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Identification of HLA-B27-Restricted Peptides from the 
Chlamydia trachomatis Proteome with Possible Relevance to 
HLA-B27-Associated Diseases

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The association of HLA-B27 with ankylosing spondylitis and reactive arthritis is the strongest one known between an MHC class I Ag and a disease. We have searched the proteome of the bacterium Chlamydia trachomatis for HLA-B27 binding peptides that are stimulatory for CD8+ cells both in a model of HLA-B27 transgenic mice and in patients. This was done by combining two biomathematical computer programs, the first of which predicts HLA-B27 peptide binding epitopes, and the second the probability of HLA-B27 peptide generation by the proteasome system. After preselection, immunodominant peptides were identified by biomathematical computer programs, the first of which predicts HLA-B27 peptide binding epitopes, and the second the probability that bacteria are also crucial for pathogenesis (9, 10). HLA-B27, a molecule that is strongly associated with diseases such as ankylosing spondylitis and reactive arthritis (ReA) (4 – 6). Both diseases belong to the group of disorders termed spondyloarthopathies.

Among the HLA-B27 subtypes, HLA-B*2705 and HLA-B2704 are the most strongly associated with spondyloarthopathies, whereas subtypes HLA-B*2706 and B*2709 are associated either weakly or not at all (7, 8). Besides HLA-B27, there is strong evidence that bacteria are also crucial for pathogenesis (9, 10). HLA-B27 transgenic rodents develop spondyloarthropathy-like manifestations only if raised in a nonsterile environment (11, 12). In humans the clearest evidence for the role of bacteria in the pathogenesis of spondyloarthopathies is found for Chlamydia trachomatis or certain enterobacteria in the pathogenesis of ReA. Furthermore, 20 – 40% of HLA-B27-positive ReA patients move on to ankylosing spondylitis after 10 – 20 years (13), suggesting that the ReA-associated bacteria can cause ankylosing spondylitis, possibly through a cross-reaction between a bacterial and a self Ag (10).

The main hypothesis advanced for the association between HLA-B27 and spondyloarthopathies is the arthritogenic peptide theory. It states that some B27 subtype alleles, due to their unique amino acid residues, bind a specific arthritogenic peptide(s) that is recognized by CD8-positive T cells (6, 14 – 16). Thus, for further investigation and verification of this hypothesis it is important to identify HLA-B27-specific peptides able to stimulate CD8-positive T cells.

Highly selective screens using, for instance, lysteriolysin for Listeria (17) and bacteria-specific T cell clones or lines are of limited value (18, 19) because they might miss the relevant epitopes. Therefore, we have studied Chlamydia trachomatis, the genome of which has recently been sequenced, using a more comprehensive approach (20). A huge number of potential HLA-B27-binding peptides occur in the genomes, indeed too many to test in functional assays such as proliferation or CD8 T cell cytotoxicity assays. To overcome this problem we combined two computer programs that allow preselection of potentially relevant HLA-B27 peptides together with flow cytometry (21) to identify HLA-B27-restricted CD8+ stimulatory chlamydial peptides. The SYFPEITHI epitope prediction program identifies peptides on the basis of anchor residues required for HLA-B27 binding (22), while the proteasome program (23, 24) is based on the proteasomal peptide cleavage properties responsible for the generation of most MHC class I peptides. Using this novel approach we screened the entire proteome with Possible Relevance to HLA-B27-Associated Diseases. The Journal of Immunology, 2001, 167: 4738 – 4746.

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3 Abbreviations used in this paper: ReA, reactive arthritis; NP, nucleoprotein; SF, synovial fluid mononuclear cells.

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C. trachomatis proteome and identified a number of B27-restricted MHC-class I CD8+ epitopes that might be potentially relevant for disease pathogenesis.

Materials and Methods

Mice and infection of mice

CBA/J mice (H-2d), 6–12 wk old and transgenic for HLA-B*2705 and human β2-microglobulin (9, 25), and nontransgenic CBA/J mice were maintained in the barrier facilities of the Klinikum Benjamin Franklin (Berlin, Germany), where all breedings were performed. The transgenic mice were immunized i.v. with 3 × 10^7 IFU purified infectious elementary bodies of C. trachomatis serovar L2. Three weeks later the mice were boosted with 5 × 10^8 inclusion forming units elementary bodies. One week after the second immunization, spleen cells from three to five mice were harvested, pooled, and used for either cytokine secretion experiments or cytotoxicity assays. The elementary bodies were a gift from Dr. M. Hartmann, Friedrich Schiller University (Jena, Germany) and were prepared as previously described (26). The local review board for animal studies approved these experiments.

