

# EVIDENCE FOR A SHARED HLA-A INTRALOCUS DETERMINANT DEFINED BY MONOCLONAL ANTIBODY 131

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Major histocompatibility complex (MHC)<sup>1</sup> class I antigens are composed of a polymorphic 45 kilodalton (kD) glycoprotein heavy chain noncovalently associated on the cell surface with a nonpolymorphic 12 kD protein,  $\beta_2$ -microglobulin ( $\beta_2$ -m) (1). These proteins are expressed on nearly all cells, and they play an essential role in the immune response of cytotoxic T lymphocytes (CTL) to foreign antigens (reviewed in 2). Most species express more than one of these proteins. The mouse MHC, known as H-2, encodes at least three class I molecules, H-2K, H-2D, and H-2L; HLA, the human MHC, encodes at least three class I gene products, HLA-A, HLA-B, and HLA-C.

One interesting aspect of MHC class I gene proteins, which may be related to their immune function, is their high degree of polymorphism; in the mouse, the H-2K and H-2D loci have at least 50 alleles (3), and the extent of polymorphism in humans, as detected serologically, seems to be of the same order (4). This polymorphism is localized mainly to three regions (residues 62–83, 95–121, and 135–177) in the first two amino-terminal extracellular domains of the class I molecule (3). Structural analysis of HLA-A2 variants in the human (5) and H-2 mutant strains of mice (6) have shown that determinants defined by CTL recognition reside in the first two external domains, a suggestion that it is the polymorphism of these regions that is important for the immune function of these molecules.

An intriguing aspect of the polymorphism of the murine H-2 class I molecules is the apparent lack of shared intralocus determinants, i.e., there is little to indicate that the products of the K alleles are structurally different from those of the D alleles (1). There is some possibility that this may not be true for the

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<sup>1</sup> *Abbreviations used in this paper:* Az, sodium azide;  $\beta_2$ -m,  $\beta_2$ -microglobulin; CTL, cytotoxic T lymphocyte; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; IEF, isoelectric focusing; mAb, monoclonal antibody; MHC, major histocompatibility complex; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; RIA, radioimmunoassay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

products of HLA loci. Yang et al. (7) and Billing and Lucero (8) have both reported monoclonal antibodies (mAb) specific for all or most HLA-B-encoded gene products. Here, we show that a recently described (9) mAb, 131, recognizes a determinant unique to HLA-A locus-encoded proteins. Taken together, these data suggest that shared intralocus determinants, definable by mAb, do in fact exist in the human HLA system.

### Materials and Methods

**Cell Lines.** Human cell lines were maintained at 37°C in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 25 mM Hepes, and 2 mM glutamine. All cell lines are B lymphoblastoid cell lines with the exception of Molt 4, a T cell line, and K562, an erythroleukemia cell line. The HLA-A,B type for each cell line is provided in Table I. Mouse L cells transfected with human class I genes were kindly provided by J. Barbosa (Harvard University, Cambridge, MA). The L cell transfectants were maintained at 37°C in Dulbecco's  $\alpha$  minimum essential medium supplemented with 10% FBS, 25 mM Hepes, and 2 mM glutamine. LJE1 has been transfected with the HLA-A2 and the human  $\beta_2$ -m genes; LJG3 with HLA-B7 and human  $\beta_2$ -m; LB.40.10 with HLA-B40; and LJCM has been transfected only with the human  $\beta_2$ -m gene (10).

**Antibodies.** mAb 131 was produced in our lab and has been described before (9); it reacts with a subset of class I proteins. W6/32 (11) recognizes a nonpolymorphic determinant shared by all HLA class I gene products. mAb 4E, specific for all HLA-B gene products and HLA-A29, -A30, -A31, and -A32 (7), was kindly provided by S.-Y. Yang (Memorial Sloan-Kettering Cancer Center, New York); control antibody P3 was provided by M. Hemler (Harvard University). Mouse mAb PA2.1 (12) and BB7.1 (13) recognize allospecific determinants found on HLA-A2 and HLA-B7 gene products, respectively. mAb 187.1, a rat antibody specific for mouse  $\kappa$  light chains, and which binds protein A, was grown from hybridoma cells obtained through the American Type Culture Collection, Rockville, MD. Rabbit antiserum raised against mouse F(ab) fragments was produced in our lab.

