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Research Article

MHC class I allele, HLA-B27, is strongly associated with a group of human diseases called spondyloarthropathies. Some of these diseases have an onset after an enteric or genitourinary infection. In the present study, we describe spontaneous disease in HLA-B27 transgenic mice where endogenous beta2-microglobulin (beta2m) gene was replaced with transgenic human beta2m gene. These mice showed cell surface expression of HLA-B27 similar to that of human peripheral blood mononuclear cells. In addition, free heavy chains (HCs) of HLA-B27 were also expressed on thymic epithelium and on a subpopulation of B27-expressing PBLs. These mice developed spontaneous arthritis and nail changes in the rear paws. Arthritis occurred primarily in male animals and only when mice were transferred from the pathogen-free barrier facility to the conventional area. Transgenic mice expressing HLA-B27 with mouse beta2m have undetectable levels of free HCs on the cell surface and do not develop arthritis. In vivo treatment with anti-HC-specific antibody delayed the onset of disease. Our data demonstrate specific involvement of HLA-B27 'free' HCs in the disease process.

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HLA-B27 Heavy Chains Contribute to Spontaneous Inflammatory Disease in B27/human β_2 -Microglobulin (β_2 m) Double Transgenic Mice with Disrupted Mouse β_2 m

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Abstract

MHC class I allele, HLA-B27, is strongly associated with a group of human diseases called spondyloarthropathies. Some of these diseases have an onset after an enteric or genitourinary infection. In the present study, we describe spontaneous disease in HLA-B27 transgenic mice where endogenous β_2 -microglobulin (β_2 m) gene was replaced with transgenic human β_2 m gene. These mice showed cell surface expression of HLA-B27 similar to that of human peripheral blood mononuclear cells. In addition, free heavy chains (HCs) of HLA-B27 were also expressed on thymic epithelium and on a subpopulation of B27-expressing PBLs. These mice developed spontaneous arthritis and nail changes in the rear paws. Arthritis occurred primarily in male animals and only when mice were transferred from the pathogen-free barrier facility to the conventional area. Transgenic mice expressing HLA-B27 with mouse β_2 m have undetectable levels of free HCs on the cell surface and do not develop arthritis. In vivo treatment with anti-HC-specific antibody delayed the onset of disease. Our data demonstrate specific involvement of HLA-B27 'free' HCs in the disease process. (*J. Clin. Invest.* 1996. 98:2746–2755.) Key words: HLA-B27 • spondyloarthropathy • Reiter's disease • animal model • heavy chain

Introduction

The MHC class I gene, HLA-B27, is strongly implicated in a group of human diseases called spondyloarthropathies (1). In a subgroup of B27-linked diseases, such as reactive arthritis, the changes are seen after an enterobacterial infection of the small or large intestine or genitourinary tract (2). The importance of the HLA-B27 gene and unknown environmental factors in disease pathogenesis has now been confirmed in transgenic animals (3, 4). How HLA-B27 interacts with enteric bacteria to trigger the disease is not yet clear. Several hypotheses have been proposed to explain the role of enterobacteria in HLA-B27-associated diseases including: (a) molecular mimicry of HLA-B27 with enterobacteria, (b) presentation of arthritogenic peptides from environmental antigen by HLA-B27 to CD8+ T cells, and, (c) presentation of exogenous antigen by empty HLA-B27 (5). A possible role of polymorphic genes coding for molecules involved in antigen processing/presentation, such as proteasomes (Lmp2 and Lmp7) and peptide transporters (Tap-1/Tap-2 heterodimer), has also been suggested but not substantiated (6–9).

While studies on the crystal structure of HLA-B27 showed that a 9 amino acid long peptide can bind to this molecule (10), longer peptides have also been eluted from a MAR-B4 reactive B27 molecule (11). Further, peptide elusion and binding studies showed arginine at P2 as a primary anchor for binding to HLA-B27 and an additional secondary anchor at P9 (12, 13). Recently, sequence analysis of hypervariable regions of HLA-B27 with disease-implicated enterobacteria with B27 binding motif showed that a number of peptides fulfill this criteria and could be important in the initiation of autoimmune response (14). More recently, these investigators showed binding of such peptides with HLA-B27 in an in vitro assembly assay (15).

Role of CD8+ vs. CD4+ T cells in B27-linked diseases has also been a controversial issue. Even though HLA-B27 molecule as a class I antigen should be expected to present antigens to CD8+ T cells, most of the reports describe CD4+ T cells in the diseased joint (16–19). Only a few reports are available that describe isolation of bacteria-specific, B27-restricted CD8+ T cells from involved joints of patients with B27-associated reactive arthritis and ankylosing spondylitis (20, 21). A β_2 -microglobulin (β_2 m)¹ free HLA-B27 molecule presenting a longer peptide could 'mimic' a class II molecule and stimulate CD4+ T cells.

We have recently reported spontaneous arthritis and nail changes in B27+ β_2 m^{-/-} mice (4). This study suggested a role of 'free' HLA-B27 molecules in the disease process. To make this disease model more analogous to human disease, the mouse β_2 m gene was replaced with the human β_2 m transgene. This resulted in a higher cell surface expression of HLA-B27 molecules in these mice similar to human PBLs. When these mice were brought out of the specific pathogen-free (SPF) area, they developed spontaneous arthritis and nail changes. Staining of thymus and PBLs of these mice showed expression of free heavy chains (HCs) of HLA-B27 on cell surface. Furthermore, in vivo treatment with anti-HC-specific antibody delayed development of disease in these double transgenic/knock-out mice. Our study provides strong evidence for a specific role of 'free' heavy chains of HLA-B27 in the disease process.

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1. Abbreviations used in this paper: β_2 m, β_2 -microglobulin; HC, heavy chain; SPF, specific pathogen-free.

Methods

Mice. To produce mice with HLA-B27 (B*2705) and human β_2m transgenes lacking endogenous β_2m (B27+H β_2m +M $\beta_2m^{-/-}$), β_2m -deficient mice (reference 22; gift of Dr. Beverly Koller, University of North Carolina, Chapel Hill, NC) were mated with human β_2m (H β_2m) transgenic mice (reference 23; gift of Dr. Hidde Plough, Massachusetts Institute of Technology, Boston, MA). Human β_2m transgene-positive littermates were intercrossed to generate a mouse having homozygous mutation of endogenous β_2m gene replaced with human β_2m gene (H β_2m +M $\beta_2m^{-/-}$). The H β_2m +M $\beta_2m^{-/-}$ mice were mated with previously described B27+ $\beta_2m^{-/-}$ animals (4) to obtain B27+H β_2m +M $\beta_2m^{-/-}$ mice. A flow chart describing the generation of these mice is shown in Fig. 1. A similar strategy was used to produce control double transgenic mice with HLA-Cw3 and human β_2m lacking endogenous β_2m (Cw3+H β_2m +M $\beta_2m^{-/-}$). All mice used in this study were bred in our specific pathogen-free barrier facility.

