Identification of Antibodies to DQβ:DRα Interisotypic Heterodimers in Human Sera

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Background. HLA class II antigens, DR, DQ, and DP, comprised an α and β chains, which typically combine, within the same isotype, to form the major histocompatibility complex:peptide complex. Interisotypic pairing is not commonly observed. Although reports of DQβ:DRα heterodimers exist, the pairing was reported to be unstable and, therefore, not studied to any extent. **Methods.** DQβ:DRα single antigens were produced through transfectant cell lines and used to identify and characterize positive reactive human sera by a multiplex bead-based assay. **Results.** Stable DQβ:DRα transfectants were constructed. Cell surface staining with class II–specific monoclonal antibodies revealed that some DQB1 alleles appear to be more efficient in expressing DQβ:DRα heterodimers. Interestingly, alleles within the same serological group varied in their efficiency of forming dimers on the cell surface. For example, DQβ0601:DRα had the highest transfection and cell membrane expression efficiency among 16 common DQB1 alleles tested. In contrast, DQβ0603:DRα-positive transfectants demonstrated minimal surface expression. Assembly of DQβ0601:DRα was not affected by the presence of a DQα chain. DQβ0601:DRα and DQβ0603:DRα single-antigen beads were used to screen human sera. Positive sera were identified that reacted to the unique epitopes of DQβ0601:DRα protein on the cell surface of the transfectants. **Conclusions.** Our studies have demonstrated that unique DQβ:DRα heterodimers can be formed and are stably expressed on the cell surface. Such antigenic combinations, presented on single-antigen beads, demonstrated that patient sera can react with such heterodimers. Investigations on the potential clinical roles of antibodies against such interisotypic heterodimers are now possible.

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INTRODUCTION

The HLA gene cluster is located within the major histocompatibility complex region on the short arm of human chromosome 6. HLA class I molecules comprise a membrane-bound heavy chain and a noncovalently associated β_2 microglobulin, whereas HLA class II molecules are comprised of distinct α and β chains, both of which are integral membrane proteins. However, both classes of molecules have a similar tertiary structure with the 2 α helix sidewalls and a β sheet creating a natural groove to bind peptides from either self or nonself antigens.[1] Class I and II antigens are highly polymorphic and, therefore, capable of binding a wide array of peptides. As such, they can stimulate a wide range of immune responses, especially when presenting exogenous peptides. HLA class I and class II molecules that present immunogenic peptides serve as ligands for the T-cell receptor (TCR) and CD8 and CD4 molecules, respectively. HLA class I proteins are expressed on the cell surface of all nucleated cells and platelets, whereas class II protein expression is limited to B cells and professional antigen-presenting cells.

Three different HLA class II proteins, DR, DQ, and DP, are expressed on antigen-presenting cells, and all are extremely polymorphic (https://www.ebi.ac.uk/ipd/imgt/hla/). In addition, certain *DRB1* haplotypes are associated with specific *DRB3*, *DRB4*, or *DRB5* genes. These proteins are also dimerized with the DRα chain to form a complete DR protein with the ability to present immunogenic peptides. Specific *DQA1* and *DQB1* or *DPA1* and *DPB1* gene products assemble into functional DQ and DP

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proteins on the cell surface. Because of the highly polymorphic nature of HLA genes, individuals can generate antibodies to nonself HLA antigens through pregnancy, transfusion, or organ transplantation. Such HLA-specific alloreactive antibodies can create a major barrier for patients in need of a transplant or transfusion. HLA antibodies are most often determined via solid-phase, bead-based assays. The sources of HLA antigens for these assays are either purified from human cell lines or derived from recombinant cells transfected with unique DR, DQ, or DP α and β chains.[2]

The most common association between class II molecules is DRα with DRβ, DQα with DQβ, and DPα with DPβ, respectively. Lotteau et al[3] first identified DRα and DQβ heterodimers in Epstein-Barr virus–transformed B-cell line through immunoprecipitation and Western blot and further demonstrated that only cells expressing high levels of *DRA* mRNA produced this mixed heterodimers suggesting an inefficient assembly of the heterodimer pair.[4] Kwok et al[5] later found that the DQw1β(DQβ0501) chain can form heterodimers with DRα but not the DQw3β(DQβ0301) chain and suggested that the DQw1β molecule cannot compete effectively with the DRβ chain for binding of the DRα chain. There has been no evidence to show that such hybrid molecules are assembled, in vivo, into functional and stable dimers capable of eliciting an immune response.

