

Genetic Evidence for a New Type of Major Histocompatibility Complex Class II Combined Immunodeficiency Characterized by a Dyscoordinate Regulation of HLA-D α and β Chains

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Summary

Major histocompatibility complex (MHC) class II combined immunodeficiency (CID), also known as type II bare lymphocyte syndrome, is an autosomal recessive genetic disorder characterized by the complete lack of expression of MHC class II antigens. The defect results from a coordinated lack of transcription of all class II genes. Cell fusion studies using many patient- and experimentally derived class II-negative cell lines have identified four distinct genetic complementation groups. In this report, we present genetic evidence that cell lines derived from two newly described MHC class II-deficient patients, KER and KEN, represent a fifth complementation group. In addition, the KER and KEN cell lines display a unique pattern of dyscoordinate regulation of their MHC class II genes, which is reflected in a new phenotype of *in vivo* promoter occupancy as revealed by *in vivo* genomic footprinting. These data point to a new defect that can result in the MHC class II-deficient phenotype.

MHC class II antigens are highly polymorphic cell surface glycoproteins that are intimately involved in the generation of the immune response (1). In the human, three isotypes (HLA-DR, -DQ, and -DP) of class II antigens are encoded by the HLA-D locus on chromosome 6. Each isotype consists of an α and β chain, which together form a heterodimeric structure capable of binding exogenously derived peptides and presenting them to CD4⁺ Th cells (2). The expression of class II molecules is limited to APC, such as B cells, macrophages, and dendritic cells, and to thymic epithelial cells, where expression is involved in the "education" of naive T cells before their exportation to the periphery (2, 3). In addition to the constitutive and inducible expression on cells of the immune system, class II levels can also be induced on otherwise class II-negative cells by exposure to cytokines such as IFN- γ (2).

The lack of expression of MHC class II molecules results in an immunodeficiency called MHC class II combined immunodeficiency (CID),¹ or the bare lymphocyte syndrome. The disease is characterized by the complete lack of

constitutive and inducible expression of class II antigens (4). Patients with MHC class II CID demonstrate failure to thrive, subsequently develop overwhelming bacterial and viral infections, and usually die in childhood unless treated with bone marrow transplantation (4, 5). The defect in gene expression is at the level of transcription and affects all class II genes coordinately (6). Genetic studies of affected families have shown that the defect does not segregate with the MHC and suggest that the class II structural genes in such patients are intact (7). The defect, rather, appears to be due to a malfunctioning *trans*-acting factor(s), as fusion of a class II-negative derivative of the Burkitt's lymphoma Raji to a class II-positive murine B lymphoma results in the restoration of expression of human class II antigens (8). More extensive cell fusion studies, using a panel of CID patient EBV-transformed B lymphoblastoid cell lines (B-LCL), have identified four complementation groups, suggesting that four separate genetic lesions can give rise to the class II-deficient phenotype (9-11). In complementation groups I and IV, the lack of class II expression is thought to be due to a deficiency in a DNA-binding protein complex (RF-X) that binds to the conserved X box in class II promoters (12, 13). A cDNA for one member of the RF-X family, RF-X5, has recently been reported to restore class II expression

¹Abbreviations used in this paper: B-LCL, B lymphoblastoid cell line; CID, combined immunodeficiency; CIITA, class II *trans*-activator; RF-X, DNA-binding protein complex.

in cell lines from complementation group IV (14). The gene defective in complementation group II, termed class II *trans*-activator (CIITA), has recently been isolated by expression cloning and has been shown to be sufficient to restore class II antigen expression in all cell lines from this complementation group (15). The gene(s) defective in complementation group III remains to be identified.

Recently two new patients with MHC class II deficiency have been identified based on the lack of surface expression of class II antigens on their peripheral B cells and monocytes (16). Unlike other class II CID patients, these histologically identical twin brothers have normal numbers of CD4⁺ cells and are able to mount both cellular and humoral immune responses, presumably because of extremely low levels of class II antigen expression in certain adherent mononuclear cells. Analysis of the transcription of class II genes in EBV-transformed B-LCL derived from these patients revealed a previously undescribed dyscoordinate pattern of expression: whereas levels of DR α , DQ α , and DP β mRNA were approximately normal compared with controls, DR β , DQ β , and DP α mRNA were completely absent (17). Taken together, these results suggested that these patients represent a novel phenotype of MHC class II deficiency.