RMA cell lines and culture media

RMA is a Rauscher leukemia virus-induced cell line of C57BL/6 (H-2b) origin; RMA-S is a mutant of this cell with a defect in peptide loading of class I molecules due to a mutant TAP-2 locus (27). RMA-S-B27 cells are transfected expressing the HLA-B*2705 subtype; the α1 and α2 domains are human, whereas the α3 domain is of the H-2b haplotype. The cells were a gift from Dr. H.-G. Rammensee (Institute for Cell Biology, Tübingen, Germany). RMA-S cells and their transfectants express very low amounts of class I molecules, which are unstable on the cell surface when cultured at 37°C due to their TAP defect. However, when cultured at 28°C the RMA-S cells and their B27 transfectants can express the empty class I heavy chains, or, alternatively, the heavy chains may be occupied by low affinity peptides (28). At 37°C surface expression of the heavy chains can only be stabilized by adding exogenous peptide ligands. The cells were cultured at 37°C in 5% CO2 in RPMI 1640 culture medium, supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 5 × 10^-3 M 2-ME (Sigma, St. Louis, MO). For stable transfection cells were periodically supplemented with 0.5 mg/ml G-418 sulfate. If not stated otherwise, all media and supplements were obtained from Life Technologies (Karlsruhe, Germany).

Epitope stabilization assay

For peptide loading, RMA-S-B27 cells were preincubated in RPMI 1640 culture medium with 2% FCS at 28°C for 6 h and cultured for an additional 8 h with 100 μM selected chlamydial or control peptides. The cells were then transferred to 37°C for 10 h. Cell surface staining of HLA-B27 on the RMA-S-B27 cells was performed by direct staining in 45°C n5 % CO2 in RPMI 1640 culture medium, supplemented with 4% human serum, and 5 μl of mouse anti-human HLA-B27-FITC (HLA-ABCm3; Serotec, Eching, Germany) for 20 min at room temperature; subsequently, the cells were washed twice in PBS/1% BSA, resuspended in FACS buffer, and analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA).

Search for HLA-B27-binding peptides derived from C. trachomatis

A search for HLA-B27 binding nonamer peptides from C. trachomatis proteins was conducted with an epitope prediction program described by Rammensee and colleagues (22). The SYFPEITHI program, (http://www.uni-tuebingen.de/uni/kki/) combines knowledge about MHC class I binding patterns and functional data (T cell responses to MHC class I/peptide complexes). A scoring system for HLA-B27 binding was used, giving highest weights to anchor positions with arginine (R) at position 2, second highest numbers for amino acids at auxiliary anchor positions (position 9 for HLA-B27), and lower numbers for preferred amino acids (29, 30). Peptides were selected for a binding score of ≥25 and in an extra round for a binding score between 20 and 24.

Prediction of cleavage-determining amino acid motifs of the 20S proteasome

A second mathematical program mimicking functions of the cleavage process was used to model positions of particular cleavages of amino acid motifs in 20S proteasomes (23, 24) was used to investigate whether peptides selected by the epitope prediction program would be cut by proteasomes. The main assumption of this program is that the mutual recognition of peptide substrates and active sites of the proteasome is determined by the presence of distinct amino acid residue motifs in the vicinity of the scissile bond. For this reason, each nonamer of interest was divided by the 10 natural amino acids at the N- and the C-terminal ends next to the presumptive cleavage site. On this basis, the likelihood for the preselected nonamer peptides to be cleaved by the 20S proteasome was analyzed.

Pepptide synthesis

Nonamer peptides were synthesized according to standard F-moc solid phase synthesis methods on a Syro-Synthesizer (MultiSynTech, Witten, Germany), purified by HPLC (Shimadzu LC-10; Shimadzu Scientific Instruments, Duisberg, Germany), and identified by mass spectroscopy (LCQ ion trap; Thermosquest, Eberbach, Germany). The purity of the peptides was >95%. Peptides were dissolved in DMSO and further diluted with serum-free culture medium at a concentration of 5 mg/ml. The peptides were stored frozen at −20°C.

Identification of B27-restricted chlamydial nonamer peptides by Ag-specific flow cytometry

