HLA (A*0201) Mimicry by Anti-Idiotypic Monoclonal Antibodies


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HLA (A*0201) Mimicry by Anti-Idiotypic Monoclonal Antibodies


Soluble MHC Ags and anti-Id (anti-anti-MHC) Abs have both been shown to inhibit MHC alloantigen-specific B cell responses in vivo. We hypothesized that some anti-idiotypic Abs function as divalent molecular mimics of soluble HLA alloantigen. To test this idea, we studied two well-defined anti-idiotypic mAbs, T10-505 and T10-938, elicited in syngeneic BALB/c mice by immunization with CRll-351, an HLA-A2,24,28-specific mAb. Each anti-Id induced “Ab-3” Abs in rabbits that cross-reacted with HLA-A2 but not with HLA-B Ags. Furthermore, each anti-Id could bind to and block Ag recognition by Ha5C2.A2, a human homologue of mAb CRll-351. Both anti-Id mAb displayed weak reactivity with the human mAb SN66E3, which recognized an overlapping but distinct determinant of HLA-A2 Ags; neither reacted with human mAb MBW1, which recognized a nonoverlapping HLA-A2 determinant. Amino acid sequence comparison of mAb CRll-351 heavy and light chain variable region complementarity-determining regions (CDRs) with those of mAb Ha5C2.A2 and SN66E3 revealed short regions of homology with both human mAb; a large insert in the light chain CDR1 of mAb SN66E3 distinguished it from both CRll-351 and Ha5C2.A2. The amino acid sequences of mAb T10-505 and T10-938, which differed markedly from each other, revealed no homology to the α2 domain sequence of HLA-A*0201 that contains the CRll-351 mAb-defined epitope. We conclude that structurally different anti-Id Abs can mimic a polymorphic conformational epitope of an HLA Ag. In the case of T10-505 and T10-938 mimicry was not based on exact replication of the epitope by the hypervariable loops of the anti-Id mAb. The Journal of Immunology, 1998, 161: 6705–6714.

The semiallogeneic fetus in normal mammalian pregnancy and allogeneic blood transfusions used in clinical transplantation for preconditioning the allograft recipient can induce the formation of either a) Abs to polymorphic epitopes of MHC Ags (1) or b) anti-Id Abs that specifically block their binding to the polymorphic MHC ligand (2, 3). Development of anti-MHC alloantibodies constitutes a major obstacle to successful engraftment of organ transplants in persons with end stage organ failure (4) and contributes significantly to the problem of chronic rejection, the major cause of late allograft loss (5, 6). Production of anti-anti-MHC (anti-Id) Abs, on the other hand, has been associated with the beneficial effects of blood transfusion on allograft survival as well as the absence or reversal of alloantibody sensitization (7, 8). The functional and structural relationship of these anti-Ids to soluble forms of allogeneic MHC Ags, which are also present in serum and which play a role in the outcome of allografts, (9–14) remains largely unexplored.

It has been shown in transgenic mice that soluble Ags can functionally inactivate an Ag-specific B cell, provided that a sufficient level of soluble gene product is expressed (15). Studies in MHC class I alloantigen H-2Dk transgenic mice now indicate that, while the native, membrane-bound (44-kDa heavy chain) form tolerizes both CTL and B cell responses, B cell but not CTL responses to alloantigen are tolerized in mice made transgenic for a soluble (36-kDa heavy chain) form expressed at high (>100 µg/ml in serum) levels (16). Similarly, a sufficient dose (i.e., mice given repeated daily i.p. injections) of a recombinant H2 O-soluble form of HLA-B7 inhibited Ab responses specific for HLA-B7 polymorphic epitopes when the mice were later challenged with an immunogenecell membrane-bound form of the Ag (17). A naturally occurring form of humoral CTL+ “split tolerance” in mammals may be induced by exposure of the fetus and neonate to soluble forms of noninherited maternal Ags (18–21). New evidence suggests that this form of tolerance contributes to long term success of kidney transplants in living-related sibling renal transplant recipients (22).

The powerful tolerogenic effects of soluble MHC proteins upon alloantibody responses may be mediated partly through direct effects on B cells and partly through indirect effects on T helper cells. For example, the IgG response to allogeneic MHC proteins is completely CD4+ T helper cell dependent (23), and this T helper cell effect has recently been shown to require recognition of reprocessed forms of the alloantigen presented as peptides bound to host MHC class II (24). In contrast, primary IgM responses to MHC class I alloantigens appear to be relatively T helper cell independent (25, 26) and minimally deviated from germline (27).

The relationship between soluble forms of MHC Ag and humoral “split” tolerance (16) prompted us to reexamine the question of the nature of anti-Id (anti-anti-MHC) Abs. Because Abs are

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multivalent and are present at $\mu$g/ml levels in serum, any anti-Id Ab that could mimic a soluble MHC Ag, even with less than complete fidelity to the original alloantigen in structure, might be a highly effective antagonist of alloresponsive B cells in vivo. Jerne (28) hypothesized that an anti-Id Ab could provide in its variable regions the "internal image" of a particular antigenic epitope. In the case of MHC Ab-defined polymorphisms, this hypothesis predicts that anti-Id Abs could act at the clonal B cell level in a way analogous to a soluble foreign MHC Ag, leading either to sensitization or tolerance in specific subsets of anti-MHC B cells, depending upon the dose, route, and timing of exposure to the anti-Id. The extent to which a given epitope is immunodominant would determine the range of efficacy of a given soluble HLA "epitope mimic" anti-Id.