Identification of transgenes by PCR. In the absence of β_2m , MHC class I molecules are rarely expressed on the cell surface. The presence of MHC class I transgenes in $\beta_2m^{-/-}$ mice was analyzed by PCR. DNA was extracted from the peripheral blood according to manufacturer's instructions using an Isoquick nucleic acid extraction kit (MicroProbe Corp., Bothell, WA). 10 μ l of DNA was added to 0.1 mM dNTPs, 0.25 μ M each of 3' and 5' primers in the PCR buffer in a total volume of 100 μ l. 1.0 U of Taq polymerase was added to this mixture and amplified in 30 cycles under the following conditions: 3 min at 94°C, (94°C for 1 min, annealing temp for 2 min, and 72°C for 1.5 min) \times 30, and 7 min at 72°C. PCR products were analyzed by electrophoresis and their molecular weight was compared with a standard molecular weight marker. The following primer sequences were used: HLA-B27: 3'(CTC TGC CTT GGC CTT GCA GA) and 5'(CCA CTC CAT GAG GTA TTT CCA). HLA-Cw3: 3'(GTC TGT GCC TGG GGC TTG TAC) and 5'(CCA CTC CAT GAG GTA TTT CTG).

The presence of homozygous mutation in mouse β_2m was identified by PCR. We used three primers to identify the presence of wild-type and mutated mouse β_2m genes. M β_2m -1 and -2 oligonucleotide sequences amplify wild-type β_2m . Neomycin and M β_2m -1 primer sequences amplify mutated β_2m . Presence of both PCR products indicates heterozygosity for the knock-out gene.

M β_2m -1: 3'(GAA AAC CCC TCA AAT TCA AGT ATA CTC A).

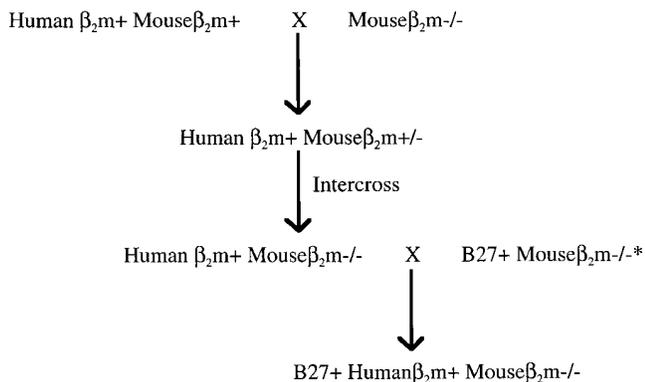


Figure 1. Schematic representation of the generation of B27+H β_2m +M $\beta_2m^{-/-}$ mice. Segregation of the HLA-B27 gene was analyzed by PCR and/or flow cytometry in the F₁ population. Segregation of H β_2m and HLA-B27 in the F₂ population was examined by flow cytometry using L368 and ME1 mAbs, respectively. The presence of mutated and wild-type M β_2m genes was confirmed by PCR using three primers as described in Methods. *Generation of B27+Mouse $\beta_2m^{-/-}$ mice has been described previously (4).

M β_2m -2: 5'(GAC GGT CTT GGG CTC GGC CAT ACT).

Neomycin: 5'(TCG AAT TCG CCA ATG ACA AGA CGC T).

Immunofluorescence and flow cytometry. Identification of transgenes HLA-B27 and human β_2m was carried out by immunofluorescence using ME-1 and L-368 monoclonal antibodies (American Type Culture Collection, Rockville, MD), respectively. Briefly, mononuclear cells from peripheral blood were incubated with antibodies for 30 min at 4°C. After washing with FACS[®] buffer (PBS containing 1% bovine serum albumin and 0.1% sodium azide) (Becton Dickinson & Co., San Jose, CA), cells were incubated with fluorescence-labeled secondary antibody (IgG goat anti-mouse Fab'2; Accurate Chemical and Scientific Corp., Westbury, NY). Expression of cell surface molecules was analyzed on 10,000 gated lymphocytes on forward and side scatter by flow cytometry. Two-color analysis was carried out for the detection of free heavy chains of HLA-B27 using the H chain-specific antibody, HC10 (24). Isolated cells were incubated with HC10, followed by secondary antibody (IgG goat anti-mouse). After washing, cells were incubated with ME1-biotin, and then Streptavidin R-phycoerythrin conjugate (Tago Inc., Burlingame, CA). Each incubation period was 30 min at 4°C. Cell surface expression of 20,000–40,000 gated lymphocytes was analyzed on FL-1 and FL-2 channels using dot plot or contour plots on the PC-Lysis program (Becton Dickinson & Co.).

Three-color analysis for V β populations among CD4 and CD8 cells was done as previously described by Nabozny et al. (25). Briefly, isolated PBLs were incubated with one of the following V β 8 TcR-specific mAbs for 30 min at 4°C: B20.6, rat anti-V β 2; KT4-10, rat anti-V β 4; MR9-8, mouse anti-V β 5.1; MR9-4, mouse anti-V β 5.1.2; 44-22-1, rat anti-V β 6; TR310, rat anti-V β 7; F23 1, mouse anti-V β 8.1.2.3; KJ16-133, rat anti-V β 8.1.2; F23.2, mouse anti-V β 8.2; MR10-2, mouse anti-V β 9; RR3-15, rat anti-V β 11; 14.2, rat anti-V β 14; KJ23a, mouse anti-V β 17a. Cells were washed and then incubated with FITC-conjugated Fab'2 fragment of the appropriate isotype of anti-mouse or anti-rat secondary antibody (Accurate Chemical & Science Corp.). After washing, 100 μ l of 1:100 diluted 1:1 mixture of mouse anti-CD4-RPE and CD8-RD613 was added to cells and incubated for 30 min at 4°C. Cells were finally washed and fixed with 1% paraformaldehyde solution. Finally, expression of TcR V β was determined on 40,000 cells using FACS[®] Vantage flow cytometer.

