EVIDENCE FOR A NEW SEGREGANT SERIES OF B CELL ANTIGENS THAT ARE ENCODED IN THE HLA-D REGION AND THAT STIMULATE SECONDARY ALLOGENEIC PROLIFERATIVE AND CYTOTOXIC RESPONSES

By STEPHEN SHAW, ARMEAD H. JOHNSON, and GENE M. SHEARER

From the Metabolism and Immunology Branches, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205; and the Division of Immunology, Duke University Medical School, Durham, North Carolina 27710

Gene products of the major histocompatibility complex (MHC)[1] are important in many, if not all, cell-cell interactions involving immune responses of T cells (1). These MHC antigens can be classified into two kinds of molecules based on functional and structural considerations (2). Class I molecules (e.g., H-2K and HLA-A) are composed of two chains of 44,000 and 12,000 mol wt; they are expressed on virtually all cells and are the predominant antigens recognized by cytotoxic T cells. Class II molecules (e.g., H-2IA and HLA-DR) are composed of two chains of ~34,000 and ~28,000 mol wt; they are preferentially expressed on B cells and macrophages and are the predominant antigens that stimulate T cell proliferation. There is extraordinary diversity of both the class I and class II molecules, which generally are expressed on a single donor's cells. This has apparently been achieved during the course of evolution by two distinct mechanisms: gene duplication, and generation of polymorphism for each of those duplicated genes (3, 4). Thus, for example, in humans, the HLA-A, -B, and -C loci each code for class I molecules, and there are at least seven alleles for each locus; so that many donors have six distinct class I molecules. It is difficult to explain this evolutionary acquisition of a diverse repertoire of structurally similar cell surface antigens by random drift. Instead, it is attractive to hypothesize that because these antigens are involved in immune recognition, the possession of such diverse self structures confers on the individual a better immune response to a wide variety of foreign antigens (5, 6).

Polymorphism has been shown, and gene duplication suggested, for class I molecules in man (HLA-A, -B, and -C), for class I molecules in the mouse (H-2K, -D, and -L), and for class II molecules in the mouse (H-2IA and H-2E/C) (4, 7, 8). In contrast, for class II molecules in the human, the major emphasis has been on a single segregant series of B cell alloantigens: HLA-DR. Recently, serologic (9–12) and structural (13, 14) evidence have suggested the existence of several distinct kinds of human B cell antigens. This study provides strong functional evidence for two distinct segregant

[1] *Abbreviations used in this paper:* complete medium, RPMI-1640 medium with glutamine that was supplemented with penicillin (10 U/ml) and streptomycin (10 μg/ml); CML, cell-mediated cytotoxicity; MHC, major histocompatibility complex; PHA, phytohemagglutinin; PLT, primed lymphocyte typing; SB, secondary B cell.

series of B cell alloantigens. Allogeneic mixed lymphocyte responses have been exploited to identify five new histocompatibility antigens. These determinants, which we have designated secondary B cell (SB) antigens, are distinct from the DR antigens; however, both the SB and the DR antigens are encoded in the same region of the MHC, both are preferentially expressed on B cells, and both stimulate secondary allogeneic proliferative and cytotoxic responses. These data are consistent with the interpretation that the SB antigens and DR antigens are products of duplicated ancestral genes.

Materials and Methods

Human Reagents. Human peripheral blood mononuclear cells were obtained by batch leukapheresis, separated by flotation on Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) -Hypaque (Winthrop Laboratories, New York), and cryopreserved as previously described (15). Plasma from five or more normal males was pooled, frozen in aliquots at −20°C, and used as a normal plasma pool. Serotyping and HLA-D typing were performed by standard procedures (16).

Lymphocyte Priming. Responder and stimulator cells were thawed, washed, and resuspended in RPMI-1640 medium with glutamine (Grand Island Biological Co., Grand Island, N. Y.) that was supplemented with penicillin (10 U/ml) and streptomycin (10 μg/ml) (complete medium). 6×10^6 responding cells were mixed with 3×10^6 irradiated (2,000 rad; cesium source) stimulator cells in a 25-cm^2 tissue culture flask in 8 ml of complete medium supplemented with 0.5 ml of normal human plasma. The flask was incubated upright in 5% CO_2-95% air at 37°C for 10 d. Primed cells were either used fresh or after cryopreservation.

Primed-Lymphocyte Typing (PLT). 2×10^4 primed cells were cocultured with 1×10^5 irradiated stimulator cells in 0.2 ml of complete media with 15% heat-inactivated human plasma. After 48 h, 1 μCi of [^{3}H]thymidine was added, the cells were harvested 18 h later, and [^{3}H]thymidine incorporation was evaluated by counting beta emissions.