To determine whether anti-Ids could serve as a surrogate soluble MHC Ag, we tested two anti-Id mAb, T10-505 and T10-938, which recognize distinct idiotopes (idiotypes) in the Ag binding site of the anti-HLA-A*0201 mouse mAb CRIL-351 (29, 30). Our data support the hypothesis that an anti-Id may function as a divalent mimic of an $\alpha$-helical, conformational epitope of HLA-A*0201, despite a difference in secondary structure between the epitope ($\alpha$ helical) and the epitope mimic (complementarity-determining region (CDR)$^3$-connecting loops and $\beta$-pleated sheets of Ab V regions).

Materials and Methods

Mouse mAbs and antisera

The mouse anti-Id mAb T10-505 and T10-938, which recognize distinct idiotopes in the Ag recognition site of the immunizing mAb CRIL-351, were generated as described (30). The murine hybridomas producing A2/24/28-cross-reactive mouse mAb CRIL-351 (31) and the A2/B17-cross-reactive mouse mAb MA2.1 (32) were grown as ascites in BALB/c mice or maintained in culture with RPMI complete medium (RPMI 1640; Bio-whittaker, Walkersville, MD) and medium with 10% FCS (HyClone Laboratories, Gaithersburg, MD). All mouse mAbs were purified by affinity chromatography using Sepharose 4B (Sigma, St. Louis, MO) beads coupled with goat anti-mouse IgG1 Abs as described previously (30), or by sequential precipitation with caprylic acid and ammonium sulfate (33). The purity of mAb preparations was monitored by SDS-PAGE (34). Purified mAb were coupled to biotin using the N-hydroxysuccinimidyl-biotin reagent (Pierce Chemicals, Rockford, IL), as described (35). Rabbit anti-human $\beta_2$-microglobulin Abs were purchased from Accurate Chemical (Westbury, NY).

Production and purification of rabbit anti-(anti-Id) Abs

One-month-old male New Zealand white rabbits were purchased from Hazelton Laboratories (Madison, WI). Anti-Id mAb T10-505 or T10-938 (2.5 mg) was chemically conjugated to 2.5 mg keyhole limpet hemocyanin (KLH) (Sigma) using 2.5% glutaraldehyde (Sigma) in PBS (10 mM phosphate, 150 mM NaCl, pH 7.0). At the end of a 60-min incubation at room temperature, the reaction was stopped with 1 M glycine (50 $\mu$M), and the solution was dialyzed against PBS. Each Ab-2-KLH conjugate (0.75-1.5 mg) emulsified in CFA was injected s.c. into two rabbits. Rabbits were boosted twice at 4-week intervals with s.c. injections of mAb T10-505-KLH conjugate (0.75-1.5 mg) in IFA. Sera were collected by venipuncture of the ear pinna 2 weeks after each injection. Mouse anti-human (anti-Id) Abs (Ab-1) were purified from rabbit serum by affinity chromatography over an mAb T10-505 or T10-938-conjugated Sepharose 4B column following initial absorption with mouse IgG-coupled Sepharose 4B. Abs eluted from the Ab-2 affinity columns were further extensively absorbed with CRIL-351-conjugated Sepharose 4B to remove residual mouse-specific Ab as well as any possible "Ab-4" that might bind to CRIL-351 in subsequent inhibition or blocking assays. Abs were isolated from rabbit preimmune sera utilizing a similar procedure, except that the secondary absorption step with CRIL-351 was omitted.

Human hybridomas, cell lines, and HLA class I Ag preparations

The human hybridoma secreting mAb SN6663 (IgM, $\kappa$) was constructed with B lymphocytes from a multiparous female (SN) who had developed anti-HLA-A2 and anti-HLA-B17 Abs following two pregnancies miscarried at 10 weeks. Following the second pregnancy, B lymphocytes isolated from peripheral blood were EBV transformed and cultured using a modification of the anti-CD40 system (36). An IgM-secreting B-LCL was electrofused (37) with the hypoxanthine-aminopterin-thymidine (HAT), ouabain-resistant heteromyeloma cell line SHM-D33 (38) to stabilize Ab production. The hybridoma was then subcloned by limiting dilution. Testing in complement-dependent cytotoxicity (CDC) assays with HLA-typed cultured human lymphoid cell lines and PBL has shown that mAb SN6663 recognizes a determinant expressed on HLA-A2 and -A28 allotypes.

Human hybridoma clones secreting mAb SN6663, anti-HLA-A2 mAb MBW1 (IgM, $\lambda$; kindly provided by Dr. Carl Grumet) (39), and anti-HLA-A2 and -A28 mAb HaSC2.A2 (IgM, $\kappa$) (37) were grown in RPMI complete medium. Human mAb either were used as tissue culture supernatants, or were purified by size exclusion chromatography using a Sephacryl 200 gel filtration column (Pharmacia, Uppsala, Sweden). Hmy2.C1R human lymphoblastoid cells (HMY) transfected with a wild-type HLA-A*0201 gene or with mutagenized HLA-A*0201 genes as previously described (40) were cultured in RPMI complete medium. The R170G variant-expressing cell line was kindly provided by Dr. Jeffrey Frelinger (University of North Carolina, Chapel Hill).