Peptide-specific CD8+ T cells from HLA-B27 transgenic mice primed in vivo with C. trachomatis were stimulated in vitro with pools of peptides. The 199 peptides were created in two peptide matrices comprising 10 pools (pools 1–10 and 11–20 each) in a horizontal direction, while the respective pools 21–30 and 31–40 were arranged in the vertical direction. Each pool contained a mixture of 10 different peptides. Thus, both matrices comprised 40 pools in total to ensure that each peptide was represented by a horizontal and a vertical pool. An individual stimulating peptide was identified by crossing of two stimulating pools. To determine whether selected peptides could stimulate CD8+ splenic T cells from Chlamydia-primer HLA-B27-transgenic mice, intracellular cytokine staining was used after Ag-specific T cell stimulation (21). Briefly, 2 × 10^6 primed cells were stimulated for 6 h in 1 ml of culture medium in 10-ml tissue culture tubes (Greiner, Nurtingen, Germany) with anti-CD28 Ab (1 μg/ml) for optimal stimulation (31) plus mixed peptide pools (5 μg/ml peptide each), individual peptides (10 μg/ml), or no antigen peptide (negative control). Stimulation was stopped with brefeldin A (5 μg/ml) after 2 h, and after an additional 4 h cells were harvested and stained with anti-CD69-FTTC (5 μg/ml; BD PharMingen, San Diego, CA) and anti-CD8-APC (1 μg/ml; BD PharMingen). Next, the cells were fixed in 2% formalin for 15 min and washed, and the pellet was resuspended for 10 min in saponin buffer followed by staining with PE-conjugated rat anti-mouse IFN-γ (1 μg/ml; BD PharMingen). After gating on CD8-positive T cells, cells that were double positive for the early activation marker CD69 and for intracellular IFN-γ were regarded as Ag-specific. Analysis was performed using a BD Biosciences FACScan flow cytometer with CellQuest software.

In vitro expansion of Chlamydia-specific CTL

To investigate CD8+ T cells for cytotoxicity against the selected chlamydial peptides, splenocytes from three to five mice, primed in vivo with Chlamydia, were resuspended in RPMI 1640 culture medium. To increase the number of Ag-specific cytotoxic CD8+ T cells, 1 × 10^7 primed splenic cells in 7 ml of culture medium were transferred into 50-ml upright flask each (Nunc, Wiesbaden, Germany) and restimulated in vitro with 10 μg/ml respective chlamydial peptide or control peptide. After 6 days of incubation at 37°C in 5% CO2, cells were harvested, Ficoll-Paque (Pharmacia, Freiburg, Germany)-purified, resuspended in culture medium, counted, and used as effector cells for cytotoxicity assays.

Cytotoxicity assay against chlamydial peptides

Cytotoxic activity was measured on peptide-loaded RMA-S-B27 cells. For this, RMA-S-B27 cells were incubated and loaded with different peptide fractions each at 28°C in RPMI 1640/2% FCS and then shifted to 37°C as described. The cells were then washed with PBS, and the loaded cell pellets were labeled at 37°C with 100 μCi of Na2-Chromat (Amersham, Braunschweig, Germany) for 1 h. After washing three times in PBS the labeled target cells were resuspended in RPMI 1640 supplemented with 1% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 5 × 10^-3 M 2-ME (Sigma) for 4 h in 96-well U-bottom plates (Nunc) along with different numbers of effector cells in a total volume of 200 μl. Spontaneous release was determined in wells with target cells and medium alone. Maximum release was determined by adding 100 μl of 2% Triton X-100 (Sigma) to wells containing 100 μl of target cells without CTL. Target cells were tested for lysis in triplicate in a standard 6-h chromium release assay. Specific lysis was...
calculated as: 100 × (cpm experimental release − cpm spontaneous release)/cpm maximal release − cpm spontaneous release). Data presented are the means of triplicate determinations.

**Identification of B27-restricted chlamydial peptides in patients with Chlamydia-induced ReA**

In three HLA-B27<sup>+</sup> patients with *Chlamydia*-induced ReA (patient 1: male, 44 years old, 2-wk duration of the arthritis; patient 2: male, 34 years old, 5-wk duration of arthritis; patient 3: male, 36 years old, 4-wk duration of arthritis) synovial fluid mononuclear cells (SF) were investigated as described for B27-transgenic mice. Briefly, SF of the patient were separated through Ficoll gradient. Cells were then stimulated for 6 h with pools of peptides and analyzed as indicated for the mouse experiments. Cells were stained with self-conjugated anti-CD3-FITC, anti-CD8-PerCP, and tetramers for 30 min at room temperature in 0.5% BSA/0.05% azide, followed by incubation with the HLA-B27/rat CD8<sup>+</sup> T cell line (26). SF from frozen samples of these patients were analyzed as indicated for the mouse experiments. Approval of the local ethical committee of the Benjamin Franklin Hospital was approved for the investigation of patients’ synovial fluid.

