Isolation of Heavy Chain Class Switch Variants of a Monoclonal Anti-DC1 Hybridoma Cell Line: Effective Conversion of Noncytotoxic IgG_1 Antibodies to Cytotoxic IgG_2 Antibodies

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ABSTRACT: *Spontaneously arising class switch variants of the Genox 3.53 hybridoma cell line were isolated. They secrete IgG_{2a} or IgG_{2b} monoclonal antibodies of anti-DC1 specificity identical to that of the IgG_1 secreting parental cell. In contrast to the parental monclonal antibody, those secreted by the variants are cytotoxic to peripheral blood B lymphocytes of DC1 positive individuals and are thus compatible with existing HLA typing techniques. This provides a general method for converting noncytotoxic anti-HLA antibodies into cytotoxic typing reagents.*

ABBREVIATIONS

PBS	phosphate buffered saline pH 7.4	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
FACS	fluorescence activated cell sorter	FITC	fluorescein isothiocyanate
GAM1	goat anti-mouse IgG_1	PI	propidium iodide
GAM2	goat anti-mouse IgG_2		
RAM	$F(ab')_2$ rabbit anti-mouse IgG		

INTRODUCTION

Monoclonal antibodies are potentially useful reagents for HLA typing. In order to be compatible with existing typing methods they must mediate complement dependent cytotoxicity. Mouse immunoglobulins of the IgG_{2a} or the IgG_{2b} isotype do fix complement, and monoclonal anti-HLA antibodies of these classes are generally cytotoxic to human peripheral blood lymphocytes of the appropriate HLA type. However, many mouse monoclonal antibodies are of the IgG_1 isotype, an immunoglobulin class unable to fix complement. One way of making such

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antibodies cytotoxic is to use them in conjunction with a complement fixing anti-immunoglobulin reagent [1,2]. Another approach is to isolate spontaneous variants of hybridoma cells that secrete an immunoglobulin of different antibody heavy chain isotype [3] but have the same variable region and combining site specificity as the original antibody. Such "switch variant" hybridomas are obtained by staining a large number of parent hybridoma cells with fluorescent antibodies specific for the variant heavy chain isotype and then selecting for fluorescent cells with the fluorescence activated cell sorter (FACS). In this manner families of hybridomas have been generated, each producing immunoglobulins with identical combining sites but with different heavy chain constant regions [4,5].

Application of such switch variant technology may thus be useful for generating monoclonal antibodies for HLA typing from the noncytotoxic anti-HLA antibody producing hybridomas that have been characterized. Here we describe the successful application of this approach to the Genox 3.53 producing hybridoma cell line [6]. The Genox 3.53 antibody recognizes the polymorphic specificity DC1 associated with products of the second series of human class II MHC antigens. The DC1 antigenic specificity is alternatively called MB1 or MT1 by different laboratories. The DC1 antigen is often found on cells expressing the antigens DR-1,2, w6, and w10 of the first series of human class II MHC antigens. For this reason, it was initially thought that Genox 3.53 recognized a supertypic specificity of DR antigen [6]. However, structural characterization of the molecules recognized by this antibody have shown they are different from DR and have amino acid sequence homology with mouse I-A chains [7,8]. This antibody has been useful in establishing the existence and identity of the second series of human Ia-like antigens [7]. The availability of IgG_2 variants increases its utility both as a cytotoxic HLA typing reagent and as immunochemical tool. IgG_2 binds more strongly to Staphylococcus protein A than IgG_1 and thus the switch variants are more easily used in standard immunoprecipitation procedures and may be readily purified on columns of protein A.

MATERIALS AND METHODS

Preparation of goat anti-mouse IgG_1 and IgG_2. Individual goats were immunized with IgG_1, IgG_{2a}, and IgG_{2b} myeloma proteins: 50 ml of anti-IgG1 serum and a pool of anti-IgG_{2a} (50 ml) plus anti-IgG_{2b} (150 ml) serum were used as starting materials. The antisera were clarified by centrifugation at 100,000 g for 1 hr and the supernatant made 50% saturated in ammonium sulphate. After centrifugation at 6000 g for 30 min the supernatant was discarded and the pellet resuspended in 100 ml of PBS. These preparations were then applied to various affinity columns. Twenty ml affinity columns of myeloma proteins coupled to cyanogen bromide activated Sepharose 4B were used. Activation of Sepharose and coupling with purified IgG at a concentration of 10 mg/ml were by the method of March et al. [9]. The efficiency of coupling was $\simeq$ 50%.

