Brief Definitive Report

Coordinate Defects in Human Histocompatibility Leukocyte Antigen Class II Expression and Antigen Presentation in Bare Lymphocyte Syndrome

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Summary

The human immunodeficiency, type II bare lymphocyte syndrome (BLS), has been attributed to a defect in the transcription of class II histocompatibility genes. Immunocompetence, as assessed by functional exogenous antigen presentation, was not restored in immortalized B cells, derived from a BLS patient, after transfection with HLA-DR class II structural genes. Incubation of protein antigens, as well as infectious virus, with DR-transfected BLS cells failed to induce activation of antigen-specific helper T lymphocytes. Peptide antigens were presented by class II molecules displayed on BLS cells, although the conformation of these class II proteins was altered as indicated by epitope mapping. This defect in antigen presentation was independent of the specific class II DR allele transfected into BLS cells. Genetic complementation analysis has been used with BLS cells to demonstrate that the defect in class II gene transcription is linked to the absence of a *trans*-acting factor. Similarly, functional class II dimers were restored after in vitro fusion of cells derived from two distinct BLS complementation groups, implying that specific transcriptional control elements are shared by a gene critical for antigen presentation and genes encoding HLA class II antigens. Thus, two important functionally linked pathways of class II molecules, structural gene expression and antigen presentation, share a common regulatory pathway defective in BLS.

Type II bare lymphocyte syndrome (BLS) or HLA class II–deficient combined immunodeficiency is an autosomal recessive disorder in which HLA class II structural genes are not transcribed due to a mutation in a gene encoded outside of the MHC (1). This lack of HLA class II expression compromises patient immunity, leading to recurrent bacterial and viral infections. Several lines of evidence suggest that the BLS phenotype results from a defect in a *trans*-acting factor necessary for transcription of the coordinately regulated class II genes (reviewed in reference 2).

Clustering of genes for histocompatibility antigens and accessory molecules required for antigen presentation may ensure the coordinate expression and function of these molecules. The HLA class I and class II structural genes on chromosome 6 are interspersed with genes necessary for antigen responsiveness (3). The ability of class I molecules to present antigens is dependent upon functional transporters associated with antigen processing (TAP) genes which are required to supply peptides to class I molecules in the endoplasmic reticulum (reviewed in reference 3). The TAP genes map between the HLA-DQ and -DP structural genes; in addition to this genetic proximity, they are coregulated with class I α structural genes by IFN-γ (3). Similarly, genes essential for appropriate class II–mediated antigen presentation map within this same region of the MHC (4, 5). Transcriptional regulation of genes encoded outside the MHC may also be coordinated with histocompatibility antigens to facilitate immune function. Class II antigens and their intracellular chaperone, the invariant chain, are upregulated in response to IFN-γ (6). Whether transcriptional elements are shared by additional genes required for class II–restricted antigen presentation has not been addressed.

Restoration of cellular immune responses in BLS is dependent upon the expression of functional HLA class II antigens in these patients. To determine whether stable transfection of class II DR α and β subunits would restore immunocompetency in BLS, studies of class II antigen structure and function were initiated using BLS-1, an immortalized B cell line derived from a BLS patient (7). BLS-1 cells expressing abundant amounts of cell surface DR4w4 or DR5 molecules were unable to present exogenous antigens to T lymphocytes. In addition, conformationally altered class II $\alpha\beta$ complexes were

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detected by epitope mapping in BLS-1 cells transfected with the HLA alleles, DR3 or DR4w4. Complementation analysis indicated that a *trans*-acting factor was necessary to restore a wild-type APC phenotype to BLS-1 cells. Our data suggest that the mutated gene that controls class II structural gene transcription also regulates the expression of gene(s) controlling class II–mediated antigen presentation.

