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HLA antibody responses in HLA class I transgenic mice

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Abstract. In a previous report we described how cross-immunizations of pairs of transgenic mice expressing different HLA class I antigens led to the production of antibodies directed exclusively at polymorphic epitopes. This was ascribed to self-tolerance of HLA that prevents immune responses to monomorphic epitopes and focuses responses on polymorphic ones. In the present report we extend our findings and demonstrate that immunizations of class I transgenic mice with HLA transfected mouse fibrosarcoma as well as with human lymphoblastoid cells also preferentially yield antibodies to polymorphic epitopes. This was the case whether or not immunizations were carried out across locus barriers [e.g., Tg*(HLA-A *0201)* or Tg*(HLA-Cw*0301)* transgenic mice immunized with HLA-B27 transfectants] or within the same locus [e.g., Tg*(HLA-B*1302)* transgenic mice immunized with HLA-B27 transfectants or B27-expressing lymphoblastoid cells]. Use of an extended immunization protocol with four or more booster injections favored antibodies of IgG isotype with affinities high enough to lyse normal peripheral blood lymphocytes (PBLs) in complement-dependent cytotoxicity assays and to immunoprecipitate HLA antigens. The specificities covered by the monoclonal antibodies (mAbs) could be either broad or narrow, depending on the genetic distance of the HLA antigens or alleles involved. For instance, a Tg*(HLA-B*1302)* transgenic mouse immunized with B27 produced both broad B7/B27-specific antibodies, Bw4-specific antibodies, and one antibody reacting with all *B* alleles except *B13* and with some *C* alleles. On the other hand, a Tg*(HLA-B*1302)* transgenic mouse immunized with Bw47 transfectants responded narrowly with an antibody to Bw60 and Bw47. Thus it appears that by choosing appropriate recipient mice and closely related or more distant HLA antigens, antibodies of a programmed specificity can be generated.

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Introduction

Serological analysis of the human major histocompatibility complex antigens continues to be a legitimate effort towards understanding the structure/function relationship governing these immunologically important antigens. The recent explosion in primary HLA sequence data (Parham et al. 1988; Marsh and Bodmer 1990) was fueled by improvements in the polymerase chain reaction-based sequencing methods. A parallel development, based on the use of HLA transgenic mice, promises a renaissance of HLA serology by enabling the generation of custom-tailored monoclonal antibodies (mAbs). We have found that HLA class I transgenic mice expressing different HLA isotypes, or different alleles of the same isotype, can be cross-immunized by tissue graft exchanges resulting in humoral responses with narrow specificities commensurate with the genetic distances of the HLA antigens involved (Hämmerling et al. 1990). The underlying reasons for the restricted responses in HLA transgenic mice, as compared with non transgenic ones, have been attributed to self-tolerance to the shared epitopes, including especially the monomorphic epitopes which dominate the responses in normal mice. For this latter reason, the production of *HLA* allele-specific mAbs in normal mice has been difficult, although not impossible, showing that in principle the mouse B-cell repertoire contains the diversity for HLA epitopes. Cross-immunizations of HLA transgenic mice, on the other hand, favor alloantibody production. They reproduce the circumstances governing incidental immunizations during pregnancy or after organ transplantation that still furnish the bulk of reagents used for histocompatibility testing. HLA transgenic mice offer the possibility of programming the antibody responses by appropriate choices of donor-recipient pairs. Using this approach, we have previously demonstrated (Hämmerling et al. 1990) that Tg*(HLA-B*0702)* transgenic mice immunized with cells of Tg*(HLA-Cw*0301)* transgenic mice produced allospecific antibodies to *Cw3* and related

alleles, some of which could be retrieved as mAbs. The reciprocal immunization, Cw3 anti-B7, yielded a set of mAbs, some of which had private B7 specificity, others a broader specificity to the B7 cross-reactive group, and a third group some public *B*-locus specificity. In no case was there an mAb to a monomorphic epitope, showing that transgenic mouse immunizations are of advantage. However, to establish transgenic mice in each instance when an immunizing tissue is needed would be an exacting task. It was therefore of interest to investigate the use of HLA transfectants as immunogens. We report here the production and serological analysis of several mAbs raised by the immunization of HLA transgenic mice with HLA transfectants of the mouse fibrosarcoma cell line Bc20.

Materials and methods

Transgenic mouse strains. The C57BL/6Tg*(HLA-A*0201)*, C57BL/6 Tg*(HLA-B*0702)*, C57BL/6Tg*(HLA-B*1302)*, and C57BL/6Tg*(HLA-Cw*0301)* strains have been derived by E. Lacy (Sloan-Kettering Institute, New York, New York; Le et al. 1989) and G. J. Hämmerling (West German Cancer Center, Heidelberg, FRG; Dill et al. 1988).

HLA transfected cell lines. The Bc20 cell line was derived from a fibrosarcoma that arose in a C57BL/6 mouse. Transfections of HLA cDNAs were carried out either by calcium phosphate precipitation (Wigler et al. 1979) or by lipofection (Felgner et al. 1987). Cell lines were cloned repeatedly, and high expression variants isolated by sorting in a fluorescence-activated cell sorter (FACS). Several HLA transfectants of L cells, termed Ela (HLA-B14), E5ab2 (HLA-B38), S13ba3 (HLA-B39), and JII 1 (HLA-B51), were obtained from C. Müller (University of Tübingen, FRG). The HLA-A26 transfectant was obtained from P. Pascale (Hopital St. Louis, Paris, France) and the L-Cw6 transfectant was derived by one of us (S.Y.Y.).

