Free HLA Class I Heavy Chain-Carrying Monocytes — A Potential Role in the Pathogenesis of Spondyloarthropathies

WEN CHAN TSAI, CHUNG JEN CHEN, JENG HSIEN YEN, TSANG TENG OU, JIH JIN TSAI, CHIANG SHIN LIU, and HONG WEN LIU

ABSTRACT. ***Objective.*** A fully complexed HLA-B27 molecule consists of a heavy chain, a peptide, and ß$_2$-microglobulin (ß$_2$m). The heavy chain can also exist free of ß$_2$m. It has been proposed from animal and *in vitro* experiments that the free heavy chain is responsible for disease. We wanted to determine the following for patients with ankylosing spondylitis (AS): (1) are there cells expressing cell surface free heavy chains; (2) if so, which subset of cells has such capacity; (3) does expression vary with disease activity; (4) can we find free heavy chain-expressing cells at the site of inflammation that is characteristic of the disease; and (5) can such expression be induced in healthy subjects.

Methods. Quantitative flow cytometry was carried out using antibodies directed separately against HLA class I complex, free heavy chain A or B alleles. Antibodies directed against other cell surface markers were used to identify cell types. Immunohistochemical staining was used to stain synovial tissue.

Results. There was a high level of surface expression of free heavy chains in monocytes of patients with AS. The level exceeded those of normal controls and patients with rheumatoid arthritis. The level of expression correlated with the inflammation marker, erythrocyte sedimentation rate. The level of expression was enhanced when monocytes from healthy controls were driven to differentiation by longterm culture. Free heavy chain-expressing monocytes infiltrated the synovium of an involved hip joint of a patient with AS.

Conclusion. This is the first patient-related evidence that surface free heavy chains of HLA-B27 have to be considered as potential disease-causing molecules. (J Rheumatol 2002;29:966–72)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS HLA-B27 FREE HEAVY CHAIN MONOCYTE

Ankylosing spondylitis (AS) is a chronic inflammatory disease of spine. It is characterized by disordered immunological responses causing tissue-specific inflammation and damage, and by its striking familial aggregation[1]. The strong association between HLA-B27, an MHC class I gene, and AS[2] has prompted enormous interest in the structure of HLA-B27. The odds ratio for HLA-B27 in AS is in excess of 100, and is fairly constant across most ethnic groups[3,4]. Although the presence of the HLA-B27 allele marks a strong predisposition for AS, its role in disease pathogenesis is unclear.

Newly synthesized HLA class I heavy chains bind to ß$_2$-microglobulin (ß$_2$m) and peptides in the endoplasmic reticulum, and the trimeric complex is transported to the surface of the cell[5]. A strong interaction between ß$_2$m and heavy chains is critical for a stable complex. Recent knowledge about the 3-dimensional structure of HLA-B27 molecules and peptide elution studies suggest that (1) when expressed on the cell surface, HLA-B27 molecules usually complex with ß$_2$m and peptides of approximately 9 amino acids in length with arginine at position 2[6,7]; and (2) compared with other HLA class I alleles, low affinity binding of ß$_2$m and peptides with B27 heavy chain may shift the equilibrium to favor dissociation of ß$_2$m from the complex[8]. Free HLA class I heavy chains are rarely expressed on the cell surface, and when present, behave more like class II molecules that can bind longer, extracellular peptides[9,10]. Furthermore, Allen and co-workers have recently shown that HLA-B27 can form disulfide-bound heavy chain homodimers, termed HC-B27, capable of binding to and being stabilized by a human immunodeficiency virus derived peptide known to bind HLA-B27[11]. Thus ß$_2$m free heavy chains can in theory contribute to disease susceptibility by presenting exogenous antigens to T cells. Transgenic rats expressing copies of HLA-B27 and human ß$_2$m genes develop spontaneous inflammatory disease, which has many similarities to

From the Department of Internal Medicine, Kaohsiung Medical University, Kaohsiung City, Taiwan.

W.C. Tsai, MD, Assistant Professor of Internal Medicine; C.J. Chen, MD, Associate Professor of Internal Medicine; J.H. Yen, MD, PhD, Associate Professor of Internal Medicine; T.T. Ou, MD, Visiting Staff of University Hospital; J.J. Tsai, MD, Assistant Professor of Internal Medicine; C.S. Liu, DDS, Assistant Professor of Pathology; H.W. Liu, MD, Professor of Internal Medicine.