**FACS analysis of Chlamydia peptide-specific T cells with HLA-B27 tetramers**

HLA-B27 tetramers were generated as previously described (32) with some modifications. The expression vector pLMI-HLA-B27 (a gift from E. Collins, D. Garboczki, and D. Wiley, Boston University, Boston, MA) was modified by tagging with the BirA recognition sequence as previously described (33) and by mutating the Cys<sup>S</sup> to Ser<sup>S</sup> (34). Correct folding and biotinylation of HLA-B27 were analyzed by gel filtration and gel electrophoresis, HLA-B27 monomers were tetramerized with PE-labeled streptavidin for FACS analysis. We generated HLA-B27 tetramers with the EP peptide nucleoprotein (NP); aa 25–266 (35), the rat peptide L5 (aa 36–44) (36), and chlamydial peptides 146 and 195. Mononuclear cells from a B27<sup>+</sup> donor with EBV infection were stained with the HLA-B27/EP NP (aa 25–266) tetramer immediately after harvesting the cells and 14 days after stimulation with 1 μg/ml EBV NP (aa 25–266) peptide in the presence of IL-2 and IL-7.

For B27/tetramer analysis SF from patient 3 were available. Furthermore, SF from two other patients (patient 4: female, 22 years old, B27<sup>+</sup>, 4-wk duration of *Chlamydia*-induced ReA; patient 5: male, 22 years old, B27<sup>+</sup>, 5-wk duration of oligoarthritis, arthritis not triggered by *Chlamydia*<sup>+</sup>) were investigated in parallel. SF from frozen samples of these patients were incubated overnight in culture medium. After performing a Ficoll gradient to get rid of the dead cells, mononuclear cells were incubated with anti-CD3-FITC, anti-CD8-PerCP, and tetramers for 30 min at room temperature in 0.5% BSA/0.05% azide, followed by flow cytometry. CD3-positive <T> cells were gated. As a negative control the HLA-B27/rat L5 (aa 36–44) tetramer was used.

**Results**

**Screening of the proteome of C. trachomatis for HLA-B<sup>*</sup>2705-binding nonamer peptides**

Analysis of the chlamydial genome by Stephens and colleagues (20) resulted in the identification of 894 likely protein-coding genes. The protein sequences deduced from this database were the basis for our screening approach for candidate nonamer peptides with binding to HLA-B<sup>*</sup>2705. In the first step all nonamer peptides with arginine (R) at position 2 (29, 30) were selected. This selection step resulted in about 18,000 nonamer peptides. Selecting for only those peptides with an arbitrary binding score of ≥25 to HLA-B<sup>*</sup>2705 reduced the number of peptides to 1881. The biological reason for selecting a binding score of 25 or greater was that most of the published natural peptides identified in the HLA-B<sup>*</sup>2705 molecule showed a binding score of ≥25 when we checked these natural ligands with the binding program.

**Peptide generation by the proteasome program**

In a second step we asked which of the 1881 selected peptides would also be processed by the proteasome system (Fig. 1). One hundred and ninety-nine of the 1881 nonamer peptides fulfilled this criterion (cutting probability, ≥4.5). All of these nonamer peptides were then synthesized, and their function tested.

**Identification of B27-restricted nonamer peptides from C. trachomatis based on cytokine secretion**

To identify peptide-specific CD8<sup>+</sup> T cells derived from HLA-B27 transgenic mice that were infected in vivo with *C. trachomatis*, primed splenocytes were stimulated with pools of the selected 199 peptides. Table I summarizes the results of four different mouse experiments with pools of peptides for the stimulation of CD8<sup>+</sup> T cells. Eleven of 40 pools were stimulatory in all four experiments, and the CD8<sup>+</sup> T cell response could be attributed at the level of a single peptide to nine peptides (Table I). In Fig. 2 two examples of single positive peptides (131 and 133) with their corresponding pools (14/31 and 14/33, respectively) are shown. The same experiment performed in parallel at the same time gave nearly identical results (data not shown), proving that the results are reproducible. For the remaining 28 pools the percentage of CD69/IFN-γ-positive CD8<sup>+</sup> T cells was comparable to the control value (≤0.02%) in the presence of anti-CD28 but without Ag. The sequences of these stimulatory peptides, their source in the database, and the proteins from which they are derived are shown in Table II. When we performed the same experiment with nontransgenic mice after immunization with *Chlamydia*<sup>+</sup> and in vitro peptide stimulation, the percentage of IFN-γ-positive cells was not higher than that with the negative controls (without peptide; data not shown).