HLA-A2 and a pool of HLA-B,C proteins devoid of HLA-A2 activity were purified from human splenocytes as described (41). Recombinant soluble HLA-A2/Q10$^A$ and HLA-B$^7$ proteins secreted by transfected cells were purified as described elsewhere (42), and the purity of Ab preparations was monitored by SDS-PAGE (34).

Sero logical assays

The complement-dependent cytotoxicity (CDC) assay to measure specific mAb reactivity with cell surface-bound HLA-A*0201 was performed as described elsewhere (43). End point titer (EPT) was determined as the reciprocal of the highest dilution of mAb giving $\geq$30% lysis of target cells within a well. The ELISA to measure binding of Abs to HLA-A2 Ag was performed using a modification of a protocol described elsewhere (44). Briefly, microtiter were precoated with 2.5 $\mu$g/ml purified HLA-A2 native detergent-solubilized protein, or with a mixture of purified HLA-B,C-B proteins devoid of HLA-A2 activity. After an overnight incubation at 4°C and blocking with 2% BSA in pH 7.1 PBS for 30 min, wells were washed twice and incubated at 22°C for 2 h with dilutions of primary Ab human IgM, mouse IgG, or rabbit purified "Ab-3" Ig. After washing three times in PBS containing 0.5% Tween 20 (PBS/Tween) between steps, HRP-conjugated extravidin, HRP-conjugated goat anti-human IgM Abs, and HRP-conjugated goat anti-rabbit IgG Abs (all from Sigma) were added to detect binding of biotinylated mouse IgG, human IgM, and rabbit IgG Abs, respectively. Chromogen addition, color development, and detection were as described (13).

To detect binding of human IgM mAbs to mouse Ab-2, purified T10-505 or T10-938 was coated onto microtiter wells at a concentration of 2.5 $\mu$g/ml in 10 mM Tris (pH 9.0). After three washes in PBS/Tween, spent hybridoma medium containing human IgM mAbs was added at varying dilutions to the Ab-2-coated wells. After an additional wash step, anti-human IgM-HRP was added to each well and processed as for the HLA-A2-binding assay, as described above.

Ab-blocking assays

Blocking of Ab-1 binding to Ab-2 by affinity-purified Ab-3. Serial dilutions (50 $\mu$l) of affinity-purified rabbit Ab-3 or of unconjugated Ab-1 (mAb CRIL-351) were incubated in microtiter wells coated with Ab-2 mAb T10-505 or T10-938. After a 90-min incubation at room temperature, an equal volume of biotinylated Ab-1 (mAb CRIL-351-biotin conjugate: 80 ng/ml) was added and incubated for an additional 90 min. After three washings, HRP-conjugated extravidin was used for the detection of plate-bound biotinylated mAb.

CDC blocking assays. Serial dilutions of non-complement-fixing mouse IgG1 mAbs were mixed with a selected dilution of complement-fixing human IgM mAb. Following the addition of the mixture to HLA-A2$^+$/target cells, the assay was continued as in the conventional CDC assay.
Kin-Elmer, Emeryville, CA) and either a) the V_H forward and reverse primers. V_L region DNA was cloned directly into the pGEM-T vector (Promega) and the human V_L region DNA was amplified at 94°C, 1 min; 52°C, 1 min; and the human V_H region of mouse IgG1; 2) the reverse primer, “R1” (5'-GGG AAT TC(R) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = G or T) and “R3” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = C or G, S = A or C, and Y = A or G), derived from the sequence in the constant region proximal to the V_H region of mouse IgG1; 1) the forward primer, “F1” (5'-GGG AGT GGA TAG AC-3'), complementary to the sequence in the constant region proximal to the V_H region of mouse IgG1; 2) the reverse primer, “R1” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = G or T) and “R3” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = C or G, S = A or C, and Y = A or G), derived from the sequence in the constant region proximal to the V_H region of mouse IgG1; 3) the reverse primer, “R2” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = G or T) and “R3” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = C or G, S = A or C, and Y = A or G), derived from the sequence in the constant region proximal to the V_H region of mouse IgG1; 4) the reverse primers “R2” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = G or T) and “R3” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = C or G, S = A or C, and Y = A or G), derived from the sequence in the constant region proximal to the V_H region of mouse IgG1; and 4) the reverse primers “R2” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = G or T) and “R3” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = C or G, S = A or C, and Y = A or G), derived from the sequence in the constant region proximal to the V_H region of mouse IgG1; and 4) the reverse primers “R2” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = G or T) and “R3” (5'-GGG AAT TCG(A)T(G) AGG T(R)(S) A(Y)C TGC AG(R) CT-3, in which R = C or G, S = A or C, and Y = A or G), derived from the sequence in the constant region proximal to the V_H region of mouse IgG1.

The human and mouse mAb V_H and V_L region sequences were analyzed utilizing Microgenie (version 7.0, Beckman Instruments, Fullerton, CA) for nucleic acid sequence, and nucleic acid sequences were translated into deduced amino acid sequences. Nucleic acid sequences were compared for homology with Ig sequences in GenBank. Amino acid sequence comparisons were performed at the National Center for Biotechnology Information (NCBI) using BLAST. The V_H and V_L region sequences of mouse mAb CRll-351 and MA2.1 and of human mAb MBW1 SN66E3 and Ha5C2.A2 were deposited in GenBank (accession No. AF052618 and No. AF069004–07). Canonical class and conformational cluster assignments for the hypervariable loops were determined using an algorithm that takes into account both the CDR length and composition and key framework residues contributing to CDR loop conformation (48).