Immunizations. Groups of HLA transgenic mice received bi-weekly injections of cultured Bc20 transfectants. The initial dosage was 2×10^7 cells, and this was gradually increased to 5×10^7 cells over the course of four to five immunizations. Since several instances occurred where HLA-negative Bc20 cells formed progressively growing tumors, later immunizations were performed with cell membranes obtained by hypotonic shock in distilled water for 30 min in the presence of deoxyribonuclease (Sigma Chemicals, St. Louis, Missouri) to prevent clumps. Animals were bled periodically and serum antibody titers to the immunizing HLA antigen monitored by cytotoxicity assay. Mice exhibiting low autoantibodies and high anti-HLA titers (preferentially $> 1{:}500$) were chosen for hybridoma production. For immunization with lymphoblastoid cell lines (LCLs), mice received seven injections of 20×10^b cells each.

Cell fusion. The method of Köhler and Milstein (1975) was used with the Sp2/0 myeloma cell line.

Screening for antibody formation. Cell culture supernates were tested by cytotoxicity assays on human homozygous LCLs consigned to the test cell panel of the Tenth International Workshop (Yang et al. 1989). The criteria for choosing a particular mAb were positive reaction (> 90 % lysis) on an LCL exhibiting the same *HLA* allele as the immunizing HLA transfectant, and negative reaction on a second LCL with the *HLA* allele of the transgenic recipient mouse. Hybridomas were recloned at least twice.

Serological analysis. The mAbs were tested by the standard National Institutes of Health (Bethesda, Maryland) cytotoxicity test on the entire LCL Workshop panel and on randomly selected but well-typed peripheral blood lymphocytes (PBLs). In addition, mAbs were tested by immunofluorescence on our panel of Bc20 and L-cell transfectants expressing single HLA heavy chains. The transgenic mouse strains, the cell lines used as immunogens, and the mAbs that resulted from the immunizations are listed in Table 1.

Results

Immunizations of HLA transgenic mice across HLA locus barriers with Bc20 HLA transfectants. Bc20 is a fibrosarcoma of the C57BL/6 mouse strain, fully histocompatible with our C57BL/6-derived HLA transgenic mouse strains (see Table 1). It also performs well in cDNA transfection and expression by the calcium phosphate (Wigler et al. 1979) or lipofection (Felgner et al. 1987) methods. B6 Tg*(HLA-A*0201)* and B6Tg*(HLA-Cw*0301)* mice were immunized with cloned live Bc20.B27.hβ2M cells. In spite of the presence of the human transplantation antigen, known to induce rejection, several mice developed tumors. These tumors proved to be HLA-negative. Since even the tumor-bearing mice possessed high cytotoxic antibody titers, they were used for hybridoma production. The results were not encouraging overall, as several fusion experiments became hopelessly overgrown with Bc20 cells which had metastasized to the spleen. Two mice, however, yielded four satisfactory mAbs. Table 2 summarizes the results obtained by these four mAbs in cytotoxicity assays on a panel of homozygous LCLs assembled by the Tenth International HLA Workshop (Yang et al. 1989). Although they were obtained from different immunizations, their reactivity patterns were similar: mAb TT4, obtained from the B6Tg*(HLA-A* 0201)* immunization, and mAbs 63, 186, and 367 from the B6Tg*(HLA-Cw*0301)* immunization show the highest correlation with the Bw4 epitope, A23 and A25. The A24 reactivity commonly seen with Bw4 antisera is missing (see Table 3). The last two mAbs were identical in specificity and isotype and since they originated from the same fusion, they probably constitute repeat isolates and therefore only mAb 186 is listed.

To verify the specificity further, randomly selected but well-typed PBLs were tested by microcytotoxicity. Once again, the four mAbs yielded high correlation with Bw4. Table 3 summarizes the results for mAb TT4, showing the cross-reactivity to A23 and A25, but not to A24. The four mAbs were tested by immunofluorescence microscopy on a panel of transfectants. The results in Table 4 confirm that the epitope in question is encoded by the *HLA-B* locus, since neither untransfected Bc20, nor HLA-A or -C transfectants bound these mAbs. It is worth noting that the A24 transfectant did not bind mAbs TT4, 63, and 186, confirming the results of cytotoxicity assays.

Table 1. Immunizations and mAbs.

HLA transgenic mouse		Immunogen		mAb	Isotype	Specificity
Designation	*HLA* allele	Cell	*HLA* allele			
C57BL/6Tg (*HLA-A*0201*)	*A*0201*	Bc20.B27.β2M	*B*2702*	TT4	IgG2a	Bw4
C57BL/6Tg (*HLA-Cw*0301*)	*Cw*0301*	Bc20.B27.β2M	*B*2702*	63	IgG2a	Bw4
,,	,,	,,	,,	186	IgG2a	Bw4
,,	,,	,,	,,	367	IgG2a	Bw4
C57BL/6Tg (*HLA-B*1302*)	*B*1302*	Bc20.B27.β2M	*B*2702*	22E1	IgG2b	Bw4
,,	,,	,,	,,	8A3	IgG†	all B minus B13 & C
,,	,,	,,	,,	5C12	IgM	broad B
,,	,,	,,	,,	5G2*	IgG2b	B7/B27/A28
,,	,,	,,	,,	15C12*	,,	,,
,,	,,	,,	,,	9D2*	,,	,,
,,	,,	,,	,,	20D10*	,,	,,
,,	,,	,,	,,	24A5*	,,	,,
,,	,,	,,	,,	25B1*	,,	,,
C57BL/6Tg (*HLA-B*1302*)	*B*1302*	Bc20.Bw47	*Bw*4701*	TT7	IgM	Bw47/Bw60
C57BL/6Tg (*HLA-B*1302*)	*B*1302*	HOM-2	N/A	15C3	IgG2b	broad B
,,	,,	,,	,,	2A1**	,,	Bw4
,,	,,	,,	,,	17A10**	,,	Bw4
,,	,,	,,	,,	17A12**	,,	Bw4
,,	,,	,,	,,	22C4**	,,	Bw4
,,	,,	,,	,,	3H5	,,	B7/B27/Bw62