In this study, we have examined the expression of HLA-DR in transient heterokaryons formed by fusion between the patients' EBV-transformed B-LCL, KEN and KER, and cell lines from each of the four established complementation groups. Our data provide genetic evidence for a fifth complementation group of MHC class II deficiency characterized by a dyscoordinate expression of the class II genes. In addition, direct examination of the promoter regions of the class II genes in KEN and KER by *in vivo* genomic footprinting reveals that this dyscoordinate expression is associated with a new pattern of promoter occupancy different from those previously described for class II-negative cell lines.

Materials and Methods

Cell Lines. Experimentally derived class II-negative cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, 10 mM HEPES, 2 mM glutamine, 50 μ M 2-ME, penicillin (100 U/ml), and streptomycin (100 μ g/ml). 721.174 (18) is an EBV-transformed B-LCL that bears a homozygous deletion of the DR, DQ, and part of the DP region. RM3 (19) is a class II-negative derivative of the human Burkitt lymphoma Raji, whereas 6.1.6 (20) is derived from the class II-positive EBV-transformed B-LCL T5.1. All three cell lines are negative for DR, DQ, and DP expression.

The patient-derived EBV-transformed B-LCL KEN, KER, Nacera (21), BLS-1 (22), TF (23), and SJO (24) were maintained in RPMI 1640 medium supplemented with 20% FCS along with the additives listed above. EBV-transformed B-LCL derived from the mother and father of KER and KEN were grown in the same medium.

Cell Fusion. For the preparation of transient heterokaryons, 10⁶ cells of each type were mixed in a 5-ml centrifuge tube and washed three times in serum-free medium. The cells were dis-

gregated by three passages through an 18-gauge needle and were washed once more in serum-free medium. Cell pellets were gently dispersed and warmed to 37°C. Fusion was initiated by the dropwise addition of 0.1 ml PEG 1500 (50% wt/vol) (Boehringer-Mannheim Corp., Indianapolis, IN) over the course of 1 min. 5 ml of serum-free medium was then added slowly over a 5-min period. Cells were rested at room temperature for 5 min, pelleted at 250 *g* for 3 min, and plated in 5 ml of complete medium. After 48 h, the cells were analyzed for the expression of HLA-DR by incubation with FITC-conjugated anti-HLA-DR mAb L243 (Becton Dickinson & Co., San Jose, CA).

***In Vivo* Genomic Footprinting.** Preparation of *in vitro* and *in vivo* methylated genomic DNA and piperidine treatment were as described (25). Piperidine-treated genomic DNA (5 μ g) was used in ligation-mediated PCR as described (26). Primers specific for the DR α , DR β , and the DQ β promoter coding strands have been described (25, 27).

RNA Analysis. Total cellular RNA was extracted as described (28). 20 μ g of RNA was electrophoresed on 1.2% agarose gels, blotted to GeneScreen (DuPont-NEN, Boston, MA), and hybridized with radioactive probes generated by random-hexamer labeling.

Results

Complementation Analysis. To determine if the MHC class II-deficient cell lines KER and KEN belong to any of the four previously defined CID complementation groups, transient heterokaryons were prepared and analyzed 48 h after fusion for expression of HLA-DR antigens. Cells from both KEN and KER were fused to the following EBV-transformed B-LCL representing all four known CID complementation groups: Nacera and BLS-1 (group I), RM3 (group II), 6.1.6 (group III), and TF (group IV). In addition, 721.174, an experimentally derived EBV-transformed B-LCL that lacks the class II structural genes, was used as a further control. Each fusion experiment also included fusions between pairs of cells from groups I, II, III, and IV.

