect of consecutive substitutions at P1 to P9 with 19 amino acids on B27.M2 reactivity.** A set of analogs based on the sequence of peptide Ch2, synthesized by the pin method, were incubated with B27-T2 cells. In the first series, the arginine at P2 was substituted by 19 other amino acids. As expected from the known motif of HLA-B27-binding peptides (23, 27, 34), reactivity was highest with arginine in P2 (Fig. 2a). The reactivities of residues other than arginine were <30% of the value with the parent peptide. Taking into account the variability of individual experiments, in subsequent analyses with peptides synthesized by this pin method, all values <40% of those with the parent peptides were arbitrarily regarded as negative.

The above strategy was extended to all of the other residues of Ch2 (Fig. 2). Almost total promiscuity was observed with P3, P5, and P7, in which 19 to 20 of the analogs induced positive reactivity. A slightly higher degree of stringency was observed with P1 and P4, in which 16 of 20 analogs induced positive reactivity. The highest degree of stringency was observed with P6, P8, and P9; however, at each of these positions, substitutions that led to positive reactivity were not related in a predictable manner to the properties of the amino acids.

TABLE 5. Sequences of peptides that induced high reactivity

| Peptide | Sequence |
|---|---|
| Induction of >30% lysis by auto-1 | |
| 214 | IRRGVMLAV |
| 219 | KRIQEIIEQ |
| 224 | KRTLKIPAM |
| 238 | GRNVVLDKS |
| 1 | DRGIDKAVI |
| 12 | IRAASAITA |
| Inf | SRYWAIRTR |
| Induction of positive reactivity with Ye-2 | |
| Endo15 | RRYQKSTEL |
| HIVgp120 | RIQRGSGRAFVTIGK |
| Induction of positive reactivity with B27.M2 | |
| Ch2 | RRKAMFEDI |
| Endo14 | RRMGPPVGGHR |

As negative controls, all of the analogs were incubated with T2 cells that had not been transfected with HLA-B27. When assayed with the B27.M2 antibody, the reactivity was uniformly negative. Positive controls were reported in those previous experiments in which peptide analogs were incubated with B27-T2 cells and tested with the peptide-independent ME1 antibody. They showed stringency for arginine at P2, as expected, but almost total promiscuity at other positions, including P6, P8, and P9 (34).

**Testing both Ye-2 and B27.M2 antibodies with HLA-B27 mutants.** We reported earlier that the Ye-2 antibody became more reactive with HLA-B27-transfected L cells when they were cultured in the presence of peptides Endo15 and HIV (Table 5). To assess the role of the HLA-B27 heavy chain in B27.M2 and Ye-2 reactivity, we generated four heavy-chain mutants designated R62E, K70Q, R79G, and R83G. These positions were chosen because previous experiments suggested that Ye-2 reacts with cationic residues (35). After completing the mutagenesis procedures, during sequencing for verification, we found that in the particular HLA-B27 subclone used to generate the mutants, the codon coding for L82 has a spontaneous mutation to one coding for V82. Because of that, in all the above four mutants, residue 82 was valine instead of leucine. The spontaneous mutant itself was designated L82V. These mutant genes were transfected into β2m-L cells.

Results of testing these mutants with antibodies are shown in Fig. 3. The cells reacted differently with B27.M2 and Ye-2. Ye-2 did not react with the parent or any of the mutants. B27.M2 reacted negatively with R62E and weakly with R79G; the other mutants gave a more intense reaction. The effect of peptides on antibody reactivity was also different. Endo15 peptide (RRYQKSTEL) induced reactivity of Ye-2 only in the