Materials and Methods

Cell Lines. Class II DRα (DRA1) and DRβ (DRB1*0401, DRB1*1101, DRB1*0301) cDNA encoding DR4w4, DR5w11, and DR3w17 molecules were introduced into BLS-1 using retrovirus-mediated gene transfer (8). T2 (.174×CEM.T2) is a human cell hybrid that lacks all four copies of the MHC class II region on chromosome 6 (9). T2.DR4w4 (provided by W. Kwok, Virginia Mason Research Center, Seattle, WA) and T2.DR3 and T1.DR3 (10) were generated by transfer of the DRA1 and DRB1*0401 or DRB1*0301 genes. T1 is the progenitor of T2 and retains one unmutated copy of chromosome 6. Cell lines were grown in IMDM plus 10% FCS.

Assays with T Cell Clones. The anti-HAR and BCHA59 human T cell clones (provided by J. Krieger and A. Sette, Cytel, San Diego, CA) were isolated by limiting dilution cloning after stimulation of DR5 or DR4w4 PBL with HA 307-319 (11). To measure T cell proliferation, APCs (3×10^4) were pulsed for 3 h with fixed A/Mississippi/1/85 (H3N2) virus (Connaught Laboratories, Swiftwater, PA) or HA 307-319 peptide (PKYVKQNTLKLAT), washed, irradiated, plated in microwells with an equal number of T cells, and incubated for 72 h, with [^{3}H]thymidine (1 μCi/well) present during the last 15 h. Live A/Bangkok/1/79 virus (200–300 HAU/ml) (provided by P. Cresswell, Yale University, New Haven, CT) was incubated with cells in serum free medium for 1 h at 37°C to allow viral adherence; nonadherent virus was washed away and cells were resuspended in complete medium for 24 h before incubation with T cells.

Assays with T Cell Hybridomas. The DR4w4-tetanus–specific T cell hybridoma 49.23.2 was generated after tetanus immunization of a DR4w4 transgenic mouse (12) as described (13). Formalin fixed tetanus toxoid (TT) (Wyeth Laboratories, Philadelphia, PA) was digested with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) trypsin. APC (10^5), TT antigen (native or trypsin digested) (0.4 μM) and 49.23.2 T cells (10^5) were incubated together for 24 h. T cell IL-2 and IL-4 production was determined by the survival of an IL-2/IL-4–dependent cell line, HT-2, measured using 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT) (14). The presence of antigenic peptides in the tryptic digest of TT was confirmed by incubating the digested preparation with fixed APCs. The T cell hybridoma did not produce lymphokines in response to APC's in the absence of antigen.

Antibody Binding Assays. For flow cytometry experiments, the anti-DR mAb L243 (15) and the anti-DR3 mAb 16.23 (16) were used at saturating concentrations and binding was detected with FITC-conjugated goat anti–mouse Ig using a FACScan® (Becton Dickinson & Co., Mountain View, CA). Binding of anti-DR4w4 mAbs was detected with a live cell enzyme-linked immunoassay (CELISA) (17).

Cell Fusions. Fusions between BLS-1 (DR3,5) and SJO (DR5, 7) cells were carried out using 50% polyethylene glycol-1500, 5% DMSO (18). Before cell hybridization, hygromycin-resistance or neomycin genes were introduced into BLS-1 or SJO, respectively; the fused cells were selected for using hygromycin B (150 μg/ml) and G418 (1 mg/ml). Four independently fused drug-resistant populations were analyzed for cell surface expression of the endogenous DR molecules.

Results and Discussion

BLS-1 cells expressing high levels of cell surface DR molecules were assessed for their ability to process and present foreign protein antigens to T cells. The BLS-1.DR5 transferent and a wild-type DR5 homozygous B cell line presented