Results of multiple fusion experiments are summarized in Table 1. Fusion of KEN to KER yielded no class II-positive heterokaryons, whereas fusion of either cell line to 721.174 caused reexpression of HLA-DR. These results suggest that both the transcribed (DR α , DQ α , and DP β) and the nontranscribed (DR β , DQ β , and DP α) class II structural genes in KER and KEN are functionally intact and that the nontranscribed genes can be expressed in the presence of the appropriate *trans*-acting factor. Fusion of KEN or KER to cell lines from groups I, II, and IV invariably gave rise to heterokaryons that showed expression of HLA-DR and thus clearly demonstrate that both KER and KEN do not belong to any of these complementation groups. Although the only cell line in group III, 6.1.6, has a small percentage of class II-positive revertants, repeated fusions, scored blindly, indicated that both KER and KEN can complement 6.1.6 and thus do not belong to complementation group III. Fig. 1 shows the results from representative fusion experiments between KER and 721.174, Nacera (group I), RM3 (group II), and TF (group IV). Based on this analysis, KER and KEN clearly define a new com-

Table 1. Complementation Analysis of KER and KEN

	721.174	KEN	KER	Nacera	BLS-1	RM3	6.1.6	TF	SJO
721.174	-								
KEN	+	-							
KER	+	-	-						
Nacera	+	+	+	-					
BLS-1	+	+	+	+	-				
RM3	+	+	+	+	+	-			
6.1.6	+	+	+	+	+	+	-		
TF	+	+	+	+	+	+	+	-	
SJO	+	+	+	+	+	+	+	-	-

Transient heterokaryons were formed between the pairs of cell lines listed above and analyzed for HLA-DR expression 48 h after fusion. (+) denotes HLA-DR-positive fusion products; (-) denotes no HLA-DR-positive fusion products observed.

plementation group of MHC class II deficiency, which we call complementation group V.

Promoter Occupancy in Group V CID. The occupancy of the MHC class II promoters in both class II-positive B cells and class II-negative CID cells as analyzed by *in vivo* genomic footprinting has revealed two predominant promoter phenotypes: (a) an "occupied" promoter is characteristic of class II-positive B lymphoblastoid cells as well as those cell lines that belong to complementation group II, and (b) a "bare" promoter is found in all cell lines examined from complementation groups I, III, and IV (27). Although the patterns of protection of the conserved X1, X2, and Y elements in the proximal promoters of the class II genes of CID cell lines can show either an occupied or bare phenotype, all the class II genes in a given cell type display a coordinated pattern of occupancy reflecting the fact that all the class II genes are either transcriptionally active or inactive. Given the dyscoordinate expression of class II mRNAs in KER and KEN ($DR\alpha^+DR\beta^-$, $DQ\alpha^+DQ\beta^-$, $DP\alpha^-DP\beta^+$), we examined the promoters of three class II genes in these cells by *in vivo* genomic footprinting. In both KER and KEN, the $DR\alpha$ gene is transcribed at normal levels, and indeed, the $DR\alpha$ promoter is occupied in both. The pattern of protection of the X1, X2, Y, and the $DR\alpha$ -specific octamer elements was identical to that seen in a class II-positive EBV-transformed B-LCL derived from the mother of the patients (Fig. 2, left). In contrast, the promoter of the nontranscribed $DR\beta$ gene is not protected in either KER or KEN (Fig. 2, right). Footprint analysis of the $DQ\beta$ promoter, which is nontranscribed in KER and KEN, also revealed the bare phenotype (data not shown). These *in vivo* genomic footprinting studies suggest that in group V CID, those class II genes that are actively transcribed ($DR\alpha$, $DQ\alpha$, and $DP\beta$) have occupied promoters, whereas those genes that are not transcribed ($DR\beta$, $DQ\beta$, and $DP\alpha$) have bare promoters. Thus, in addition to defin-