Anti-IgG_2 (GAM2). The sample was applied to a column of IgG_1 Sepharose with a 1 ml precolumn of Sephadex G50 and washed through with PBS. The flow-through was saved, the G50 column discarded, and the IgG_1 column eluted with 50 ml of 50 mM diethylamine-HC1, pH 12.0 [10]. The eluted material was neutralized and frozen, the column was re-equilibrated with PBS. This procedure was repeated seven times with the flow-through fraction taken as sample for the next cycle. In this way the anti-IgG_2 serum was depleted of anti-IgG_1 cross-reactivity. In a similar fashion, anti-IgM cross-reactivity was depleted by two cycles over an IgM column. The next step was to apply the sample to a G50

precolumn followed by a mixed IgG_{2a}, IgG_{2b} column. The sample was washed through with PBS and the column then washed with 100 ml PBS, 50 ml PBS + 0.5 M NaCl, and 100 ml PBS. After elution with 50 mM diethylamine-HC1 pH 12.0 the previously bound material was neutralized with 0.5 M tris-HC1 pH 7.5 and dialyzed against PBS. The dialyzed material was centrifuged at 6,000 g for 30 min and the supernatant passed through an IgG_1 column to remove any residual cross-reactive material. The flow-through was concentrated to 1 mg/ml by ultrafiltration. The flow-through from the IgG_2 column was recycled three times over the IgG_2 columns to ensure complete removal of all anti-IgG_2 antibodies. The material eluted with pH 12.0 buffer was further processed as described above. Only the first two eluates gave significant amounts of protein. Sixteen mg of anti-IgG_2 was thus obtained.

Anti-IgG_1 (GAM1). The procedure was as for anti-IgG_2 except that the sample was depleted on IgG_2 and IgM columns and then positively selected for an IgG_1 column. Thirty-four mg of anti-IgG_1 was obtained.

Specificity of GAM1 and GAM2. The GAM1 and GAM2 were >90% IgG as assessed by SDS-PAGE. Fifty μg aliquots were iodinated and tested for binding to monoclonal antibodies of different isotypes in solid phase and cell binding assays. As shown in Figure 1 the cross-reactions of the anti-IgG_1 onto IgG_2 and of anti-IgG_2 onto IgG_1 were weak. Anti-IgG_2 reacted 2–3 times more strongly with IgG_{2b} than with IgG_{2a}. About 40% of the radioactivity in each preparation bound to IgG of the appropriate isotype.

Fluorescence conjugation. GAM1 and GAM2 were conjugated to fluorescein isothiocyanate (FITC) by a modification of the method of Goding [11]. The purified goat antibody preparation was applied to a Sephadex G-25 column equilibrated with 0.5 M carbonate buffer pH 9.5. Antibody fractions found in the excluded volume were pooled prior to reacting with FITC. Because of the relatively low antibody concentrations (0.6–1 mg/ml), FITC dissolved in dimethylsulfoxide (10 mg/ml) was added in excess at 140 μg FITC per milligram of protein. The reaction was allowed to proceed for 4 hr at room temperature with end-over-end shaking. The reaction was terminated by passing the mixture over a Sephadex G-25 column equilibrated with phosphate buffered saline at pH 7.4 (PBS). Antibody fractions in the excluded volume were again collected. Fluorochrome-protein conjugates

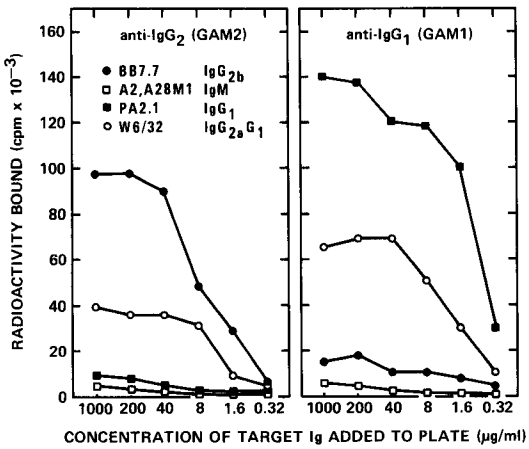


FIGURE 1 Specificity of ^{125}I-GAM1 and ^{125}I-GAM2 in solid phase radioimmune assay. Monoclonal antibodies of different isotype were adsorbed to wells of microtitre plates and then incubated with 3×10^5 cpm ($\simeq 10^7$ cpm/μg) of ^{125}I-GAM1 or ^{125}I-GAM2 for 1 hr at 4°C. The plates were washed, the wells separated and assayed for bound radioactivity. W6/32 was drived from a fusion with an IgG_1 secreting myeloma cell line and the anitbody preparation contains nonspecific IgG_1 heavy chain and κ light chain in addition to the W6/32 chains [25].

were analyzed spectrophotometrically at 280 and 493 nm and the fluorescence to protein conjugation ratios calculated using the formula and nomogram given in Goldman [12]. The fluorescence to protein ratios were ~ 2.8 for the FITC-GAM1 and ~ 3.4 for the FITC-GAM2 preparations. These reagents were titrated against mouse hybridoma cells of known isotype and analyzed for cell surface staining with a fluorescent activated cell sorter (FACS) (FACS II Becton Dickinson, Mountain View, CA).