**Cell-surface Binding Assays.** Radioimmunoassays (RIA) to test mAb binding to cell-surface antigens were carried out at 4°C in phosphate buffered saline (PBS), pH 7.4, containing 1% FBS and 0.05%  $\text{NaN}_3$  (henceforth termed PBS-FBS-Az). Washed cells were plated in 96-well U-bottom plates at  $10^5$  cells/100  $\mu\text{l}$ /well. Antibodies were added (25  $\mu\text{l}$ /well culture supernatants) and the plates were incubated for 1 h. After three washes in PBS-FBS-Az,  $10^5$  cpm/25  $\mu\text{l}$ /well of  $^{125}\text{I}$ -labelled goat anti-mouse IgG,A,M was added, followed by another 1-h incubation. After five washes in PBS-FBS-Az, cells were harvested, and the number of counts bound was determined. In all cases, background radioactivity bound to cells in the absence of a primary mAb have been subtracted.

Indirect immunofluorescence flow cytometry was carried out as described previously (9). Cells were examined with a FACS IV fluorescence-activated cell sorter analyzer.

**Protein Radiolabelling and Immunoprecipitation.** Cells ( $2\text{--}4 \times 10^6$  cells in 2 ml) were biosynthetically labelled in methionine-deficient medium (RPMI 1640 Met, Gibco Selectamine Kit; Gibco, Grand Island, NY) supplemented with 15% dialyzed FBS, 25 mM Hepes, 2 mM glutamine, and 250  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine (Amersham Corp., Arlington Heights, IL). After a 12–18-h incubation at 37°C, radiolabelled cells were washed twice in 10 ml ice-cold Hank's balanced salt solution. The cell pellets were suspended in ice-cold lysis buffer (1 ml of 10 mM Tris, pH 7.0, containing 1% Nonidet P-40 (NP-40), 10 mM iodoacetamide, and 1 mM phenylmethylsulfonylfluoride for 40 min after vigorous vortexing. The lysates were then centrifuged at 10,000  $g$  for 10 min to remove cellular debris, and were dialyzed overnight against two changes of 40 mM NaCl in 10 mM Tris, pH 7.8.

Cell-surface proteins were labelled with  $^{125}\text{I}$  by the lactoperoxidase method (14).  $2\text{--}3 \times 10^7$  cells (>96% viable by Trypan Blue dye exclusion) were labelled, washed three times in 10 ml of PBS containing 2.1 gm KI per 100 ml, and lysed in 1 ml of 1% NP-40 in 10 mM Tris, and treated as described above.

Before immunoprecipitation, 1 ml of lysates were incubated with two cycles of heat-inactivated, formalin-fixed *Staphylococcus aureus* (packed pellet from 250  $\mu$ l of a 10% wt/vol suspension) and 20  $\mu$ l of normal rabbit serum to remove nonspecifically bound proteins. Precleared lysates were used immediately or stored at  $-70^{\circ}\text{C}$ . All steps were carried out at  $4^{\circ}\text{C}$ .

