Alloantibody to a Bw4 Epitope in a Bw4$^+$ B*27:05 Patient

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Background. Alloantibodies to the Bw4 epitope are known to be heterogeneous, but it is widely assumed that anti-Bw4 alloantibodies arise only in individuals who do not express a Bw4 epitope.
Methods. Bw4 expression was confirmed by DNA sequence analysis. Anti-Bw4 reactivity was confirmed by absorption with transfected cells.
Results. A Bw4$^+$ (B*27:05 or B*27:13) patient expressed antibody that bound all Bw4$^+$ human leukocyte antigen-A and human leukocyte antigen-B antigens tested, except B*27:05 and B*44:02. Serum absorbed with B*51:01-transfected HYM2.C1R cells left only reactivity to B17 (B57, B58), but not to any other Bw4$^+$ antigens.
Conclusion. A Bw4$^+$ patient made antibody to a Bw4 epitope. This finding indicates that apparent anti-Bw4 or anti-Bw6 antibody should not be ignored even in patients who express a common Bw4$^+$ or Bw6$^+$ antigen, respectively.

Keywords: Transplantation, Histocompatibility, Antibody, Bw4 epitope.

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Anti–human leukocyte antigen (HLA) antibodies are a major cause of organ transplant rejection. Antibodies to public epitopes, such as Bw4 and Bw6, preclude transplantation with most potential donors. Based on sequence comparisons, the Bw4 and Bw6 epitopes are located on residues 79 to 83 or residues 77, 80 to 83 of the α-1 α-helix (*1, 2*). Bw4$^+$ proteins express heterogeneous amino acid sequences at residue 77 (N, D, or possibly S) and at residues 80 to 83 (isoleucine-alanine-leucine-arginine [IALR], threonine-alanine-leucine-arginine [TALR], and threonine-leucine-leucine-arginine [TLLR]). In addition to epitope heterogeneity, anti-Bw4 and anti-Bw6 antibodies vary and bind subsets of the Bw4 group or Bw6 group. Partially overlapping Bw4 and Bw6 subsets are recognized by mouse and human monoclonal antibody (mAb) and alloantibodies found in human sera (*2–6*).

Using site-directed mutagenesis of Bw6$^+$ HLA-B*07:02, researchers found that substitution of residues 80, 82, and 83 for amino acids found in Bw4$^+$ HLA-B*27:05 conferred reactivity to all six anti-Bw4 mAb and all three anti-Bw4 human alloantibodies tested (*6*). The same triple mutation destroyed the Bw6 epitope as detected by all three anti-Bw6 mAb and three of four anti-Bw6 human alloantibodies tested. Substitution of residues 79 alone (R79G) or 82 alone (R82L) abrogated binding by one anti-Bw6 mAb (SFR8-B6), but not by the other two anti-Bw6 mAb tested. Modeling provided a strong rationale for these findings. Bw6 amino acids R79 and R82 are oriented outward and could form energetic contacts with anti-Bw6 antibodies (*7*). Substitution of residue 83 alone (G83R) abrogated binding by a different anti-Bw6 mAb (BB7.6), but not by the other two anti-Bw6 mAb (*6*). The HLA-B*07:02 R82L and G83R single amino acid substitutions each largely eliminated binding by both of the human anti-Bw6 alloantisera tested. Interestingly, the R82L and G83R single amino acid substitutions each conferred significant binding by four and five of six anti-Bw4 mAb, respectively. Furthermore, the G83R single amino acid substitution was able to bind one of two anti-Bw4 alloantisera tested (*6*). A major lesson from this study is that the Bw4 and Bw6 epitopes are heterogeneous. Antibodies recognizing these epitopes are specific to individual amino acids in the 77 to 83 region.

Bw4 epitope heterogeneity was confirmed by El-Awar et al. (*4*), who described three mouse mAb that reacted with Bw4 epitopes. In each case, the mAb reacted with nearly all Bw4$^+$ proteins tested. The exceptions correlated with amino acid sequences outside of the 77 to 83 region, including connecting loop residue 90 and α-2 α-helix residues 144/145.