Cell-mediated Cytotoxicity. 3×10^6 primed cells were cocultured with 10×10^6 irradiated stimulator cells in 6 ml of complete media with 15% heat-inactivated human plasma in 25-cm^2 tissue culture flasks for 5 d. The target cells and blocking cells used were either: (*a*) lymphoblastoid B cell lines that had been transformed with Epstein-Barr virus (17), or (*b*) peripheral blood mononuclear cells that had been precultured for 3 d in complete medium with 1:200 dilution of phytohemagglutinin (PHA) (M form; Grand Island Biological Co.)—these cells were generally >90% T cells by analysis of E-rosette binding, surface immunoglobulin, and esterase staining and are therefore referred to as T lymphoblasts. Targets were labeled by a 90-min incubation with Na[^{51}Cr]O_4 (New England Nuclear, Boston, Mass.). Cytotoxicity was determined in a standard 6-h chromium-release assay (15) using 1×10^4 target cells and effector:target ratios of 20:1 and 7:1. For cold-target-cell inhibition assays, effector cells and unlabeled blocking cells were preincubated for 10 min before the addition of the labeled targets; the effector:target cell ratio used was 20:1 and the blocker:target cell ratios were 60:1 and 20:1.

Data Analysis. Proliferative data were calculated as the geometric mean and standard error. Data were expressed as percent relative response. For primary proliferative responses, the percent relative response was calculated as [(counts per minute for experimental)/(counts per minute for a pool of 10 allogeneic cells)] × 100. For secondary proliferation, the percent relative response was calculated as [(counts per minute for unknown restimulating cell)/(counts per minute for the specific restimulating cell)] × 100. The geometric SE for this ratio was <1.2 for all data shown. For cytotoxic assays, percent specific lysis was calculated as [(counts per minute experimental) − (counts per minute media)]/[(counts per minute detergent) − (counts per minute media)]. Spontaneous release was always <18% for B cells and <27% for T lymphoblasts. The arithmetic SE for percent specific release never exceeded 5%. To assure objectivity in scoring positives and negatives in proliferative and cytotoxic assays, assignment was made by cluster analysis (18). This analysis tests all possible assignments of donors as positive and

TABLE I
HLA Phenotype of Principal Donors Studied

Donor	HLA-						
	A	B	C	D	DR	MB	SB
H9	1,2	7,8	−*	w2,3	w2,3	1,2	1,2
M14	1,2	7,8	−	w2,3	w2,3	1,2	3,4
PM1	1,2	7,8	−	w2,3	w2,3	1,2	2,4
S11	1,2	7,8	−	w2,3	w2,3	1,2	2,5
W7	1,2	7,8	−	w2,3	w2,3	1,2	1,4
R5	1,2	7,8	−	w3	w2,3	ND‡	2,3
B17	1,2	7,8	−	w2,4	w2,4	1,3	2,4
F2	1,2	7,8	−	w2,4	w2,4	1,3	4,5
L10	1,2	7,8	−	w2,2	w2	1	4
P6	1,2	7,8	3	ND	w2,5	ND	4
H7	1,2	7,8	−	ND	w3,?6	ND	4
H10	1,2	7,8	−	ND	w3	ND	4
K4	1,2	7,8	−	ND	w1,3	2,?1	1
F1	1,2	7,8	−	ND	w4,6	?3	2,4
FB6	25,31	15,40	3	w4,-	w4,4	3,?2	1,-
FB11	25,31	15,40	3	w4,-	w4,4	3,?2	4,-

* −, indicates that no specificity was identified (blank); for donors FB6 and FB11, haplotype assignment was made by family study, and therefore blanks could be assigned in presence of another defined specificity.
‡ ND, not done.

negative and chooses the assignment that minimizes the variability within the positive and negative clusters.

Results

In order to use cellular responses to define new histocompatibility antigens, responder lymphocytes were primed in vitro with stimulator cells with which they were matched for all known HLA antigens (A, B, C, D, DR, and MB). Table I summarizes the HLA phenotype of the principal donors used: 14 individuals of phenotype A1,2,B7,8 and two siblings from an informative HLA recombinant family (19). HLA-A, -B, and -C, and DR antigens were analyzed by serotyping: MB antigens were also assigned by serotyping, as proposed by Duquesnoy et al. (12). Assignment of HLA-D was by primary proliferative responses to homozygous typing cells. Assignment of SB antigens is based on studies described in this report and included here as a summary.

Definition of Five New Antigens That Stimulate Secondary Proliferative Responses. Among the A1,2,B7,8 donors, 22 different combinations of responders and stimulators were identified in which the cells also shared D, DR, and MB. Lymphocytes were primed in vitro in all 22 combinations. As expected on the basis of HLA-D matching, there was little primary proliferative response in these combinations; the mean relative response in these pairs was 8% compared to a mean autologous response of 3%, and a mean response of 48% among unselected combinations of the A1,2,B7,8 donors. These 22 populations of primed lymphocytes were restimulated with cells from each of the 14 A1,2,B7,8 donors (10 such combinations are shown for a representative