For immunoprecipitation, precleared lysates corresponding to  $10^6$  cells (250–500  $\mu$ l) for  $^{35}\text{S}$ -labelled material, or  $3\text{--}5 \times 10^6$  cells (100  $\mu$ l) for  $^{125}\text{I}$ -labelled lysates were added to equal volumes of wash buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.1% Az, 0.5% NP-40, 0.5% deoxycholic acid, 10% glycerol). Antibodies (2  $\mu$ l ascites per sample) were added, followed by an overnight incubation. For mAb 131, rabbit anti-mouse F(ab) (25  $\mu$ l) or 187.1 (10  $\mu$ l ascites) was added to facilitate *S. aureus* binding. *S. aureus* (100  $\mu$ l of a 10% wt/vol suspension) was added, followed by a 1-h incubation. Immune complexes were washed three times in 0.6 ml wash buffer. For isoelectric focusing (IEF), O'Farrell method (15) (see below), *S. aureus* pellets were resuspended in 40  $\mu$ l IEF sample buffer, heated to  $60^{\circ}\text{C}$  for 15 min, and centrifuged. For IEF by the method of Krangel et al. (16), *S. aureus* pellets were resuspended in 100  $\mu$ l of Laemmli's sample buffer, boiled for 2 min, and centrifuged. Supernatants containing class I proteins were carefully removed and analyzed as described below.

**Two-dimensional IEF Analysis.** Proteins were resolved according to the method of O'Farrell (15). Briefly, class I antigens in 20  $\mu$ l IEF sample buffer were separated in the first dimension by IEF using 2% ampholines (one part pH 3.5–10, two parts pH 4–6, two parts pH 6.8). After equilibrating the gels for 45 min, proteins were further resolved in a second dimension by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide.

Proteins were also resolved as described by Krangel et al. (16). Immunopurified class I antigens (50  $\mu$ l) were resolved by SDS-PAGE using 7.5% acrylamide according to Laemmli (17). Using dansylated alcohol dehydrogenase (41 kD) as a molecular marker, the portion of the gel corresponding to 40–45 kD was excised, equilibrated, and polymerized into the IEF gel containing 2% ampholines (one part pH 3.5–10, one part pH 4–6, one part pH 6–8). Electrophoresis was carried out for 7 h at 1,000 V.

After electrophoresis, gels were fixed, enhanced (New England Nuclear, Boston, MA), and dried. Radioactive bands were detected by fluorography at  $-70^{\circ}\text{C}$  using Kodak XR-5 film.

## Results

Previous sequential immunoprecipitation and preclearing studies have shown that mAb 131, made against the human natural killer cell line 3.3, detects a subpopulation of class I molecules detected by the pan-HLA-reactive mAb W6/32 (9). That mAb 131 detects only some of the class I proteins precipitated by W6/32 is further shown in Fig. 1. Here,  $^{125}\text{I}$ -labelled JY (HLA-A2, B7) cell lysates were immunoprecipitated with a panel of class I-specific mAb and resolved on two-dimensional O'Farrell gels (15). The results show that W6/32 reacts with both the HLA-A2 and HLA-B7 45 kD proteins (Fig. 1A), whereas 131 immunoprecipitates only the more basic HLA-A2 protein (Fig. 1B). mAb 4E, which recognizes a determinant shared by all HLA-B locus proteins as well as HLA-A29, -A30, -A31, and -A32 proteins (7), reacts only with the more acidic HLA-B7 protein (Fig. 1D). The relative positions of the HLA-A2 and HLA-B7 spots were confirmed by analysis of proteins immunoprecipitated with mAb PA2.1 (anti-HLA-A2) (Fig. 1C) and BB7.1 (anti-HLA-B7) (Fig. 1E).

NK3.3, the immunizing cell line, and JY share the HLA-B7 allele, but express different HLA-A alleles, thus raising the possibility that mAb 131 may be specific for a number of different HLA-A locus-encoded gene products. To examine