Because only very limited *DQB1* alleles were studied in these reports,[3-5] we were interested in understanding whether any of the common *DQB1* allele could assemble into a stable dimer with DRα chain and their potential clinical implications in organ transplants. Here, we demonstrate the stable assembly of DQβ and DRα hybrids in B-cell lines and the successful isolation of the hybrid proteins from recombinant human cell lines for screening patient sera.

MATERIALS AND METHODS

Ethic Statement

Institutional Review Board (IRB) approved using human serum in this study. Written consent from patients was waived by the Methodist Specialty and Transplant IRB Board of Directors.

Cell Lines and Antibodies

Epstein-Barr virus–transformed lymphoblastoid B-cell line (LCL)3023[6] and lymphoblastoid B-cell line HLA class II knockout (LCLKO) were kindly provided by Dr William Burlingham and One Lambda, respectively. T2 cell line[7] was purchased from American Type Culture Collection. LCL3023 was derived from LCL721.174, which has deletions from DRA to DPB1 in both copies of the chromosomes and later incorporated *DMA*, *DMB*, and *DRA* genes through transfections.[6] LCL3023 has no detectable expression of HLA class II on the surface of the cell membrane. LCLKO is derived from LCL721.221[6] and has no detectable expression of HLA class II except *DRA* gene. HLA monoclonal antibodies FJ5109 and FM5148 were gifts from One Lambda. HL-37 was purchased from Thermo Fisher Scientific (Waltham, MA); L243, LN3, TU36, and TU169 were purchased from BioLegend (San Diego, CA).

Transfection and Cell Culture

The *DQB1* and *DRA* cDNA sequences were synthesized according to the published sequences in the IPD-IMGT/HLA Database (https://www.ebi.ac.uk/ipd/imgt/hla/). Each *DQB1* allele and *DRA* cDNA were cloned into the pEF6 mammalian expression vector (Thermo Fisher Scientific) and cotransfected into either T2 or LCL3023 human B-cell lines using a Neon Transfection System (Thermo Fisher Scientific) according to the manufacturer's protocol. Cells were pulsed twice with a voltage of 1100 and a width of 30 ms. Stable cells were established in Roswell Park Memorial Institute medium-1640 media with 10% fetal bovine serum and 5 μg/mL blasticidin-S (Thermo Fisher Scientific).

HLA Antibody Assay

HLA class II antigens were purified by cell lysis and affinity purification from cell pellets and attached to Luminex beads.[2] A solid-phase bead assay was performed as per the LABScreen antibody detection protocol[8] and analyzed by a LABScan3D flow analyzer (One Lambda, West Hills, CA).

Antigen Beads Denaturation

Antigen beads were resuspended in 0.3 M glycine-HCl pH2.7 with 1% bovine serum albumin at room temperature for 1 h, washed 3 times with LABScreen wash buffer at a volume of 10X the original bead volume. The beads were resuspended in PBS at the original bead volume.

Flow Cytometry Analysis and Cell Sorting for Transfectants

Monoclonal antibodies were first labeled with Fluorochrome using Alexa Fluor Antibody Labeling Kits (Thermo Fisher Scientific) and then used to stain cells for 30 min at room temperature. Flow cytometric analyses were performed in a Quanteon Flow Cytometer (Agilent, Santa Clara, CA). Data were analyzed with FlowJo Software.[9] Stable transfected cells were sorted on an MA900 cell sorter (Sony Biotechnology, San Jose, CA). High immunofluorescent cells were sorted and plated in Roswell Park Memorial Institute medium-1640 with 10% fetal bovine serum and 5 μg/mL blasticidin-S.

Flow Cytometry With Human Sera

Approximately 10^6 *DQB1*0601-DRA* transfected cells were mixed with 50 μL of serum and incubated for 20 min at 4°C. After incubation, the cells were washed with 1X PBS and 0.1% glucose and then incubated with biotinylated F(ab')2-goat anti-human IgG Fc-specific antibody (Invitrogen) for 20 min. Samples were washed and incubated with R-phycoerythrin streptavidin (Jackson ImmunoResearch Lab). After washing, samples were acquired on a Quanteon flow cytometer (Agilent, Santa Clara, CA). Data were analyzed with FlowJo Software.[9]

RESULTS

Cotransfection of Various *DQB1* Alleles and *DRA* cDNA Into LCL3023 B-Cell Line Produced DQβ:DRα Heterodimer Surface Expression With Varying Efficiencies

Plasmids with *DRA* and various *DQB1* alleles were constructed and used to transfect LCL3023, a B-cell line

without surface-expression of any class II genes. Surface expression of DRα and DQβ on the transfectants were detected by flow cytometry using a class II (FJ5109) monoclonal antibody, as shown in Figure 1.