TABLE II

Definition of Five New Alloantigens by PLT

Priming antigens	Primed cells	Percent relative response when restimulated with cells from donor														Total counts per minute* × 10^{-3}
		H9	M14	PM1	S11	W7	R5	B17	F2	L10	P6	H7	H10	K4	F1	
SB1	PM1 H9‡	100	38	3	20	120	45	6	12	4	6	5	2	112	5	17.2
		+§*	−	−	−	+	−	−	−	−	−	−	−	+	−	
	S11 H9	100	30	14	6	120	28	13	7	18	16	13	5	100	18	19.5
		+	−	−	−	+	−	−	−	−	−	−	−	+	−	
SB2	F2 B17	75	10	72	77	4	92	100	4	9	6	6	31	34	78	16.0
		+	−	+	+	−	+	+	−	−	−	−	−	−	+	
	W7 H9	100	8	88	100	5	120	110	6	8	10	8	56	40	100	19.8
		+	−	+	+	−	+	+	−	−	−	−	−	−	+	
SB3	PM1 M14	19	100	5	8	19	110	5	5	6	6	4	3	37	6	12.6
		−	+	−	−	−	+	−	−	−	−	−	−	−	−	
	W7 M14	8	100	9	10	5	100	13	6	8	7	5	5	33	11	15.3
		−	+	−	−	−	+	−	−	−	−	−	−	−	−	
SB4	H9 W7	11	120	90	20	100	15	110	71	120	94	94	38	40	91	10.2
		−	+	+	−	+	−	+	+	+	+	+	−	−	+	
	S11 PM1	16	120	100	16	120	31	130	91	120	120	120	73	28	110	10.8
		−	+	+	−	+	−	+	+	+	+	+	+	−	+	
SB5	H9 S11	5	22	13	100	14	17	22	64	19	11	19	7	23	15	26.8
		−	−	−	+	−	−	−	+	−	−	−	−	−	−	
	B17 F2	27	13	4	120	17	16	4	100	9	6	9	5	46	6	33.6
		−	−	−	+	−	−	−	+	−	−	−	−	−	−	
DRw1	H9 K4	3	5	3	7	2	23	12	6	3	5	6	4	100	9	15.2
		−	−	−	−	−	−	−	−	−	−	−	−	+	−	
DRw2	F1 B17	85	97	59	80	67	84	100	35‖	99	65	2	2	9	3	17.8
		+	+	+	+	+	+	+	−	+	+	−	−	−	−	
DRw3 (+ SB1)	F2 W7	90	85	69	79	100	99	7	3	8	6	103	55	112	26	13.8
		+	+	+	+	+	+	−	−	−	−	+	+	+	−	
Drw4 (+ SB1)	H9 B17	3	12	9	7	10	5	100	60	20	20	17	4	21	72	17.4
		−	−	−	−	−	−	+	+	−	−	−	−	−	+	
DR antigens known		2,3	2,3	2,3	2,3	2,3	2,3	2,4	2,4	2,2	2,5	3,26	3	1,3	4,6	
SB antigens assigned		1,2	3,4	2,4	2,5	1,4	2,3	2,4	4,5	4	4	4	(4)	1	2,4	

* Total counts per minute for the secondary response to the original priming cell.

‡ Designates the responding cells and the stimulating cells that were used to generate the primed cells.

§ Designates assignment as positive (+) or negative (−) stimulation by cluster analysis.

‖ Designates the only individual in whom the PLT assignment of DR did not correlate with the serotyping.

experiment in Table II). Despite the absence of strong proliferation during priming, many of the primed lymphocyte reagents showed strong specific restimulation (i.e., by the original stimulating, but not responding, cell). In fact, many of the primed cells were highly discriminatory reagents with a bimodal distribution of positive and negative responses, suggesting that they recognized predominantly a single antigen (or a cluster of highly associated antigens).

Analysis of the responses revealed that the specificity of all 22 reagents tested (in a total of 308 different responder-stimulator combinations) could be explained in terms of recognition of five distinct antigens (either singly or in pairs). Table II illustrates

that at least two different reagents could be identified which defined each of the five antigens, designated SB1 through SB5. Four lymphocyte populations that had been primed to DRw antigens were included as controls in this experiment. Assignment of positive and negative was made on the basis of cluster analysis to achieve objectivity in the scoring of results. However, the results were sufficiently clear that inspection would have been almost as effective in making assignments; in the experiment shown, 65 out of 67 of the positive assignments were made in combinations with relative responses ≥60%, and 126 out of 129 of the negative assignments were in combinations with relative responses ≤40%.

Each SB antigen was identified with two different PLT reagents; there was concordance between the results with the two different reagents in 69 out of the 70 pairs of SB typing results. 8 of the 14 donors could be unequivocally assigned two specificities; 6 of the 14 were assigned only one specificity. Furthermore, the results were internally consistent in that the specificity of the primed lymphocytes could be predicted on the basis of the SB antigen assignment of the original responder and stimulator cells (e.g., S11 anti-H9 is an SB2,5 anti-SB1,2; and, as predicted, recognizes only SB1).

Our results confirmed the reports of others (20–22) that DR antigens (or antigens very highly associated with them) can induce stimulation in secondary proliferative responses. The data in this experiment and others (data not shown) suggest that the single apparent disparity between serotyping and PLT (donor F2) reflects difficulty in scoring positives and negatives in PLT rather than a difference between the antigens recognized in PLT and those recognized serologically. Results with D/DR-discordant donors like R5 support the conclusion that the DR antigen itself can stimulate strong secondary proliferation.