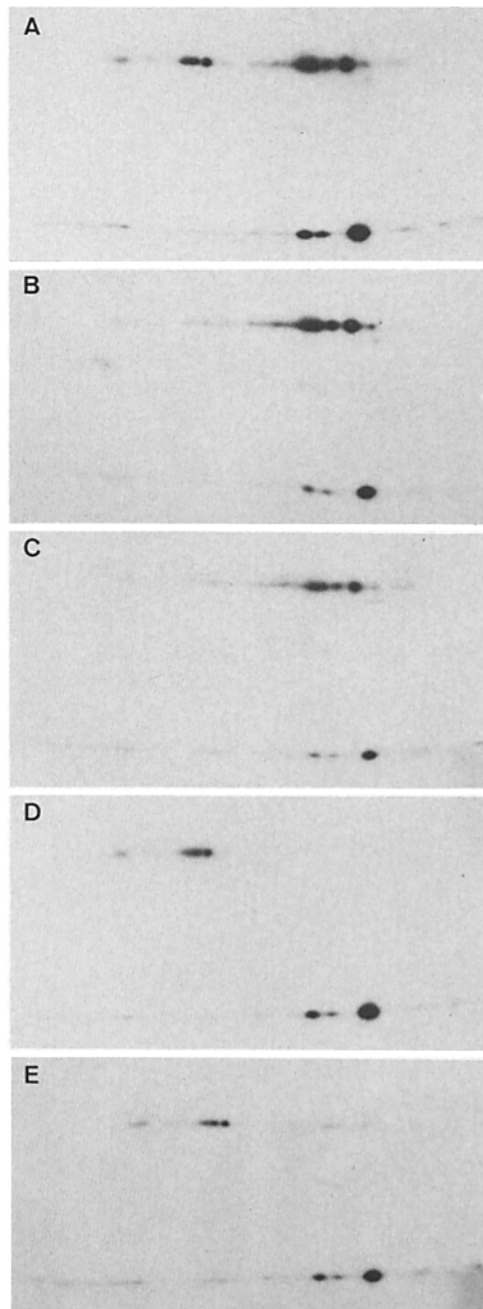


FIGURE 1. O'Farrell two-dimensional analysis of class I proteins immunoprecipitated with mAb W6/32, 131, PA2.1, 4E, and BB7.1. The first dimension is IEF, with the basic end of the gel to the right. The second dimension is SDS-PAGE. The 12 kD spot, corresponding to  $\beta_2$ -m, has been aligned in all five gels. At 45 kD, the more basic HLA-A2 and the more acidic HLA-B7 spots can be seen when using W6/32 (A). Only the more basic spot corresponding to HLA-A2 is seen when immunoprecipitating with 131 (B) or the HLA-A2-specific mAb, PA2.1 (C). The more acidic spot, corresponding to HLA-B7, can be seen with the HLA-B-specific mAb, 4E (D), or the HLA-B7-specific mAb, BB7.1 (E).