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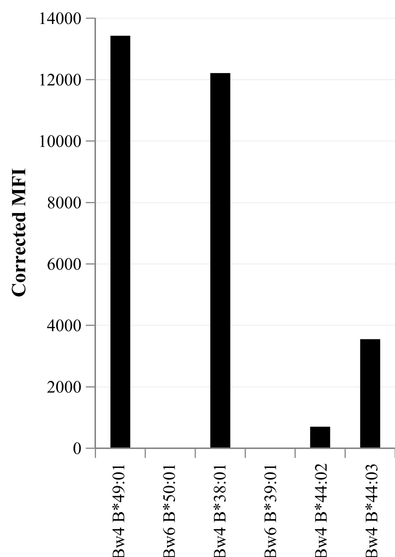

FIGURE 1. Patient serum recognized a Bw4 epitope. Serum was tested using Luminex single antigen beads. Shown are results with illustrative pairs of closely related antigens of the B21 group (Bw4$^+$ B*49:01 and Bw6$^+$ B*50:01) and the B16 group (Bw4$^+$ B*38:01 and Bw6$^+$ B*39:01). Also shown is binding to two Bw4$^+$ B44 antigens.

Similarly, Marrari et al. (*5*) described an anti-Bw4 human mAb that reacted with nearly all Bw4 members, with the exceptions correlating with residues near amino acid 144. These correlations between amino acid sequences and anti-Bw4 mAb reactivity were not confirmed by mutagenesis studies. However, these anti-Bw4 mAb reactivity patterns were reminiscent of anti-Bw6 mAb, BB7.6, which was affected by mutations in both the α-1 and α-2 α-helices, and SFR8-B6, which depended on α-1 α-helix amino acids and connecting loop residue 90 (*6*). El-Awar et al. (*4*) speculated that anti-Bw4 human alloantisera might contain multiple antibodies, each reacting with a distinct Bw4 epitope.

Tissue typing analysis generally assumes that anti-Bw4 antibodies are made only by individuals who do not express a common Bw4 epitope. Nonetheless, anti-Bw4 alloantisera have been reported in apparently Bw4$^+$ individuals. In some cases, these patients showed unusual HLA-B alleles in which amino acids 76 to 83 had been exchanged with other sequences, resulting in a Bw4-Bw6 epitope switch or in the expression of neither the Bw4 epitope nor the Bw6 epitope (*8*). Similarly, patients who expressed novel HLA molecules with atypical Bw4 epitopes made antibodies that reacted with a subset of Bw4$^+$ proteins (*9*). These rare cases do not violate the assumption that anti-Bw4 antibody is made only by individuals who do not express a typical Bw4 epitope. Anti-Bw4 reactive antibody was reported (*10*) in two B*13:02$^+$ patients with alloantisera that reacted with all Bw4 antigens tested, with the exception of B13. However, these authors did not confirm the B*13:02 typing by DNA sequence analysis, leaving open the possibility that these two patients expressed variant Bw4 epitopes, similar to those reported previously. Furthermore, absorption studies were not performed, thus failing to rule out the possibility that the observed reactivity pattern was due a collection of antibodies that were not specific for the Bw4 epitope. We describe an alloantibody from a HLA-B27$^+$ patient that reacted with all tested Bw4$^+$ proteins except B*44:02 and B*27:05. Absorption studies were performed to identify the alloantibody specificity.

RESULTS

An African American female in her sixth decade presented with emphysema and secondary pulmonary hypertension and was evaluated for possible lung transplantation. The patient had a history of pregnancy and a probable history of blood transfusion. Her HLA type by sequence-specific oligonucleotide probe hybridization was consistent with several HLA alleles, indicating the following serologic type: A2, A68, B27, B72, Bw4, Bw6, DR103, DR17. Combining these data with Sanger DNA sequence analysis, the HLA-B alleles were found to be B*15:03/B*15:220 and B*27:05/B*27:13. Neither B*27:13 nor B*15:220 is common or well documented (*11*), strongly suggesting that the patient's HLA-B type was B*15:03, B*27:05. Moreover, B*27:05 and B*27:13 have identical predicted mature amino acid sequences, differing by only one amino acid in the leader sequence (IMGT/HLA Database, Release 3.15.0, 2014-01-17; http://www.ebi.ac.uk/ipd/imgt/hla/).