Comparison of the assignments of SB antigens and DR antigens for each donor reveals that there is no consistent relationship between a donor's HLA-DR antigens and his SB antigens. The SB antigens are clearly not subsets (or splits) of the known D-region antigens, because it is impossible to make a consistent assignment of SB antigens as subgroups of Dw, DRw, or MB alleles. Furthermore, the finding that none of the five donors matched for A1,2,B7,8, and Dw/DRw2,3 are also identical for SB suggest that there may not even be a very strong linkage disequilibrium between HLA-DR and SB antigens.

The mean proliferative response for specific restimulation for the SB reagents was 16,900 cpm compared with a mean response of 15,900 cpm for the DR reagents. Thus, both series of antigens can induce a strong secondary proliferative response. It is noteworthy that when lymphocytes are primed concurrently to SB and DR, there often is a strong preferential response to the DR specificity, as illustrated by the DRw4-directed PLT (Table II); although this priming combination would also allow recognition of SB4, there is little if any response to SB4 by these primed cells. (Results of other experiments demonstrate that lymphocytes primed concurrently against a DR and SB specificity sometimes recognize both [S. Shaw, A. H. Johnson, and G. M. Shearer. Unpublished observations.].)

Failure to Find Alloantisera Identifying SB. Cells from the donors listed in Table I were analyzed with a wide variety of B cell antisera including the Eighth International Workshop (Los Angeles, 1980) genetic and disease sets plus 50 additional antisera on a local tray. No antisera were found whose pattern of reactivity correlated well with

TABLE III

The Gene(s) Encoding SB Antigens SB1, 3, and 4 Map Centromeric to HLA-B in Two HLA-B/D Recombinant Families

Priming antigens	Primed cells	Percent relative response when restimulated with cells from donor with haplotypes indicated											
		Family A					Family P						
		cd*	bc	bc/d	bd	ad	cd	ac	ac	ac	ac	a/bc	bc
SB1	S11 H9‡	107	62	3	6	3	12	108	103	86	111	16	15
		+§	+	−	−	−	−	+	+	+	+	−	−
SB2	F2 B17	3	39	64	75	1	26	12	13	9	19	13	10
		−	+	+	+	−	−	−	−	−	−	−	−
SB3	PM1 M14	2	1	1	3	2	13	13	9	8	11	84	63
		−	−	−	−	−	−	−	−	−	−	+	+
SB4	FB11 FB6	170	9	166	181	172	223	9	7	9	19	11	17
		+	−	+	+	+	+	−	−	−	−	−	−
SB5	H9 S11	17	3	5	11	5	21	11	8	5	11	17	14
		−	−	−	−	−	−	−	−	−	−	−	−

* Haplotype characteristics were as follows:

Family A.
- a A1-B7-DRw2-MB1-Dw2
- b A2-Bw51-DRw2-MB1-Dblank
- c Aw30-Bw44-DRw7-MB2-Dw7
- d Aw26-Bw38-Dub15-MBblank-DwBSK
- c/d Aw30-Bw44-Dub15-MBblank-DwBSK

Family P.
- a A1-B8-DRw3-Dw3
- b Aw24-Bblank-Dub15-Dblank
- c A2-B18-DRw2-Dw2
- d Aw23-B7-DRw6-Dblank
- a/b A1-B8-Dub15-Dblank

‡ Designates the responding cells and the stimulating cells that were used to generate the primed cells.
§ Designates assignment as positive (+) or negative (−) stimulation by cluster analysis.

the SB specificities assigned by PLT. More extensive screening of unselected antisera is in progress.

HLA-linkage Studies. To determine whether the SB antigens are coded by HLA-linked genes, segregation analysis was performed in seven families, and the results were consistent with HLA linkage in all families. Table III illustrates the findings in the two informative HLA-B/D recombinant families. The data from family A indicate segregation of SB1 with haplotype c, SB2 with haplotype b, and SB4 with haplotypes a and d; the HLA-B/D recombinant sibling has inherited the portion of the d haplotype centromeric to HLA-B and, with it, the SB4 marker from that haplotype (rather than the SB1 marker from the c haplotype). The data from family P. indicate segregation of SB1 with haplotype a, SB3 with haplotype b and SB4 with haplotype d. The recombinant acquired that portion of the b haplotype centromeric to HLA-B, and with it the SB3 marker of the b haplotype (rather than the SB1 marker of the a haplotype). These family studies indicate that SB antigens 1, 2, 3, and 4 are HLA linked and map the gene(s) encoding SB1, 3, 4 centromeric to HLA-B.

These family studies demonstrate two other features of the SB antigens. First, they confirm the conclusions from population studies that DR and SB are distinct antigens, because they demonstrate that the SB1 antigen segregates with different DRw antigens (DRw3 and DRw7) on different haplotypes. Furthermore, they demonstrate