**Binding of the selected chlamydial nonamer peptides to RMA-S-B27**

To confirm that the nine selected peptides were able to bind to HLA-B27, they were tested in an assembly assay with the TAP-defective RMA-S-B27 transfectant mouse cell line (27). RMA-S-B27 transfectants were incubated with 100 μM of each peptide at 28°C, then incubated at 37°C for 10 h. HLA-B27 molecules stabilized by peptides were monitored by direct staining with an FITC-coupled, anti-HLA-B27-specific mAb. Fig. 3 shows the different features of the transfectant cells with and without added peptides at different incubation temperatures. RMA-S-B27 cells incubated at 37°C without peptide showed no HLA-B27 expression, as expected (Fig. 3A), but did so at 28°C (Fig. 3B). However, when the temperature was shifted from 28 to 37°C (Fig. 3C) we found that after 10 h the cell line no longer showed empty heavy chain expression (thermolabile). In contrast, when the RMA-S-B27 line was incubated with exogenous peptides at 28°C, stable expression of the heavy chains was maintained at 37°C (thermostable). Incubation with a control peptide unrelated to HLA-B27 binding revealed no stabilization of HLA-B27 on the surface (Fig. 3D), whereas the B27 undecamer self-peptide (aa 169–179) derived from a polymorphic region of the α1 domain (37) and the influenza nonamer NP (aa 383–391) (38), both known to bind B27,
We next investigated whether the same approach would allow in the influenza nonamer peptide NP (aa 383-391) was comparable to that of the B27 undecamer self-peptide or the C. The binding efficiency of the nine peptides was found to stabilize HLA-B27 on the cell surface after shifting the ° cells from 28 to 37°C. The binding efficiency of the nine peptides proved also to be stimulatory on the single peptide level: four peptides (8, 68, 145, and 195) in patient 1, six peptides (8, 68, 133, 144, 146, and 194) in patient 2 and eight peptides (68, 80, 133, 138, 146, 194, 195, and 196) in patient 3 (Fig. 5). One of the peptides was seen by all three patients (68), whereas peptides 133, 146, 194, and 195 were seen by two patients (Table III). Eight of 11 human peptides were also seen by murine CD8+ T cells (Table III). Potential individual stimulating peptides were identified at the crossing point of two stimulating pools (indicated by bold numbers). By this method, nine immunogenic individual peptides (underlined numbers), which were also stimulatory on the single peptide level, could be identified in the murine system.

CD8+ T lymphocytes from B27-transgenic mice immunized with Chlamydia lyse targets loaded with selected chlamydial peptides in an HLA-B27-restricted manner

We asked next whether peptides that induce IFN-γ secretion by CD8+ cytotoxic T cell response. Eight of the nine peptides tested triggered Ag-specific lysis of target cells by CD8+ cytotoxic T cells (Fig. 4). Lysis at 10% for peptide 194 was weak, but was still regarded as positive, because lysis obtained with the control peptide was negligible. The self-derived B27 control peptide did not induce cytotoxicity, although it is a good B27 binder. No lysis was observed for peptide 80. When this experiment was performed with either unprimed splenocytes from B27-transgenic mice or splenocytes derived from Chlamydia-primed nontransgenic CBA mice, no lysis was observed after in vitro stimulation of splenic cells with the respective peptides of RMA-S-B27 peptide-loaded cells (data not shown).

Identification of B27-restricted chlamydial peptides in patients with Chlamydia-induced ReA

We next investigated whether the same approach would allow identification of peptide-specific CD8+ T cells from the SF of Chlamydia-induced ReA patients. SF of patients were stimulated with pools of peptides as described in the mouse experiments. Using the peptide matrix we could identify 11 human peptides that proved also to be stimulatory on the single peptide level: four peptides (8, 68, 145, and 195) in patient 1, six peptides (8, 68, 133, 144, 146, and 194) in patient 2 and eight peptides (68, 80, 133, 138, 146, 194, 195, and 196) in patient 3 (Fig. 5). One of the peptides was seen by all three patients (68), whereas peptides 133, 146, 194, and 195 were seen by two patients (Table III). Eight of 11 human peptides were also seen by murine CD8+ T cells (Table III). Potential individual stimulating peptides were identified at the crossing point of two stimulating pools (indicated by bold numbers). By this method, nine immunogenic individual peptides (underlined numbers), which were also stimulatory on the single peptide level, could be identified in the murine system.
III), whereas peptide 131 was only recognized by murine, but not human, T cells (Tables II and III).

**HLA-B27/Chlamydia peptide (195)-tetramer complex specifically detects CD8⁺ T cells in Chlamydia-induced arthritis**

We used tetramers of HLA-B27 with Chlamydia peptides 146 and 195 to stain the SF of two patients with Chlamydia-triggered ReA and one with B27⁺ control arthritis, not triggered by Chlamydia (Fig. 6A). The HLA-B27/rat L5 (aa 36–44) peptide tetramer was used for control experiments. For these studies frozen cells were examined after 1 night of incubation in culture medium without further expansion and were directly analyzed by flow cytometry. In Chlamydia-induced arthritis, two patients (3 and 4), but not the B27⁺ control patient (5), had CD8⁺ T cells specific for the B27/Chlamydia peptide 195 tetramer (Fig. 6A). No staining was observed with the B27/Chlamydia peptide 146 complex or with the control rat L5 peptide tetramer. The B27/tetramer of the rat L5 peptide did not bind to any T cells in all three patients, indicating the specificity of this experiment. The HLA-B27/EBV NP (aa 258–266) complex specifically detected T cells in a patient with previous EBV infection (Fig. 6B). Tetramer-specific T cells could be expanded from 0.12 to 56.3%, indicating that the HLA-B27 NP 258–266 tetramer had a biological function (Fig. 6B). Some tetramer staining was observed for CD8- negative T cells. This has also been reported in the past by other