Screening beads indicated the presence of immunoglobulin G (IgG) antibodies to multiple HLA class I antigens, but not to class II antigens. Assayed with single antigen Luminex beads, serum antibody reacted with all tested Bw4$^+$ HLA-A and HLA-B antigens with a corrected mean fluorescence intensity (MFI) of at least 2,000, with the exception

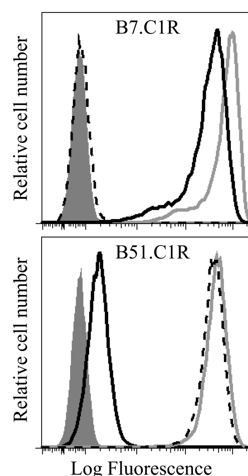

FIGURE 2. Transfectants react appropriately with mouse mAb. B7.C1R (*top*) and B51.C1R (*bottom*) cells were incubated with the indicated mouse mAb (primary antibody), washed, and stained with labeled goat anti-mouse IgG (secondary antibody). Staining with control mouse IgG (*filled histogram*) gave similar results to that of no primary antibody or no secondary antibody. Staining with 4E anti-HLA-B/C (*gray line*) served as a positive control. Staining with W6/32 (anti-HLA class I), and BBM1 (anti–β_2-microglobulin) produced similar relative levels. Anti-Bw6 SFR8-B6 (*thick black line*) and anti-Bw4 22E1 (*dashed line*) differentially bound to the transfectants. IgG, immunoglobulin G; HLA, human leukocyte antigen.

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of B*44:02 (703 MFI) and B*27:05 (0 MFI). These results were confirmed using single antigen flow beads, except that B*47:01 binding could not be detected (data not shown). A Bw4 epitope was suggested by differential binding to subtypes of the B21 and B16 groups (Fig. 1). Antibody reacted with Bw4$^+$ B*49:01, but not Bw6$^+$ B*50:01, proteins that differ only at amino acid residues 77 and 80 to 83. Likewise, antibody reacted with Bw4$^+$ B*38:01, but not Bw6$^+$ B*39:01, which differ only at positions 74, 77, and 80 to 83. Because these amino acids are critical for Bw4 and Bw6 epitope formation, the results strongly implied that the patient serum was specific for the Bw4 epitope.

To rule out the possibility that the observed antibody reactivity was caused by multiple IgG antibodies specific for epitopes other than Bw4, serum was absorbed with HLA^{low} HYM2.C1R cells that had been transfected with B*07:02 (B7.C1R) or with B*51:01 (B51.C1R). Transfected cell identity was confirmed by flow cytometry (Fig. 2 and *Materials and Methods*). Mock-absorbed serum did not significantly change antibody binding compared with nonabsorbed serum (data not shown). Absorption with B7.C1R cells did not lower antibody binding to any of the Bw4$^+$ antigens (Fig. 3). Binding by the B7.C1R-absorbed serum might be slightly higher than mock-absorbed serum, possibly by removing weak interfering substances. Absorption with B51.C1R cells, in contrast, abrogated antibody binding to all Bw4$^+$ HLA-A and HLA-B antigens, except those of the B17 group (B*57:01, B*57:03, and B*58:01), which showed reduced binding after absorption (Fig. 3). This result rules out the possibility that the serum contained multiple antibodies that reacted with various Bw4$^+$ HLA-A and HLA-B antigens, but not to the Bw4 epitope itself. Instead, this result indicates that the serum contained two antibody specificities: one to a B17 epitope and one to a Bw4 epitope. This Bw4 epitope is not expressed on the patient's self B*27:05 molecules and is relatively weakly expressed by other HLA-B molecules with threonine at residue 80 (Fig. 3).