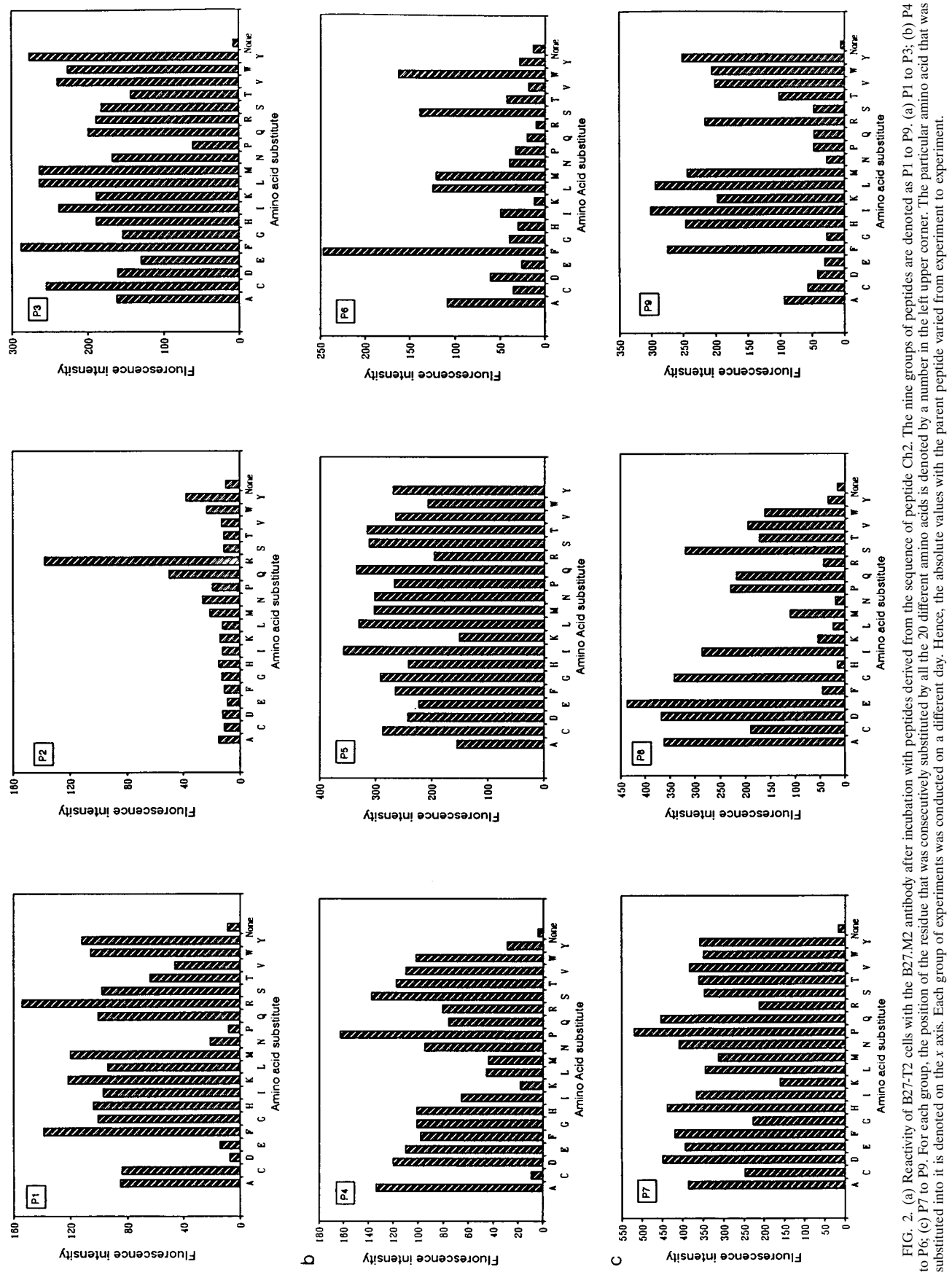


FIG. 2. (a) Reactivity of B27-T2 cells with the B27.M2 antibody after incubation with peptides derived from the sequence of peptide Ch2. The nine groups of peptides are denoted as P1 to P9. (a) P1 to P3; (b) P4 to P6; (c) P7 to P9. For each group, the position of the residue that was consecutively substituted by all the 20 different amino acids is denoted by a number in the left upper corner. The particular amino acid that was substituted into it is denoted on the *x* axis. Each group of experiments was conducted on a different day. Hence, the absolute values with the parent peptide varied from experiment to experiment.