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**Table II. Identification of B27-restricted chlamydial peptides immunogenic for CD8⁺ T cells derived from B27-transgenic murine splenic cells**

<table>
<thead>
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<th>Peptide No.</th>
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<th>Function</th>
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<td>CT339</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>131</td>
<td>KRLAETLAL</td>
<td>CT589</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>133</td>
<td>IRSSVQNLK</td>
<td>papQ</td>
<td>Invasin repeat-family-phosphatase</td>
</tr>
<tr>
<td>138</td>
<td>ARKLLDNL</td>
<td>CT610</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>144</td>
<td>MRDHTITLL</td>
<td>CT634</td>
<td>NADH-ubiquinone-oxidoreductase-α-chain</td>
</tr>
<tr>
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<td>ygeD</td>
<td>Efflux-protein</td>
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<tr>
<td>196</td>
<td>ERFLAQEQL</td>
<td>CT850</td>
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*The amino acid sequences of the nine immunodominant chlamydial peptides, their source in the database (20), and the function of the protein from which the peptides are derived are shown. Hypothetical protein means that so far no other bacterial sequence or the related protein function could be found in the database.*

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**FIGURE 3.** Peptide loading of TAP-defective RMA-S-B27 cells with the nine selected chlamydial peptides. RMA-S-B27 cells were incubated with control peptides (A–F) or with the selected chlamydial peptides (G–P). After incubation at 28°C, the peptide/cell suspensions were shifted for 10 h to 37°C for stabilization of HLA-B27 expression. Surface staining for HLA-B27 expression was performed by anti-B27 FITC-conjugated Ab and analyzed by FACSscan. A. No stable expression of HLA-B27 on RMA-S-B27 cells at 37°C, without peptide addition (thermolabile). B. Incubation of RMA-S-B27 cells at 28°C shows stabilization of the empty B27 heavy chains at this temperature. C. Switching the cells from 28 to 37°C again results in thermolability of the B27 heavy chains after a 10-h incubation. D. Incubation of the cells with the non-B27-binding control peptide reveals B27 instability, whereas the B27 self-11-mer (aa 169–179; E) and the influenza-derived NP (aa 383–391; F) stabilize the B27 heavy chains at 37°C. G–P. Finally all nine selected peptides (80, 131, 133, 138, 144, 146, 194, 195, and 196) reveal binding and thus stabilization of the B27-Ag similar to the positive control peptides.
Chlamydia found in the murine system. Finally, preliminary studies with B27/tides were identified, eight of which were identical with those HLA-B27-restricted peptides from the proteome of in vitro T cell expansion by peptide stimulation (41).

groups (39, 40), especially when cells were analyzed without prior in vitro T cell expansion by peptide stimulation (41).

Discussion
We have used a novel strategy to identify potentially arthritogenic HLA-B27-restricted peptides from the proteome of C. trachomatis. In the model of HLA-B27 transgenic mice, nine HLA-B27-restricted chlamydial nonamer peptides proved stimulatory for CD8$^+$ T cells, and eight of them raised an HLA-B27-restricted cytotoxic T cell response. Even more importantly, when investigating HLA-B27$^+$ patients with Chlamydia-induced ReA 11 peptides were identified, eight of which were identical with those found in the murine system. Finally, preliminary studies with B27/Chlamydia peptide tetrameric complexes in HLA-B27$^+$ patients suffering from ReA after Chlamydia infection revealed staining of CD8$^+$ T cells in two of three patients.

The two biomathematical computer programs (23, 24), which were combined, allowed us to reduce the number of HLA-B27-restricted peptides to a workable amount of 199 from an initial number of $\sim 18,000$ nonamers with an arginine at position 2. When we set a binding score of $\geq 25$, we still obtained a number of 1881 nonamer peptides. In the initial computerized screening step (22) peptides were selected according to the presence of canonical HLA-B27 anchor residues and the probability of their binding to HLA-B27 molecules. Although this program allows the identification of HLA-B27-binding peptides with high fidelity, it does not allow us to predict which of these, due to the given sequence environment within a protein, are actually immuno-relevant and generated by the cellular proteasome system. Therefore, we screened the 1881 theoretical peptides with a proteasome-based cleavage site prediction program based on the enzymatic properties of the 20S proteasome and the established efficiency of the system to generate MHC class I molecules (42). Using the proteasome program it is important to realize that the predicted epitopes will be generated with high probability. However, it cannot be excluded that the program, due to a still limited database, is restrictive against peptides that are generated by the proteasome but do not obey the presently known cleavage parameters. Nevertheless, both programs have a predictive accuracy of $\sim 90\%$ (22, 23).