Immunizations of HLA class I transgenic mice with the Bc20 fibrosarcoma HLA transfectants were performed as described in the text. The *HLA* genes are identified by the convention adopted by the WHO Nomenclature Committee for Factors of the *HLA* System (Bodmer et al. 1990). For definitions of specificities and further detail, see text and accompanying tables. MAbs marked with one or two asterisks may belong to hybridoma siblings. N/A, not applicable.

† Subclass not determined.

Table 2. Reactivities of mAbs with homozygous LCLs.

WS #	Name	*HLA* allele				TT4	63	186	5G2	22E1	8A3	5C12	15C3	3H5	17A10	TT7
		A	*B*	*Bw4/w6*	*C*											
9001	SA	*24*	*7*	*6*	*7*	1	4	4	8	6	8	8	6	8	6	2
9002	MZ070782	*24*	*14*	*6*	*2/8*	1	1	1	1	6	8	8	8	1	8	1
9003	KAS116	*24*	*51*	*4*	–	8	2	8	1	6	8	1	6	1	6	1
9004	JESTHOM	*2*	*27*	*4*	*1*	8	8	8	8	8	8	8	8	8	8	2
9005	HOM2	*3*	*27*	*4*	*1*	8	8	8	8	8	8	8	8	8	8	1
9006	WT100BIS	*11*	*35*	*6*	*4*	1	1	1	1	1	8	1	1	1	1	1
9007	DEM	*2*	*57*	*4*	*6*	8	6	8	1	8	8	6	8	4	8	1
9008	D0208915	*25*	*18*	*6*	–	8	1	8	1	8	8	1	8	1	8	1
9009	KAS011	*1*	*37*	*4*	*6*	8	8	8	1	8	8	1	8	2	8	1
9010	AMAL	*28*	*53*	*4*	*4*	1	2	4	8	8	8	8	8	8	8	1
9011	E4181324	*1*	*52*	*4*	–	8	8	8	1	8	8	1	8	1	8	1
9012	WJR076	*2*	*57*	*4*	*7*	8	6	8	1	8	8	8	8	4	6	1
9013	SCHU	*3*	*7*	*6*	*7*	2	2	2	8	1	8	8	8	8	1	1
9014	MGAR	*26*	*8*	*6*	*7*	1	NT	NT	1	1	8	1	8	1	1	1
9015	WT24	*2*	*27*	*4*	*2*	8	NT	NT	8	8	8	8	8	8	8	2
9016	RML	*2*	*51*	*4*	–	8	NT	NT	1	8	8	1	6	1	6	1
9017	WT8	*3*	*7*	*6*	*7*	1	2	2	8	2	8	8	8	8	2	1
9018	L0081785	*3/24*	*18*	*6*	*5*	1	2	2	2	8	8	2	8	1	8	1
9019	DUCAF	30	*18*	*6*	*5*	2	2	1	1	2	8	4	8	4	1	1
9020	QBL	26	*18*	*6*	*?5*	1	1	1	1	1	8	1	8	1	1	1
9021	RSH	*68/30*	*42*	*6*	*2*	1	1	1	8	1	8	8	6	6	1	1
9022	COX	*1*	*8*	*6*	*7*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
9023	VAVY	*1*	*8*	*6*	*7*	2	1	2	1	1	8	1	8	1	1	1