*DQB1*06:01-DRA* and *DQB1*06:02-DRA* transfectants have the highest transfection and surface expression efficiency, followed by *DQB1*06:04-DRA*, *DQB1*06:09-DRA*, and *DQB1*05:01-DRA* transfectants, then *DQB1*03:01-DRA* and *DQB1*03:19-DRA* transfectants. FJ5109 antibody-positive transfectants for *DQB1*05:02*, *DQB1*05:03*, *DQB1*06:03*, *DQB1*02:01*, *DQB1*02:02*, *DQB1*04:01*, and *DQB1*04:02* alleles with *DRA* were either very low or undetectable.

DQβ0601 Can Form Heterodimer With DRα Chain in a Cell Line Expresses Both DQα and DRα Chains

To investigate whether DQβ0601:DRα can be efficiently assembled in competition with the presence of DQα, we transfected *DQB1*06:01* and *DQA1*01:03* cDNA into the B-cell line, LCLKO, which expresses only the DRA gene and no other HLA class II genes. The DQβ0601:DQα0103-positive transfectants were expected to express DQβ0601, DQα0103, and DRα chains. If DRα did not possess a sufficient affinity for DQβ0601 compared with DQα0103, then we would not expect to observe DQβ reactivity in the DRα fraction or DRα reactivity in the total class II fraction.

HLA class II proteins were purified from the cell lysates of the DQβ0601:DQα0103-positive transfectants with affinity columns conjugated with a class II–specific (DR, DQ, and DP) monoclonal antibody, FJ5109, a DRα-specific monoclonal antibody, L243, or a DQα-specific monoclonal antibody, FM5148.

The bound proteins were eluted from the columns, attached to the Luminex beads, and characterized with a panel of monoclonal antibodies with known specificity using Luminex bead assay protocol (Figure 2). The proteins purified by the FJ5109 column reacted to DQα- and DQβ-specific monoclonal antibodies as expected; it also had strong reactivity with DRα monoclonal antibody. The protein fraction from the DQα-specific column reacted to