Address reprint requests to Dr. W.C. Tsai, Department of Internal Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung City, Taiwan.

Submitted June 12, 2001; revision accepted October 23, 2001.

human spondyloarthropathy[12]. Interestingly, HLA-B27 transgenic mice lacking ß$_2$m also developed disease similar to human spondyloarthropathies[13,14]. Cells of these mice expressed free heavy chains suggesting that they may play a major role in the pathogenesis of spondyloarthropathy not only in mice, but also potentially in humans.

In this study, we investigated the presence of free heavy chains on the cell surface in patients with AS, tested the correlation between the level of expression and disease activity, sought these cells at the disease site, and finally, investigated induction of these molecules on the cell surface of healthy subjects.

MATERIALS AND METHODS

Patients. Blood was collected from 38 consecutive Chinese AS patients and 10 patients with active rheumatoid arthritis (RA) with informed consent at the Rheumatology Outpatient Department of Kaohsiung Medical University. Synovial fluid was collected from the knee joint from a patient with AS. Synovial membrane was obtained from the hip joint from a patient with AS in whom total hip replacement was done. All patients with AS fulfilled the Modified New York Criteria for AS[15], and they all carried HLA-B27 allele. None of these patients were receiving steroids or disease modifying antirheumatic drugs. All were HLA-B27 negative.

Controls. Twenty-one healthy Chinese adults (age and gender-matched) served as a control population. Two were HLA-B27 positive.

Antibodies and other reagents. A number of monoclonal antibodies (Mab) were used in this study to evaluate antigen expression in various cell types: Mab reactive with macrophage differentiation antigen 27 E10, RM 3/1, 25 F9 were gifts from Dr. David Yu from UCLA and purchased from Accurate Chemical & Scientific Corp. (Westbury, NY, USA); CD14 and CD11b were purchased from PharMingen (San Diego, CA, USA); phycoerythrin (PE) conjugated goat anti-mouse IgG2a antibody and streptavidin-PE were purchased from Caltag (Burlingame, CA, USA); Mab HC10 (binds preferentially to a determinant on the free heavy chain of alleles of HLA-B and C) and Mab HCA2 (binds to an HLA-A allele) were kindly provided by Hidde Ploegh (MIT, Boston, MA, USA). Lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF-α) were purchased from Sigma (St. Louis, MO, USA). GM-CSF and interleukin 4 (IL-4) were purchased from Pepro Tech (Rocky Hill, NJ, USA). Purified mouse IgG2a was purchased from Immunotech (Miami, FL, USA).

Cell isolation and culture. Peripheral blood white cells were isolated from heparin treated blood by using a standard Ficoll hypaque (Pharmacia, Uppsal, Sweden) gradient. Synovial cells were separated immediately after aspiration by the same method. After harvesting, peripheral blood mononuclear cells (PBMC) were suspended in RPMI 1640 medium supplemented with L-glutamine. After incubating for 20 minutes in a culture dish at room temperature, the non-adherent cells were harvested. This fraction was designated as the lymphocyte fraction. The adherent cells were washed twice with serum-free medium to remove any residual non-adherent cells. Adherent monocytes were harvested by gently scraping with a plastic cell scraper. These adherent cells contained more than 85% of CD14+ cells and were cultured in 6-well plates (1×10^6 cells/ml) for one week with or without the addition of the following stimulants: LPS (10 μg/ml), TNF-α (10 ng/ml), GM-CSF (70 ng/ml), IL-4 + GM-CSF, IL-4 (200 U/ml) for 1 week. Synovial mononuclear cells were stained immediately without further incubation to separate subpopulations of cells.

Immunostaining and flow cytometry analysis. Isolated cells (3×10^5) and 20 μl "Quantum simply cellular bead" (Flow Cytometry Standards Corporation, NC, USA) were incubated with each Mab in 50 μl of staining buffer (Hank balanced salt solution, 1% BSA, 0.1% sodium azide) for 30 minutes at room temperature, then washed 3 times with PBS. The cells were stained with a second antibody for another 30 minutes or directly suspended in 500 μl of 1% paraformaldehyde in PBS. Quantum Simply Cellular beads were used to convert mean fluorescence intensity (MFI) to antibody-binding capacity (ABC) as detailed elsewhere[16]. Mouse purified IgG2a was used as an isotype control to stain the cells; the MFI obtained from isotype control stood for the background value for the calculation of ABC.