WS #	Name	*HLA* allele				TT4	63	186	5G2	22E1	8A3	5C12	15C3	3H5	17A10	TT7
		A	*B*	*Bw4/w6*	*C*											
9024	KT17	*2/11*	*62/35*	*6*	*9/4*	1	1	1	1	1	8	1	4	2	1	1
9025	DEU	*31*	*35*	*6*	*4*	1	1	1	1	2	8	1	1	1	1	1
9026	YAR	*26*	*38*	*4*	–	8	8	8	2	8	8	2	8	4	8	1
9027	PF97387	*29*	*44*	*4*	–	8	8	8	1	8	8	1	6	1	8	1
9028	PE117	*24*	*60/61*	*6*	*10*	1	2	4	1	6	8	2	8	1	6	8
9029	WT51	*23*	*65*	*6*	*8*	6	8	8	1	8	8	6	8	6	8	1
9030	JHAF	*31*	*51*	*4*	*8*	8	NT	NT	1	8	8	1	6	1	8	1
9031	BOLETH	*2*	*62(75)*	*6*	*10*	1	2	2	1	1	8	2	8	6	1	2
9032	BSM	*2*	*62(75)*	*6*	*9*	1	1	2	1	1	8	1	8	6	1	2
9033	BM14	*3*	*7*	*6*	*7*	1	NT	NT	6	1	8	8	2	6	1	1
9034	SAVC	*3*	*7*	*6*	*7*	1	NT	NT	8	1	8	8	8	8	1	1
9035	JBUSH	*32*	*38*	*4*	–	8	6	8	1	8	8	4	8	2	6	1
9036	SP0010	*2*	*44*	*4*	*5*	8	8	8	1	8	8	1	6	1	8	1
9037	SWEIG007	*29*	*61*	*6*	*2*	1	1	1	1	1	8	6	6	1	1	1
9038	BM16	*2*	*18*	*6*	*7*	2	NT	NT	1	1	8	1	6	1	1	1
9039	JVM	*2*	*18*	*6*	*5*	2	2	2	1	1	6	1	4	1	1	2
9040	BM15	*1*	*49*	*4*	*7*	8	8	8	2	8	8	8	2	2	8	2
9041	J0528239	*1*	*35*	*6*	*4*	1	1	1	1	1	8	1	1	1	1	1
9042	TISI	*24*	*35*	*6*	*4*	1	1	1	1	8	8	1	1	1	8	1
9043	BM21	*1*	*41*	*6*	–	2	1	2	1	1	8	2	8	1	1	1
9044	BRIP	*24*	*51/63*	*4*	–	8	6	8	1	8	8	6	8	2	8	1
9045	TUBO	*2/3*	*51*	*4*	*7*	8	6	8	1	8	8	1	8	1	8	1
9046	BH	*2*	*13*	*4*	*6*	8	2	8	1	1	1	1	1	1	1	1
9047	PLH	*3*	*47*	*4*	*6*	8	8	8	1	8	8	2	8	1	8	8
9048	LBUF	*30*	*13*	*4*	*6*	8	NT	NT	1	1	6	1	1	1	1	1
9049	IBW9	*33*	*65*	*6*	*8*	1	2	2	1	1	8	4	2	1	1	1
9050	MOU	*29*	*44*	*4*	–	8	8	8	1	6	8	2	2	1	4	1
9051	PITOUT	*29*	*44*	*4*	–	8	8	8	1	8	8	2	8	1	8	1
9052	DBB	*2*	*57*	*4*	*6*	8	4	8	NT	NT	NT	NT	NT	NT	NT	NT
9053	HOR	*33*	*44*	*4*	–	8	8	8	1	8	8	1	6	4	8	1
9054	EK	*2*	*44*	*4*	*5*	8	4	8	1	8	8	1	4	1	8	1
9055	HO301	*3*	*14*	*6*	*8*	2	2	1	1	1	8	8	8	2	1	2
9056	KOSE	*2*	*35*	*6*	–	1	1	1	1	1	8	1	1	1	1	1
9057	TEM	*26*	*38*	*4*	–	8	4	8	1	8	8	2	6	2	6	1
9058	OMW	*2*	*45*	*6*	–	1	NT	NT	1	1	8	1	1	1	1	1
9059	SLE005	*2*	*60*	*6*	*10*	1	NT	NT	1	1	8	2	8	1	1	8
9060	CB6B	*1*	*62*	*6*	*9*	1	NT	NT	1	1	8	2	8	6	1	6
9061	31227ABO	*2*	*18*	*6*	*7*	1	1	1	1	1	8	1	8	1	1	1
9062	WDV	*2*	38	*4*	–	8	NT	NT	2	8	8	6	8	2	8	1
9063	WT47	*32*	*44*	*4*	*5*	8	8	8	1	8	8	1	8	1	4	1
9064	AMALA	*2*	*62(75)*	*6*	*9*	1	1	2	1	1	8	1	8	4	1	1
9065	HHKB	*3*	*7*	*6*	*7*	NT	NT	NT	8	1	8	8	6	8	1	1
9066	TAB089	*2*	*46*	*6*	*11*	1	1	1	1	1	6	1	4	2	1	1
9067	BTB	*2*	*27*	*4*	*1*	8	8	8	8	8	8	8	8	8	8	2
9068	BM9	*2*	*35*	*6*	*4*	2	1	1	1	1	8	1	1	1	1	4
9069	MADURA	*2*	*60*	*6*	*10*	1	NT	NT	1	1	8	1	8	1	1	8
9070	LUY	*2*	*51*	*4*	–	8	NT	NT	1	8	8	1	6	8	4	1
9071	OLGA	*31*	*62*	*6*	*11*	1	1	1	1	1	8	1	6	4	1	2
9072	SPACH	*31*	*62*	*6*	*11*	1	NT	NT	1	1	8	1	6	6	1	2
9073	KT12	*24/31*	*35/52*	*4/6*	*4*	8	6	8	1	8	8	2	8	2	8	1
9074	HID	*2*	*61/60*	*6*	*10/8*	1	1	1	1	1	8	2	6	1	1	8
9075	DKB	*24*	*60*	*6*	*10*	2	1	2	1	6	8	2	8	1	8	8
9076	T7526	*2*	*46*	*6*	*11*	1	1	1	1	1	8	1	6	6	1	1
9077	T7527	*2*	*46*	*6*	*11*	1	1	1	1	1	8	1	6	6	1	1
9078	PMG075	*3/33*	*65*	*6*	*8*	1	NT	NT	1	1	8	8	8	1	1	1
9079	LWAGS	*33*	*14*	*6*	*8*	2	1	4	1	1	8	8	8	2	1	1
9080	EHM	*3*	*35*	*6*	*4*	1	1	1	1	1	8	1	1	1	1	1
9081	EA	*3*	*7*	*6*	*7*	2	2	2	8	1	8	8	4	8	1	1
9082	HO104	*3*	*7*	*6*	*7*	1	1	1	8	1	8	8	6	8	1	1