Results

Immunization of rabbits with anti-anti-HLA-A2 elicits “Ab-3” that bind to HLA-A2

Table I lists the monoclonal and polyclonal Abs used in this study, highlighting the interrelationships of Ab-1, Ab-2, and Ab-3. To obtain the Ab-3, rabbits were immunized with T10-505 or T10-938, two well-characterized monoclonal Ab-2 derived from syngeneic BALB/c mice immunized with mAb CRll-351 (30). After extensive adsorption and affinity purification, each Ab-3 preparation retained strong binding activity against the immunizing Ab-2 but lacked significant cross-reactivity with normal mouse IgG or CRll-351 (data not shown). To determine their titer, we measured the ability of each Ab-3 preparation to competitively inhibit binding of CRll-351 to the immunizing Ab-2, a specific measure of Ab-3 activity (30). As shown in Fig. 1A, anti-T10-505 was able to block the binding of biotin-labeled CRll-351 to T10-505 at a dose of 1.6 μg/ml added protein. The blocking effect was specific, in that neither rabbit Abs elicited with T10-505 nor preimmune rabbit serum, affinity-purified using T10-505-coupled Sepharose beads, inhibited the CRll-351 interaction with T10-505. Compared with the specific Ab-3, unlabeled Ab-1 (mAb CRll-351) was less effective, requiring a 10-fold higher concentration for an equivalent blocking effect. Finally, the anti-T10-505 Ab did not inhibit the binding of CRll-351 to T10-938 (Fig. 1B), indicating that a specific Ab-3 had been induced by immunization with T10-505.

A similar pattern of specific inhibition of Ab-1 binding to the Ab-2 was seen with the T10-938-specific Ab-3 (Fig. 1B). This Ab-3 was of lower titer than the T10-505-specific Ab-3 preparation; blocking of biotin-labeled CRll-351 binding to T10-938 was seen only at the highest doses of Ab-3 tested (8.0–40 μg/ml) and was comparable to the blocking effect of similar doses of unlabeled CRll-351 (Fig. 1B). The rabbit T10-938-specific Ab-3 preparation failed to block the interaction of CRll-351 with T10-505 (Fig. 1A).

Despite differences in their specificity for Ab-2, both Ab-3 reacted with HLA-A2 Ag as measured by cellular ELISA (data not shown).

<table>
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<tr>
<th>Ab-1*</th>
<th>Reactivity*</th>
<th>Isotype</th>
<th>Ab-2*</th>
<th>Isotype</th>
<th>Ab-3b</th>
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<td>A2,24,28</td>
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<td>T10-505</td>
<td>yl,κ</td>
<td>Anti-T10-505</td>
<td>Polyclonal (rabbit)</td>
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<td></td>
<td></td>
<td></td>
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<td>Anti-T10-938</td>
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Other mAbs

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<td>SN66E3*</td>
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<td>μ,κ (human)</td>
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<td>yl,κ (mouse)</td>
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* See Materials and Methods.

See this paper.
sHLA-B7 proteins were equally effective in blocking the binding of biotin-coupled CRll-351 (\(\bullet\)) and by unlabeled CRll-351 (\(\square\)) or T10-938 (\(\bigtriangleup\)) or T10-938 (\(\bigtriangleup\)) and by rabbit Ab-3 (A-1) with 1
HLA-A*0201 (49) and mAb cross-blocking (50) have demonstrated a high degree of homology in the polymorphic HLA epitopes recognized by mouse mAb and human polyclonal anti-HLA-A2 Abs. If a human mAb specific for the same epitope as CRll-351 could be identified, then mimicry of this epitope by anti-Ids T10-505 and T10-938 ought to cause them to be recognized by the human mAb.

We used both the HLA-A*0201 variant panel and Ab cross-blocking approaches to compare the fine specificity of human HLA-A*0201-reactive mAbs Ha5C2.A2 (IgM, \(\kappa\)), SN66E3 (IgM, \(\kappa\)), and MBW1 (IgM, \(\lambda\)) with that of CRll-351. For epitope mapping, the three human mAbs were tested in CDC assay with HMY.C1R (IgG, \(\kappa\)), SN66E3 (IgM, \(\kappa\)), and MBW1 (IgM, \(\lambda\)) as control. The results were compared with previously published analysis of epitope specificity of mAbs CRll-351 and the nonoverlapping mAb MA2.1 using flow cytometry (40) and radioimmunoassay inhibition tests (49). As summarized in diagrammatic form in Fig. 3, based on Bjorkman et al. (51), the reactivity of mAb Ha5C2.A2 and SN66E3 (top left and center) was unaffected by changes in five residues in the \(\alpha_2\) domain (indicated by the dotted lines in Fig. 3), or with mutated DNAs each encoding an HLA-A*0201 variant protein with a single amino acid substitutions in the \(\alpha_1\) or \(\alpha_2\) domain (see list, Fig. 3). Parental HMY.C1R cells were used as a negative control. The results were compared with previously published analysis of epitope specificity of mAbs CRll-351 and the nonoverlapping mAb MA2.1 using flow cytometry (40) and radioimmunoassay inhibition tests (49). As summarized in diagrammatic form in Fig. 3, based on Bjorkman et al. (51), the reactivity of mAb Ha5C2.A2 and SN66E3 (top left and center) was unaffected by changes in five residues in the \(\alpha_2\) domain (indicated by open circles). However, neither Ha5C2.A2 nor SN66E3 mAb reacted with the triple-substitution A149T, V152E, and L156W in the \(\alpha_2\) domain (hatched circles), which represent collectively the HLA-A*0201 subtype.