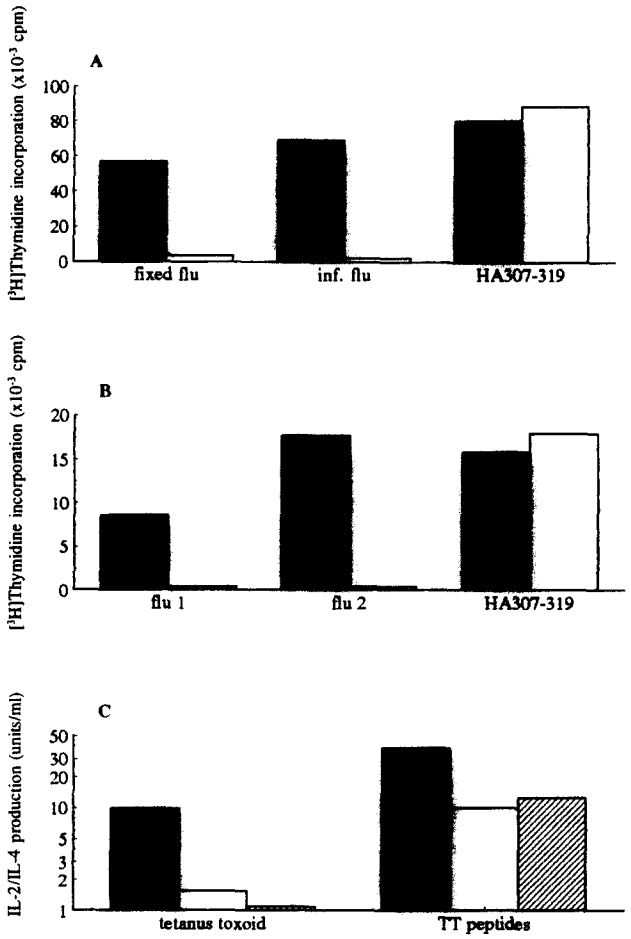

Figure 1. BLS-1.DR transferents are unable to mediate class II–restricted presentation of exogenous native protein antigens to T cells. (*A*) Response of the anti-HAR T cell clone to the DR5 B cell line Sweig (*solid bars*) and BLS-1.DR5 cells (*open bars*) after incubation with 45 μg/ml formalin-fixed A/Mississippi influenza virus (fixed flu), infection with live A/Bangkok influenza virus (inf. flu), or incubation with 1 μg/ml peptide HA 307-319. (*B*) Proliferation of T cell clone BCHA59 to the DR4w4 B cell line JAH (*solid bars*) and BLS-1.DR4w4 (*open bars*) cells after incubation with HA 307-319 or fixed A/Mississippi influenza virus. Two concentrations of influenza virus were used: flu1, 45 μg/ml; flu 2, 180 μg/ml. T cell proliferation data presented are mean values of [^{3}H]thymidine incorporation from triplicate cultures of representative experiments; standard deviation of the means was <15%. cpm values resulting from proliferation of T cells in response to APCs in the absence of antigen have been subtracted. (*C*) Lymphokine production (IL-2 and IL-4) by T cell hybridoma 49.23.2 in response to the DR4w4 B cell line Priess (*solid bars*), BLS-1.DR4w4 (*open bars*), or T2.DR4w4 (*hatched bars*) after incubation with 0.4 μM native tetanus toxoid protein or a tryptic digest of TT peptides. Data are representative of four experiments.

the influenza hemagglutinin (HA) peptide, HA 307-319, equally well to a DR5/HA 307-319–restricted T cell clone (Fig. 1 *A*). In contrast, BLS-1.DR5 incubated with formalin-fixed influenza virus did not stimulate proliferation of the T cell clone (Fig. 1 *A*), suggesting that BLS-1.DR5 was unable to mediate presentation of native HA. To increase the amount of intracellular influenza HA, APCs were infected with live influenza virus before T cell addition; the T cell clone proliferated in response to the influenza-infected DR5 B cell line, yet there was no response to virally infected BLS-1.DR5 (Fig. 1 *A*). Similarly, a BLS-1.DR4w4 transferent was unable to stimulate proliferation of a DR4w4/HA 307-319 restricted T cell clone after incubation with native influenza virus proteins, whereas the presentation of the HA 307-319 peptide by BLS-1.DR4w4 was comparable to that of a homozygous DR4w4 B cell line (Fig. 1 *B*).