WS #	Name	*HLA* allele				TT4	63	186	5G2	22E1	8A3	5C12	15C3	3H5	17A10	TT7
		A	*B*	*Bw4/w6*	*C*											
9083	LD2B	*3*	*7*	*6*	*7*	1	1	2	8	1	8	8	8	8	1	1
9084	CALOGERO	*2*	*61*	*6*	*2*	1	1	1	1	1	8	4	8	1	1	1
9085	EJ32B	*30*	*18*	*6*	*5*	1	1	1	1	1	8	1	8	1	1	1
9086	L0541265	*1*	*8*	*6*	*7*	1	2	4	1	1	8	1	6	1	1	1
9087	STEINLIN	*1*	*8*	*6*	–	8	8	8	1	8	8	1	8	1	8	1
9088	PF04015	*1*	*8*	*6*	*7*	1	1	1	1	1	8	1	8	1	1	1
9089	BOB	*24*	*51*	*4*	–	8	4	8	1	8	8	1	8	1	8	1
9090	AWELLS	*2*	*44*	*4*	*5*	8	8	8	1	8	8	1	8	1	8	1
9091	MLF	*2*	*62*	*6*	*9*	1	1	1	NT	NT	NT	NT	NT	NT	NT	NT
9092	BM92	*25*	*51*	*4*	*1*	8	6	8	1	8	8	1	8	6	8	1
9093	BER7643	*2*	*13*	*4*	*6*	8	4	8	1	1	2	1	1	1	1	1
9094	CF996	*2/3*	*14*	*6*	*8*	1	2	2	1	1	8	8	8	6	1	2
9095	WIN76241	*1*	*57*	*4*	*6*	8	6	8	1	8	8	8	8	6	8	1
9096	LBF	*30*	*13*	*4*	*6*	8	2	8	1	1	8	1	1	1	1	1
9097	EMJ	*2/3*	*60*	*6*	*10*	1	2	1	1	1	8	2	6	1	1	6
9098	MT14B	*31*	*60*	*6*	*10*	2	NT	NT	1	2	8	2	8	2	1	8
9099	LZL	*2*	*62 (75)*	*6*	*9*	1	1	2	1	1	8	2	8	6	1	6
9100	OLL	*31*	*62*	*6*	*11*	1	1	1	1	1	8	1	6	6	1	2
9101	SPL	*31*	*62*	*6*	*11*	2	2	4	1	1	8	1	6	6	1	2
9102	ARBO	*3*	*57*	*4*	*6*	8	6	8	1	8	8	8	1	1	4	1
9103	KT14	*24/26*	*51/61*	*4/6*	*8*	8	2	8	1	8	8	1	8	8	8	1
9107	LKT3	*24*	*54*	*6*	*1*	2	1	2	8	8	8	8	1	8	8	2

Reactivities of mAbs in cytotoxicity assays with homozygous LCL panel assembled by the Tenth International Workshop (WS). Entries in tables signify cytotoxicity scores as follows: 8=80%–100% lysis; 6=60%–80%; 4=40%–60%; 2=20%–40%; 1=20% or less. For interpretation of data, scores of 8, 6, and 4 were defined as positive and scores of 2 and below as negative reactions. NT, not tested.

Table 3. Tail analysis of correlations of microcytotoxicity indices of Bw4 mAbs with HLA class I specificities.

Panel	mAb	*HLA* haplotype	+/+	+/−	−/+	−/−	N	R
LCL	TT4	*Bw4*	39	3	1	59	102	0.92
		A23	1	2	0	59	62	0.57
		A25	1	1	0	59	61	0.70
	63	*Bw4*	29	3	5	46	83	0.80
		A23	1	2	0	46	49	0.57
		B8	1	1	3	43	48	0.31
	186 and 367	*Bw4*	34	8	0	40	82	0.82
		A9	3	5	3	37	48	0.34
		A25	1	4	0	37	42	0.42
		B8	2	2	2	35	41	0.45
PBL	TT4	*Bw4*	77	19	5	51	152	0.69
		A25	6	13	0	51	70	0.50
		A23	5	8	1	50	64	0.50
		A32	1	5	0	50	56	0.39
		Aw36	1	4	0	50	55	0.43

The cytotoxicity scores were analyzed by a theorem developed by J. D'Amaro (University of Leiden, The Netherlands). +/+, number of positive reactions of mAb on antigen-positive test cells; +/−, number of positive reactions of mAb on antigen-negative test cells; −/+, number of negative reactions of mAb on antigen-positive test cells; −/−, number of negative reactions on antigen-negative test cells. N, total number of cells tested; R, correlation coefficient calculated by the formula $R = \sqrt{\chi^2/N}$. The probability values were 1×10^{-3} or smaller and were omitted for simplicity. LCL, homozygous test cell panel of Table 2; PBL, raw data were obtained from 152 randomly selected PBL samples.

Table 4. Immunofluorescence analysis of mAbs on mouse cell HLA transfectants.