Cytometry analysis was performed with an EPICS Elite ESP flow cytometer (Coulter). A total of 5000 gated cells were used in each analysis. Two-color analysis was used to obtain the percentage of each population. Compensation was adjusted by the use of synthetic beads from Coulter.

Sections of paraffin-embedded synovial tissue were subjected to immunohistochemical staining by using DAKO LSAB®2 System, horse radish peroxidase (HRP) (DAKO, CA, USA). Briefly, tissue slides were deparaffinized in xylene and later in absolute ethanol. The slides were then rehydrated in distilled H_20 before staining. Specimens were incubated for 10 minutes with Mab HC10. After washing, slides were incubated with biotin labeled antibody for 10 minutes and washed again and incubated with streptavidin peroxidase. DAKO AEC substrate-chromogen was used to develop the reaction sites. Finally, the slides were counterstained by hematoxylin.

Statistical analysis. Statistical analysis was performed using the SPSS Pro 9.0 statistical package (SPSS, Chicago, IL, USA). Correlations between ABC and erythrocyte sedimentation rate (ESR) were analyzed using Spearman's rank correlation, p values > 0.05 were considered biologically significant. Statistical analysis of the free heavy chain expression was performed by one way analysis of variance (ANOVA) and adjusted by Scheffe's test.

RESULTS

Expression of free heavy chains. We first stained the cell fractions from patients with AS immediately after isolation with Mab HC10. As shown in Figure 1, only monocytes had a high level of HC10 reactivity on their surface. The mean ABC of HC10 from 38 AS patients was 12,940 per cell, which was significantly higher than that on the granulocytes and lymphocytes ($p = 0.0001$ between groups, when multiple comparisons were used; $p = 0.367$ between granulocytes and lymphocytes). Figure 2 shows that the mean ABC of HC10 expressed on the monocytes from patients with AS was significantly higher than that of patients with

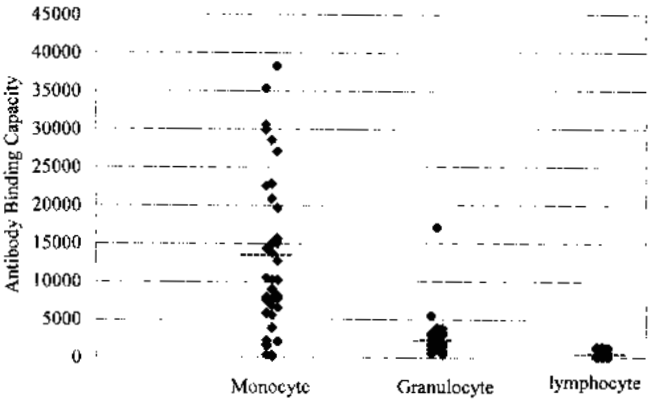

Figure 1. Free heavy chains on 3 WBC subpopulations isolated from 38 AS patients were stained with Mab HC10. Mean fluorescence intensity was converted to antibody binding capacity (ABC) expressed as molecules per cell. The dotted line shows the mean ABC of each subpopulation.

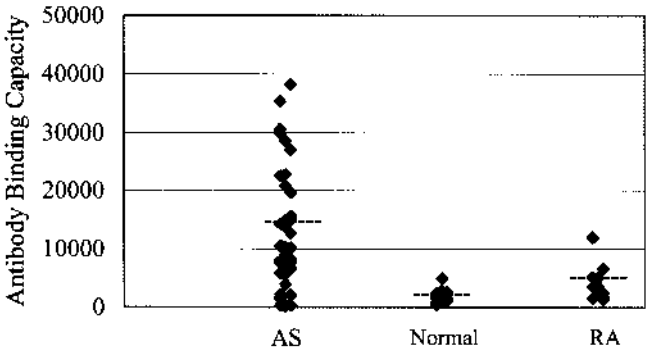

Figure 2. Monocytes isolated from patients with AS (n = 38), RA (n = 10) and healthy controls (n = 21) were stained with Mab HC10. Mean fluorescence intensity was converted to ABC. The dotted line shows the mean ABC of each group.