The defect in exogenous antigen presentation by BLS-1 was also observed with another native antigen, TT. Upon incubation with native TT, BLS-1.DR4w4 was ineffective in stimulating a murine T cell hybridoma that is specific for tetanus presented in the context of DR4w4 (Fig. 1 *C*). T cell responses to BLS-1.DR4w4 and tetanus were reduced 10-fold when compared with a homozygous DR4w4 B cell line. Incubation of these APCs with proteolyzed TT (containing antigenic peptides) resulted in activation of the T cell hybridoma (Fig. 1 *C*). Thus, BLS-1 cells expressing two different DR alleles, DR4w4 or DR5, were unable to mediate class II–restricted antigen presentation to antigen-specific T cells after incubation with the native protein antigens, TT and influenza HA; however, BLS-1.DR transferents could present preprocessed antigenic peptides derived from these proteins as efficiently as wild-type B cell lines. The inability of BLS-1 cells to present native protein antigens was not due to a defect in internalization of exogenous antigens. Infection of BLS-1 with influenza did not facilitate presentation of the viral HA antigen (Fig. 1 *A*). In addition, BLS-1 and the wild-type B cell lines were equally able to internalize TT or influenza virus present in the culture medium (data not shown).

This defect in class II–mediated antigen presention is similar to the phenotype of the in vitro generated mutant cell lines 9.5.3 (19), 721.174 (5), and the related cell T2 (10). These mutant cell lines each lack a gene required for the processing and presentation of native exogenous class II–restricted antigens. A comparison of BLS-1.DR4w4 and the mutant T2.DR4w4 indicated that the ability of each of these cells to present TT to T cells was similarly reduced (Fig. 1 *C*). The mutation in 721.174 and 9.5.3 maps between the LMP2 and DNA genes in the class II region of the HLA gene complex (4, 5).

A characteristic feature of the in vitro–generated mutant APC is the expression of structurally aberrant class II $\alpha\beta$ dimers lacking specific epitopes (5, 19). To assess the conformation of the DR molecules present on the surface of BLS-1.DR transferents, BLS-1.DR3 and BLS-1.DR4w4 cells were analyzed for binding of allele-specific, conformation-dependent anti-DR3 and anti-DR4w4 mAbs using flow cytometry and CELISA (Fig. 2). The mAb 16.23, which recognizes a conformation-dependent epitope on DR3 molecules, binds to DR3 molecules on wild-type DR3 cell lines but not on BLS-1.DR3 or mutant T2.DR3 cells (Fig. 2 *A*). Expression of the epitope bound by another anti-DR mAb, L243 is unaffected by the mutations in these cells.

A panel of anti-DR4w4–specific mAbs (20, 21) was used to probe the structure of DR4w4 on BLS-1.DR4w4 cells and a DR4w4 homozygous B cell line (Fig. 2 *B*). Two of these mAbs, NFLD.D1 and NFLD.D10, bound to DR4w4 molecules on both cell lines equally well, as did the anti-DR