TABLE IV

CML Against Two Distinct Series of B Cell Antigens

Priming antigens	Effectors	Percent lysis on B cell lines from donors with HLA-DRw serotype indicated												Percent lysis of stimulators' T lympho-blasts
		H9 2,3	M14 2,3	PM1 2,3	W7 2,3	R5 2,3	B17 2,4	F2 2,4	L10 2,2	P6 2,5	H7 3,26	K4 1,3	F1 4,6	
SB1	S11 anti-H9	40	7	4	50	8	2	5	5	1	3	27	1	7
		+*	−	−	+	−	−	−	−	−	−	+	−	
	FB6 anti-FB11	46	16	10	56	20	5	17	7	4	7	36	4	11
		+	−	−	+	−	−	−	−	−	−	+	−	
SB2	W7 anti-H9	33	3	51	3	37	34	3	4	0	3	5	48	4
		+	−	+	−	+	+	−	−	−	−	−	+	
	F2 anti-B17	45	10	64	14	40	46	6	12	5	6	10	55	14
		+	−	+	−	+	+	−	−	−	−	−	+	
SB3	W7 anti-M14	12	33	13	6	48	8	10	13	5	2	5	1	6
		−	+	−	−	+	−	−	−	−	−	−	−	
	PM1 anti-M14	8	44	1	5	56	2	4	3	0	2	6	0	6
		−	+	−	−	+	−	−	−	−	−	−	−	
SB4	FB11 anti-FB6	13	22	51	35	12	32	39	38	22	42	3	43	10
		−	−‡	+	+	−	+	+	+	−‡	+	−	+	
SB5	B17 anti-F2	10	8	2	11	4	4	52	3	3	2	7	5	11
		−	−	−	−	−	−	+	−	−	−	−	−	
DRw2	F1 anti-B17	53	42	58	75	50	53	57	57	33	5	4	3	15
		+	+	+	+	+	+	+	+	+	−	−	−	
DRw3 (+SB1)	F2 anti-W7	50	40	65	65	50	3	3	2	2	50	37	22	17
		+	+	+	+	+	−	−	−	−	+	+	−	

* Designates assignment as positive (+) or negative (−) cytotoxicity by cluster analysis.

‡ Designates the only combinations in whom the PLT assignment of SB did not correlate with the assignment by cytotoxicity.

that the proliferative response to SB is not restricted by DR antigens. The fact that cells of cd haplotype in family A. stimulate the S11 anti-H9 reagent, even though they share no DR antigens with the original stimulating cell, demonstrates that SB1 need not be recognized in conjunction with the DRw2 or DRw3 present on the original stimulating cell.

Evidence That SB Antigens Are Target Antigens for Cell-mediated Cytotoxicity (CML). We analyzed these antigens to determine if they can be recognized as target antigens in CML, using principally the same donors as in the proliferative studies. Cytotoxic activity was assayed in secondary responses on target cells from Epstein-Barr virus-transformed lymphoblastoid cell lines (Table IV). As controls, cytotoxic effector cells were also generated against DR differences. Assignment of positive and negative was made on the basis of cluster analysis, although the results were sufficiently clear that inspection would have been almost as effective in making assignments.

Cytotoxicity was generated in the same combinations of responder and stimulators that had generated SB-specific proliferation—i.e., those matched for HLA-A, -B, -C, -D, -DR, and MB. Killing by these effector cells differed among target cells that were identical for HLA-A, -B, -C, -D, -DR, and MB. The distribution of the CML antigens among the donors was similar to the distribution of the antigens defined by proliferation. Assignment of antigens by cytotoxic assay agreed with that by PLT in 58 out of 60 comparisons. If the two examples of apparent discordance between CML and

PLT (Table IV, footnote ‡) were consistent, that would suggest that the two assays detected different antigens; instead, the discordances were limited to the SB4 antigen (which is hardest to define) and were not confirmed by other experiments (Table IV; and S. Shaw, A. H. Johnson, and G. M. Shearer. Unpublished observations.).

There was a perfect correlation (24 out of 24) between the DR assignment of cells and their susceptibility to lysis by effector cells generated by DRw2-specific priming and DRw3-specific priming. This confirms previous reports (23–25) that HLA-DR antigens per se (or antigens highly associated with them) can be target antigens for allogeneic CML. Again, results relating to the D/DR-discordant donor R5 indicated that the DR antigen itself (in the absence of the concordant D specificity) might function as the cytotoxic target antigen.

Another feature of the cytotoxicity results is that the SB-specific killing is not restricted either by HLA-A, -B, and -C or by HLA-DR. This is evident in the typing for SB1 by CML reagents derived from two completely independent responder and stimulator cell combinations. The first five targets share all previously defined HLA antigens with the stimulator cell in the first combination (donor H9) but none of them with the stimulator cell in the second combination (donor FB6). The magnitude of the killing by these effector cells and their specificity are equivalent; therefore matching for A, B, C, D, DR, and MB is not necessary for SB-specific killing.

To estimate the expression of SB antigens on B cells relative to that on T cells, cytotoxicity was compared on B lymphoblastoid cell lines and on PHA-stimulated lymphoblasts (Table IV). Both DR-specific and SB-specific CML effector cells preferentially lysed the appropriate B cell lines. The low level of lysis of the T lymphoblasts by effectors of both specificities could be explained by the expression of Ia antigens on a small fraction of lymphoblasts 3 d after stimulation with PHA (26, 27) or by limited contamination of the mitogen-stimulated cells with B cells and macrophages. The poor lysis of the T lymphoblasts is not a result of their being insusceptible to lysis, because they were well lysed by control effector cells specific for HLA-A and -B antigens (data not shown). Confirmation that the SB antigens are B cell antigens is provided by studies showing that the SB-specific CML is inhibited by a monoclonal antiserum specific for an Ia-like molecule (S. Shaw and L. Lampson. Manuscript in preparation.).