RA and healthy controls (12,940 ± 10,408 *vs* 4699 ± 3055 and 1859 ± 1016) (p = 0.0001 between groups, when multiple comparisons were used; p = 0.709 between RA and healthy controls). We then investigated whether the expression of HC10 reactivity correlated with disease activity. We chose CRP, ESR and Bath AS Disease Activity Index (BASDAI) as activity indicators and compared them with the ABC of HC10 from each patient with AS. CRP and BASDAI failed to show any correlation with ABC of HC10 (data not shown), but as shown in Figure 3, the level of ABC was linearly correlated with ESR ($R^2 = 0.443$, p = 0.042). In Figure 4, more than 90% of CD14+ synovial cells showed HC10 reactivity.

Findings of immunohistochemistry. To further determine whether HC10+ cells play an important role in the disease process, we stained synovial tissue obtained from a patient with AS (during total hip replacement surgery) with Mab HC10. We identified many HC10+ mononuclear cells infiltrating the synovial tissue, especially around the blood vessels (Figure 5).

The free heavy chain is inducible on monocytes from healthy controls. We had already found that monocytes from healthy controls did not show HC10 reactivity. When monocytes were cultured *in vitro* for 1 week, they matured into macrophages. If cultured in GM-CSF and IL-4, they transformed into dendritic cells[17]. We hypothesized that these free heavy chain-carrying monocytes reflect a matured monocyte subpopulation. After isolation of monocytes from 3 healthy controls (all HLA-B27 negative), we cultured the cells in a 6-well plate for 1 week with the addition of one of the following stimulants in each well: LPS, IL-4, GM-CSF, IL-4 + GM-CSF, TNF-α. After incubation, the cells were stained with each one of the following Mab: HC10, HCA2, 27E10, RM3/1, 25F9, CD11b. As shown in Figure 6A, after incubation for 1 week, the HC10 reactivity increased dramatically even in the absence of any stimulants added. The HCA2 reactive molecules also increased at the end of incubation although not as dramatically as that of HC10. Figure 6B shows the increased expression of macrophage differentiation antigens and the downregulated expression of CD11b at the end of incubation.

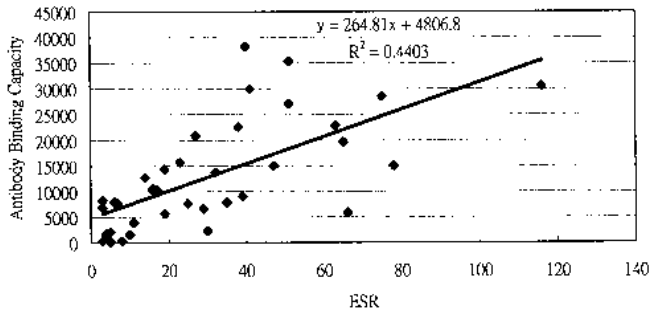

Figure 3. Correlation between ESR and mean ABC for each of 38 AS patients.

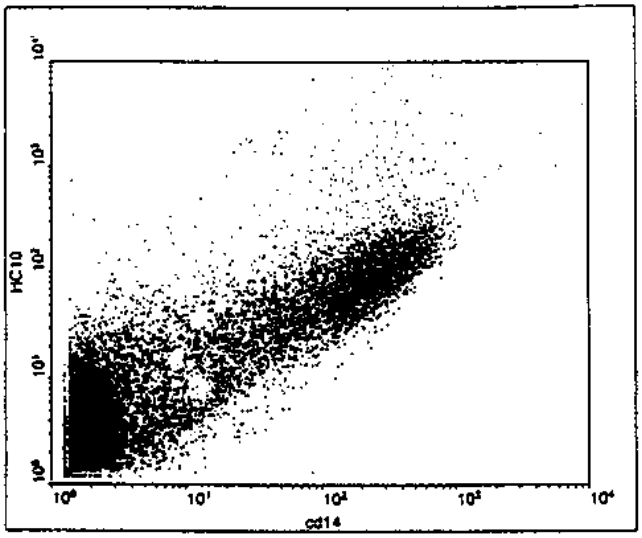

Figure 4. Free heavy chain expression in HC10-stained synovial mononuclear cells from the swollen knee joint of a patient with AS. More than 90% of CD14+ cells are HC10 reactive.