HLA transfectant		mAb designation										
HLA specificity	*HLA* allele	TT4	63	186	5G2	22E1	17A10	8A3	5C12	15C3	3H5	TT7
A2/hβ2M	*A*0201*	−	−	−	−	−	−	−	−	−	−	−[≠]
A3	*A*0301*	−	−	−	−	−	−	−	−	−	−	−
A24	*A*2401*	−	−	−	−	+	+	−	−	−	−	−
A26	*A*2601*	−	−	−	−	−	−	−	−	−	−	−
A29.1	*A*2901*	−	−	−	−	−	−	−	−	−	−	−
A33	*A*3301*	−	−	−	−	−	−	−	−	−	−	−
B7(w6)	*B*0702*	−	−	−	+	−	−	+	+	−	+	−
B8(w6)	*B*0801*	−	−	−	−	−	−	+	−	−	−	−
B13(w4)	*B*1301*	+	−	+	−	−	−	−	−	−	−	−
B14(w6)	N/A	−	−	−	−	−	−	+	−	+	−	−
B27/hβ2M (w4)	*B*2702*	+	+	+	+	+	+	+	+	+	+	−
Bw47(w4)	*Bw*4701*	+	+	+	−	+	+	+	−	+	−	+
B38(w4)	N/A	+[†]	−	+[†]	−	+[†]	+[†]	+	−	+	−	−
B39(w6)	N/A	−	−	−	−	−	−	+	−	+	+	−
B51(w4)	N/A	+	−	+	−	+	+	+	−	+	−	−
Cw1/hβ2M	*Cw*0101*	−	−	−	−	−	−	+	−	−	+	−
Cw3	*Cw*0301*	−	−	−	−	−	−	+	−	−	−	−[≠]
Cw5	*Cw*0501*	−	−	−	−	−	−	−	−	−	−	−
Cw6/hβ2M	*Cw*0601*	−	−	−	−	−	−	+	−	−	−	−
CwBL	*Cw*1301*	−	−	−	−	−	−	+	−	−	−	−[≠]

HLA transfectants with genes indicated (Bodmer et al. 1990) represent either Bc20 or L-cell fibroblasts stably expressing class I antigens in combination with mouse β2M. Exceptions with human β2M are indicated. L-cell transfectants expresing B14, B38, B39, and B51 were obtained from C. Müller (University of Tübingen, Federal Republic of Germany) and the A26 transfectant was from P. Pascale (Hopital St. Louis, Paris, France). The *Cw5* and *CwBL* genes were obtained from T. Delovitch (University of Toronto, Canada; Tibensky et al. 1989). Positive and negative reactions were scored by fluorescence microscopy. N/A, not applicable.

[†] Partial positive reactions on a subpopulation of transfectants.

[≠] Data obtained by cytotoxicity assay.

Attempts to corroborate the assignments to HLA-B by immunoprecipitation were unsuccessful.

Immunizations of HLA transgenic mice with Bc20 HLA transfectants within the B locus. To avoid tumor formation, B6Tg*(HLA-B*1302)* mice were immunized with osmotically lysed Bc20.B27.hβ2M cells. The fusion of spleen cells of one mouse selected for high serum HLA antibody yielded nine mAbs with specificity for various *HLA-B* alleles, and for that reason these were analyzed in detail. Testing on the homozygous LCL panel revealed four patterns; a group of six mAbs (see Table 1), exemplified by mAb 5G2, were identical and the hybridomas are possibly siblings. The principal correlations calculated from the reactivity pattern on LCLs of Table 2 are with B27, B7, and A28 (Aw68; see Table 5). The reactions of the 5G2 group of mAbs with B7 and B27 were confirmed by immunofluorescence analysis on Bc20 and L-cell HLA transfectants (see Table 4), but since an HLA-A28 transfectant is unavailable, we could not verify reactivity with A28. However, A28 is not in linkage disequilibrium with B7 or B27, and the reaction with A28 is probably real. The results of PBL panel testing were in good agreement with those of LCL testing (see Table 5). They revealed the additional reactivity with Bw22, Bw42, and Bw57. The LCL panel indeed signalled positive reactions with Bw22 and Bw42, but with only one cell line for each these scores were not deemed significant. On the other hand, Bw57 is represented four times on the LCL panel, and all four cell lines were negative. The reason for this discrepancy with PBLs is not known. MAb 5G2 immunoprecipitated B7 as well as B27 antigens as verified by an analysis of the precipitates by one-dimensional isoelectric focusing electrophoresis (data not shown). Immunoprecipitation of HLA-A28 was attempted but failed.

The second reaction pattern is provided by mAb 22E1. It does not show a strong correlation with any particular allele, indicating that the epitope in question is widely shared (see Tables 2 and 5). The highest correlation is with Bw4, reminiscent of the Bw4-related pattern generated by mAb 63 (see Tables 2 and 3), with extra reactivities to A24 and A25. The extra reactivity of 22E1 with A24 was confirmed by immunofluorescence analysis with the Bc20.A24 transfectant (see Table 4). The broad Bw4 reactivity, inclusive of A24, is well known for other Bw4 antisera and mAbs (Arnaiz-Villena et al. 1989). MAb

Table 5. Tail analysis of correlations of microcytotoxicity indices with HLA class I specificities.