Thus, the first experimental combination of the two computerized epitope prediction programs allowed us to tackle an entire C. trachomatis proteome and to overcome the pitfalls of biochemical approaches to elute pathogen-derived peptides from the MHC molecules on the cell surface in sufficient amounts for analysis.

To test the selected peptides for their immunogenicity we used Ag-specific flow cytometry based on the staining of cell surface expression markers and intracellular cytokine secretion (21, 43, 44). This method identifies Ag-specific T cells directly ex vivo after short-term stimulation, preventing a change in the Ag specificity by long term cultures of T cells. Furthermore, Ag-specific CD8$^+$ T cells can be detected even at low frequencies of $\sim 0.1\%$, a sensitivity that is not reached by assays previously used. A recent study used a related approach for the identification of peptides derived from the tumor-associated Ag preferentially expressed Ag of melanoma presented by HLA-A*0201. However, this investigation did not use Ag-specific flow cytometry as a readout for CD8 response and was limited to the analysis of just one protein (45).

The chlamydial peptides from proteins with known function are mainly derived from either secretory or conserved proteins (20). Secretory proteins are of special interest because they might obtain easier access to the MHC class I pathway. Conserved Ags shared by various bacterial species might also be of relevance because not only C. trachomatis, but also enterobacteria such as Yersinia, Salmonella, or Shigella, can induce ReA. Furthermore, conserved bacterial Ags might be crucial for cross-reactivity with human self Ags. Some of the peptides identified are derived from hypothetical proteins with unknown function. Immunodominant peptides from hypothetical proteins have also been reported for Borrelia burgdorferi (19).
Until now there have been no data about how many peptides derived from a whole bacterium are stimulatory to CD8+ T cells when presented by a specific MHC class I molecule. About 10 peptides found in our study present a rather small number. However, for viruses, although containing ~100-fold fewer proteins compared with bacteria, it had been found that only a single peptide can be immunodominant for the CD8 response in the context of an MHC class I molecule (46, 47).

Because the chosen restriction scores for both programs were arbitrary, it appeared possible that we might have missed potentially positive peptides due to the applied stringent score values. Therefore, we searched in an additional approach for peptides with a binding score between 20 and 24. To restrict the resulting number of peptides two additional selection steps had to be used, choosing groups of candidate proteins and excluding good binders that all peptides were indeed B27 binders, and we confirmed the immunogenicity of eight of nine identified peptides in a cytotoxic T cell assay (Fig. 4), while the B27 self-derived peptide was tolerated and thus did not induce cytotoxicity. Earlier studies reported a good HLA-B27 binding of chlamydial heat shock protein 60-derived peptides (48), and a recent study demonstrated HLA-A2- and HLA-B51-restricted CD8+ CTLs for the major outer membrane protein of C. trachomatis in humans (49). However, no stimulatory, HLA-B27-restricted chlamydial major outer membrane protein- or 60-kDa heat shock protein-derived peptide was identified.

Our first and main aim in this study was to establish a method for the identification of HLA-B27-restricted, bacteria-derived peptides that are immunogenic for CD8+ T cells using the HLA-B27-transgenic mouse, which gave us reproducible and stable experimental conditions. Although similar epitopes in mice and humans had been described in the past, it has been doubted whether peptide processing and T cell repertoire are the same in mice and humans, because auxiliary molecules that help to load MHC class I might be not identical (50). Therefore, it was of great importance that we could also investigate three HLA-B27-positive patients with Chlamydia-induced ReA. The access to synovial fluid, directly from the site of inflammation, is an advantage compared with studies using peripheral blood, because we have shown recently that the Ag-specific frequency is about 10 times higher in synovial fluid than in peripheral blood (43). In these patients we identified 11 peptides, three of which were only seen by human T cells, one only by

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**Table III. Identification of B27-restricted chlamydial peptides immunogenic for human CD8+ T cells derived from reactive arthritis patients**