Finally we studied the dynamics of free heavy chain expression. Again, we cultured the monocytes from 3 healthy controls without any stimulants for 1 week. Each day we isolated and stained monocytes with Mab HC10 and HCA2. As shown in Figure 7, after the third day of incubation, the HC10 and HCA2 reactivity increased dramatically.

DISCUSSION

Mononuclear phagocytic cells are key elements in the development of chronic arthritis[18]. In addition to presenting antigens to T cells, these cells also secrete various proinflammatory cytokines like TNF-α and transforming growth factor-ß. Braun, *et al* found separate areas with high levels of these 2 cytokines in sacroiliac tissue from AS patients[19].

In this study, we used immunostaining and quantitative flow cytometry to quantify the status of free heavy chain

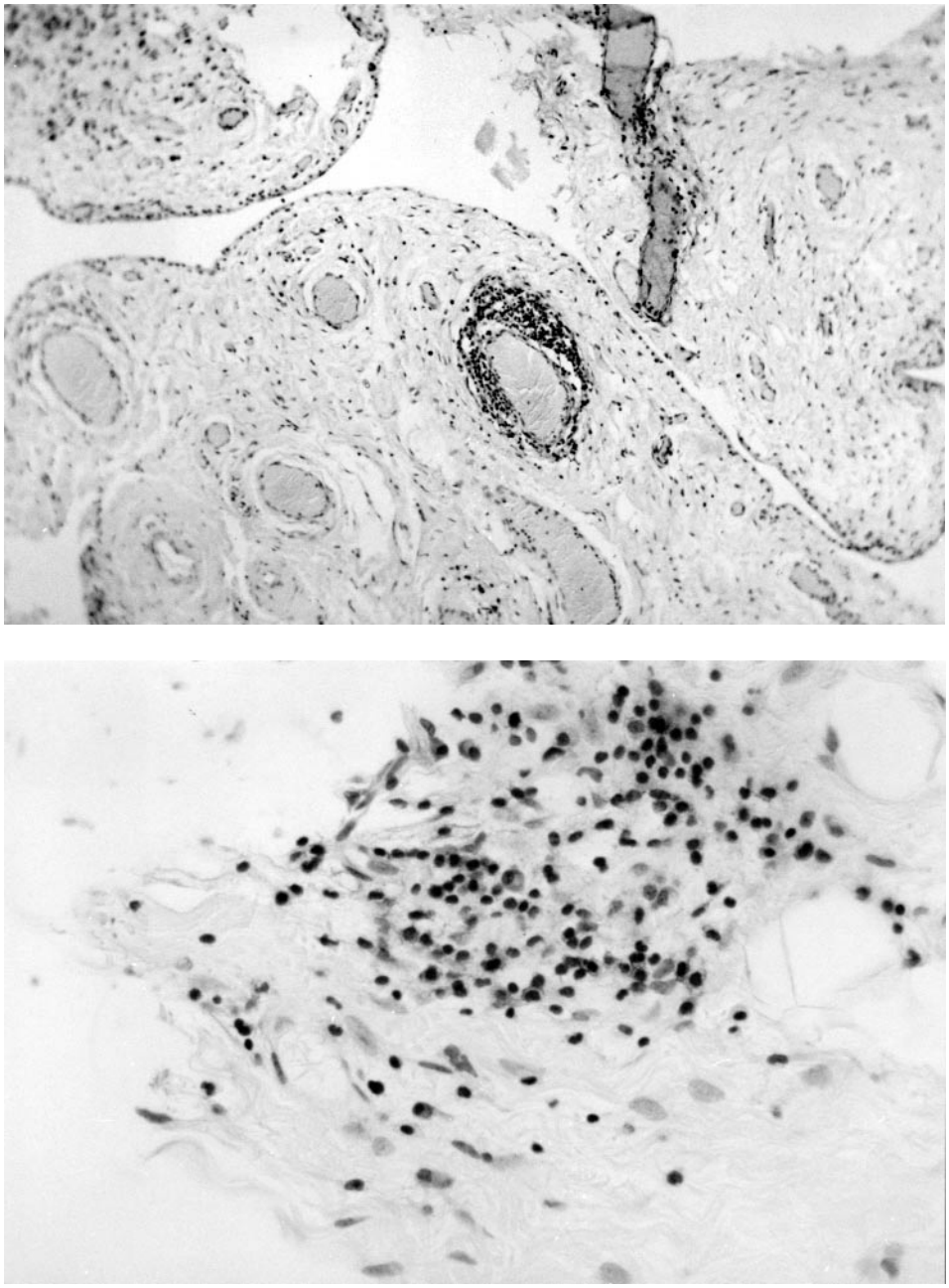

Figure 5. Detection of free heavy chain-carrying mononuclear cells in the synovial membrane by immunohistochemical staining. Darker staining indicates HC10+ cells. Note the heavy infiltration of these cells in some areas of synovium, especially around the blood vessel. (A): low power field (100 ×); (B): high power field (400 ×).