Panel	mAb	*HLA* haplotype	+/+	+/−	−/+	−/−	N	R
LCL	5G2	*B7*	9	7	0	85	101	0.72
		B27	4	3	0	85	92	0.74
		A28	2	1	0	85	88	0.81
		Aw68	1	2	0	85	88	0.57
PBL	5G2	*B7*	33	21	3	95	152	0.65
		Bw22	7	14	2	93	116	0.45
		B27	4	10	0	93	107	0.51
		Bw57	4	6	4	89	103	0.39
		Bw42	1	5	0	89	95	0.40
LCL	22E1	*Bw4*	35	10	4	52	101	0.72
		A9	8	2	0	52	62	0.88
		A24	7	3	0	52	62	0.81
		A25	1	1	0	52	54	0.70
PBL	22E1	*Bw4*	76	33	6	37	152	0.50
		A9	18	15	2	35	70	0.54
		A24	14	19	0	37	70	0.53
		A25	4	11	0	35	50	0.45
		A32	1	8	0	35	44	0.30
		Aw36	1	7	0	35	43	0.32
LCL	5C12	*B7*	9	25	0	67	101	0.44
		B14	7	18	0	67	92	0.47
		Cw2	4	11	0	52	67	0.47
		B17	4	10	0	67	81	0.50
		Cw1	4	3	1	51	59	0.64
		A28	1	5	0	66	72	0.39
		Bw63	1	4	0	66	71	0.43
		B21	1	3	0	66	70	0.49
		B16	2	1	2	64	69	0.56
PBL	5C12	*B7*	33	21	3	95	152	0.65
		Bw22	8	13	1	94	116	0.53
		B27	4	9	0	94	107	0.53
LCL	17A10	*Bw4*	35	10	4	52	101	0.72
		A9	8	2	0	52	62	0.88
		A24	7	3	0	52	62	0.81
		A25	1	1	0	52	54	0.70
PBL	17A10	*Bw4*	73	30	9	40	152	0.49
		A9	17	13	3	37	70	0.54
		A24	13	17	1	39	70	0.51
		A25	4	9	0	37	50	0.50
		A32	1	6	0	37	44	0.35
		Aw36	1	5	0	37	43	0.38
LCL	3H5	*B7*	9	29	0	63	101	0,40
		Bw62	9	20	1	62	92	0.44
		A28	2	13	0	62	77	0.33
		Cw1	5	12	0	47	64	0.48
		B17	3	10	1	61	75	0.36
		A23	1	7	0	60	68	0.33
		B27	4	34	0	63	101	0.26
PBL	3H5	*B7*	34	29	2	87	152	0.60
		Bw22	8	21	1	86	116	0.43
		B27	4	17	0	86	107	0.40
		B39	4	13	2	84	103	0.34
		Cw1	2	11	1	83	97	0.28
		Bw42	1	10	0	83	94	0.28
		Bw46	1	9	0	83	93	0.30
LCL	TT7	*Bw60*	7	4	0	90	101	0.78
		Bw47	1	3	0	90	94	0.49

For explanation of entries, see legend to Table 3.

22E1 is also closely related to mAb TT4, but the following two differences are worth noting: 22E1 does not react with the Bw4 epitope of the *B13* allele (owing to the fact that this mAb is derived from a B6Tg*(HLA-B*1302)* mouse); mAb TT4 consistently missed A24 whereas mAb 22E1 did not. The converse was true for A23, which was seen by mAb TT4 but missed by mAb 22E1.

MAb 5C12 created the third distinct pattern. The appearance from the typing data on the homozygous LCL panel is that of a broadly cross-reactive mAb, with the most frequent reactivities with B7, B27, Bw22, B14, Cw2, B17, and Cwl (Tables 2 and 5). Analysis on the PBL panel supported B7, Bw22, and B27, but did not concur with B14, B17, and Cw2 reactivities. Analysis on the transfectant panel (Table 4) allowed a more narrow interpretation, eliminating the B14 and Cwl reactivities. A decision on Cw2 could not be made since no Cw2 transfectant is available. Cw2 reactivity is unlikely to be correct since Cw2 is in linkage disequilibrium with Bw61, and reactivity occurred with Bw61-expressing cells. Therefore the observed cross-reactivity could actually involve Bw61. Once again a clarification by transfectants was not possible due to the lack of a respective transfectant in the panel.

The last pattern, generated by mAb 8A3, is remarkable in that it encompasses all *B*-locus alleles, except *B13*. For this reason, no useful positive correlations were obtained by testing on LCLs and PBLs, although the negative correlation with *B13* was perfect. On the other hand, the analysis on the HLA transfectants was informative, showing additional reactivities with *Cw1*, *Cw3*, and *Cw6*, but not with any *HLA-A* allele (see Table 4); thus, mAb 8A3 sees an epitope shared by most *B* alleles and some *C* alleles.

*Immunizations of B6Tg(HLA-B*1302) mice with human lymphoblastoid cells.* Since human homozygous LCLs from the procurement standpoint are a more convenient source of immunogen than the HLA transfectants, we attempted to demonstrate that these, too, give rise to allo-HLA antibodies in HLA transgenic mice. B6Tg*(HLA-B *1302)* mice were immunized with the HOM-2 cell line (A3, B27, Cwl) and hybridomas prepared from one mouse selected on the basis of the highest serum titer. Screening on HOM-2 cells, eliminating cultures whose supernatants reacted with BH (A2, B13, Cw6), and then confirming B27 reactivity with the Bc20.B27 transfectant yielded six mAbs with HLA specificity (see Table 1). Screening with HLA-A3 or HLA-Cw1 transfectants was not performed, and therefore other HLA mAbs may have been missed. Of the six mAbs found, four show identical reactivity patterns, and they are presumed to be siblings. This pattern, exemplified by mAb 17A10, is highly correlated with Bw4, including A24, and also is identical with the one generated by mAb 22E1 (discussed above) on LCLs and HLA transfectants (see Tables 2 and 5).

The fifth mAb, 15C3, according to the reactivity pattern with transfectants, showed specificity for several *B*-locus alleles. The reactivity pattern on the homozygous LCLs and PBLs was too broad to allow the calculation of correlations with known specificities. It is significant that 15C3 did not react with B13-positive cells. Sequence comparisons identified a region of the HLA-B molecule around residue 103 which correlated with 15C3-positive and negative reaction. Site-directed mutagenesis of that site to convert 15C3-negative to a positive phenotype by exchange of 103-Leu to Val is planned.