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>Sequence</th>
<th>Source</th>
<th>Function</th>
<th>Peptide-Recognition by Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>NRFISVAYML*</td>
<td>pmpC</td>
<td>Putative outer membrane protein</td>
<td>1, 2</td>
</tr>
<tr>
<td>68</td>
<td>NRAKQVIKL*</td>
<td>clpC</td>
<td>Protease-ATPase</td>
<td>1, 2, 3</td>
</tr>
<tr>
<td>80</td>
<td>IRMFKLPL</td>
<td>CT339</td>
<td>Hypothetical protein</td>
<td>3</td>
</tr>
<tr>
<td>133</td>
<td>IRSSVQNL</td>
<td>papQ</td>
<td>Invasin repeat family phosphatase</td>
<td>2, 3</td>
</tr>
<tr>
<td>138</td>
<td>ARKLLDLNL</td>
<td>CT610</td>
<td>Hypothetical protein</td>
<td>3</td>
</tr>
<tr>
<td>144</td>
<td>MRDHTITL</td>
<td>CT634</td>
<td>NADH-ubiquinone oxidoreductase-α-chain</td>
<td>2</td>
</tr>
<tr>
<td>145</td>
<td>DRLALLANL*</td>
<td>recC</td>
<td>Exodeoxyribonucleoside-β-γ-chain</td>
<td>1</td>
</tr>
<tr>
<td>146</td>
<td>YRLLLTRVL</td>
<td>ygeD</td>
<td>Efflux protein</td>
<td>2, 3</td>
</tr>
<tr>
<td>194</td>
<td>EREQTNLQL</td>
<td>ftsH</td>
<td>ATP-dependent zinc protease</td>
<td>2, 3</td>
</tr>
<tr>
<td>195</td>
<td>NRELIQQEL</td>
<td>CT847</td>
<td>Hypothetical protein</td>
<td>1, 3</td>
</tr>
<tr>
<td>196</td>
<td>ERFLAQEQL</td>
<td>CT850</td>
<td>Hypothetical protein</td>
<td>3</td>
</tr>
</tbody>
</table>

* Description of 11 stimulatory peptides for CD8+ T cells derived from human synovial fluid of three patients suffering from ReA due to chlamydial infection. Three peptides (indicated by *) were only recognized by human CD8+ T cells of both patients, whereas eight peptides are identical to peptides also found in the B27-transgenic murine system.

**Figure 6.** Analysis of the binding of two HLA-B27/Chlamydia peptide (146 and 195) tetramer complexes to synovial fluid-derived CD8+ T cells stained with PerCP from three B27+ patients. Patients 3 and 4 were suffering from Chlamydia-triggered ReA; control patient 5 had non-Chlamydia-induced oligoarthritis. A. Two of the three patients (patients 3 and 4, but not patient 5) revealed binding of their CD8+ cells to the B27/peptide 195 tetramer PE-stained complex. None of the three patients showed binding for the B27/peptide 146 tetramer-PE complex. As a negative control, the HLA-B27 rat L5 (aa 36–44) tetramer was used. B. As a positive control, CD8+ T cells from peripheral blood of a B27+ donor after EBV infection were stained with the B27/EBV NP (aa 258–266) tetramer before and after Ag-specific in vitro expansion, confirming that the tetramer had a biological function.
mice, and eight by both human and murine T cells. The relevance of the single peptides for the pathogenesis has to be investigated in future studies.

The arthritogenic peptide hypothesis is currently the one most likely to explain the presentation of peptides to CD8-positive T cells and the striking association of HLA-B27 with human diseases. Evidence for this is found in transgenic animal systems (51, 52) as well as in human studies (15, 16, 53, 54). Furthermore, the fact that the disease not-associated subtypes HLA-B*2706 and B*2709 differ from the associated subtypes by only one or two amino acids located in the peptide binding groove (7, 8) can best be explained by this hypothesis. A CD8+ HLA-B27-restricted T cell response against Chlamydia or against ReA-associated bacteria might not be essential for an effective anti-bacterial immune response. However, we have postulated (6, 10) that this small response might be sufficient to induce an autoimmune response in HLA-B27-positive patients who move on to ankylosing spondylitis (13). Thus, although the number of Ag-specific CD8+ T cells in our assay is relatively small compared with the background, we think that this response is relevant. It has been a general problem in the past to identify Ag-specific T cells of low frequencies, for example directed against autoantigens (55). The immunogenicity of the peptides identified in this study has to be confirmed in future experiments. For mice, we already did this by using a cytotoxic assay, and for humans we presented data using B27/peptide-specific tetramers. We will expand on these approaches in the future by separating Ag-specific CD8+ T cells using B27/chlamydial peptide-specific tetramers, as described recently for HLA-A2- and HLA-B51-restricted chlamydial major outer membrane protein peptide motifs (56), and by using the cytotoxic secretion assay (57) to prove the relevance of the peptides identified here for the pathogenesis of disease. In this study we present a feasible approach for the identification of immunodominant peptides from a complex bacterium.

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References