Surface labeling and immunoprecipitation. Cell surface proteins were labeled by using sulfo-NHS-biotin reagent (Pierce Chemical Co., Rockford, IL) as previously described by Altin and Pagler (26). Briefly, mononuclear cells isolated from spleen were washed three times with cold PBS (pH 7.6). Cells were suspended in PBS at a density of 50 \times 10⁶ cells/ml and labeled with 1 mg/ml sulfo-NHS-biotin at room temperature. Cell viability was > 90%. Cells were washed with cold PBS to remove excess sulfo-NHS-biotin. Biotinylated splenocytes were now solubilized in lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 20 mM iodoacetamide (Sigma Chemical Co., St. Louis, MO), and protease inhibitors (10 μ g/ml pepstatin, 10 μ g/ml antipain, 10 μ g/ml leupeptin) at 4°C for 30 min with occasional shaking. The lysate was centrifuged to remove particulate material and nucleic acid. The supernatant was precleared by incubating with protein A-agarose (Boehringer Mannheim, Mannheim, Germany) for 2–3 h in cold on an end-to-end rotator.

Purified monoclonal antibody was coupled to protein A-agarose by incubating on a rotator for 2 h. Immunoprecipitation was performed by incubating beads with sample lysate for 2 h at 4°C on a rotator. Immune complexes were pelleted and washed five times with lysis buffer. Now SDS-PAGE sample buffer containing 2-mercaptoethanol was added directly on the pellet. Samples were boiled for 5 min before analysis by SDS-PAGE.

Electrophoresis, Western blotting and chemiluminescence. Protein samples were electrophoresed on 9% SDS-polyacrylamide gel and blotted to nitrocellulose membrane. Blot was washed with 0.05% Tween-20 containing PBS and blocked with PBS containing 5% skimmed milk. The membrane was washed four times, and then incubated with streptavidin-HRP (Biosource International, Camarillo,

CA) at 1:2,000 dilution for 60 min. The membrane was washed five times and the protein detection was done using ECL protein detection system (Dupont-NEN, Boston, MA). The chemiluminescent blot was blotted and then exposed to film (Eastman Kodak Co., Rochester, NY).

Disease examination and quantification of arthritis. Arthritis was monitored twice a week for a period of 6 wk to 2 mo in the conventional area as described previously by Wooley et al. (27).

Histology and immunostaining. Hematoxylin and eosin staining was done by a standard method on previously decalcified paraffin-

embedded tissues. For immunostaining, fresh tissue was embedded and immediately frozen. 5- μ m-thick cross-sections were cut with a carbide tungsten knife. After fixing tissue with acetone, sections were stained with biotinylated antibody or isotype-matched irrelevant antibody. After a 40-min incubation at 4°C, sections were washed with PBS, and then incubated with streptavidin-conjugated diaminobenzidine for 40 min at 4°C. Sections were carefully washed and examined under the microscope.

In vivo antibody treatment. Mice were injected with 0.5 ml (2 mg/ml) of purified antibody within 30 min of transfer into the conven-