Other amino acid substitutions in the \(\alpha_2\) domain had different effects on the reactivity of mAb Ha5C2.A2 vs SN66E3. Specifically, single replacements of either alanine with threonine at position 149 (A149T), glutamic acid with lysine at position 154 (E154K), or arginine with glycine at position 170 (R170G) completely abolished Ha5C2.A2 reactivity, as indicated by the filled circles (Fig. 3). In contrast, only the A149T substitution, and a conservative substitution of alanine for glycine at position 162 (G162A), affected SN66E3 reactivity, and these changes reduced but did not completely abolish its reactivity (Fig. 3). The reactivity pattern of mAb Ha5C2.A2 with HLA-A*0201 variants, especially

**FIGURE 1.** Ab-3 titer and cross-reactivity with HLA-A2 Ags of rabbit Ab-3 elicited with mAbs T10-505 and T10-938. Top. Dose-dependent inhibition of binding of biotin-coupled CRll-351 (\(\bullet\)) to immobilized mouse mAb T10-505 (\(\bullet\)) or T10-938 (\(\square\)) by rabbit Ab-3 elicited with T10-505 (\(\bigtriangleup\)) or T10-938 (\(\bigtriangleup\)) and by unlabeled CRll-351 (\(\bigtriangleup\)). Medium alone (\(\triangle\)) and normal rabbit serum affinity-purified protein (\(\bigtriangledown\)) were used as specificity controls. y-axis, Ab binding expressed in OD units. x-axis, Protein concentration of Ab tested as an inhibitor. Bottom, Binding of affinity-purified rabbit Ab-3 Abs anti-T10-505 (\(\bigtriangleup\)) or anti-T10-938 (\(\bigtriangledown\)) to HLA-A2 Ag-coated wells (\(\bigotimes\)). An HLA-B,C Ag mixture devoid of HLA-A2 Ag (\(\bigcirc\)) was used as a specificity control. Binding of preimmune rabbit serum affinity-purified proteins to HLA-A2 (\(\Delta\)) and to HLA-B,C (\(\triangle\)) Ags was used as control. y-axis: Ab binding expressed in OD units. x-axis, Protein concentration of Ab.

**FIGURE 2.** HLA-A2 specificity of rabbit Ab-3 elicited with mouse anti-Id mAb T10-505. Rabbit Ab-3 elicited with mAb T10-505 (8 \(\mu\)g/ml, \(\bigtriangleup\)) and rabbit anti-human \(\beta_m\) (1 \(\mu\)g/ml, \(\bigtriangleup\)) were mixed with varying concentrations of soluble recombinant HLA-A2/Q10\(^b\) (\(\bigcirc\), Expt. 1; \(\bullet\), Expt. 2), sHLA-B7\(^b\) (\(\square\), Expt. 1), or OVA (\(\bigtriangleup\), Expt. 2). The mixtures were then tested in ELISA for binding to HLA-A2 Ag-coated microwells. y-axis, Ab binding expressed in OD units. Protein concentration of inhibitor.

**FIGURE 2.** HLA-A2 specificity of rabbit Ab-3 elicited with mouse anti-Id mAb T10-505. Rabbit Ab-3 elicited with mAb T10-505 (8 \(\mu\)g/ml, \(\bigtriangleup\)) and rabbit anti-human \(\beta_m\) (1 \(\mu\)g/ml, \(\bigtriangleup\)) were mixed with varying concentrations of soluble recombinant HLA-A2/Q10\(^b\) (\(\bigcirc\), Expt. 1; \(\bullet\), Expt. 2), sHLA-B7\(^b\) (\(\square\), Expt. 1), or OVA (\(\bigtriangleup\), Expt. 2). The mixtures were then tested in ELISA for binding to HLA-A2 Ag-coated microwells. y-axis, Ab binding expressed in OD units. Protein concentration of inhibitor.

Identification of a human homologue of mAb CRll-351

In addition to eliciting Ab-3 that recognize Ag, an Ab-2 that mimics Ag would also be predicted to bind any Ab that recognizes the same epitope as the original Ab-1. Previous epitope mapping studies using B-LCL expressing single amino acid variants of HLA-A*0201 (49) and mAb cross-blocking (50) have demonstrated a
the abolition of binding by the substitutions of A149T and R170G, and by a nonconservative substitution at position 163 (i.e., by T163R, but not T163M) closely resembled that of the mouse mAb CRll-351 (Fig. 3, lower right) (40, 49, 52).

In contrast, the reactivity of the mAb MBW1 with HLA-A2 Ags was abolished by substitutions in the α1 domain, specifically the substitutions of alanine for glutamic acid at position 58 (E58A) and either a valine or arginine substitution for glycine at position 62 (G62V or G62R), as indicated in Fig. 3, upper left. These substitutions in the α1 domain have been previously shown to abolish or diminish the binding of the mouse anti-HLA-A2, -B17 mAb MA2.1 (Fig. 3, lower left) as well as that of human IgG λ Abs purified from two different HLA-A2, -B17-reactive antisera (40, 49) and one human IgG λ mAb, SN66E3. However, three- to fourfold higher concentrations of CRll-351 were required to achieve 1/2 maximum inhibition, and, at the highest dose of CRll-351 (1000 ng/ml), a small amount of residual cytotoxicity by SN66E3 (CDC score = 2) remained.