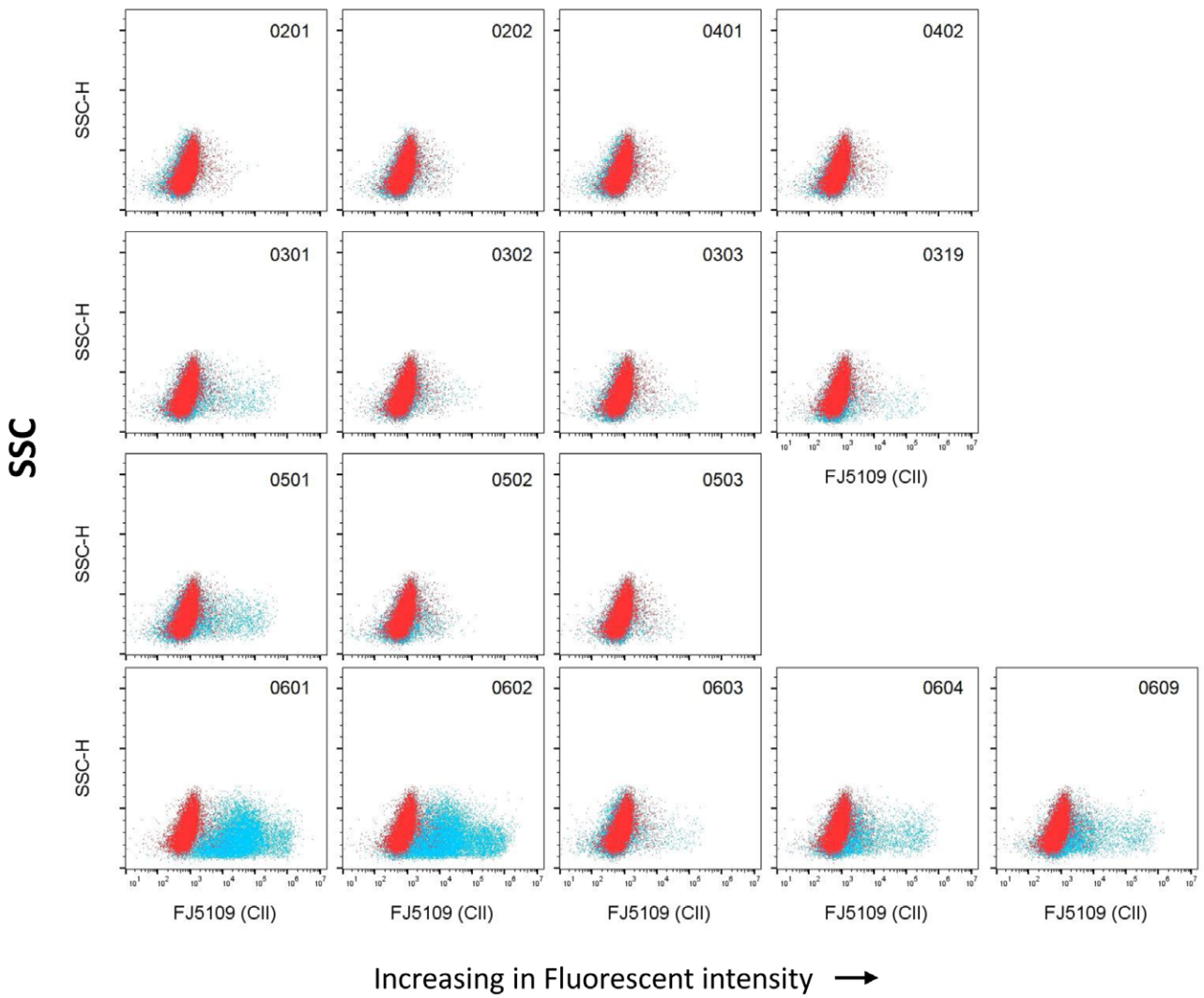


FIGURE 1. Differential surface expression of stable transfected LCL3023 with various *DQB1* allele and *DRA* complementary DNA clones. Alexa Fluor 647-conjugated FJ5109 and L243 were used to detect the surface expression of either DQβ or DRα chain after cotransfection of various *DQB1* and *DRA* complementary DNA. Blue dots represent the events from transfectants, and red dots represent the events from the LCL3023 host. LCL, lymphoblastoid cell line; SSC, side scatter; SSC-H, side scatter height.

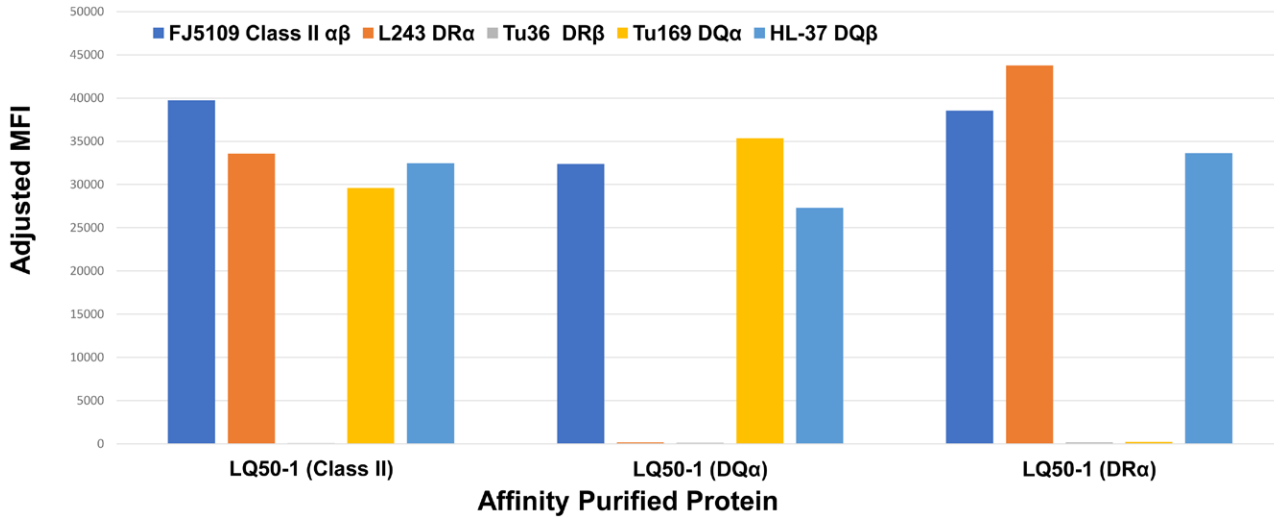
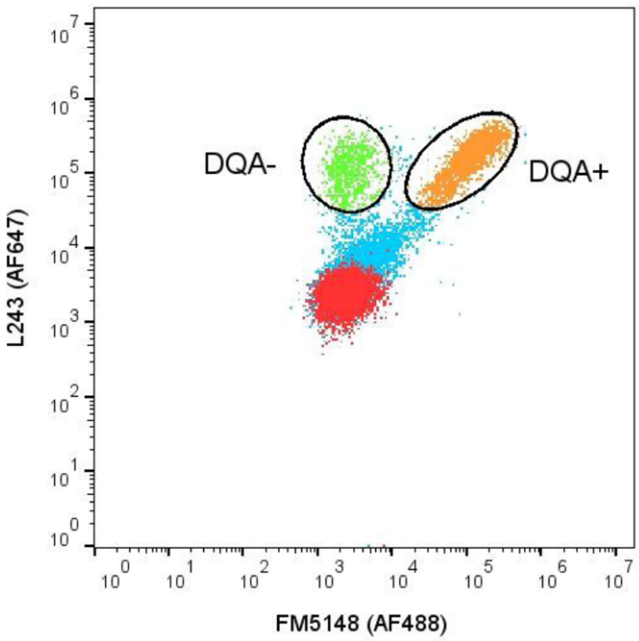