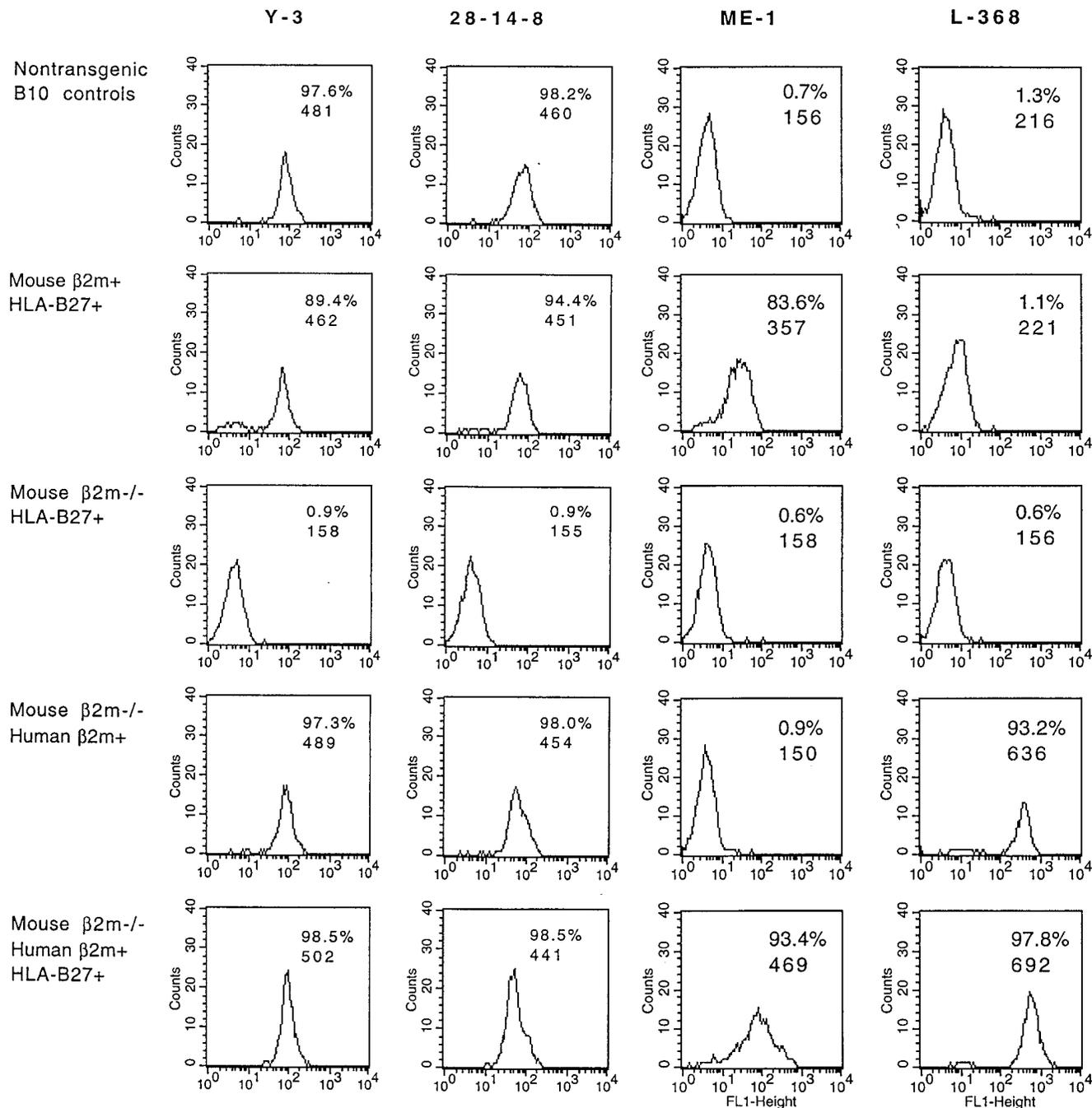


Figure 2. Expression of mouse endogenous MHC class I genes (K^b and D^b), transgenes HLA-B27 and $H\beta_2m$ on peripheral blood lymphocytes. Two mAbs for each mouse endogenous MHC class I gene K^b (Y3 and B8-24-3) and D^b (B22-249 and 28-14-8) were used. Expression of HLA-B27 and $H\beta_2m$ transgenes was analyzed using ME1 and L368 mAbs, respectively. Identified mice are shown in the extreme left (Y-3) column. The level of expression and percent positive cells are shown individually on each histogram.

Table I. Expression of HLA-B27 with Mouse and Human β_2m in Transgenic PBLs: a Comparison with B27 Expression on Human PBLs

mAb	B27+M β_2m + mouse PBLs	B27+H β_2m + M β_2m -/- mouse PBLs	B27+ human PBLs
ME1	++	++++	++++
HC10	-	++	++
B27.M1	-	+++	++
B27.M2	-	+++	+++
Ye-2	-	++	++
W6/32	-	++++	++++

tional colony from the barrier facility. Mice in the experimental group were intravenously injected with purified HC10 (HC-specific) antibody. Control groups of mice were given the same amount of either ME1 (HC+ β_2m specific), isotype-matched 2F13 monoclonal antibody or PBS. Animals were monitored 2–3 times/wk for the development of disease.

Results

Generation of mice and expression of MHC class I molecules. Generation of human β_2m and HLA-B27 double transgenic mice lacking mouse β_2m (M β_2m -/-) is shown in Fig. 1. The B27+H β_2m +M β_2m -/- mice restored cell surface expression of transgene HLA-B27 and endogenous mouse class I (K^b and D^b) molecules (Fig. 2). As evident from Fig. 2, expression of HLA-B27 was 10-fold or higher with H β_2m as compared with its expression with M β_2m . Such increased expression of (B27+H β_2m) complex was noticed in several independent experiments. An increase in the expression of HLA-Cw3 was also noticed when expressed with H β_2m . The level of expression of endogenous class I molecules (K^b and D^b) was unaffected. By using several previously defined anti-HLA-B27 antibodies, expression of the B27 molecule on PBLs from B27+H β_2m +M β_2m -/- mice was found to be similar to that of its expression on human PBLs (Table I). Only ME1 antibody showed reactivity to B27 molecules paired with M β_2m .

Reconstitution of CD8+ T cells and V β repertoire. We have previously demonstrated a negligible number of CD8-positive T cells in B27+M β_2m -/- mice (4). The B27+H β_2m + M β_2m -/-

mice showed restoration of CD8+ T cells (Fig. 3), similar to that from a mouse with wild-type β_2m . Analysis of V β TcR among CD4 and CD8 showed no significant difference between B27+H β_2m +M β_2m -/- mice and B27 negative littermates (Table II). Further, no difference in TcR V β s were found between mice maintained in the SPF facility vs. a conventional colony (data not shown).

Spontaneous erosive arthritis in B27+H β_2m + β_2m -/- mice. The mice were bred in the pathogen-free area and 8–12-wk-old animals were transferred to the conventional colony for experiments. None of the B27+H β_2m +M β_2m -/- mice and negative littermates had any clinical disease inside the barrier. However, B27+ mice developed spontaneous inflammatory disease (Fig. 4 A) and nail changes (Fig. 4 B) within 4 wk of their transfer to the conventional area. Approximately 70% of males and 33% of females developed disease. The disease was similar to a previously described disease in B27+M β_2m -/- mice (Table III). The disease began with discoloration and hyperkeratosis in nails, primarily affecting the rear paws. In the next 3–5 wk, nails disintegrated after reappearance of thicker nails. Some mice showed repetition of these features. The mild arthritis led to severe ankylosis in another 2 mo in B27+H β_2m +M β_2m -/- male mice. Histological examination of involved joints showed the presence of mononuclear cells in the joints, cartilage destruction, and pannus formation (Fig. 4 C). Female mice, in general, developed a milder form of arthritis. A mild and transient arthritis was also observed in ~10% of B27 negative control animals and mice expressing HLA-Cw3 transgene (HLA-Cw3+H β_2m +M β_2m -/-). These results indicate that the presence of a B27 molecule in these mice influences disease susceptibility. The low incidence of mild and transient disease in the control mice is probably due to endogenous mouse class I molecule, H-2D^b/H β_2m .

Cell surface expression of free HCs and [HC+ β_2m] complex on PBLs. Cell surface expression of free HCs of HLA-B27 was analyzed on transgenic PBLs using HC-10 mAb by flow cytometry and biotin labeling of cell surface proteins. As shown in Fig. 5, A and B, 20–50% of transgenic PBLs of B27+H β_2m +M β_2m -/- mice express free HCs of HLA-B27. No such expression of HCs on PBLs from single HLA-B27 transgenic mice was observed. The data suggest that β_2m is required for the active transport of HLA-B27 to the cell surface. After [HC+ β_2m] complex reaches the cell surface, some H β_2m may dissociate from B27, but probably not M β_2m . Indi-