The sixth mAb, 3H5, produced a pattern distinct from those of the two preceding mAbs, with the principal specificities B7, B27, B15 (Bw62), and Cw1. Tests on the LCL panel (see Tables 2 and 5) showed that 3H5 reacted with all nine B7 cell lines represented, all B27 cell lines, and nine of ten Bw62 cell lines. Two Cw1-positive cell lines that are neither B7, B27, nor Bw62 were also positive. B7, B27, and Cw1 reactivities were confirmed on the HLA transfectant panel, but neither B15 nor its split specificity Bw62 could be confirmed since the respective transfectants are unavailable. When tested on PBLs, an additional reactivity with Bw22 was seen with high frequency (eight of nine cells). Bw22 was probably overlooked in the LCL analysis because this specificity is represented only once. The respective cell line, however, was positive. In summary, 3H5 is a broadly reactive mAb, detecting an epitope shared among the three *HLA-B* alleles, *B7*, *B27*, *B15* (*Bw62*), and cross-reactive to *Cw1*. Immunoprecipitations and one-dimensional isoelectric focusing electrophoresis were carried out with ^{35}S-labeled lysates of HOM-2 cells. MAb 3H5 generated a band pattern characteristic of the *B27* allele expressed in HOM-2 cells (data not shown), but an attempt to precipitate Bw62 was unsuccessful.

Immunizations across a narrow allelic difference. B6 Tg*(HLA-B*1302)* mice were immunized with the Bc20.Bw47 transfectant. The single antibody obtained, mAb TT7, was positive on *Bw47* and *Bw60* but negative on all other *HLA-B* alleles and isotypes (Tables 2 and 5). Reactivity with Bw47 in the LCL panel was uncertain, since Bw47 is represented by only one cell, PLH, but the Bc20.Bw47 transfectant reacted specifically.

Discussion

Immunizations of HLA transgenic mice with live Bc20 HLA transfectants in most cases gave rise to serum HLA alloantibodies, showing that in principle the immunization system is applicable. It fulfills the premise that responder mice, self-tolerant to HLA, can respond to different *HLA*

alleles without giving a dominant response to monomorphic epitopes. However, when using live Bc20 transfectants there is a high incidence of tumors at the local injection site, with metastasis to spleen. When analyzed by FACS, the majority of the tumors were HLA-negative, despite the fact that the Bc20 transfectants were cloned repeatedly. It is likely that under the selective pressure of an immune response, HLA-negative variant tumors arise. They escape immunosurveillance because the ancestral Bc20 fibrosarcoma originated in a C57BL/6 mouse and is therefore fully histocompatible with our HLA transgenic mice. This trait is of course desirable to avoid antibodies to mouse antigens. When spleen cells were used for hybridoma fusions despite the presence of tumors, the resultant mAbs were predominantly of IgM isotype and displayed low affinity (data not shown). It is conceivable that a large tumor burden exerts a suppressive influence that prevents maturation of the humoral immune response. Evidently the immunization scheme with live Bc20 transfectants is risky. Nevertheless, some useful mAbs were obtained from immunizations of B6Tg*(HLA-A *0201)* and B6Tg*(HLA-Cw*0301)* mice with live cells of the doubly transfected cell line Bc20.*HLA-B27*.hβ2M.

To prevent the formation of tumors, we used osmotically lysed Bc20 HLA transfectants as immunogen, and had success. Not only did we obtain high HLA antibody titers in serum following four to six immunizations, but the quality of antibodies was improved with regard to higher proportions of IgG antibodies and to higher affinities. Previous immunization schemes were plagued by the low affinities of HLA mAbs that frequently reacted in LCL cells but failed to lyse PBLs in cytotoxicity assays, and also failed to immunoprecipitate class I antigen (Hämmerling et al. 1990). With the improved immunization scheme, the proportion of mAbs reacting with satisfactory strength with PBLs and precipitating HLA is substantially higher. Since these "good" mAbs are usually of IgG isotype, it appears that the immunization scheme with repeated booster injections of osmotically lysed Bc20 HLA transfectants promotes affinity maturation and class switch.

We also explored the use of whole human cells as immunogen. With a large number of well-characterized LCLs available, these cells would constitute a more convenient source of immunogen than the somewhat laboriously prepared Bc20 transfectants. Their use would come at the expense of numerous antibodies to human cellular antigens; however, since simple screening systems can be devised to distinguish HLA-specific allo-mAbs from human-specific hetero-mAbs this complication is acceptable. With the human homozygous LCL HOM-2, for instance, B6Tg*(HLA-B*1302)* mice produced a complex mixture of antibodies, but upon dual screening of mAbs with HOM-2 (B27) and BH (B13) and elimination of all those reactive with both, but retention of the HOM-2-specific ones, we still garnered six mAbs from one mouse that proved to have various HLA-B specificities. Thus, as was the case with immunization with Bc20 HLA transfectants and the previously reported HLA transgenic mouse tissues (Hämmerling et al. 1990), the responder HLA transgenic mice were tolerant to the expressed HLA-B13 antigen, but were capable of responding to the allelic B27 antigen. We did not pursue the question of whether antibodies to *A*or *C* locus-encoded antigens were directed at polymorphic or monomorphic epitopes and thus cannot comment from these experiments on whether B13 cross-tolerizes to HLA-A and -C monomorphic antigens. However, in experiments reported in this paper as well as in a previous one (Hämmerling et al. 1990), HLA-Cw3 expression in mice cross-tolerized effectively for HLA-B monomorphic sites, and HLA-B7 for -C monomorphic sites.

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