Cold-Target Blocking Studies Confirm That Effectors Can Be Generated against Both DR and SB. CML effector cells were generated against individual DR or SB antigens and assayed on B cells of the stimulator cell donor. In a typical experiment (Table V), each such effector:target combination was assayed both unblocked and in the presence of blocking B cells from 12 donors. As in previous studies, the presence or absence of blocking was determined by cluster analysis, although inspection would have resulted in the same assignments. The results confirmed those observed in direct lysis. First, effector cells raised against DRw2 or DRw3 differences were inhibited by all blocking cells that had the appropriate DRw specificity and only by those cells. Second, effector cells raised against individual SB differences were inhibitable only by blocking cells that expressed the appropriate SB specificity (assigned by PLT); the concordance in assignment of antigen between direct killing and cold-target inhibition is complete and strengthens the evidence that the proliferation-inducing antigen and the CML antigen are either identical or in very strong linkage disequilibrium. The differences

TABLE V

Cold-Target Competition Confirms Two Distinct Series of Antigens

Priming antigens	Effectors	Percent lysis unblocked	Percent lysis when blocked with B cell lines from donors with HLA-DRw serotype indicated:											
			H9 2,3	M14 2,3	PM1 2,3	S11 2,3	W7 2,3	B17 2,4	F2 2,4	L10 2,2	P6 2,5	H7 3,26	K4 1,3	F1 4,6
SB1	S11 anti-H9	47	3	47	41	49	4	46	39	45	46	40	8	44
			+*	−	−	−	+	−	−	−	−	−	+	−
	FB6 anti-FB11	59	5	48	52	49	3	51	43	48	51	41	6	47
			+	−	−	−	+	−	−	−	−	−	+	−
SB2	W7 anti-H9	59	3	54	1	12	50	4	49	49	52	45	52	2
			+	−	+	+	−	+	−	−	−	−	−	+
	F2 anti-B17	60	6	49	7	18	43	1	38	43	43	35	46	5
			+	−	+	+	−	+	−	−	−	−	−	+
SB3	W7 anti-M14	57	61	8	53	54	48	56	50	51	52	43	56	51
			−	+	−	−	−	−	−	−	−	−	−	−
	PM1 anti-M14	65	62	5	67	70	68	70	56	64	62	58	65	65
			−	+	−	−	−	−	−	−	−	−	−	−
SB5	B17 anti-F2	66	65	66	61	20	64	65	4	63	60	60	68	67
			−	−	−	+	−	−	+	−	−	−	−	−
DRw2	F1 anti-B17	66	27	29	21	26	22	5	3	17	15	52	65	62
			+	+	+	+	+	+	+	+	+	−	−	−
DRw3 (+ SB1)	F2 anti-W7	59	19	23	17	30	18	60	56	54	55	16	20	52
			+	+	+	+	+	−	−	−	−	+	+	−

* Designates assignment as positive (+) or negative (−) blocking by cluster analysis.

in SB-specific blocking by cells that are DR matched and vice versa confirm the independence of these two antigens.

DR- and SB-specific Killing are Both Inhibited Better by B Cell Lines Than by T Lymphoblasts. To confirm that SB and DR antigens were similar with respect to greater expression on B cells than on T cells, B and T cells were compared in cold-target inhibition of DR- and SB-specific CML. Lymphoblastoid B cell lines from the stimulator inhibted SB- and DR-specific lysis almost completely, but blockers from the responder donor did not (Table VI). In contrast, PHA lymphoblasts from the stimulator donor blocked poorly, only a little better than did cells from the responder donor.

Analysis of SB-specific Killing in an HLA-Recombinant Family. SB-specific killing was analyzed in an unusual HLA-recombinant family, in which we first identified SB1 and SB4 in secondary proliferative responses (19). CML effector cells raised against SB1 and SB4 among donors of phenotype A1,2,B7,8 were assayed on lymphoblastoid B cell lines from five family members and from three control donors (Table VII). The data demonstrate two findings. First, the target antigens for CML, like the SB-proliferative antigens in this family (19), segregate with HLA-A and -B markers, except in the recombinant donor. SB1 segregates with the a haplotype, and SB4 segregates with the b and d haplotype; the recombinant, who has inherited the HLA-A and -B antigens from the b haplotype, has inherited the SB antigen from the a haplotype. Thus, the SB1 and 4 antigens map to a region of HLA outside of the HLA-A/B segment; although homozygosity for other HLA markers (C2,C4,DR,D,GLO) precludes precise mapping in this family, these cytotoxicity data

TABLE VI

Cold-Target Inhibition Studies Indicate Preferential Expression of SB and HLA-DR Antigens on B Cells

Priming antigen	Effector	Percent lysis unblocked	Percent lysis when blocked with: B cell lines autologous* to		T lymphoblasts autologous* to	
			Stimulator	Responder	Stimulator	Responder
SB1	S11 anti-H9	47	3	48	21	32
	FB6 anti-FB11	59	5	52	37	47
SB2	F2 anti-B17	60	1	37	36	46
	W7 anti-H9	59	3	50	27	46
SB3	W7 anti-M14	57	8	48	32	48
	PM1 anti-M14	65	5	67	42	58
SB5	B17 anti-F2	66	4	65	36	51
DRw2	F1 anti-B17	66	5	62	47	60
DRw3 (+ SB1)	F2 anti-W7	59	2	56	41	51

* Autologous except for FB6 which was substituted by B17 and FB11 which was substituted by H9.