These results indicate that the human mAb SN66E3 recognizes an epitope that is distinct from, but spatially close to, that defined by CRll-351. In contrast, CDC mediated by human mAb MBW1 was unaffected by mAb CRll-351 but was completely blocked by mAb MA2.1 (Fig. 4B), consistent with binding patterns seen in the analysis of HLA-A*0201 variants.

Cross-reactivity of human anti-HLA-A*0201 mAbs with T10-938 and T10-505

The identification of a human mAb, Ha5C2.A2, recognizing a similar epitope to that seen by CRll-351, and a second human mAb, SN66E3, recognizing a partially overlapping epitope, enabled us to test the prediction that a human mAb could recognize a mouse anti-anti-HLA-A2 mAb as an HLA-A*0201 epitope mimic. The human IgM mAbs MBW1, SN66E3, and Ha5C2.A2 were titrated onto microwells coated with T10-505 or T10-938. Fig. 5 shows that, of the three human mAbs tested, only Ha5C2.A2 bound with mAb CRll-351 (≥125 ng/ml) completely blocked CDC by mAb Ha5C2.A2. The inhibition was specific, since preincubation of PBL with up to 1000 ng/ml of mAb MA2.1 had no effect on CDC by mAb Ha5C2.A2 (Fig. 4B). Similarly, CRll-351 (Fig. 4A), but not MA2.1(Fig. 4B), inhibited CDC mediated by mAb SN66E3. However, three- to fourfold higher concentrations of CRll-351 were required to achieve 1/2 maximum inhibition, and, at the highest dose of CRll-351 (1000 ng/ml), a small amount of residual cytotoxicity by SN66E3 (CDC score = 2) remained.

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strongly to both mouse Ab-2. The measured OD of IgM binding by Ha5C2.A2 to microwells coated with T10-505 or T10-938 was similar at all doses tested to the measured OD of its binding to HLA-A2 Ag-coated microwells (data not shown). The binding of Ha5C2.A2 to T10-938 was higher than binding to T10-505 at the three highest doses (2, 4, and 8 \( \mu g/ml \)) of mAb tested. In contrast, MBW1 failed to bind significantly to either anti-Id, while the SN66E3 mAb bound weakly to both anti-Id at the highest dose of human mAb tested.

A competitive ELISA inhibition assay was used to determine whether the selective binding of human mAb Ha5C2.A2 to T10-505 and T10-938 was similar at all doses tested to the measured OD of its binding to HLA-A2 Ag-coated microwells (data not shown). The binding of Ha5C2.A2 to T10-938 was higher than binding to T10-505 at the three highest doses (2, 4, and 8 \( \mu g/ml \)) of mAb tested. In contrast, MBW1 failed to bind significantly to either anti-Id, while the SN66E3 mAb bound weakly to both anti-Id at the highest dose of human mAb tested.

A competitive ELISA inhibition assay was used to determine whether the selective binding of human mAb Ha5C2.A2 to T10-505 and T10-938 reflected an interaction with the HLA-A*0201 Ag binding site of Ha5C2.A2 or an interaction with a non-Ag binding region. As a positive control, both Ab-2 were tested for dose-dependent inhibition of biotin-labeled CR11-351 binding to Ag; biotin-labeled MA2.1 was used as a negative control. The results are shown in Fig. 6. Both Ab-2 strongly and completely inhibited the binding of mAb CR11-351 to HLA-A2 Ag-coated wells: a 1/2 maximal inhibitory effect was seen at approximately 80 ng/ml for T10-505, equivalent on a molar basis to the dose of divalent biotin-conjugated CR11-351 ligand used in this experiment. A twofold higher concentration, 160 ng/ml, was required for a 1/2 maximal inhibitory effect of T10-938 (Fig. 6A and data not shown).

In agreement with the Ag mimicry hypothesis, mAb T10-938 caused a strong and complete inhibition of Ha5C2.A2 binding to HLA-A2 Ag at the highest dose tested (2 mg/ml; Fig. 6B). T10-505 also inhibited binding of Ha5C2.A2 to Ag, but only partially. The inhibition of Ha5C2.A2 was specific, since neither anti-Id mAb inhibited the binding of mouse mAb MA2.1 (Fig. 6C) or human mAb SN66E3 (Fig. 6D) to immobilized HLA-A2 Ags. These results were confirmed by CDC inhibition tests using HLA-A2+ target cells; CDC mediated by Ha5C2.A2 was inhibited in a dose-dependent manner by added T10-505 and T10-938, whereas MBW1 and SN66E3 were not (data not shown).

It should be noted that much higher doses (0.03–2.0 mg/ml) of T10-938 inhibitor were required for inhibition of Ha5C2.A2, as compared with CR11-351 binding to Ag (compare Figs. 6A and 6B). This result was not surprising; CR11-351, because of its high affinity for HLA-A*0201, could be used at a much lower starting concentration (80 ng/ml) than mAb Ha5C2.A2 (8000 ng/ml). Also, since the T10-938 is an IgG, a molar equivalence at a fivefold lower protein concentration of inhibitor would be expected in the case of an IgG ligand (CR11-351), as compared with an IgM ligand (Ha5C2.A2).