FIGURE 2. Antibody reactivity of LQ50-1 cell lysates purified with different affinity columns. LQ50-1 cell lysates were loaded and eluted from HLA class II–specific (FJ5109), DQα-specific (FM5148), and DRα-specific (L243) columns individually. Affinity-purified proteins were tested with a panel of monoclonal antibodies on Luminex bead-based assay following the LABScreen assay protocol. The antibody specificity is listed following the antibody ID. The specificity of the affinity column used is listed within parentheses following the cell ID. MFI was adjusted by subtracting the goat anti-mouse IgG reactivity for each respective bead. MFI, mean fluorescent intensity.



	Sample ID	Subset	Count	Mean:FL1-H FM5148 (AF488)	Mean:FL4-H L243 (AF647)
■	LCLKO Host	Total	6716	2463	2524
■	DQβ0601:DRα Transfectant	Total	10679	31755	55877
■		DQA-	818	2792	124152
■		DQA+	2556	119516	174609

FIGURE 3. Surface expression of DQβ0601:DQα0103 and DQβ0601:DRα on lymphoblastoid cell line HLA class II knockout (LCLKO) cell line transfected with *DQB1*06:01* and *DQA1*01:03* complementary DNA. Stable transfectants of LCLKO transfected with *DQB1*06:01* and *DQA1*01:03* cDNA were stained with Alexa Fluor 647-conjugated L243 and Alexa Fluor 488-conjugated FM5148. The fluorescent intensities of both L243 and FM5148 from the gated cell population were graphed. Blue dots represent the events from the transfectant population and red dots represent the events from the LCLKO host.

DQα- and DQβ-specific monoclonal antibodies and almost undetectable DRα antibody reactivity. Finally, the protein fraction from the DRα-specific column reacted strongly to the DQβ-specific monoclonal antibody and undetectable DQα reactivity, demonstrating the efficient assembly of the DRα chain with the DQβ chain. Undetectable DQα reactivity rules out the assumption that DQβ reactivity in the DRα fraction was caused by contamination.

Surface expression of both forms of heterodimers on the transfectants was also examined (Figure 3). Alexa Fluor 647-labeled DRα antibody, L243, and Alexa Fluor 488-labeled DQα antibody, FM5148, were used to stain the *DQB1*06:01* and *DQA1*01:03* cDNA transfectants simultaneously and the untransfected host LCLKO. There was no detectable DRα chain on the cell surface of LCLKO because without associating with a β chain DRα will not be exported to the cell surface. There were 3 populations of cells from the transfectants based on the fluorescent intensities of L243 and FM5148 staining. The negative population was superimposed with the stained

untransfected host; there were 2 fluorescent positive populations: a small L243^{+}FM5148^{-} population, representing the cells that express only the DQβ1*06:01:DRα protein because of the incorporation of *DQB1*06:01* cDNA but without *DQA1*01:03* cDNA, and a large L243^{+}FM5148^{+} population, representing cells expressing both the DQβ0601:DRα and DQβ0601:DQα0103 proteins. The L243 staining intensity of the population that expressed both versions of heterodimers was similar to the population that expressed only the DQβ06:01:DRα heterodimer, suggesting that the assembly and export of common DQβ0601:DQα0103 heterodimer had no significant advantage over the assembly and export of the uncommon DQβ0601:DRα heterodimer.

Human Sera Recognize DQβ0601:DRα Heterodimers Expressed on the Cell Surface of a *DQB1*06:01-DRA* Transfectant

To determine the immunogenicity of DQβ:DRα heterodimers, randomly selected sera from our past collection of human allosera were screened against individual Luminex beads bearing either DQβ0601:DRα or DQβ0603:DRα heterodimers. The DQβ0601:DRα reactive sera were further tested and confirmed to be nonreactive against either the DQβ chain from a DQβ0601:DQα0103 antigen or the DRα chain by testing against a series of DR-bearing beads. Sera that reacted only to DQβ0601:DRα or DQβ0603:DRα bearing beads were selected for further study.