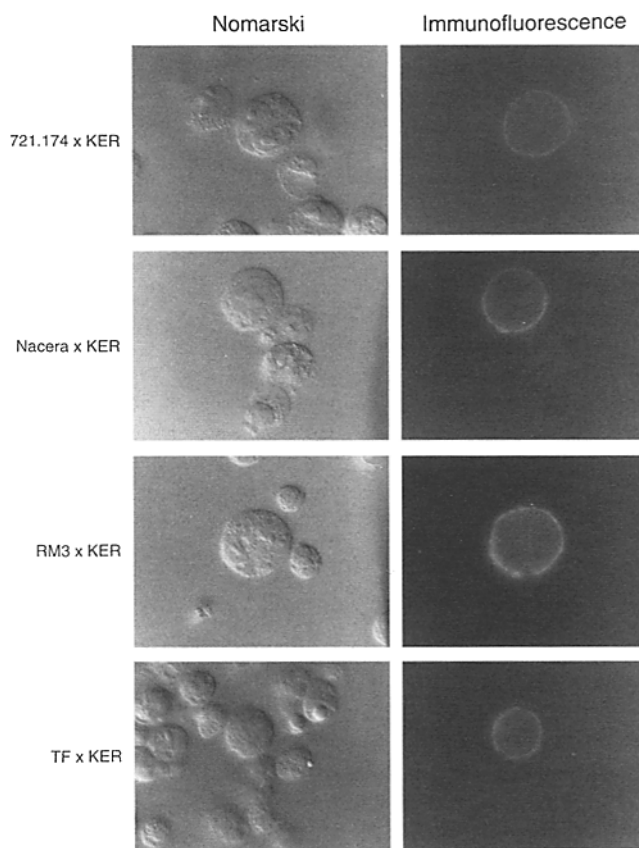


Figure 1. Reexpression of HLA-DR on the surface of transient heterokaryons formed between group V CID and other class II-negative cell lines. Transient heterokaryons were formed by PEG mediated fusion of KER and 721.174, KER and Nacera, KER and RM3, and KER and TF and analyzed 48 h later for expression of surface HLA-DR by immunofluorescence. Normarski (left) and immunofluorescence (right) micrographs are shown ($\times 400$).

ing a new genetic defect that can lead to MHC class II deficiency, cell lines in complementation group V exhibit a novel, mixed *in vivo* promoter phenotype characterized by a dyscoordinate pattern of promoter occupancy between the α and β chain genes of a given HLA-D isotype.

Transcription of Other Genes in the MHC. Since the cell lines KER and KEN displayed a completely new pattern of expression of the MHC class II genes, we examined the expression of two other class II-related genes whose promoters share some of the conserved elements common to all class II genes. DMA and DMB, which map within the class II region of the MHC between the DP and the DQ loci, have been shown to be essential for class II-mediated antigen presentation (29). Two CID cell lines, SJO (group IV) and BLS-1 (group I) have recently been found to lack expression of both DMA and DMB, suggesting a coordinate regulation of the class II structural genes and the related DMA and DMB genes (30). Northern blot analysis of cellular RNA derived from KER and KEN (Fig. 3) probed for DMA and DMB reveal that neither cell line expresses either of these two transcripts, whereas class II-positive EBV-transformed B-LCL derived from the patients' mother

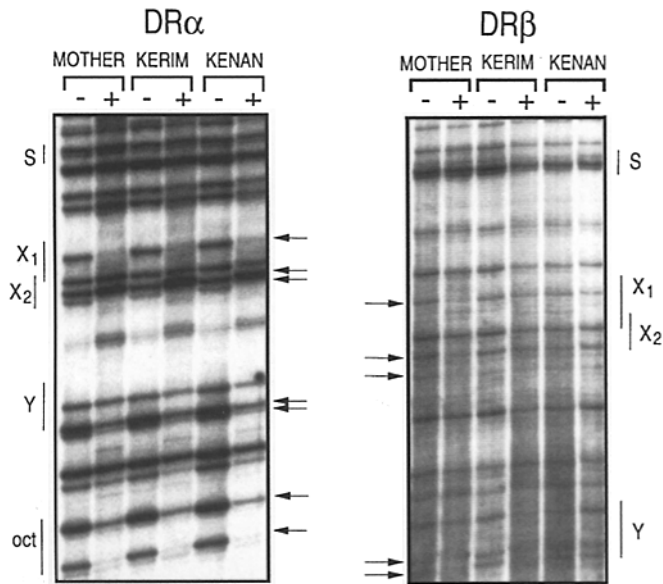


Figure 2. The DR α but not the DR β promoter is occupied in group V CID cell lines. In vitro and in vivo methylated genomic DNA was prepared from KER, KEN, and an EBV-transformed B-LCL derived from the patient's mother and analyzed as described in Materials and Methods. In vitro methylated DNA is in lanes marked (-); in vivo methylated DNA is in lanes marked (+). Regions encompassing the regulatory elements X1, X2, Y, and octamer (*oct*) are indicated by bars. Residues protected in vivo are indicated by arrows.

and father express normal amounts of mRNA for both DMA and DMB. Interestingly, TAP1 and TAP2, two genes also located in the MHC class II locus that are clearly regulated differently than the class II genes (31, 32), are expressed normally in both KER and KEN (data not shown).