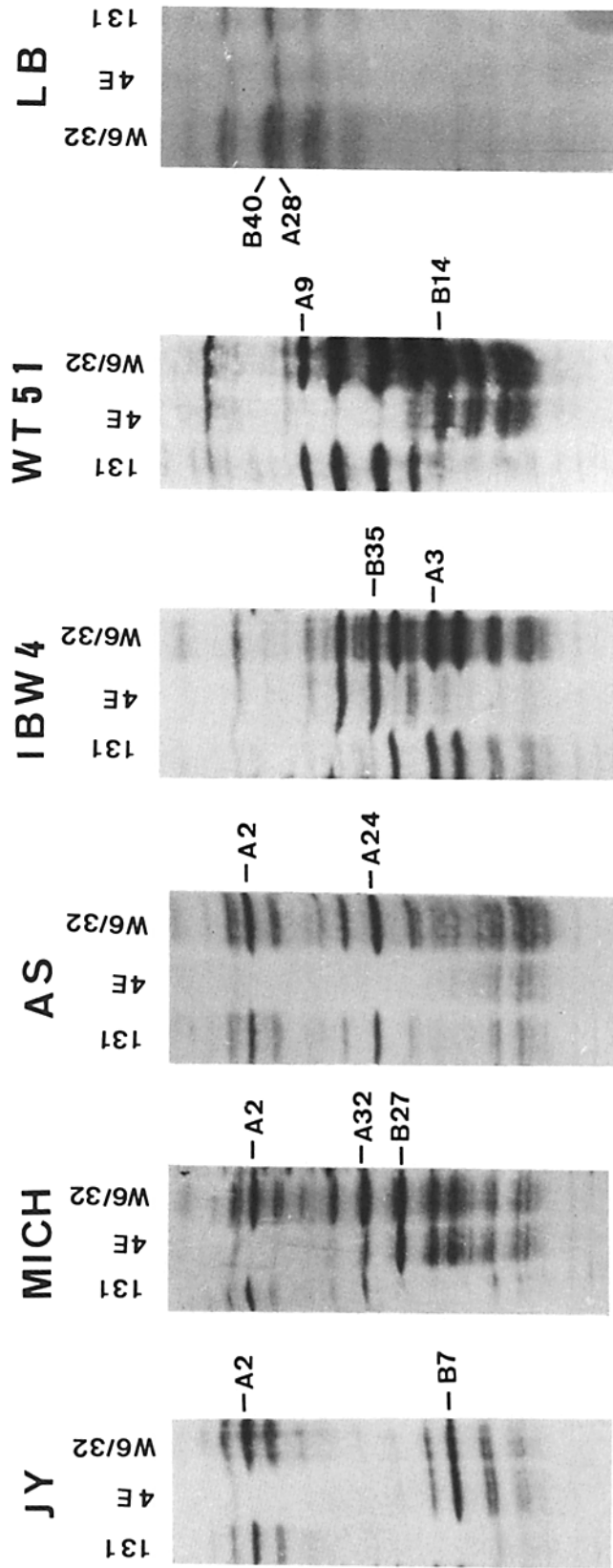


FIGURE 2. Two-dimensional IEF analysis of class I antigens immunoprecipitated from [ $^{35}$ S]methionine-labelled NP-40 cell lysates. Samples were first resolved by SDS-PAGE. The region of the gel corresponding to 40–45 kD was excised, equilibrated, polymerized into the second (IEF) dimension, and further resolved. Gels were fixed and fluorographed for the detection of radioactive bands. For each radiolabelled cell line, lysates were divided into three aliquots and immunoprecipitated with 131, 4E, or W6/32. Cell lines are denoted at the top of the figure (see Table I for HLA typing). Major bands corresponding to the HLA alleles are designated. The gel is oriented with the basic end at the top of the figure.

TABLE I  
*Binding Profiles of mAb on Panel of Human Cell Lines as Assessed by  
 Cell-surface RIA*

Cell line	HLA-A	HLA-B	<sup>125</sup> I-Goat anti-mouse Ig bound (cpm)		
			131	W6/32	P3
Daudi	—	—	147	244	44
K562	—	—	142	156	0
Molt-4	1, 10	NT	934	2,542	14
MICH	2, 32	27, 15	3,239	6,992	192
JY	2	7	4,810	7,038	148
IBW 4	3	35	3,647	4,455	66
Herluff	2	12, 35	3,375	3,842	99
THAL B	3,10	5,35	5,103	7,933	135
IBW 9	33	14	2,187	3,840	98
WT51	9	14	3,912	7,408	80
23.1	2	27	3,150	4,438	0
LB	2	40	1,891	3,133	0
WT46	32	13	2,011	2,507	0
AS	2,24	27	4,564	5,417	20
Preiss	2	15	2,322	666	0
WT49	2	17	4,673	4,862	30
LBF	30	13	4,627	5,807	140
Mann	29	12	3,679	4,324	0
HOM-2	3	27	3,483	3,807	49

this possibility, class I proteins from a number of different [<sup>35</sup>S]methionine-labelled B lymphoblastoid cell lines were immunoprecipitated with mAb 131, 4E, and W6/32, and analyzed on two-dimensional IEF gels as described by Krangel et al. (16). The results (Fig. 2) indicate that, for each of the different cell lines, the IEF banding profiles seen with 131 and 4E can be combined to produce the W6/32 profile. Note also that mAb 131 and 4E, as expected, both react with HLA-A32 from MICH. From these data, we conclude that mAb 131 reacts with all of the HLA-A proteins tested (HLA-A2, -A3, -A9, -A24, -A28, and -A32), but not with the HLA-B proteins (HLA-B7, -B14, -B27, -B35, and -B40).