expression. The Mab HC10 reacts preferentially with free heavy chains of HLA-B, and C alleles. As shown in Figures 1-3, free heavy chains were expressed only on circulating monocytes from patients with AS. We also found that almost all the monocytes in synovial fluid expressed free heavy chains. Furthermore, we could identify free heavy chain-expressing mononuclear cell infiltrates in the synovium, the disease-specific site of inflammation.

In subsequent experiments, we found that after longterm culture, monocytes from healthy controls could be induced to express free heavy chains. In addition, they also expressed macrophage differentiation antigens that were stained by the Mab 27E10, CD163 and 25F9. Supporting previous reports that when peripheral monocytes mature into macrophages, the level of CD11b is downregulated[20], we also found the level of CD11b downregulated in this

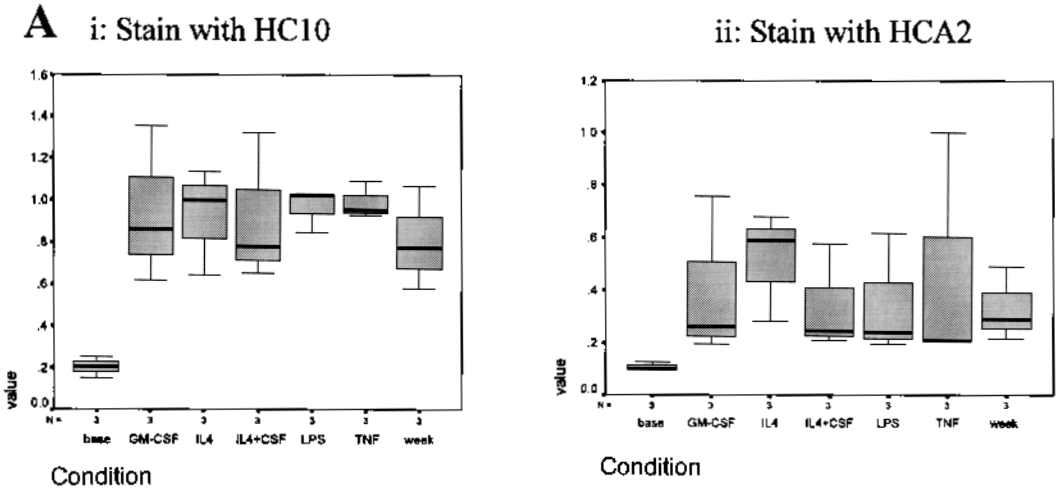

Figure 6A. HLA class I free heavy chain–expression after a 1-week incubation with one of the following: GM-CSF (70 ng/ml), IL-4 (200 U/ml), IL4 (200 U/ml) + GM-CSF (70 ng/ml), LPS (10 μg/ml), or TNF-α (10 ng/ml). Base indicates the cells were stained immediately after isolation. Week indicates the cells were cultured for one week in the absence of any stimulants. After incubation, the cells were harvested and stained with (1) Mab HC10 or (2) Mab HCA2.