Discussion

The immunological profile and the unique pattern of expression of class II mRNA in the class II-deficient patients KER and KEN suggested that they represent a new phenotype of MHC class II CID. Previously identified class II-negative cell lines, both patient derived and experimentally generated, have been placed into four complementation groups by somatic cell fusion analysis, implying that at least four separate genetic lesions can lead to the class II-negative phenotype. We extended that type of genetic analysis to KER and KEN and showed that they define a new CID complementation group and presumably represent

a mutation in another gene critical for class II antigen expression. KER and KEN also exhibit a mixed pattern of in vivo MHC class II promoter occupancy that correlates completely with the transcriptional status of the various class II genes: those genes that are transcribed (DR α , DQ α , and DP β) have an occupied promoter, whereas those genes that are transcriptionally silent (DR β , DQ β , and DP α) have bare promoters. This dyscoordinate regulation between the α and β chains of a given HLA isotype is the hallmark of complementation group V.

There are numerous examples of cell lines, both normal and malignant, that show a dissociation of expression among the three class II isotypes. A derivative of the Burkitt's lymphoma Jijoye, clone 13, for example, expresses DQ but not DR and DP (33). In human dermal fibroblasts, treatment with IFN- γ induces detectable DR and DP but not DQ (34). Malignant B cells have been described that fail to express one or more specific class II isotypes (35, 36). These studies, taken together, suggest that certain class II genes may be regulated differentially at the transcriptional level. None of these examples, however, reflect the pattern of dyscoordinate regulation seen in KER and KEN: the transcription of a given MA chain but not the β chain (or vice versa) for a specific HLA-D isotype.

Analysis of cell lines from the four previously described CID complementation groups by in vivo genomic footprinting has suggested two different mechanisms for the lack of transcription of class II genes (25, 27). The bare promoter phenotype points to a defect in promoter accessibility since factors recognizing some or all of the promoter elements conserved among class II genes can be detected in vitro. The occupied promoter phenotype suggests a defect in an activation domain of either a promoter-binding protein or a protein that interacts with promoter-binding factors. Indeed, CIITA, the gene defective in complementation group II, does not itself bind DNA but appears to function as a coactivator (15). In cells of the B lineage, where the expression of MHC class II antigens is developmentally regulated, the occupied promoter phenotype correlates with transcriptionally active class II genes, as in mature B cells, whereas the bare promoter phenotype is observed in pre-B cells and in plasma cells, where the class II genes are transcriptionally silent (37). These results imply that promoter accessibility is a primary determinant of class II expression and that such accessibility may be developmentally regulated.

The dyscoordinate regulation of the class II genes as well as the mixed pattern of promoter occupancy observed in KEN and KER suggest that the defect in complementation group V may have unmasked a previously unrecognized level of regulation of the MHC class II genes. Of the many factors known to bind to the highly conserved W, X1, X2, and Y elements common to all class II promoters, not all factors bind with equal affinity to all promoters. One of these factors, RFX, shows a distinct hierarchy of binding to the α chain promoters: a high affinity for DR α , a reduced affinity for DP α , and a very low affinity for DQ α . Furthermore, binding of RFX to the β chain promoters is weak or non-

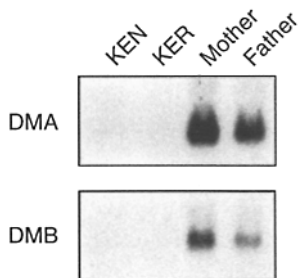


Figure 3. Lack of expression of DMA and DMB in group V CID cell lines. Northern blot analysis of expression of the DMA (*top*) and DMB (*bottom*) genes in KER, KEN, and in EBV-transformed B-LCL derived from the patient's parents (*Mother, Father*).