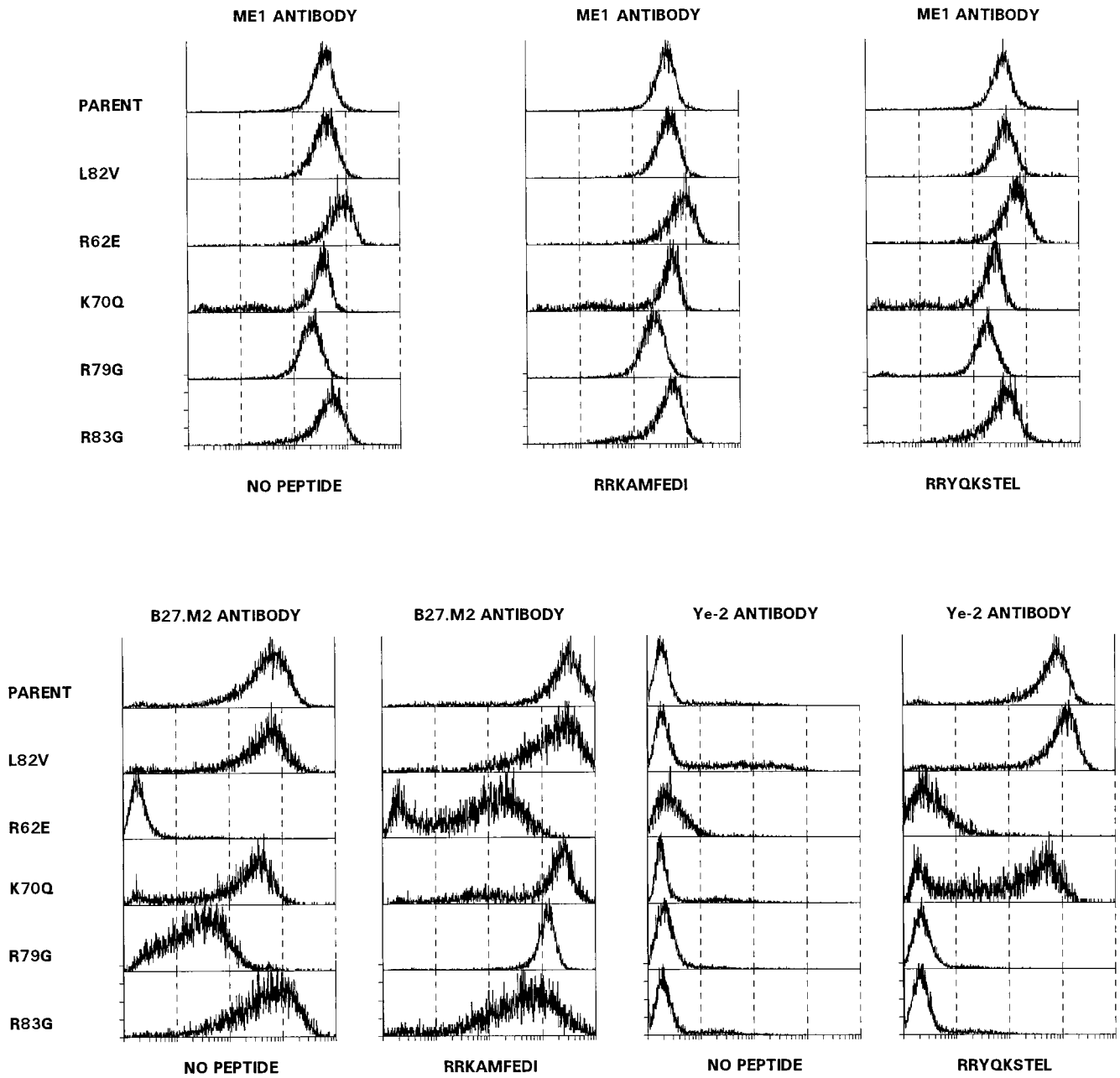


FIG. 3. Histograms of flow cytometry analysis of L cells transfected with HLA-B27 mutants and then preincubated with peptide Ch2 (RRKAMFEDI) for testing the B27.M2 antibody and peptide Endo14 (RRYQKSTEL) for testing the Ye-2 antibody. As controls, cells incubated with these peptides were also tested with the ME1 antibody.

parent and the L82V and K70Q mutants. Ch2 peptide (RRKA MFEDI) induced increased reactivity of B27.M2 in the parent and all the mutants. The reactivity was lowest with R62E. All positive control cells were highly reactive with the ME1 antibody.

## DISCUSSION

In this study, we describe an HLA-B27-restricted CTL clone derived from the synovial fluid of an HLA-B27-positive arthritis patient. It is peculiar among most described CTL clones because of its very low stringency for antigenic peptides. Such low-stringency recognition seems to be common among anti-MHC class I antibodies. Using the anti-HLA-B27 monoclonal antibody B27.M2, we analyzed the principles governing an analogous type of stringency. Our strategy was based on the observation that in complexes between antibodies and protein antigens and between HLA-DR1 and the *Staphylococcus aureus* enterotoxin B superantigen (19), pairs of contact residues derived from the two individual components are highly complementary. The complexes will be disrupted even if a single critical residue is substituted. We designed the sequential substitutions in the Ch2 peptide to identify such critical residues for interaction between HLA-B27 and the B27.M2 antibody. The positions of highest stringency were identified to be at P6,