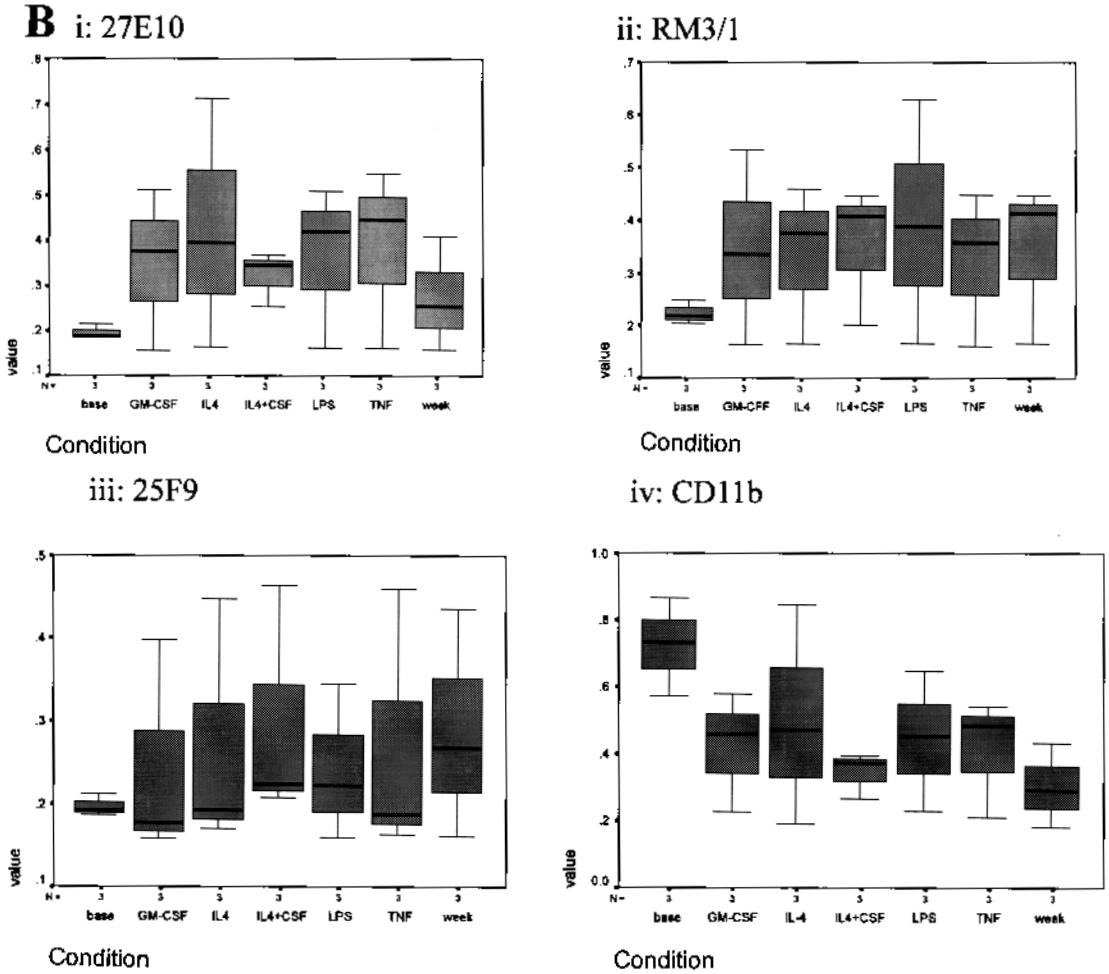

Figure 6B. Macrophage differentiation antigen-expression on monocytes isolated from 3 healthy controls cultured for 1 week with one of the following: GM-CSF (70 ng/ml), IL-4 (200 U/ml), IL-4 (200 U/ml) + GM-CSF (70 ng/ml), LPS (10 μg/ml), or TNF-α (10 ng/ml). Base indicates the cells were stained immediately after isolation. Week indicates the cells were cultured for one week in the absence of any stimulants. After one week, the cells were harvested and stained with (1) Mab 27 E10; (2) Mab RM3/1; (3) Mab 25F9; (4) Mab Anti-CD11b. The values on the y-axis indicate relative fluorescence intensity expressed in arbitrary units.