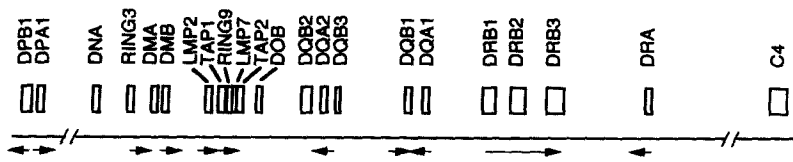


Figure 4. Transcriptional orientation of genes within the MHC class II region. Leftward- or rightward-pointing arrows indicate the direction of transcription of the HLA-DR, -DP, and -DQ α and β genes as well as the DMA and DMB genes.

existent (38, 39). Another factor that binds to the X box motif, X2BP, shows a completely different pattern of promoter-binding affinities with $DP\beta > DR\alpha > DR\beta$, whereas no binding is seen to $DQ\alpha$, $DQ\beta$, or $DP\alpha$ (40). Finally, hXBP1 binds only to the X2 boxes of the $DR\beta$ and $DP\beta$ promoters (41). These studies suggest that the combination of factors necessary to achieve coordinate regulation of the six class II α and MA chain genes may in fact differ from gene to gene. Thus, it is possible that the dyscoordinate regulation of the class II genes in complementation group V might be due to a mutational inactivation of a promoter-binding factor required for the expression of $DR\beta$, $DQ\beta$, and $DP\alpha$, but not for $DR\alpha$, $DQ\alpha$, and $DP\beta$. None of the factors described to date, however, fit this particular pattern of binding affinities.

Another explanation for the pattern of class II gene expression in group V is suggested by the striking fact that all of the transcriptionally active class II genes in KER and KEN share the same transcriptional orientation within the MHC class II locus. Conversely, those genes that are transcriptionally silent share the opposite transcriptional orientation. Thus, $DR\alpha$, $DQ\alpha$, and $DP\beta$ are transcribed from right to left, whereas $DR\beta$, $DQ\beta$, $DP\alpha$ are all transcribed from left to right, as diagrammed in Fig. 4. In addition to the defect in the transcription of certain class II genes, KER and KEN also fail to express DMA and DMB, genes whose promoters are quite homologous to the those of the class II structural genes and are thought to share a common regulatory mechanism (29, 30, 42). The orientation of these two nontranscribed genes within the HLA-D region of the MHC is the same as those class II genes not expressed in KER and KEN; that is, they are transcribed from left to right. It seems unlikely that the lack of transcription of $DR\beta$, $DQ\beta$, $DP\alpha$, DMA, and DMB could result from in-

dividual mutations in the promoters of these genes. Rather, this global lack of transcription is more reminiscent of the effects of a locus control region, albeit one that exerts its effects directionally on class II-like promoters. To our knowledge, no example of such a unidirectional locus control region has been reported.

The defect in group V CID, in addition to its effects on isotype-specific dysregulation, may also play a role in cell type-specific expression of the class II genes. Although, there is no class II protein detectable on the surface of either primary or EBV-transformed B-LCL from KER and KEN, the fact that they have normal levels of $CD4^+$ cells suggests that these patients must express class II antigens in the thymus (43). Furthermore, since both patients are able to mount specific antibody responses, some class II must be expressed in the periphery (16). Indeed, we have suggested that there may be HLA-DR present on certain adherent monocyte populations at levels too low to be detected by flow cytometry based on the finding of apparently intact intracellular $DR\alpha$ and $DR\beta$ in these cells (17). This would suggest that the regulation of the class II genes in B cells, thymic epithelial cells, and macrophages may differ. Further, such cell type-specific transcriptional differences might also be attributable to the effects of the gene defective in group V CID.

Identification of the gene(s) responsible for the dyscoordinate regulation and the differences in tissue-specific expression of the MHC class II genes in group V CID may provide insight into the aberrant expression of class II antigens in certain autoimmune diseases. The ability to manipulate the immune system by regulating patterns of expression of class II in an isotype- and cell type-specific manner suggests more gently tuned immunomodulatory treatment options than those currently available.

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