In addition, the DQβ0601:DRα and DQβ0603:DRα bearing beads were incorporated into the class II single-antigen bead panel, LS2A01, and used to characterize the HLA class II antibody profile for the selected sera. The DQβ0601:DRα antibody reactivity was observed either as the only HLA class II antibody (Figure 4A) or with other DR, DQ, and DP antibodies (Figure 4B).

Three sera that were positive to DQβ0601:DRα were then used to stain the transfectants expressing DQβ0601:DRα proteins on the cell surface. A serum nonreactive to DQβ0601:DRα beads, served as the baseline fluorescence for the DQβ0601:DRα transfectant (Figure 5).

Serum #2 exhibited the highest mean fluorescent intensity (MFI) value (85 169) against DQβ0601:DRα beads and generated approximately 20-fold higher mean channel shift than the negative serum. Sera #1 and #3 with MFI values of 28 546 and 34 889 on DQβ0601:DRα beads, respectively, generated approximately a 4-fold higher mean channel shift compared with the negative serum. These results clearly demonstrate that certain individuals could produce antibodies against assembled and exported DQβ0601:DRα protein.

To further understand the nature of the epitopes recognized by the DQβ0601:DRα-positive sera, these 3 sera were tested against pH2.7 treated beads. A monoclonal antibody that recognizes DQβ0601:DRα protein was included, and only minor changes in MFI before and after acid treatment showed that there was a minimum loss of DQβ0601:DRα protein from the acid treatment. Reactivity was lost for all 3 sera suggesting that these sera recognize tertiary structure epitopes and not linear sequence epitopes (Table 1).

DISCUSSION

Since the first report of DQβ:DRα interisotypic heterodimer in 1987,[3] there has been little progress in understanding their functions and clinical implications because of the rareness and instability of those heterodimers.[4,5] In

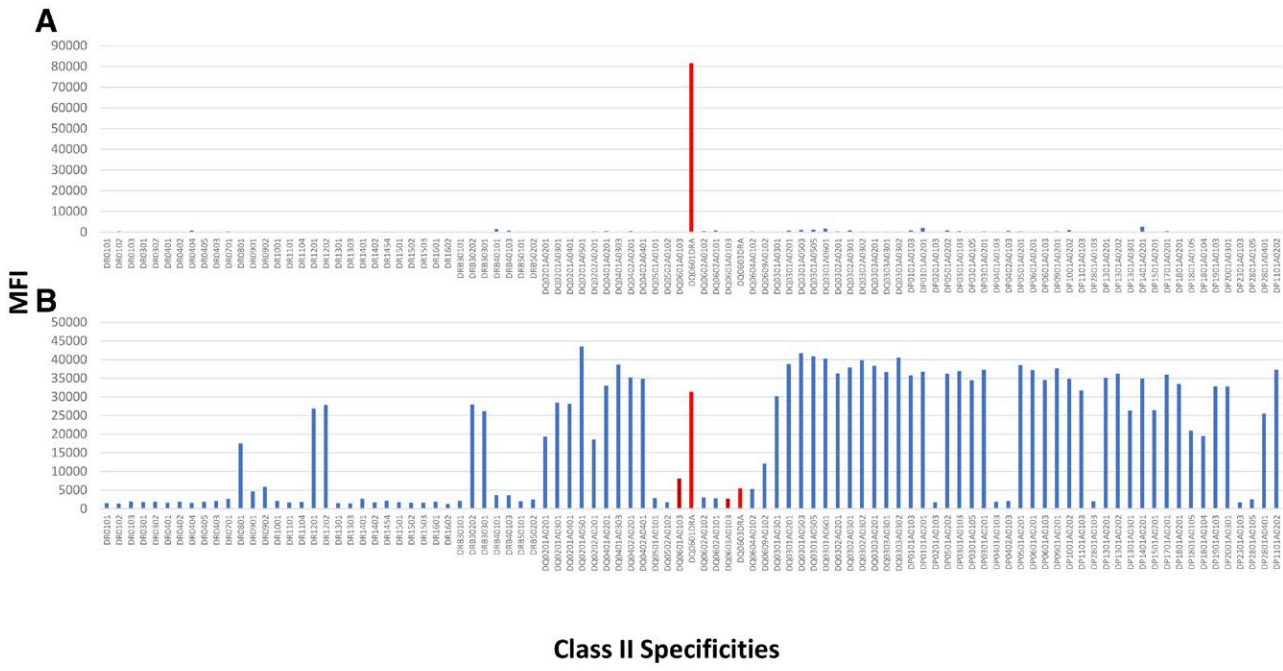


FIGURE 4. Examples of DQβ0601:DRα antibody reactivity in allosera in relation to other HLA class II antibodies (A and B). DQβ0601:DRα and DQβ0603:DRα proteins were attached to the nonoverlapping Luminex beads and incorporated into the class II single-antigen bead panel, LS2A01. Allosera that have high DQβ0601:DRα antibody reactivity were tested on the bead pool using the LABScreen assay protocol. The DQβ0601:DRα and β0603:DRα antibody reactivity were highlighted in red. MFI, mean fluorescent intensity.