P8, and P9. Unlike P6 and P8, 95% of the main-chain atoms of P9 are buried. The remaining 5% are also inaccessible (23). Hence, P9 cannot be a residue in contact with the B27.M2 antibody. Since P6 and P8 are partially exposed, they are theoretically in direct contact with the antibody. However, our results indicate that this is also unlikely, because a considerable number of substitutions were tolerated at each of these two positions. The amino acids which led to positive reactivity did not share uniform physical characteristics and thus did not offer the complementarity expected for the contact residues in the antibody molecule. These findings parallel those we reported for the Ye-2 antibody, in which we observed peptide stringency with the exposed P8 and the buried P9. Again, the several substituted amino acids at P8 which led to positive Ye-2 reactivity did not share uniform physical characteristics (13).

These peptide residues probably influence antibody reactivity by inducing conformational changes. The induced changes are either in the orientation of the exposed side chains of the heavy-chain residues neighboring the peptide residue or in the conformation of other peptide residues such as P5 and P6. In HLA-B27, these two peptide residues make very few hydrogen bonds with the heavy chain and so are freer to adopt various conformations. Both types of conformational changes were described when MHC class I molecules complexed with several different peptides were compared by crystallography (11, 22).

Peptide conformation alone may not completely explain how peptides of very different sequences can induce reactivity with the same antibody. The experiments with HLA-B27 mutants addressed an alternative possibility. Some antibodies may be influenced by peptides, because at certain positions, a variety of exposed and bulky peptide residues lead to steric hindrance, preventing the Fab from contacting the heavy-chain residues (18). On the basis of the observation that Ye-2 reacts with free synthetic peptides containing clusters of cationic amino acids (35), we generated mutants designed to change the positively charged R62, K70, R79, and R83 on the $\alpha$1 helix. None of these residues were oriented to affect peptide-binding ability. The reactivity profiles with these mutants indicate that all three arginine residues might be important to Ye-2. In contrast, only R62 and R79 led to changes in reactivity with B27.M2. The importance of the "right-hand side" of the $\alpha$1 helix in B27.M2 reactivity was reported previously (30). In both cases, these changes were greatly affected by addition of peptide, suggesting the importance of both heavy-chain and peptide residues.

The principles discussed here may be similar to those involving reactivity of the CTL clone auto-1, which was derived from a patient with HLA-B27-related arthritis and recognized HLA-B27 in the context of several different peptides. The HLA-B27 antigen is prevalent in patients with ankylosing spondylitis and bacterially induced reactive arthritis. Because of this prevalence and because HLA-B27 transgenic rats can also develop arthritis (16), it is accepted that HLA-B27 itself is a disease-causing gene. Equally important, in some patients, fragments of bacteria have been found in the inflamed joints (14). It has been postulated that the disease is induced by CTLs which fail to distinguish HLA-B27 complexed with pathogen-derived peptides from those complexed with self peptides. This leads to the hypothesis that some pathogen-derived peptides are very similar in sequence to certain self peptides (1). In such a case, HSP60-derived peptides would be likely candidates, because there is a high degree of homology of the HSP60 family among species. However, if disease-related CTLs recognize HLA-B27 with the lack of stringency described here, the pathogen-derived peptides and the self peptides might share little sequence similarity. The degree of predictability is probably even smaller than our experiments suggest. We selected peptides based on the presence of arginine at P2, which is usually required for nonamers to bind to HLA-B27. A study of longer peptides indicates that a subset of HLA-B27-bound peptides does not follow this motif (32). Also, the use of peptides based on sequence analysis criteria does not take into account whether the peptides occur naturally as a result of antigen processing in vivo. Factors that affect processing mechanisms might bias the repertoire of peptides associated with HLA-B27 on the basis of criteria not apparent in sequence analysis. All of these factors should be taken into consideration in future studies of disease-related HLA class I-restricted CTLs.