DISCUSSION

To our knowledge, this is the first well-documented case of anti-Bw4 alloantibody in a person encoding a DNA sequence-confirmed "typical" Bw4$^+$ epitope on B*27:05 (or possibly B*27:13, which has identical mature protein sequence). Evidence in the current case for antibody truly being Bw4 specific includes dramatically distinct reactivity with B21 and B16 family proteins that differed only in amino acids 77 and 80 to 83 and amino acids 74, 77, and 80 to 83, respectively. Multiple sequence comparison and mutagenesis studies have shown that these positions determine Bw4-Bw6 epitope formation (*1, 2, 6*). We ruled out the possibility that the reaction pattern was caused by multiple alloantibodies that were not Bw4 specific. Absorption with B*51:01 removed all anti-Bw4 reactivity on both HLA-A and HLA-B antigens, leaving only antibody to a B17 epitope. The finding of Bw4 and B17 antibody specificities suggest that the immunizing antigen was Bw4$^+$ B57 or B58. Our patient may not be unique because anti-Bw4 antibody in apparent Bw4$^+$ individuals has been reported (*10*), but in those cases, the anti-Bw4 antibody was not confirmed by absorption studies, and the Bw4$^+$ donor was not confirmed by DNA sequence analysis. Although formation of anti-Bw4 antibody in a Bw4$^+$ individual is unusual, it is not surprising from an immunologic perspective. The immune system is able to distinguish nonself molecules that differ from self by only a single amino acid residue (*12*). The anti-Bw4 alloantibody described here did not react with self B*27:05 protein.

Nearly all Bw4$^+$ antigens reacted with the patient's antibody, but there was a wide range of binding. Some of the differences in antibody binding may have been caused by variable density in the antigen coated on the distinct allele-specific beads. However, sequence differences both within and outside of the 80 to 83 region suggest an alternative explanation. The highest binding was to HLA-B antigens with amino acids IALR at residues 80 to 83. HLA-B antigens with TALR and TLLR sequences tended to have lower

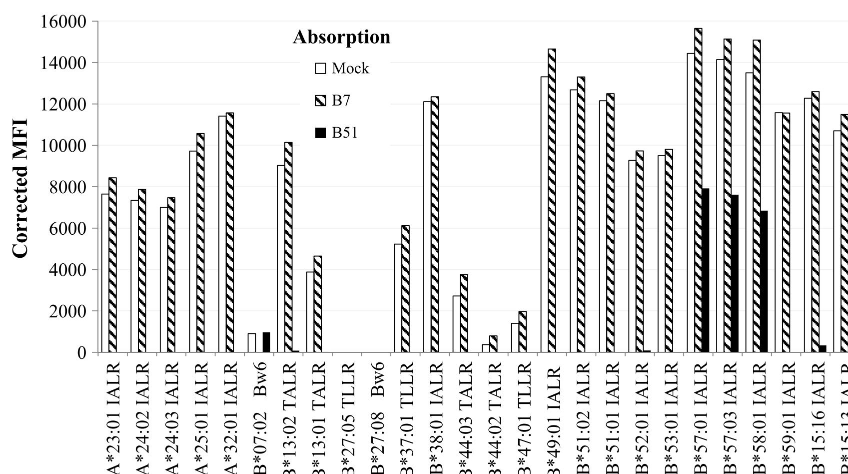

FIGURE 3. Patient alloantibodies recognize Bw4 and B17 epitopes. Serum was mock-absorbed (Mock) or absorbed with B7.C1R (B7) or B51.C1R (B51) cells as indicated. Shown is the corrected MFI of selected Luminex single antigen beads. Bw4$^+$ antigens are denoted by their residue 80 to 83 amino acid sequences; also noted are relevant Bw6$^+$ antigens. Other antigens reacted with low MFI. A complete list of results is presented in (**Table S1, SDC,** http://links.lww.com/TP/A996). MFI, mean fluorescence intensity.