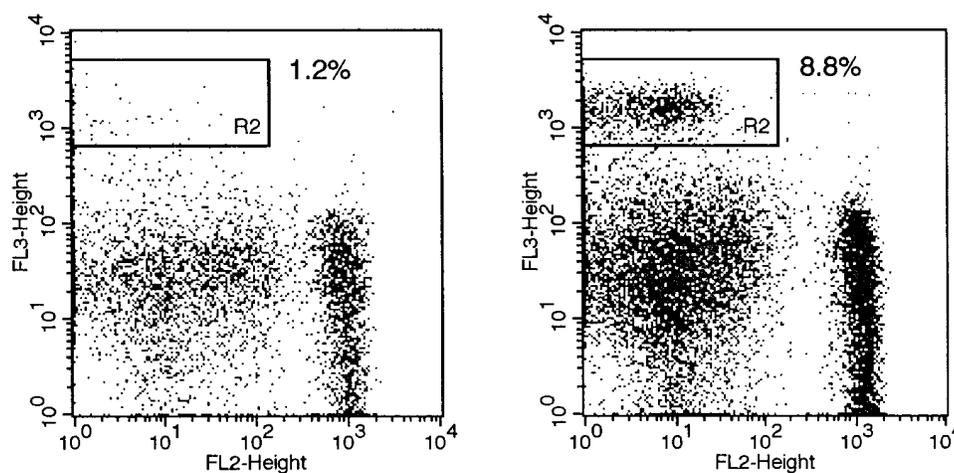


Figure 3. Two-color analysis of CD4+ (x-axis) and CD8+ (y-axis) T cells on PBLs of mice coexpressing MHC class I molecules without mouse β_2m (left) or human β_2m replacing M β_2m (right). Center dot plot shows few CD8+ T cells on PBLs of B27+ β_2m -/- mice.



Figure 4. Clinical and histological changes in B27+H β ₂m+M β ₂m^{-/-} mice. (A) Ankylosis of rear paw in an arthritic B27+H β ₂mM β ₂m^{-/-} mouse compared with the nonarthritic paw of a B27-H β ₂m+M β ₂m^{-/-} littermate. (B) Upper left: normal nail from nonarthritic mouse; upper right, lower left, and lower right: progression of nail disease in B27+H β ₂m+M β ₂m^{-/-} mice showing discoloration and hyperkeratosis. (C) Histological changes in the rear paw of an arthritic B27+H β ₂m+M β ₂m^{-/-} mouse showing infiltration of mononuclear cells within the synovium, cartilage destruction, and subchondral bone erosion. The lower panel is a higher magnification of the histology shown in the upper panel.

rectly, this suggests that [HC+M β ₂m] complex is more stable than [HC+H β ₂m] complex. Further, a negligible amount of free HCs were found on PBLs of Cw3+H β ₂m+M β ₂m^{-/-} mice. The presence of heavy chains of HLA-B27 on the cell surface of splenocytes from B27+H β ₂m+M β ₂m^{-/-} mice was

also confirmed by biotinylating surface proteins and immunoprecipitation with HC10 monoclonal antibody. Analysis of immunoprecipitated protein was done on SDS-PAGE and Western blotting (Fig. 6). Furthermore, the presence of HC was also confirmed on a HLA-B27-transfected MHC class I-defi-

Table II. V β TcR in Transgenic Mice

	B27+m β_2 m+		B27-h β_2 m+m β_2 m-/-		B27+h β_2 m+m β_2 m-/-	
	CD4	CD8	CD4	CD8	CD4	CD8
Total	18.14 \pm 1.07	10.18 \pm 0.74	20.42 \pm 1.15	11.09 \pm 1.03	14.43 \pm 1.47	11.38 \pm 0.52
V β 2	5.78 \pm 0.15	3.31 \pm 0.37	4.94 \pm 0.05	2.40 \pm 0.05	5.99 \pm 0.38	3.03 \pm 0.09
V β 3	6.73 \pm 0.31	3.23 \pm 0.10	7.91 \pm 0.45	4.05 \pm 0.03	9.21 \pm 0.37	4.30 \pm 0.37
V β 5.1	1.87 \pm 0.16	6.85 \pm 0.44	0.82 \pm 0.06	5.23 \pm 0.57	1.38 \pm 0.77	5.40 \pm 0.76
V β 5.1.2	2.44 \pm 0.12	12.91 \pm 0.76	1.21 \pm 1.01	11.84 \pm 1.07	1.53 \pm 0.19	13.04 \pm 0.06
V β 6	8.37 \pm 0.45	5.75 \pm 0.68	9.39 \pm 0.59	6.11 \pm 0.11	8.61 \pm 0.09	5.94 \pm 3.09
V β 7	3.20 \pm 0.09	5.03 \pm 0.92	3.05 \pm 0.08	6.06 \pm 0.40	3.19 \pm 0.28	5.13 \pm 1.35
V β 8.1.2	16.32 \pm 0.14	10.78 \pm 1.49	16.90 \pm 1.01	11.90 \pm 0.78	18.26 \pm 0.03	12.19 \pm 0.75
V β 8.1.2.3	21.15 \pm 0.01	17.52 \pm 0.18	20.88 \pm 0.49	19.18 \pm 0.09	24.57 \pm 0.65	18.83 \pm 0.60
V β 8.2	9.27 \pm 0.34	9.47 \pm 0.28	8.28 \pm 0.41	9.12 \pm 0.19	6.27 \pm 0.23	9.60 \pm 2.31
V β 9	0.54 \pm 0.24	2.91 \pm 0.08	1.06 \pm 1.00	3.21 \pm 0.36	0.84 \pm 0.17	2.38 \pm 1.29
V β 14	5.02 \pm 1.09	5.99 \pm 0.49	6.34 \pm 1.23	6.90 \pm 0.90	7.34 \pm 1.36	6.49 \pm 2.94

V β 11 and V β 17 are deleted in all groups.