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## REFERENCES

1. **Benjamin, R. J., and P. Parham.** 1990. Guilt by association: HLA-B27 and ankylosing spondylitis. Immunol. Today **11:**137–142.
2. **Bluestone, J. A., S. Jameson, S. Miller, and R. Dick II.** 1992. Peptide-induced conformational changes in class I heavy chains alter major histocompatibility complex recognition. J. Exp. Med. **176:**1757–1761.
3. **Bluestone, J. A., A. Kaliyaperumal, S. Jameson, S. Miller, and R. Dick II.** 1993. Peptide-induced changes in class I heavy chains alter allorecognition. J. Immunol. **151:**3943–3953.
4. **Catipovic, B., J. D. Porto, M. Mage, T. E. Johanson, and J. P. Schneck.** 1992. Major histocompatibility complex conformational epitopes are peptide specific. J. Exp. Med. **176:**1611–1618.
5. **Cerrone, M. C., J. J. Ma, and R. S. Stephens.** 1991. Cloning and sequence of the gene for heat shock protein 60 from *Chlamydia trachomatis* and immunological reactivity of the protein. Infect. Immun. **59:**79–90.
6. **Chattopadhyay, S., M. Theobald, J. Biggs, and L. A. Sherman.** 1994. Conformational differences in major histocompatibility complex-peptide complexes can result in alloreactivity. J. Exp. Med. **179:**213–219.
7. **Chen, J.-H., D. H. Kono, Z. Yong, M. S. Park, M. B. A. Oldstone, and D. T. Y. Yu.** 1987. A *Yersinia pseudotuberculosis* protein which cross-reacts with HLA-B27. J. Immunol. **139:**3003–3011.
8. **Chen, W., J. McCluskey, S. Rodda, and F. R. Carbone.** 1993. Changes at peptide residues buried in the major histocompatibility complex (MHC) class I binding cleft influence T cell recognition: a possible role for indirect conformational alterations in the MHC class I or bound peptide in determining T cell recognition. J. Exp. Med. **177:**869–873.
9. **Claverie, J. M., A. Prochnicka-Chalufour, and L. Bougueleret.** 1989. Implications of a Fab-like structure for T cell receptor. Immunol. Today **10:**10–14.
10. **Ellis, S. A., C. Taylor, and A. J. McMichael.** 1982. Recognition of HLA-B27 and related antigen by a monoclonal antibody. Hum. Immunol. **5:**49–59.
11. **Fremont, D. H., M. Matsumura, E. A. Stura, P. A. Peterson, and I. A. Wilson.** 1992. Crystal structure of two viral peptides in complex with murine MHC class I H-2K$^b$. Science **257:**919–927.
12. **Fukazawa, T., E. Hermann, M. Edidin, J. Wen, F. Huang, H. Kellner, J. Floege, D. Farahmandian, K. M. Williams, and D. T. Y. Yu.** 1994. The effect of mutant $\beta$2-microglobulins on the conformation of HLA-B27 detected by antibody and by cytotoxic T lymphocytes. J. Immunol. **153:**3543–3550.
13. **Fukazawa, T., J. Wang, F. Huang, J. Wen, D. Tyan, K. M. Williams, R. B. Raybourne, and D. T. Y. Yu.** 1994. Testing the importance of each residue in a HLA-B27-binding peptide using monoclonal antibodies. J. Immunol. **152:** 1190–1196.
14. **Granfors, K., S. Jalkanen, A. A. Lindberg, L. Maki-Ikola, R. von Essen, R. I. Lahesmaa-Rantala, H. Isomaki, R. Saario, W. J. Arnold, and A. Toivanen.** 1990. *Salmonella* lipopolysaccharide in synovial cells from patients with reactive arthritis. Lancet **335:**685–688.
15. **Grumet, F. C., B. M. Fendly, L. Fish, S. Foung, and E. G. Engleman.** 1982. Monoclonal antibody (B27.M2) subdividing HLA-B27. Hum. Immunol. **5:** 61–72.
16. **Hammer, R. E., S. D. Maika, J. A. Richardson, J. P. Tang, and J. D. Taurog.**