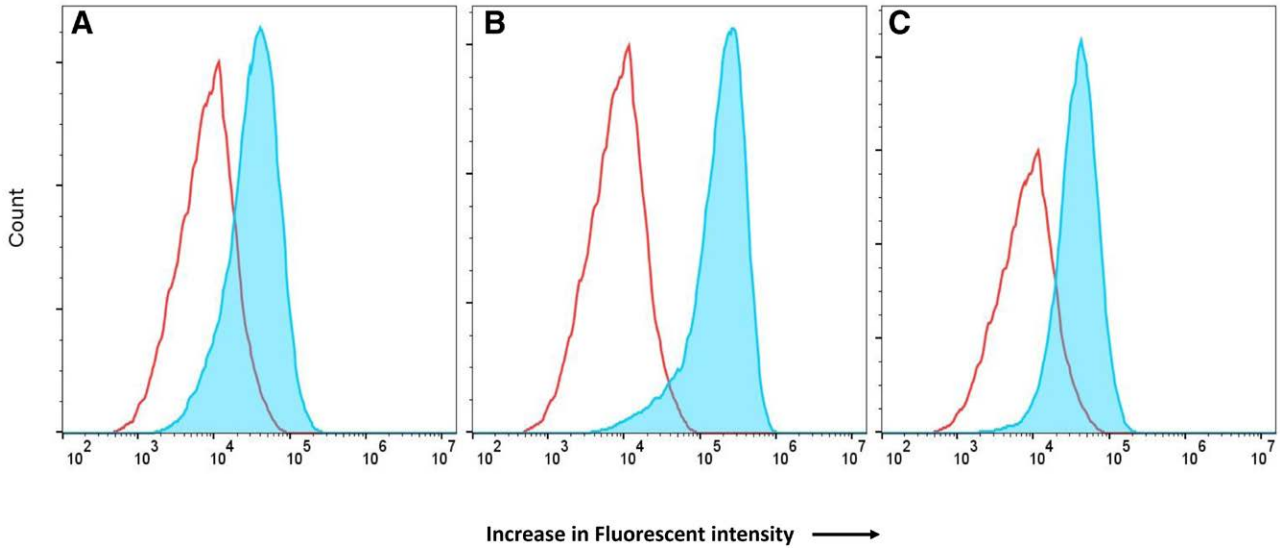


FIGURE 5. Reactivity of DQβ0601:DRα antigen positive sera on DQβ0601:DRα transfectant cells. Three DQβ0601:DRα-positive sera with MFI of 28 546 (A), 85 169 (B), and 34 889 (C) on the Luminex bead assay were tested on LCL3023 host transfected with *DQB1*06:01* and *DRA* complementary DNA. Cells were stained with biotinylated goat anti-human IgG followed by streptavidin phycoerythrin after serum incubation. A negative serum was also tested on the transfectant as the control (red line). LCL, lymphoblastoid cell line; MFI, mean fluorescent intensity.

TABLE 1.

Reactivity (mean fluorescent intensity) of DQβ0601:DRα–positive sera to the acid-denatured protein

	DQβ0601:DRα0101	
	pH 2.7 treatment	
Serum ID/antibody	No	Yes
#1	25 296	303
#2	107 417	255
#3	35 245	241
LN3	48 419	37 015

The reactivity (mean fluorescent intensity) of sera to pH 2.7 treated and untreated DQβ0601:DRα beads were listed. The mean fluorescent intensity of a positive-reactive mouse monoclonal antibody (clone LN3) on the same set of beads is listed to confirm the presence of the antigen on the beads after the treatment.

this report, we tested the surface expression of DQβ:DRα heterodimers using different *DQB1* alleles by transfecting *DQB1* alleles and *DRA* cDNA on a single-expression vector into LCL3023, a cell line has no expression of class II genes.[8] In so doing, we were able to reproduce the observation of differential surface expression of certain *DQB1* alleles.[5] We were also able to identify certain *DQB1* alleles that can produce stable DQβ:DRα heterodimers on the cell surface (Figure 1). To our surprise, there was an allelic preference for surface expression even within the same serological group (Figure 1). For example, the *DQB1*06:01* allele demonstrated the greatest surface expression with *DRA* among 5 *DQB1*06* alleles and 11 other *DQB1* alleles tested. In contrast, the *DQB1*06:03* allele had very low to undetectable transfection efficiency with DRA.