Table I shows the cell-surface binding profiles of mAb 131 and W6/32, determined by RIA, on a panel of 19 human cell lines displaying 11 different HLA-A proteins and 12 different HLA-B proteins. As expected, 131 and W6/32 do not bind to Daudi and K562; neither cell line expresses class I proteins. All other cell lines bind both of these antibodies.

The specificity of mAb 131 for HLA-A2, and lack of reactivity with HLA-B7, -B40, and  $\beta_2$ -m is shown by FACS analysis of mouse L cell lines transfected with these genes (Fig. 3). As expected, mAb W6/32, 131, and 4E bind to JY (A2<sup>+</sup>, B7<sup>+</sup>), W6/32 and 131 bind to LJE1 (A2<sup>+</sup>), W6/32 and 4E bind to LJG3 (B7<sup>+</sup>) and LB.40.10 (B40<sup>+</sup>), but not to LJCM ( $\beta_2$ -m). The bimodal peaks seen with the LB.40.10 line indicate a heterogeneous population of cells; subclones have arisen lacking expression of the transfected class I B40 gene. Negative FACS staining by 131 on LJG3 and LB.40.10 rules out detectable low-affinity binding by this antibody to HLA-B7 and -B40.

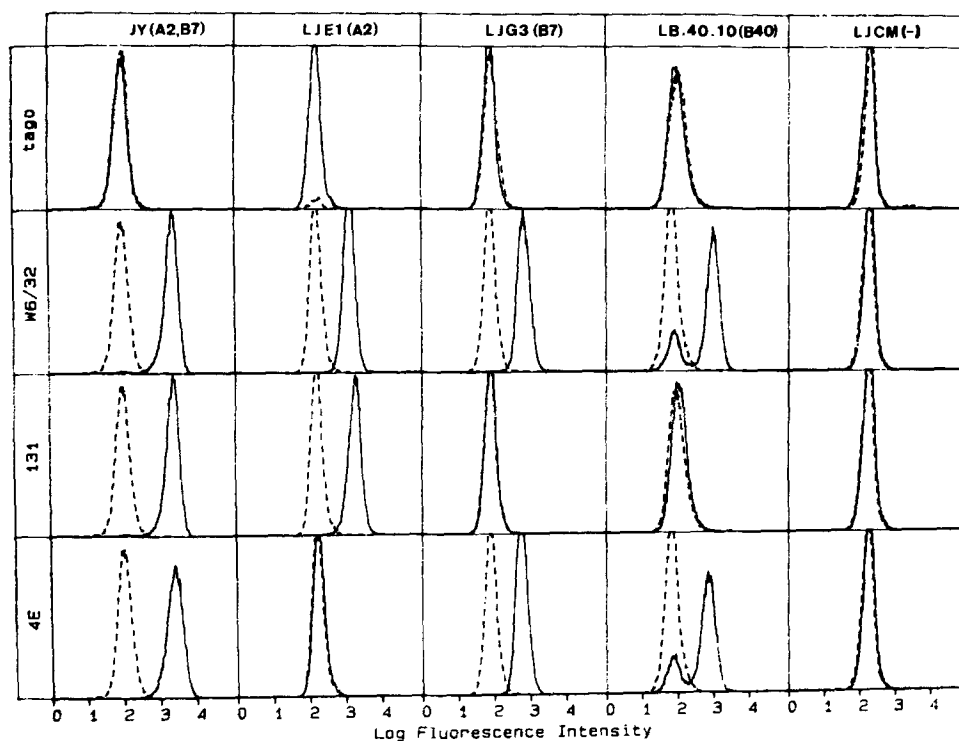


FIGURE 3. Indirect immunofluorescence of JY and mouse L cells transfected with human class I genes.  $10^6$  cells were incubated for 1 h at  $4^{\circ}\text{C}$  with the primary mAb (denoted on the left side of the figure). Cells were washed, incubated for another hour with a fluoresceinated goat anti-mouse Ig, washed again, and analyzed with a FACS IV. JY is typed as HLA-A2,B7. LJE1 has been transfected with the HLA-A2 and human  $\beta_2\text{-m}$  genes; LJG3 with HLA-B7 and human  $\beta_2\text{-m}$ ; LB.40.10 with HLA-B40; LJCm has been transfected only with the human  $\beta_2\text{-m}$  gene. *tago* denotes negative controls; hatched lines indicate autofluorescence, solid lines indicate staining with only the fluoresceinated second antibody. For W6/32, 131, and 4E, the solid lines indicate staining with those mAb; hatched lines corresponded to the *tago* control. The HLA genes expressed by the cell lines tested are given in parentheses at the top of the figure.

### Discussion

The results presented above indicate that mAb 131 recognizes a determinant expressed on a number of different HLA-A proteins. mAb 131 is therefore a candidate for a pan-reactive HLA-A antibody, and to our knowledge, this is the first description of such an antibody. From IEF analysis, we have demonstrated that 131 reacts with HLA-A2, -A3, -A9, -A24, -A28, -A32, and -A33, and shows no apparent reactivity with HLA-B7, -B14, -B27, -B35, and -B40 allelic products. FACS analysis on mouse L cells transfected with HLA class I genes confirms reactivity with HLA-A2 and the absence of binding to HLA-B7 and -B40. Cell-surface binding assays with 19 different cell lines show that mAb 131 reacts with all HLA<sup>+</sup> cells tested. IEF studies are currently in progress with a larger panel of cells to determine whether the determinant detected by mAb 131 is unique only for HLA-A proteins.