TABLE VII

CML Studies of SB Antigens in an HLA Recombinant Family: HLA Linkage and Lack of Restriction by HLA-A and -B

Priming antigens	Effector	E:T* ratio	Percent lysis on B cell lines from donors: Unrelated			Family B				
			H9	L10	B17	ac‡	a/bc	bc	ad	bd
SB1	S11 anti-H9	40:1	23	4	2	40	47	11	31	9
		10:1	19	2	1	32	38	6	22	5
			+§	−	−	+	+	−	+	−
SB4	H9 anti-L10	40:1	3	62	49	6	9	66	46	63
		10:1	1	48	33	2	7	51	26	49
			−	+	+	−	−	+	+	+

* E:T, effector:target.

‡ Haplotype characteristics were as follows:

a A24-B7-DRw4
b A25-B15-DRw4
c A31-B40-DRw4
d A33-B17-DRw5
a/b A25-B15-DRw4 (recombination outside of A-B segment)

§ Designates assignment as positive (+) or negative (−) cytotoxicity by cluster analysis.

are consistent with the proliferative studies showing that the SB antigens are encoded by genes centromeric to HLA-B.

Second, these data confirm that HLA-A and -B do not restrict SB-specific killing. The responder and stimulator cells used to generate the CML effectors share no HLA-

A and -B antigens with the family members. Furthermore, the killing of control cells totally HLA-A- and -B-matched with the effectors is comparable to the killing of cells from HLA-A- and -B-mismatched family members, indicating that there is no preferential lysis attributable to HLA-A- and -B-matching.

Discussion

This study identifies five new HLA-linked antigens by secondary allogeneic proliferative responses. Each of these antigens can be assigned by at least two discriminatory PLT reagents with almost complete concordance between the typing results with the two reagents. This study also defines five antigens that can be detected by CML and that are preferentially expressed on B cells. The discussion will focus on the following issues: (*a*) What precedents are there in the literature for such SB antigens? (*b*) What is the relationship between the proliferation-inducing antigens and the CML antigens? (*c*) Are the SB antigens part of a single segregant series? (*d*) What is the relationship between the DR antigens and the SB antigens? (*e*) What may be the functional roles of the SB antigens in physiologic immune responses?

First, what other investigations suggest the existence of antigens similar to those described in this study? Since the development of techniques for secondary human allogeneic responses (28–30), it has become apparent that several different antigens stimulate secondary proliferative responses. Both D and DR antigens can independently induce secondary responses (20–22). Family studies suggest that antigens distinct from the D and DR antigens can be recognized by PLT (for an A/B recombinant family [31], a B/D recombinant family [32], and a D/GLO recombinant family [33]). In addition, several laboratories have demonstrated that priming between donors who are phenotypically identical for D/DR either in families, or between unrelated donors can result in generation of PLT reagents that appear to recognize HLA-linked antigens distinct from D and DR (22, 34, 35).

There is no direct precedent for the antigens we have defined by cytotoxic responses. There are CML antigens distinct from the known serologically defined HLA-A, -B, and -C antigens (36), but these have generally been found on T cell targets, and are thus probably distinct from the SB antigens (which are not well expressed on T cells). Studies of primary cytotoxic responses assayed on appropriate targets (monocytes [23], Epstein-Barr virus-transformed B cells [24], and mitogen-stimulated B lymphoblasts [25]) demonstrated CML specific for B cell alloantigens, which could be largely explained by reactivity with DRw antigens. The failure to detect SB antigens in these experiments probably reflects the fact that cytotoxic responses to SB antigens are seldom seen in primary sensitization (S. Shaw, A. H. Johnson, and G. M. Shearer. Unpublished observations.) and the DR-specific CML may overshadow SB-specific CML during concurrent sensitization (Tables IV and V, DRw3 reagent).

Serologic and structural studies provide evidence for two segregant series of B cell antigens. Two groups have recently reported isoelectric focusing studies indicating two similar but distinct species of polymorphic B cell antigens (13, 14). Serologic studies with alloantisera (9, 10) and monoclonal antisera (11) have also suggested more than one kind of human Ia molecule. Collaborative studies are in progress to test whether the SB antigens defined functionally in this report are the same as those being defined serologically by other laboratories.

Second, what is the relationship between the proliferation-inducing antigens and

the CML target antigens? The data suggest that they are identical antigens; however, the data do not exclude the possibility that they are distinct but very strongly associated. If proliferation-inducing antigens and CML antigens are distinct but linked, they might not be distinguishable in the A1,2,B7,8 donors because of the strong linkage disequilibrium of A1-B8-DRw3 and A2-B7-DRw2 haplotypes; however, studies of other donors (such as the family in Table VII and six other unrelated donors [data not shown]) demonstrate no convincing discordance. Because the data fail to demonstrate any differences, we make the working hypothesis that they are identical.