Analysis of the molecular basis of HLA-A2 Ag mimicry by anti-Id

Primary amino acid sequence identity between a variable domain complementarity determining region (CDR) loop of an Ab-2 and a polypeptide sequence within the Ag itself may in certain special
cases account for the cross-reactivity of an Ab-3 with the original Ag (53, 54). In the case of T10-505 and T10-938, this kind of direct mimicry could not explain the findings of Ab-2 cross-reactivity with Ha5C2.A2 or of Ab-3 cross-reactivity with HLA-A2 Ag, since the $\alpha_2$ domain $\alpha$-helical polypeptide in the CRll-351-defined epitope region, i.e., residues HLA-A*0201 147–170, EEAAHVAEQLRAYLEGTCVEWLRR, had no significant degree of homology to T10-505 or T10-938. No homology was found when either CDRs alone (Fig. 7) or CDR plus framework sequences of both heavy and light chains were compared with the HLA-A*0201 147–170 sequence using the BLAST program (data not shown).

An alternative explanation for our findings is that there is a degree of structural homology of the variable regions of the heavy and light chains of human, rabbit, and mouse Abs specific for the same epitope of HLA-A*0201. To test this hypothesis, we compared the predicted amino acid sequences of the human and mouse anti-HLA-A2 mAbs to identify relevant homologies. Fig. 7 shows the predicted amino acid sequences of the $V_L$ (A) and $V_H$ (B) CDRs of mAbs CRll-351, Ha5C2.A2, SN66E3, MA2.1, and MBW1, as well as the previously published CDR sequences of the anti-Id mAbs T10-505 and T10-938 (30).

**FIGURE 7.** Comparison of the amino acid sequences of $V_L$ and $V_H$ CDR of human and mouse anti-HLA-A2 mAb and of anti-Id mAbs. $V_L$ (A) and $V_H$ (B) CDR1, -2, and -3 amino acid sequences of human mAbs Ha5C2.A2, MBW1, and SN66E3 of mouse mAb MA 2.1 and of anti-Id mAbs T10-505 and T10-938 are compared with those of mouse mAb CRll-351. Sequence identity with mAb CRll-351 is indicated by dashed line. Numbers above letters indicate the Kabat numbering system for each CDR residue from the N $\rightarrow$ C terminus. The conformational cluster assignment of each CDR loop is listed in the right hand column: in the case of $V_H$ CDR3, the CDR length is listed. The Ig variable gene segment assignments for the human mAbs were as follows: Ha5C2.A2 = VH4/D7/ JH6, Vk1/Je4; SN66E3 = VH1/DXP4/ JH4, V$\kappa$4/Je4; and MBW1 = VH3/D7/ JH4, V$\lambda$2/JA2.
The two other regions of sequence homology between CRll-351 and Ha5C2.A2 were shared with SN66E3, but not with MBW1, the A5 sequence at positions 51–52 of the CDR2 \( \text{V}_{\text{H}} \) (Fig. 7A) and the GGT sequence at positions 55–57 of the \( \text{V}_{\text{L}} \) CDR2 (Fig. 7B). The critical residue in the latter sequence appears to be the glycine at position 56, which was unique to the mAb Ha5C2.A2, SN66E3, and CRll-351, as compared with MBW1, MA2.1, and seven previously published human anti-HLA (non-A2-specific) mAb sequences (27, 55). In terms of canonical structure, the CRll-351 and SN66E3 \( \text{V}_{\text{H}} \) CDR2 loops were the most homologous (cluster 2/10A) and shared the sequence INP at positions \( \text{V}_{\text{H}} \) 51–52A, whereas the shorter Ha5C2.A2 \( \text{V}_{\text{H}} \) CDR2 loop lacked a 52A proline residue (cluster 1/9A). No other striking structural homologies with CRll-351 were noted; while the SN66E3 and Ha5C2.A2 had similar CDR3 lengths (9 for \( \text{V}_{\text{L}} \), 17 for \( \text{V}_{\text{H}} \) CDR3) and certain sequence homologies to each other (especially in \( \text{V}_{\text{L}} \), 7/9 residues identical), neither human mAb closely resembled CRll-351 in the critical CDR3 regions.

As previously reported, the two anti-Id mAbs T10-505 and T10-938 differed considerably in their primary \( \text{V}_{\text{L}} \) and \( \text{V}_{\text{H}} \) sequences (Fig. 7; Ref. 30). For example, T10-505 had a longer CDR3 \( \text{V}_{\text{H}} \) loop than the Ab-1 (14 vs 11 residues), whereas T10-938 had a shorter length \( \text{V}_{\text{H}} \) CDR3 (10 residues). Underscoring their fundamental differences in structure was an unusual triple tyrosine motif in the \( \text{V}_{\text{H}} \) CDR3 sequence of T10-505 (YYY \( \text{V}_{\text{H}} \) 95–97) that was absent in T10-938. CRll-351 also had a triple tyrosine in its \( \text{V}_{\text{H}} \) CDR3, but its position was shifted by two residues in the shorter \( \text{V}_{\text{H}} \) CDR3 loop of the latter (YYY \( \text{V}_{\text{H}} \) 97–99).