Two HLA-B-specific mAb, 4E (7) (4E crossreacts with some HLA-A alleles),

and 4G (8), have been reported. Cresswell and Ayres (18) produced rabbit antisera against purified HLA-A2 and -B7/-B12 antigens that displayed reactivity with other HLA-A and HLA-B alleles, respectively. These findings, along with our description of 131, provide serological evidence for the existence of some structural feature that distinguishes HLA-A and -B locus products. Evidence for such "A-ness" and "B-ness" is also supported at the molecular level. Arnot et al. (19) have recently described cDNA probes that define shared HLA intralocus sequences in class I mRNA. Whether such shared intralocus sequences correspond to epitopes detected by locus-specific mAb remains to be determined.

The possibility of shared intralocus determinants raises questions concerning the molecular nature of these epitopes and what biological function, if any, they may have. We are now attempting to biochemically characterize the epitope seen by 131. mAb 131 reacts with isolated class I heavy chains in protein blots, but only under nonreducing conditions (B. Spear, J. Kornbluth, and D. Wilson, manuscript in preparation). It therefore appears that the structural integrity of the epitope defined by mAb 131 on class I heavy chains does not depend on association with  $\beta_2$ -m, but intact intrachain disulfide bonds are necessary.

It is clear that HLA-A and -B locus proteins have similar functions of MHC-restricted antigen recognition; however, the conserved intralocus differences in structure may also indicate that these molecules have some different functions. This possibility is supported by the recent findings (9, 20) that mAb 131 and 4E have different effects on the lytic activity and interleukin 2-dependent proliferation of some human NK clones. If these findings can be extended, it will become an important issue to identify the structural features of HLA-A and -B locus proteins that correlate with their differences in function.

### Summary

We describe here a monoclonal antibody, 131, which appears to recognize a determinant shared by HLA-A locus-encoded gene products. Isoelectric focusing analysis demonstrates that 131 reacts with the products of at least seven different HLA-A alleles but none of the five HLA-B allelic products tested. Together with evidence provided by other studies, this finding indicates the existence of A-unique and B-unique determinants, which may have different biological functions. Monoclonal antibody probes, such as the one described here, specific for shared intralocus determinants, may be valuable for assessing these possible functional differences.

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