Third, are the SB antigens part of a single segregant series? Evidence in this report and to be reported subsequently suggests strongly that they comprise a single series. Family studies indicate that SB markers can be identified on 29 out of 45 haplotypes tested, with no haplotype having two markers. In populations studies, 129 SB antigens have been identified on 92 donors with no donor typing for more than 2 antigens. Although the data are still too limited to be tested meaningfully for Hardy-Weinberg equilibrium, they strongly suggest these antigens are part of a single segregant series. In addition, attempts to distinguish between the SB antigens in terms of tissue distribution and their function in allogeneic responses have failed to provide any evidence that they are different.

Fourth, what is the relationship between the SB antigens defined in this report, and the HLA-DR antigens? There are four similarities between these two series of antigens. First, both are coded in the same region of HLA—centromeric to HLA-B. Second, both are preferentially expressed on B cells rather than T cells. Third, both induce strong secondary proliferative responses in mixed lymphocyte culture. Fourth, both SB and DR appear to be target antigens for CML (although in both cases it cannot be ruled out that the target antigen is distinct and closely associated).

The only functional difference that we currently recognize between the function of DR and SB is quantitative not qualitative: DR-specific CML and D/DR-induced proliferation are regularly seen in primary responses, whereas SB-specific CML and proliferation is minimal in primary responses (S. Shaw. Unpublished observations.). The quantitative rather than qualitative differences in the responses to these functionally similar antigens can be explained by the hypotheses that the amount of the SB antigen present on the B cells is substantially less than the amount of DR, or that the size of the T cell repertoire that recognizes SB antigens is smaller than the repertoire that recognizes DR antigens.

Despite the functional similarities between the DR and SB antigens, they are clearly distinct. Population studies (Table II; and S. Shaw, A. H. Johnson, and G. H. Shearer. Manuscript in preparation.) establish that SB antigens are not invariably present with any particular DR antigen. Furthermore, family studies (such as the ones in Table III) demonstrate that a single SB antigen segregates with different DR antigens on different haplotypes. However, the molecular relationship between these antigens remains unclear. We favor the hypothesis that DR and SB are located on distinct molecules, but further studies will be required to test the alternate possibility that DR and SB are independent determinants on the same molecule.

Fifth, what may be the role of SB antigens in responses to modified-self antigens? This report demonstrates that the SB antigens are major histocompatibility antigens not only because they are encoded by MHC genes, but also by the functional criteria

that the proliferative and cytotoxic responses to the SB antigens are not restricted by HLA-DR or HLA-A, -B. The striking similarities between the SB and DR antigens suggest that these two segregant series of antigens are products of duplicated ancestral genes. We have already established that they function in qualitatively similar ways in allogeneic proliferative and cytotoxic responses. We expect that DR and SB antigens will be found to have functionally similar roles in responses to modified self antigens. Specifically, we anticipate that SB antigens will restrict clones of T cells proliferating in response to foreign antigen, as has been shown for DR antigens (37, 38). Furthermore, SB antigens will probably restrict certain clones of cytotoxic T cells responding to foreign antigen, as we have recently suggested for DR in CML specific for the hapten trinitrophenyl (39). We expect that in modified-self responses, as in allogeneic responses, the SB-restricted component will be smaller than the HLA-DR-restricted component. However, in selected responses, SB may be the dominant restriction antigen. Finally, SB antigens, like DR antigens (40), may prove to be important markers of disease risk, or even to be involved directly in the pathogenesis of certain diseases.

Summary

Five new histocompatibility antigens, designated secondary B cell or (SB) antigens, have been identified by secondary allogeneic proliferative and cytotoxic responses. The reagents used to define the SB antigens are lymphocytes primed between donors matched for all known HLA antigens. The SB antigens stimulate weak primary allogeneic proliferative responses (a mean relative response of 8%) but strong secondary proliferative responses. Strong secondary cell-mediated cytotoxicity is generated against target antigens that are indistinguishable from the SB antigens defined by proliferation. Studies by direct lysis and by cold-target inhibition indicate that these target antigens are preferentially expressed on B cells relative to T cells. The SB antigens segregate with HLA, and the gene(s) encoding the SB1, 3, and 4 antigens maps centromeric to HLA-B. The SB antigens are major histocompatibility antigens not only because they are encoded by major histocompatibility complex (MHC) genes, but also by the functional criteria that the proliferative and cytotoxic responses to SB antigens are not restricted by HLA-DR or HLA-A, -B. Parallel studies of the SB antigens and the DR antigens indicate that there are striking functional similarities between SB and DR antigens with respect to: (*a*) their preferential expression on B cells, (*b*) their function in secondary allogeneic proliferative and cytotoxic responses, and (*c*) the location of their structural gene within the MHC. However, the SB antigens and the DR antigens are clearly distinct antigens, because population studies indicate that they can occur independently, and family studies indicate that specific SB antigens segregate with HLA haplotypes having different D and DR specificities. Our data are consistent with the hypotheses that the SB antigens are a new segregant series of B cell alloantigens, and that the SB gene and the DR gene derive from a duplicated ancestral gene.

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