**Discussion**

Two lines of evidence support the hypothesis of HLA-A*0201 epitope mimicry by the anti-Id mAbs T10-505 and T10-938. The first is that immunization of rabbits elicited Ab-3 that bound not only specifically to the immunizing anti-Id mAb but also to cell membrane-bound and purified soluble HLA-A*0201 Ag. In this type of experiment, mimicry of the chemically dissimilar Ag by the anti-Id mAb used as immunogen is expected to be imperfect; i.e., by in vivo selection and somatic mutagenesis, the Ab-3 will tend to have a higher affinity for Ab-2 and a lower affinity for the Ag than does the Ab-1, as was the case for the rabbit anti-T10-505 Ab (this paper) and for two previously reported monoclonal Ab-3 (56). Despite the in vivo selection for binding to Ab-2 and not HLA-A2 Ag, the data indicate that the rabbit Ab-3 elicited by immunization with T10-505 and T10-938 recognized a polymorphic region of the HLA-A*0201 Ag (Fig. 1, C and D, and Fig. 2).

The second line of evidence for epitope mimicry by T10-505 and T10-938 was their ability to specifically compete for the HLA-A*0201 mAb binding site of a human anti-HLA mAb is even more remarkable given the rather limited degree of amino acid sequence homology (37%) between the CDRs of CRll-351 and Ha5C2.A2. While homology cannot be fully analyzed at the structural level by sequence-based comparisons alone, it was clear that certain regions of structural homology between the two were present that correlated with their largely overlapping specificity for HLA-A*0201 Ag (Fig. 7). On the other hand, the weak binding interaction of SN66E3 with T10-938 and T10-505 (Fig. 5), as well its epitope specificity, which partially overlapped that of Ha5C2.A2 and CRll-351 (Figs. 3 and 4), was also reflected in a degree of sequence homology with CRll-351. We speculate that certain unique features of the amino acid sequence of variable regions of mAb SN66E3, particularly a large insert in the CDR1 of the \( \text{V}_{\text{L}} \) (Fig. 7), provide important structural differences that account for its lower degree of homology with CRll-351 in Ag and anti-Id reactivity.

Evidence for cross-reactivity of a variety of anti-MHC Abs with the same anti-Id has been previously reported in mice (57), rats (8), and humans (2, 3, 58). If epitope mimicry is a common feature of such anti-Id, as is the case of the mAbs T10-505 and T10-938 described in this paper, this has implications for the pattern of anti-Id cross-reactivity; i.e., only those anti-MHC Abs recognizing the same epitope of the polymorphic MHC Ag would be predicted to be inhibited by the corresponding anti-Id epitope mimic. The specificity of HLA Ag mimicry by anti-Id is illustrated in the current study by the contrast between Ha5C2.A2 and SN66E3. By extension, one would predict that the epitope “mimic” T10-938 mAb would not inhibit a polyclonal anti-HLA-A2 antisera with a dominance of the SN66E3 clonotype but would inhibit one with a dominance of the Ha5C2.A2 clonotype.

It could be argued that the cross-reactive inhibition of a human mAb by mouse anti-Id mAb specific for a syngeneic anti-HLA-A*0201 mAb was due to the close relatedness to germline of the \( \text{V}_{\text{H}} \) and \( \text{V}_{\text{L}} \) genes utilized by mAb Ha5C2.A2, as previously reported for other human anti-HLA IgM mAbs (27). This would result in the retention of certain germline-encoded Ids that might be shared even across species. However this possibility is refuted by the data; all three human mAbs tested (MBW1, SN66E3, and Ha5C2A2) were highly homologous (93–98%) to reported germline gene sequences in both H and L chains (J. Fechner and W. Burlingham, unpublished observations), but only mAb Ha5C2.A2 was highly homologous to CRll-351 in epitope and anti-Id specificity.

Soluble mimics of cell-surface proteins, like soluble forms of the actual protein, may be less immunogenic than cell-bound forms. For example, cell-bound HLA Ag is known to be markedly more immunogenic than cell-free soluble HLA (59). In a previous study (30), mAbs T10-505 and T10-938 failed to induce anti-HLA-A*0201 Abs in syngeneic hosts in spite of a strong and specific Ab-3 response. Similarly, in the HLA-DQ3 antigenic system, the mouse anti-Id mAb KO3–34 was very effective in eliciting anti-HLA-DQ3 Abs in rabbits but poorly effective in syngeneic hosts (S. Ferrone, unpublished results). The lack of an Ab-3 response cross-reactive with the original Ag in syngeneic as compared with xenogeneic combinations (60) may reflect the weaker immunogenicity of the anti-Id or a genetic restriction of the immune response, which has been described in some antigenic systems (61–63).

Genetically engineered divalent forms of MHC class I/\( \text{IgG} \) fusion proteins have been shown to have enhanced affinity for TCR of alloreactive CTL as compared with monovalent soluble MHC (64). Because of the unique sensitivity of the humoral response to tolerization by soluble forms of MHC class I Ag (16), our findings suggest a potential therapeutic role for genetically engineered soluble MHC Ag, and divalent (e.g., \( \text{IgG} \) anti-Id mAb) mimics thereof, as specific B cell tolerogens in the setting of allo- and xeno-transplantation. Anti-Id epitope mimics could be particularly useful in the treatment of individuals who have antibodies with a highly restricted epitope specificity.
(49), while soluble divalent MHC fusion proteins may be applicable to persons with a broader range of alloantibody response.

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