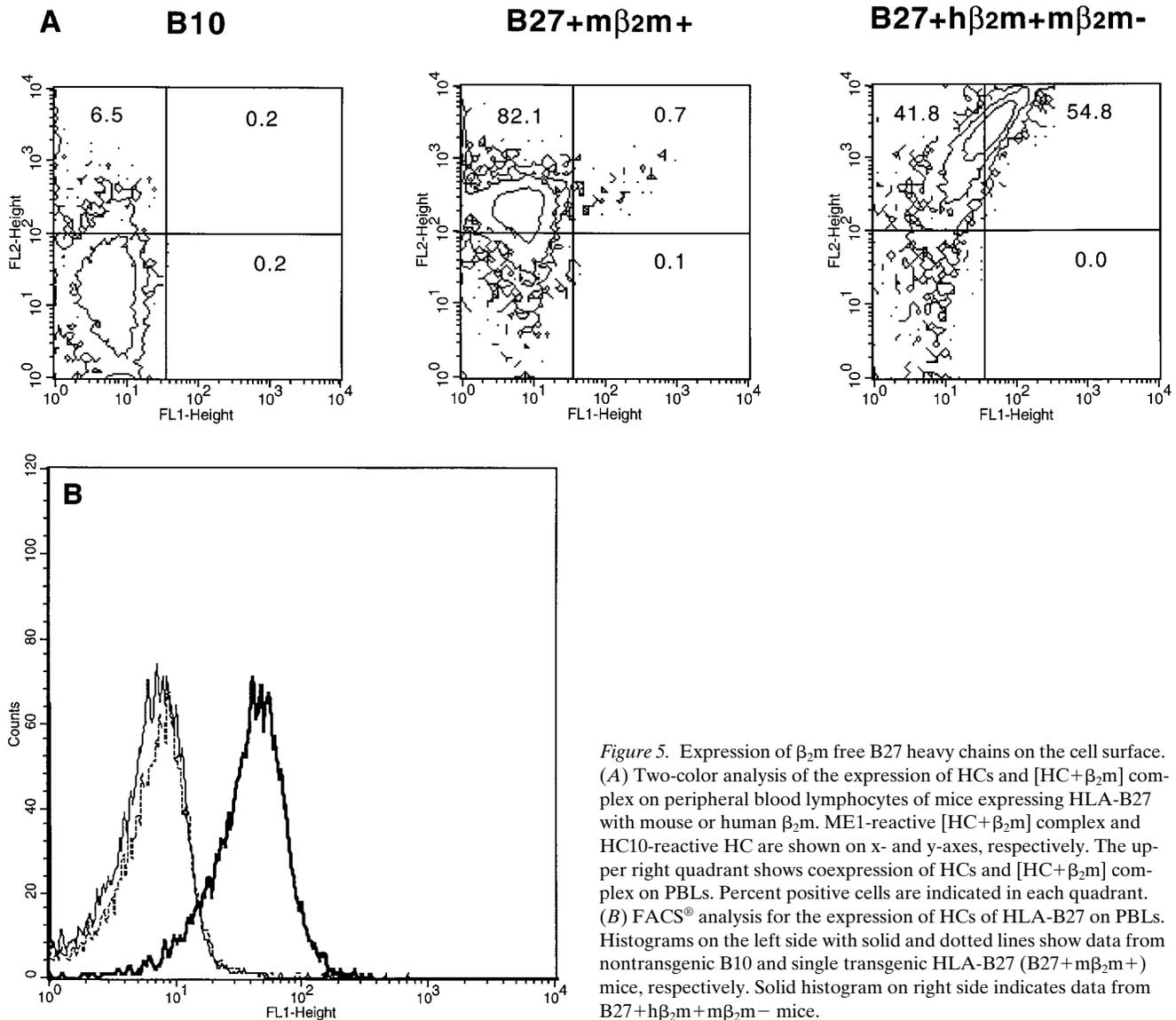


Figure 5. Expression of β_2 m free B27 heavy chains on the cell surface. (A) Two-color analysis of the expression of HCs and [HC+ β_2 m] complex on peripheral blood lymphocytes of mice expressing HLA-B27 with mouse or human β_2 m. ME1-reactive [HC+ β_2 m] complex and HC10-reactive HC are shown on x- and y-axes, respectively. The upper right quadrant shows coexpression of HCs and [HC+ β_2 m] complex on PBLs. Percent positive cells are indicated in each quadrant. (B) FACS[®] analysis for the expression of HCs of HLA-B27 on PBLs. Histograms on the left side with solid and dotted lines show data from nontransgenic B10 and single transgenic HLA-B27 (B27+m β_2 m+) mice, respectively. Solid histogram on right side indicates data from B27+h β_2 m+m β_2 m- mice.

Table III. Spontaneous Disease in B27+ Human β_2m + Mouse $\beta_2m^{-/-}$ Mice: Specificity of the MHC Class I Transgene

	No. of mice		No. of mice with arthritis		No. of mice with nail disease		Ankylosis	
	M	F	M	F	M	F	M	F
B27	39	26	26 (66%)	9 (34%)	25 (64%)	11 (42%)	5	0
Cw3	13	11	2 (15%)	1 (9%)	2 (15%)	0	0	0
Class I Tg*	57	73	5 (8%)	4 (5%)	5 (8%)	5 (7%)	0	0
Neg H β_2m +								

*This group includes HLA transgene negative littermates.

cient C1R-B27 cell line. The presence of free HCs was not observed on the cell surface of C1R cells transfected with HLA-A3, HLA-Bw58, and HLA-Cw3 molecules (data not shown), suggesting an allele-specific phenomenon for the presence of β_2m -free heavy chains on the cell surface.

Tissue specific expression of HCs in the thymus. To determine expression of free HCs and [HC+ β_2m] complex on thymic epithelium, we stained frozen sections of thymus of B27+H β_2m +M $\beta_2m^{-/-}$, B27+M β_2m +, and nontransgenic controls. As shown in Fig. 7, tissue-specific expression of HC and [HC+ β_2m] complex was observed in the thymic epithelium from B27+H β_2m +M $\beta_2m^{-/-}$ mice. Staining of the thymus from a single transgenic B27 (expressed with M β_2m) showed cell surface expression of only [HC+ β_2m] complex. As expected, intracellular staining of free HC of B27 was observed. Cell surface expression of free HC on thymic epithelium of single transgenic B27 mice was undetectable. A similar pattern of staining was observed in the spleens. Nontransgenic mice and negative controls showed no staining with W6/32, ME1, and HC10 antibodies.

In vivo treatment with anti-HC-specific antibody delays onset of disease in the conventional colony. To determine whether blocking the free heavy chains of B27 would have an effect on the spontaneous disease in the B27+H β_2m +M $\beta_2m^{-/-}$ mice, we treated these animals with an antibody which reacts with free HCs and specifically recognizes the $\alpha 1\alpha 2$ domain of the B27 molecule. Mice were given 1 mg of antibody intraperitoneally within 30 min after they were brought out into the conventional colony. Control groups of mice were treated with either ME1 (HC+ β_2m -specific) or isotype-matched 3F12 anti-