1990. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27-associated human disorders. Cell **63:**1099–1112.
17. **Hermann, E., D. T. Y. Yu, K.-H. M. zum Büschenfelde, and B. Fleischer.** 1993. HLA-B27-restricted CD8 T cells derived from synovial fluids of patients with reactive arthritis and ankylosing spondylitis. Lancet **342:**646–650.
18. **Hogquist, K. A., A. G. Grandea III, and M. J. Bevan.** 1993. Peptide variants reveal how antibodies recognize major histocompatibility complex class I. Eur. J. Immunol. **23:**3028–3036.
19. **Jardetzky, T. S., J. H. Brown, J. C. Gorga, L. J. Stern, R. G. Urban, Y. Chi, C. Stauffacher, J. L. Strominger, and D. C. Wiley.** 1994. Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. Nature (London) **368:**711–718.
20. **Jardetzky, T. S., W. S. Lane, R. A. Robison, D. R. Madden, and D. C. Wiley.** 1991. Identification of self peptides bound to purified HLA-B27. Nature (London) **353:**326–329.
21. **Kellner, H., J. Wen, J. Wang, R. B. Raybourne, K. M. Williams, and D. T. Y. Yu.** 1994. Serum antibodies from patients with ankylosing spondylitis and Reiter's syndrome are reactive with HLA-B27 cells transfected with the *Mycobacterium tuberculosis HSP60* gene. Infect. Immun. **62:**484–491.
22. **Madden, D. R., D. N. Garboczi, and D. C. Wiley.** 1993. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. Cell **75:**693–708.
23. **Madden, D. R., J. G. Gorga, J. L. Strominger, and D. C. Wiley.** 1992. The three-dimensional structure of HLA-B27 at 2.1 Å resolution suggests a general mechanism for tight peptide binding to MHC. Cell **70:**1035–1048.
24. **Momburg, F., V. Ortiz-Navarrete, J. Neefjes, E. Coulmy, Y. van de Wal, H. Spit, S. J. Powis, G. W. Butcher, J. C. Howard, P. Walden, and G. J. Hämmerling.** 1992. Proteasome subunits encoded by major histocompatibility complex are not essential for antigen presentation. Nature (London) **360:** 174–177.
25. **Rohren, E. M., D. J. McCormick, and L. R. Pease.** 1994. Peptide-induced conformational changes in class I molecules. Direct detection by flow cytometry. J. Immunol. **152:**5337–5343.
26. **Rötzschke, O., K. Falk, S. Faath, and H.-G. Rammensee.** 1991. On the nature of peptides involved in T cell alloreactivity. J. Exp. Med. **174:**1059–1071.
27. **Rötzschke, O., K. Falk, S. Stevanovic, V. Gnau, G. Jung, and H. G. Rammensee.** 1994. Dominant aromatic/aliphatic C-terminal anchor in HLA-B*2702 and B*2705 peptide motifs. Immunogenetics **39:**74–77.
28. **Sherman, L. A., S. Chattopadhyay, J. A. Biggs, R. Dick II, and J. A. Bluestone.** 1993. Alloantibodies can discriminate class I major histocompatibility complex molecules associated with various endogenous peptides. Proc. Natl. Acad. Sci. USA **90:**6949–6951.
29. **Taurog, J. D., and F. A. K. El-Zaatari.** 1988. In vitro mutagenesis of HLA-B27: substitution of an unpaired residue in the α1 domain causes loss of antibody-derived epitopes. J. Clin. Invest. **82:**987–992.
30. **Toubert, A., C. Raffoux, J. Boretto, J. Sire, R. Sodoyer, S. R. Thurau, B. Amor, J. Colombani, F. A. Lemonnier, and B. R. Jordan.** 1988. Epitope mapping of HLA-B27 and HLA-B7 antigens by using intradomain recombinants. J. Immunol. **141:**2503–2509.
31. **Townsend, A., and H. Bodmer.** 1989. Antigen recognition by class I-restricted T lymphocytes. Annu. Rev. Immunol. **7:**601–624.
32. **Urban, R. G., R. M. Chicz, W. S. Lane, J. L. Strominger, A. Rehm, M. J. H. Kenter, F. G. C. M. UytdeHaag, H. Ploegh, B. Uchanska-Ziegler, and A. Ziegler.** 1994. A subset of HLA-B27 molecules contains peptides much longer than nonamers. Proc. Natl. Acad. Sci. USA **91:**1534–1538.
33. **Wang, J., D. T. Y. Yu, T. Fukazawa, H. Kellner, J. Wen, X.-K. Cheng, G. Roth, K. M. Williams, and R. B. Raybourne.** 1994. A monoclonal antibody that recognizes HLA-B27 in the context of peptides. J. Immunol. **152:**1197–1205.
34. **Wen, J., J. Wang, J. G. Kuipers, F. Huang, K. M. Williams, R. B. Raybourne, and D. T. Y. Yu.** 1994. Analysis of HLA-B*2705 peptide motif, using T2 cells and monoclonal antibody ME1. Immunogenetics **39:**444–446.
35. **Yu, D. T. Y., T. Hamachi, M. Hamachi, and G. Tribbick.** 1991. Analysis of molecular mimicry between HLA-B27 and a bacterial *OmpA* protein using synthetic peptides. Clin. Exp. Immunol. **85:**510–514.

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