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binding (Fig. 3). This was expected because TLLR is the sequence encoded by the host B*27:05 allele. $Bw4^+$ HLA-A proteins demonstrated a somewhat lower antibody binding despite having IALR sequence at residues 80 to 83. This was not surprising because the binding interface of antibodies with protein antigens is potentially large and involves discontinuous stretches of amino acids (*13*). Therefore, the alloantibody footprint is almost certain to contact structures outside the 80 to 83 region and is likely influenced by amino acid sequences specific to the $Bw4^+$ HLA-A antigens. Support for this hypothesis comes from the observation that B*44:03 consistently bound more antibody than B*44:02 (Figs. 1 and 3). These molecules differ only at amino acid residue 156 in the α-2 α-helix (IMGT/HLA Database). This observation suggests that the patient's anti-Bw4 antibody resembles the BB7.6 anti-Bw6 mouse mAb in straddling the peptide binding groove to contact the 80 to 83 region in the α-1 α-helix and amino acids near residue 156 in the α-2 α-helix. Similarly, a human mAb recognizes a Bw4 epitope, correlating with sequences in the 80 to 83 region and sequences including residue 145 on the α-2 α-helix (*5*). Possible antibody contact with HLA α-1 α-helix residues outside the 80 to 83 region might explain the superior binding of B*37:01 compared with B*27:05. Although both alleles encode TLLR at residues 80 to 83, they differ by five amino acids in α-1 α-helical residues 67 to 74. This region is relevant because Marrari et al. (*5*) reported that reactivity of a human mAb correlated with polymorphisms in the 80 to 83 region and near residue 65 on the α-1 α-helix. We propose that the anti-Bw4 alloantibody described here straddles the peptide binding groove and contacts both the α-1 and α-2 α-helices.

Finally, our finding provides an important lesson for clinical histocompatibility testing. Bw4/Bw6 epitopes and associated antibodies are heterogeneous. Consequently, apparent anti-Bw4 or anti-Bw6 antibody should not be ignored even in patients who express a common $Bw4^+$ or $Bw6^+$ antigen, respectively.

MATERIALS AND METHODS

The patient was typed for HLA-A, B, and DR by sequence-specific oligonucleotide hybridization (One Lambda, Canoga Park, CA), following kit instructions. HLA-B typing was refined by Sanger DNA sequencing using BigDye chemistry and polymerase chain reaction (PCR) cycle sequencing primers following kit instructions (SBT Pack for HLA-B; Abbott Molecular, Des Plaines, IL). Products were assayed by capillary electrophoresis on an ABI 3130*xl* Genetic Analyzer and were analyzed using Assign software (Conexio Genomics version 3.5.1.45).

Immunoglobulin G anti-HLA antibodies were tested with flow cytometry single antigen beads (One Lambda) following kit instructions. Briefly, a mixture of phycoerythrin-labeled single HLA antigen beads were incubated with patient serum or appropriate controls for 30 min on a rotating platform at room temperature in the dark. After washing, beads were incubated with labeled goat $F(ab')_2$ anti-human IgG antibody as above. After washing, beads were fixed with paraformaldehyde and analyzed on a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ), gating on phycoerythrin signal to distinguish the antigen-coated beads in each mixture and analyzing fluorescein color to estimate the amount of human IgG bound to each bead. Heat inactivated (56°C for 30 min) serum was tested with Luminex single antigen beads (LABScreen Single Antigen Class I kit; One Lambda) using incubation and washing steps similar to those for single antigen flow beads except that the secondary antibody was labeled with phycoerythrin, reaction products were not fixed, and samples were analyzed on a $Luminex^{200}$ flow analyzer. Results were normalized by correcting with fluorescence produced by beads that were not coated with HLA antigen (**Table S1, SDC,** http://links.lww.com/TP/A996).

HYM2.C1R cells transfected with B*07:02 (B7.C1R) or with B*51:01 (B51.C1R) were grown in the presence of hygromycin B and tested for expression as described (*6*). As expected, 4E mAb (specific for HLA-B, C) bound both transfectants, with B7.C1R having somewhat higher HLA class I expression than B51.C1R (Fig. 2 and data not shown). As appropriate, B7.C1R cells bound SFR8-B6 anti-Bw6 mAb, but not 22E1 anti-Bw4 mAb (Fig. 2). B51.C1R cells reacted strongly with 22E1 mAb to Bw4, and only slightly above background with SFR8-B6 mAb to Bw6 (Fig. 2). Low anti-Bw6 mAb reactivity is consistent with $Bw6^+$ B*35:03 expression by HYM2.C1R cells, which was reported to be a few percent of levels on the nonmutated parental cell line (*14*). Patient serum (0.1 mL) was absorbed with 2.2×10^6 B7.C1R or B51.C1R cells for 2 hr at 37°C, followed by removal of cells by centrifugation. Mock-absorbed serum underwent the same incubation and centrifugation steps without cells.

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