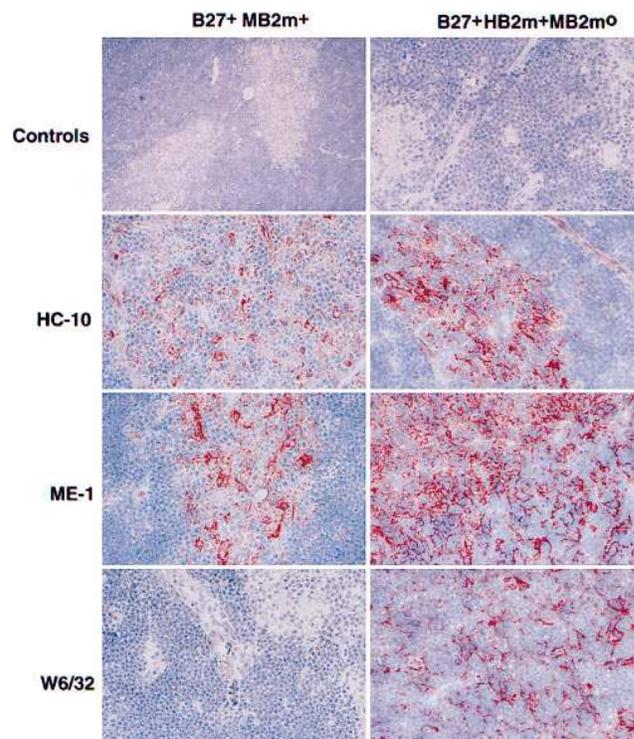
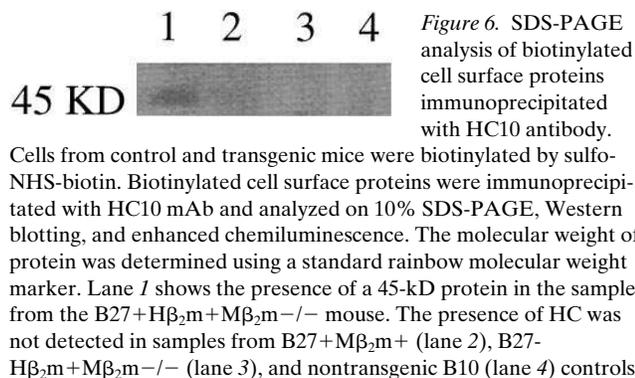


Figure 7. Immunostaining of fresh frozen sections of thymus with ME1, HC10, and W6/32 mAbs. Expression of ME1 reactive [HC of B27+ β_2m] complex is higher on B27+H β_2m +M $\beta_2m^{-/-}$ thymus compared with its expression on thymus from B27+M β_2m +. Reactivity of HC-specific HC10 mAb shows cell surface and intracellular staining on B27+H β_2m +M $\beta_2m^{-/-}$ thymus compared with intracellular staining on the thymus of B27+M β_2m +. Reactivity of W6/32 is seen only on B27+H β_2m +M $\beta_2m^{-/-}$ thymus.

body or PBS. As shown in Fig. 8 b, 9/21 (42%) of mice treated with HC10 antibody developed nail disease compared with 6/8 (75%) of ME1-treated and 14/21 (66%) of PBS-treated mice. Incidence of spontaneous development of arthritis (Fig. 8 a) was also decreased (33%) in HC10-treated animals compared with ME1-treated (50%) and PBS-treated (76%) animals. Lower incidence of disease in the ME1 antibody-treated mice could be due to the depletion of B27 molecules before dissociation from β_2m . Mice treated with isotype control antibody 3F12 also showed higher incidence of arthritis/nail disease similar to that of other control group animals. A delay of disease development was noticed in the group of HC10-treated animals. Severity of arthritis was similar in HC10-treated and control animals.

Discussion

In this report, we have shown spontaneous disease in HLA-B27 transgenic mice. These animals express B27 molecules with H β_2m and do not have any competition with endogenous M β_2m . Mice develop spontaneous disease within 4 wk of transfer from a specific pathogen-free barrier facility to a conventional colony. Disease begins with inflammation in toes of the rear paw. This mild inflammation leads to nail changes such as discoloration and hyperkeratosis. A few male mice developed ankylosis in the rear paw. These are typical characteristics of

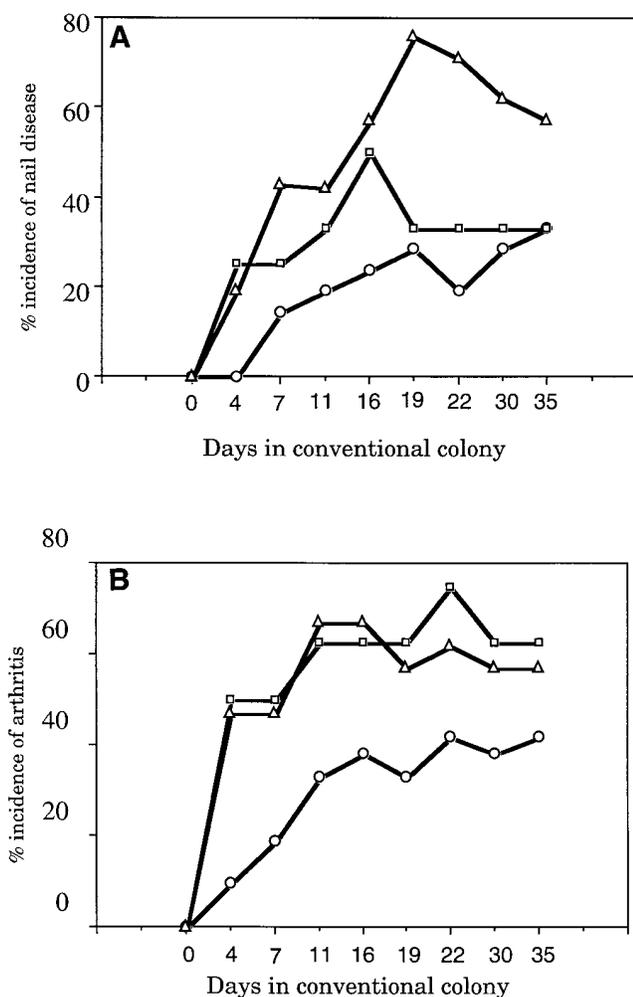


Figure 8. Incidence of arthritis and nail changes in mice treated with heavy chain-specific mAb HC10, [HC+ β_2m] complex-specific ME1, and PBS. A total of 21, 21, and 8 mice were used in the HC10, PBS, and ME1 treated groups, respectively. B27+H β_2m +M $\beta_2m^{-/-}$ mice were injected with 1 mg purified antibody, and the development of spontaneous disease was monitored two times/wk for a period of 2 mo. ○, HC10; □, ME1; △, PBS.