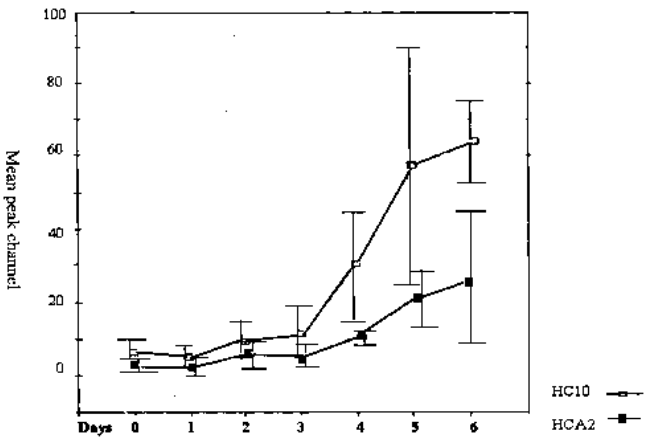

Figure 7. Monocytes isolated from 3 healthy controls were cultured in a 6-well plate without the addition of any stimulant. After each day of incubation, cells were harvested and stained with either HC10 or HCA2. The values on the y-axis are arbitrary units. Note the abrupt increase in free heavy chain expression after the 3rd day of incubation. Error bars indicate 95% CI.

population of cells. Hence, the free heavy chain-carrying monocytes represent a population of more differentiated monocytes.

To determine whether the dissociation from ß$_2$m happens only on HLA-B and C alleles or is a universal phenomenon of all HLA class I heavy chains when monocytes mature, we stained the cultured monocytes with Mab HCA2, which reacts preferentially with HLA-A alleles in addition to HC10. We found matured monocytes expressed free heavy chains of different alleles of HLA class I, although the level of expression was lower. We concluded that in patients with AS, more differentiated monocytes did circulate in the peripheral blood and infiltrate the synovium, and the number of these matured cells in the peripheral circulation correlated with the disease activity. However, there is some concern that monocytes express high levels of certain Fc receptors, which typically increase with activation and differentiation. To ensure that the molecules we measured were free heavy chains and not Fc receptors expressed by monocytes, we used isotype-specific antibody. In addition, monocytes from patients with RA cannot be stained by HC10. Indeed, it was reported that monocytes from patients with RA were activated and had increased levels of Fc receptors[21]. Furthermore, we don't consider that cell size contributes to the differential expression of HC10. Granulocytes are much bigger than lymphocytes, but there was no difference in HC10 expression between these 2 cellular populations in any of the 3 study groups.

HLA class I molecules present antigenic peptides to CD8+ T cells and trigger a cell mediated immune response. But to free heavy chains of HLA-B27, a completely different mechanism may be involved. Stable empty HLA-B27 molecules on viable human cells were first described by Parham's group[22]. They speculated that long-lived empty B27 molecules might bind extracellular arthritogenic peptides. Molecular modeling suggests that disulphide bonding through Cys67 would result in partial unwinding of α_1 helix in this region[23]. Computer simulation studies also suggest that the tightness of the class I pocket may be due to the way they are bound with ß$_2$m. In the absence of ß$_2$m, a B27 heavy chain may have a more flexible groove with the ends open, able to present longer peptides. This would not only affect peptide binding, but might result in a structure resembling MHC class II. Elution of peptides from B27 molecules shows shorter nanomer peptides, as well as longer 14–24 amino acid long peptides[24]. Presumably the longer peptides may be coming off the free heavy chain. Evidence from a study of mutant ß$_2$m also confirmed the conformational change of HLA-B27 after showing that the addition of aberrant ß$_2$m could change the CTL response[25]. These findings raise the possibility that an aberrant form of HLA-B27 molecule complex might be capable of presenting a completely different array of antigens from conventional HLA class I, and this behavior may even result in a breakdown of self-tolerance.

Only HLA-B27 can be expressed as free heavy chains. In addition to the α3 domain that makes direct contact with ß$_2$m, residue 9 in the floor of ß sheets is critical for the stability of the class I/ß$_2$m complex. All the B27 subtypes contain histidine at this site, and surprisingly, 2 other class I molecules, HLA-B73 and HLA-B40, which had been reported in a few cases with spondyloarthropathies, were also found to have histidine at residue 9. It was suggested that histidine at the bottom of the groove interacts inefficiently with human ß$_2$m[26]. Certain other class I molecules can be expressed in a free empty form, but usually they are short-lived[23].

From the above findings, we hypothesized that an unknown mechanism triggers the influx of mature monocytes in the peripheral blood circulation in a variety of patients. Only those HLA-B27 carriers with circulating mature monocytes/macrophages may express long-lived free heavy chains and present aberrant peptides to T cells (CD4 and/or CD8), leading to a spectrum of inflammatory disorders. Our study provides the first patient-related evidence that free heavy chains may be disease-causing molecules and we offer this as a new model for the pathogenesis of spondyloarthropathies.

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