We also demonstrated that the surface expression of DQβ0601:DRα heterodimer was at a level similar to transfectants that expressed DQα0103 suggesting that the affinity of DRα chain for DQβ0601 chain is comparable with the affinity of DQα0103 chain for DQβ0601 chain (Figures 2 and 3). Overall, our data showed that multiple DQβ:DRα heterodimers can assemble intracellularly and be transported to the cell surface efficiently.

Even with low surface expression efficiency compared with other *DQB1*06* alleles, we were able to isolate stable clones of DQβ0603:DRα transfectant for expansion in cell culture. Sufficient amounts of DQβ0603:DRα protein were also purified from the cell lysates for immunological studies.

Because this is the first time DQβ0601:DRα and DQβ0603:DRα proteins have been isolated in significant quantity and purity, we were interested to determine whether the immunogenicity of this protein can be detected in human sera. Using the purified protein constructs, we screened samples from our collection of human kidney transplant candidate's sera and identified some sera that reacted specifically to this protein by Luminex bead-based assay. Only sera that were completely devoid of reactivity to DQβ0601:DQα0103 or DRβ:DRα were considered to be DQβ0601:DRα positive. This postulate ruled out the possibility of cross-reactivity against the shared DQβ or DRα peptide sequences. Therefore, positive sera defined by these criteria must be reacting to a structural epitope contributed by both DQβ0601 and DRα molecules and possibly with participation of the peptide in the binding groove. Three sera with the highest bead reactivities were selected for further characterization by flow cytometry. Positive cell surface staining of DQβ0601:DRα transfectants demonstrated that they recognized the assembled and exported heterodimers. The loss of reactivity of these 3 positive sera when tested on the acid-denatured DQβ0601:DRα beads supports the assumption that these antibodies were reacting to tertiary structure epitopes and not linear epitopes on the polypeptides. Isolation of other stable DQβ:DRα proteins for the investigation of the prevalence of the DQβ:DRα antibodies and their clinical implication is underway in collaboration with multiple transplant centers.

To explore the surface expression of the heterodimer, a flow cytometric crossmatch was performed using archived lymphocytes from a healthy donor that had been high-resolution HLA typed and was homozygous for *DQB1*06:01/DQA1*01:03*. Not unexpectedly, the B-cell crossmatch with the DQβ0601:DRα positive sera was negative (data not shown). This finding was most likely due to the low abundance of the DQβ0601:DRα heterodimer expressed on the cell surface of this particular cell. Nevertheless, we cannot exclude the expression of this interisotypic heterodimer in lymphoid cells because it has been reported that the assembly of the DQβ:DRα heterodimer can be influenced by the number of DRα chains present in the cells.[4] Although our data showed that DQβ0601 was more efficient than other DQβ chains in forming a heterodimer with DRα, low expression is not unexpected in that its assembly would be in competition with the DRβ chain for the DRα chain and with DQα chain for the DQβ chain.

Polymorphism of HLA class I and II antigens affects which amino acids are anchored in the peptide-binding groove and therefore their peptide-binding capacity and specificity. Furthermore, many amino acid substitutions affect the peptide-binding groove and the site of interaction with the TCR. These amino acid substitutions account for further diversification of the immune response because more peptides can be presented to TCRs to elicit specific immune responses. Any changes in the electrostatic, hydrophobicity, and shape of the cleft by any conformational change can alter the peptide-binding property.[1]

The availability of DQβ:DRα heterodimer can facilitate addressing an important question of whether the TCRs will recognize this group of interisotypic heterodimers and the interaction with the peptides they present.

Karp et al[10] attempted to address mixed DR and DP heterodimers and were unsuccessful in identifying mixed DRα:DPβ or DPα:DRβ heterodimers. Because we have observed that DQβ0601:DRα and DQβ0602:DRα have the highest transfection efficiency and cell membrane expression followed by DQβ0604>DQβ0609>DQβ0501>DQβ0301>DQβ0319 DRα heterodimers, it might be prudent to evaluate whether certain allelic DPβ chains may form stable heterodimers with DRα chain.

In conclusion, we have documented the stable expression of certain DQβ:DRα interisotypic heterodimers and, for the first time, shown that these proteins can be purified in sufficient quantity for immunological study and for future major histocompatibility complex:peptide:TCR interaction studies. Furthermore, the presence of antibodies directed against these heterodimers was discovered in the serum of kidney transplant candidates. The potential clinical relevance of this class of HLA proteins can now be evaluated given the availability of DQβ:DRα single antigens.

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