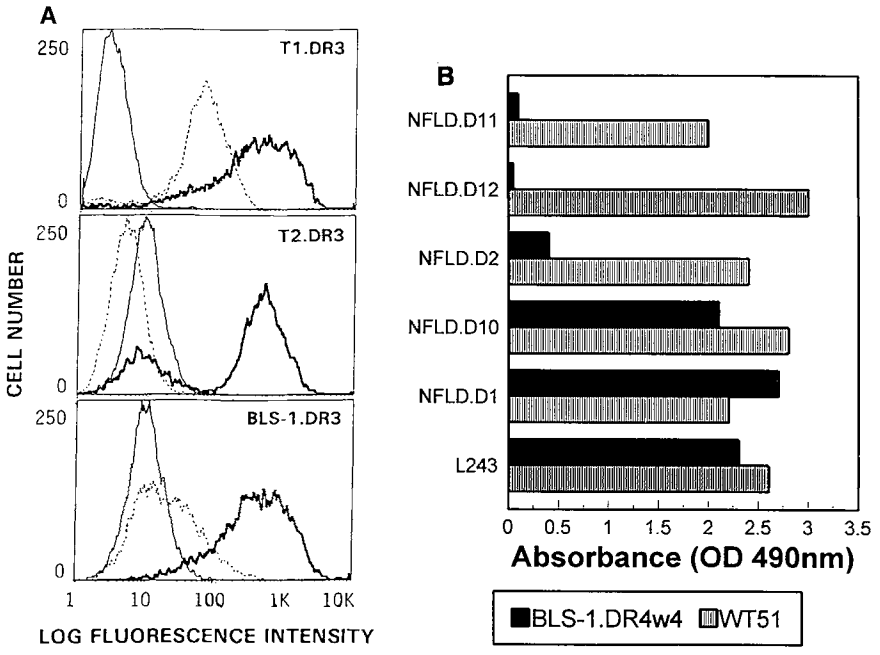

Figure 2. Binding of allele-specific, conformation dependent anti-DR3 and anti-DR4w4 mAbs to BLS-1.DR transferents is greatly reduced. (*A*) Binding of the anti-DR3 mAb 16.23 (*dotted line*), mAb L243 (*thick line*) and an irrelevant mAb NN4 (*thin line*) to the DR3 transferent cell lines T1.DR3, T2.DR3, and BLS-1.DR3. Fluorescence intensity was determined after binding of mAbs and flow cytometry. (*B*) Binding of a panel of anti-DR4w4 mAbs and mAb L243 to the DR4w4 B cell line WT51 (*hatched bars*) and BLS-1.DR4w4 (*solid bars*). Binding was detected using a live CELISA. The absorbance (OD 490 nm) resulting from incubation of cells with medium alone has been subtracted.

mAb L243. However, mAbs NFLD.D11 and NFLD.D12 bound wild-type DR4w4 B cells but not BLS-1.DR4w4 cells; these mAbs had previously been shown to bind epitopes that are dependent on DR4w4-peptide complexes and do not bind to the DR4w4 molecules on the surface of mutant T2.DR4w4 cells (20). Binding of mAb NFLD.D2, which also recognizes an epitope dependent on class II conformation, was reduced on BLS-1.DR4w4 cells. Taken together, these data with both anti-DR4w4 and anti-DR3 mAb suggest that the DR molecules on the surface of BLS-1 cells are structurally altered and thus do not display epitopes recognized by conformationally sensitive antibodies. This defect in class II antigen structure has been linked with the impaired ability of mutant cells to present peptides derived from exogenously added proteins.

Surface expression of endogenous class II DR, DQ, and DP molecules is restored upon fusion of B cell lines isolated from different BLS patients, indicating that the defect in class II gene transcription differs among BLS cell lines and allowing the definition of four genetic complementation groups (18). BLS-1 is a member of complementation group I. SJO, a B cell line derived from a patient in complementation group IV (22), was transfected with genes encoding DR4w4 molecules and found to display a mutant APC phenotype identical to that of BLS-1.DR4w4 (data not shown). To determine if the defects in antigen presentation in BLS-1 and SJO could be complemented by *trans*-acting factors present in each cell line, BLS-1 was fused with SJO. BLS-1 × SJO hybrids were isolated and screened for wild-type DR3 expression using mAb 16.23. The hybrid cells were found to express the endogenous DR3 molecule from BLS-1 with a wild-type conformation (Fig. 3), indicating that a *trans*-acting factor present in SJO can restore the production of functional class II molecules and therefore the ability to present exogenous antigens.

Our results demonstrate that BLS-1.DR transferent cells have a defect in the class II–mediated presentation of native exogenous protein antigens to T cells. The class II $\alpha\beta$ dimers formed in these cells also fail to express conserved epitopes as detected by conformationally sensitive antibodies. BLS-1, a cell deficient in the transcription of class II structural genes, is therefore remarkably similar in phenotype to a set of in vitro–generated APC mutants. Genetic studies indicate that the mutations in these two classes of cells, one a regulatory mutant and the other a structural gene defect, map to different locations in the genome (4, 5, 18).