B27-associated human diseases such as reactive arthritis and Reiter's disease (28). Several reports suggest that HLA-B27-linked diseases in humans are triggered by infection with enteric or genitourinary pathogenic bacteria (29, 30). Transgenic rats raised in a germ-free environment did not develop inflammatory intestinal or peripheral joint disease (31). To identify disease causing microorganisms, we killed a few animals housed in SPF and the conventional colony. No difference in the microbial flora of mice raised in the two facilities was observed. Microbiological examination excluded the possibility of *Yersinia*, *Salmonella*, *Klebsiella*, *Shigella*, and certain strains of mycoplasma.

Why do mice expressing HLA-B27 with M β_2m not develop disease? Species-specific replacement of the β_2m gene induces conformational changes in the transgene product of B27 and peptide binding (data not shown). Such conformational changes in MHC class I molecules are induced intracellularly, and reassembly of such molecules by providing exogenous H β_2m does

not have any influence in peptide binding (32). In H-2d mice, a single substitution in β_2m leads to changes in peptide presentation (33). By using several recombinant β_2m mutants, Fukazawa et al. (13) have described their effect on conformation of HLA-B27 and CTL recognition. Human β_2m differs from mouse β_2m at 29 amino acids (34) that may influence conformation of MHC molecule and peptide binding. We have also noticed similar effects with the species of β_2m in B27 transgenic animals (data not shown). It is possible that some arthritogenic environmental antigens cannot bind to B27+M β_2m +complex and thus are unable to trigger disease in HLA-B27 single-transgenic mice.

There are at least two potential possibilities for the development of spontaneous disease in these mice: (a) HLA-B27 with human β_2m presents an exogenous antigen, and (b) free HCs of B27 on the cell surface present an exogenous peptide. Cell surface expression of free HCs of HLA-B27 on PBLs in these mice suggest that association of HLA-B27 with mouse β_2m is more stable than its association with human β_2m . Heavy chains of HLA-B27 are also expressed on thymic epithelium in mice expressing B27 and human β_2m . As shown by others (35), it is possible that after reaching the cell surface a few human β_2m dissociate from HLA-B27 heavy chains. Since free heavy chains are not detected in the presence of M β_2m or with Bw58, Cw3, Cw4, and other unknown MHC class I molecules, this must be unique for HLA-B27 and human β_2m . It has been reported that interaction of amino acid residue at position 9 in the floor of the heavy chain beta strand with β_2m is critical for stability of the [HC+ β_2m] complex (35). All subtypes of HLA-B27 have histidine at position 9, which is part of the B pocket and may also cause weak interaction with human β_2m . Amino acid differences in human and mouse β_2m may also affect such stability of [HC+ β_2m] complex. While this may allow the B27 molecule to assemble and reach the cell surface, it may not provide enough stability to remain as a trimeric complex on the cell surface. Thus, some B27 molecules may dissociate from β_2m and lose endogenous peptides. Now, as free and 'empty' heavy chains, they may dimerize like a class II molecule (36) and be able to present an exogenous antigen. Studies in β_2m -deficient mice suggest that free HC can also present antigens to T cells (37, 38). Expression of [HC+ β_2m] complex (without peptide) on the surface of living cells was first reported by Benjamin et al. (39). This empty HLA-B27 molecule also presented exogenously added peptides from a nuclear envelope protein of influenza, NP-1. These authors speculated that like the MHC class II molecule, B27 can also present exogenous peptides. Moreover, MHC class I-restricted CD4+ T cells have also been reported (40, 41). Several investigators have reported isolation of enterobacteria-specific CD4+ T cells from the joint (18, 19).

Our studies demonstrate that free HCs contribute to disease pathogenesis in B27 transgenic mice. Spontaneous disease in these mice can be delayed by treatment with anti-HC-specific antibody. These findings suggest that HCs of B27 are critical in disease pathogenesis. Studies to determine whether CD4+ T cells are involved in this disease are currently under investigation.

On the basis of our results, we propose the following hypothesis (Fig. 9): (a) the transgene B27 molecule is more stable with mouse β_2m ; therefore, in the thymus there is high affinity interaction with T cells, which leads to deletion of self reactive T cells, and (b) poor interaction of HLA-B27 with human β_2m

Hypothesis

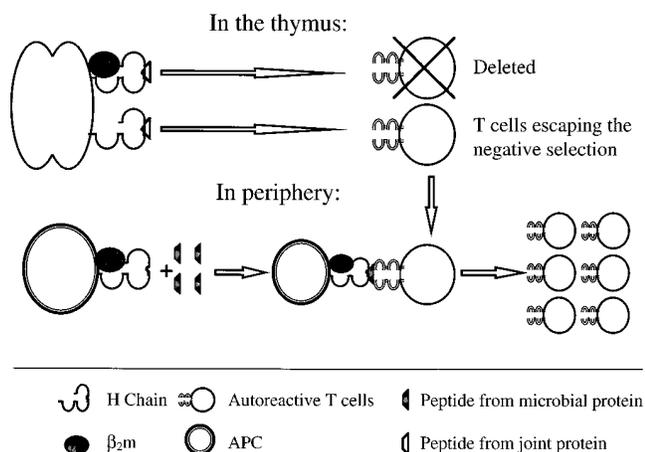


Figure 9. Diagrammatic representation of our hypothesis for the involvement of B27 heavy chain in disease pathogenesis.

leads to dissociation of HC from the β_2m and/or peptide. T cells interacting with free B27 molecules plus self peptide may either escape the negative selection or get positively selected because of low affinity interaction. Further, these self-reactive T cells may be specific for a joint-related peptide presented by B27 HC. In the periphery, these autoreactive T cells may recognize environmental antigen(s) mimicking the joint-related protein, proliferate, migrate to the joint, and cause tissue injury.

Our findings may answer many intriguing aspects of HLA-B27-linked diseases. First of all, why is HLA-B27 the only MHC class I molecule linked to an autoimmune disease? Our studies show that as a free heavy chain B27 may function similar to a class II molecule. This will explain how B27 may be able to present an exogenous bacterial antigen and how CD4+ T cells may be activated. Since a free HC could have a more flexible alpha helix, it may be able to bind longer peptides, explaining the elution of longer peptides from this molecule. If the dissociation of B27 from β_2m is the result of the mutation at residue 9 on the floor, it will explain why all B27 subtypes are linked to the disease irrespective of substitution in the alpha helix. This mouse model of B27-linked human disease offers many advantages. Using various transgenes and 'knock-out' mice, we can further dissect the mechanism of the disease process and come to a better understanding of the human disease.

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