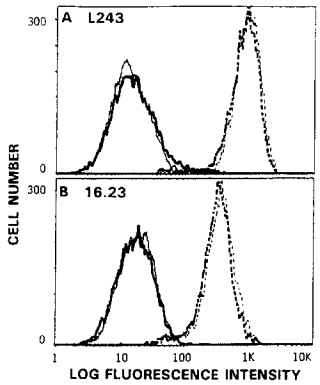

Figure 3. Fusion of class II–negative BLS-1 and SJO cells results in expression of wild-type endogenous DR3 molecules that bind mAb 16.23. Binding of anti-DR mAb L243 (*A*) and anti-DR3 mAb 16.23 (*B*) to BLS-1 (*thick line*), SJO (*thin line*), BLS-1 × SJO fusion 1 (*thick dotted line*) and BLS-1 × SJO fusion 2 (*thin dotted line*). Fluorescence intensity was determined after binding of mAbs and flow cytometry.

Complementation of endogenous class II gene expression, as well as the defect in antigen presentation, was observed upon fusion of two different BLS cell lines. Since neither BLS cell line is able to mediate presentation of exogenous antigens, the mutations resulting in an impaired APC phenotype are different in BLS-1 and SJO. Coregulation of class II structural genes and the gene(s) controlling antigen presentation at a transcriptional level would best explain these results. Thus, the two phenotypic defects in BLS cells are likely to arise as a result of mutations in a single *trans*-acting gene. Alternatively, the two phenotypic defects in BLS cells may be due to mutations in two separate genes that independently regulate class II structural gene transcription and transcription of genes required for antigen presentation. This latter possibility seems unlikely, however, since BLS is due to spontaneous mutations.

Genomic in vivo footprinting studies in BLS cell lines have shown that the promoters of class II DR and DQ genes in complementation groups I, III, and IV are unoccupied by protein factors that normally bind at multiple *cis*-acting DNA consensus elements (23). The "bare" promoters could result from the absence of a promoter binding protein that is critical for binding of other regulatory proteins to all class II structural gene promoters; alternatively, bare promoters may be due to a local defect in chromatin structure in the MHC class II region that renders the promoters of class II genes inaccessible to any factor binding (24). We assessed whether the class II region of the MHC was globally inactivated by monitoring transcription of several genes within the MHC including the TAP1, TAP2, and RING1 genes. The TAP genes (3) map between DOB and LMP2 near the putative gene controlling antigen presentation and the RING1 gene (25) maps centromeric to DPB2. Northern blotting and PCR analyses showed that TAP1, TAP2, and RING1 mRNA is present in BLS-1 (data not shown), indicating that the promoters of these genes reside in chromatin that is accessible to transcription factors; similar results with the LMP2 and LMP7 genes have been obtained with SJO (22). However, SJO does not express the DMA and DMB genes which encode a class II–like molecule and map between LMP2 and DNA (22); the DMA and DMB genes contain promoter regions that are similar to the promoters of the class II DR, DQ, and DP structural genes (26) and thus may be jointly regulated.

Regulatory protein(s) defective in BLS cell lines may control the accessibility of class II promoters within their native chromosomal environment (24). Since we found that the mutation(s) in BLS-1 influence a gene or genes that regulate both HLA class II gene expression and function, this implies a level of transcriptional coregulation, perhaps with the promoter region 5′ of the antigen presentation genes containing the same *cis*-acting consensus sequences (X, Y, and W boxes) or locus control region that are upstream of each of the class

II structural genes. To fully restore immune responsiveness in BLS patients, immunotherapeutic approaches must therefore be based on restoration of transcription factors or the expression of both class II structural genes and genes controlling antigen presentation.

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