Cell lines and culture conditions. The production of the Genox 3.53 cell line has been described [6]. It secretes an IgG_1 antibody with specificity for the DC1 allelic product of the second series of human class II MHC antigens. This specificity is alternatively known as MB1 and MT1. Monoclonal antibodies MRC OX3 [13] and BT3/4 [14] are of similar specificity. The hybridomas 190-59.2 and 197-24.63 used to test the isotype specificity of FITC-GAM1 and FITC-GAM2 secreted either IgG_1 or IgG_2 antibodies with specificity for the hapten dansyl (5-[dimethylamino]naphthalene-1-sulfonyl).

Hybridoma cells were cultured in RPM1-1640 medium (GIBCO) supplemented with 2 mM L-Glutamine, 1 mM sodium pyruvate, and 15% horse serum or 10% fetal calf serum at 37° in a 7% CO_2-in-air incubator. The medium for Genox 3.53 cells was also supplemented with 0.1 mM hypoxanthine and 1.6×10^{-2} mM thymidine. Elimination of these components resulted in slower cell growth, reduced viability, and poor cloning efficiency for this cell line.

Human B lymphoblastoid cells used as targets for indirect binding assays were LB which is DR6 and positive for DC1 and Mich which is DR5 and negative for DC1. The cell lines were grown in RPM1 1640 supplemented with 2 mM L-Glutamine and 10% fetal calf serum.

Radioimmune assays. Indirect cell binding assays were as described [15]. For solid phase radioimmune assays 25 μl of purified immunoglobulins (0–25 μg) on PBS were added to the wells of flexible microtitre plates and incubated on ice for 1 hr. The antibody was removed, the wells filled with 0.2% BSA in PBS, and incubated on ice for 1 hr. Radioiodinated GAM1, GAM2, or RAM (300,000 cpm in 25 μl) was added and incubated on ice for 1 hr. The plates were washed four times with 0.2% BSA in PBS and the wells separated with a hot wire cutting device and assayed for bound radioactivity. In experiments to distinguish IgG_{2a} from IgG_{2b} variants the monoclonal anti-mouse IgG allotype antibodies 8.3 (anti-Igh-1a) and 16.3 (anti-Igh-1a,3a) were used [16]. 8.3 has specificity for IgG_{2a} and 16.3 has specificity for both IgG_{2a} and IgG_{2b} of C3H mice from which Genox 3.53 was derived [6].

Fluorescent staining of cells. Cultured cells to be analyzed or sorted were centrifuged (200 × g for 4 min) and washed twice with staining media (RPM1 1640 medium supplemented with 10 mM HEPES and then adjusted to pH 7.5 with 5 M HC1) at 4°C. After resuspension in staining medium the cell concentration and viability were determined by staining an aliquot with a one part per million solution of acridine orange with ethidium bromide and counting the viable and dead cells in a hemacytometer. Viable cells comprised >95% of all cell suspensions that were analyzed or sorted. Cells to be stained were transferred to 12 × 75 plastic tubes (Falcon) and centrifuged at 200 × g for 4 min. Supernates were decanted and the cells resuspended in staining medium containing FITC-GAM1 or FITC-GAM2 and 0.3 μg/ml of propidium iodide (PI). FITC-GAM1 and FITC-GAM2 were titrated against a panel of monoclonal antibody producing hybridomas of different immunoglobulin isotypes at different concentrations and amounts

of antibody for a fixed number of hybridoma cells. Representative titrations of FITC-GAM2 are presented in Table 1. As can be seen both the concentration of the staining reagent as well as the absolute amount of antibody were important in determining the mean fluorescence of the stained cell populations. Similar results with FITC-GAM1 were also obtained. A concentration of 50 μg/ml of goat antibody for either isotype was chosen for subsequent stainings. Volumes were such that there was 1–1.5 μg of antibody per 10^6 cells. When staining to select for rare variant cells, FITC-GAM2 was used at 0.3 μg per 10^6 cells. Control cell samples were incubated with staining medium containing 0.3 μg/ml PI. Staining was for 20 min at 4°C. The cells were washed twice by centrifugation with

TABLE 1 Binding of FITC-GAM2 to Hybridoma Cells Secreting Anti-Dansyl IgG of Different Isotype

Hybrid cell line	Isotype	Antibody concentration (μg/ml)	Amount of antibody (μg/10^6 cells)	Mean fluoresence (arbitrary units)
190–59.2	IgG_{2a}	80	10	116
			3	113
			1	108
			0.3	95
		40	10	114
			3	112
			1	107
			0.3	92
		8	10	97
			3	96
			1	86
			0.3	75
			0.1	74
		No stain	—	57
197–24.63	IgG_1	80	10	67
			3	64
			1	61
			0.3	61
		40	10	66
			3	62
			1	61
			0.3	60
		8	10	62
			3	60
			1	61
			0.3	60
			0.1	59
		No stain	—	59

excess cold staining medium and resuspended at $\simeq 10^6$/ml for FACS analysis and sorting.

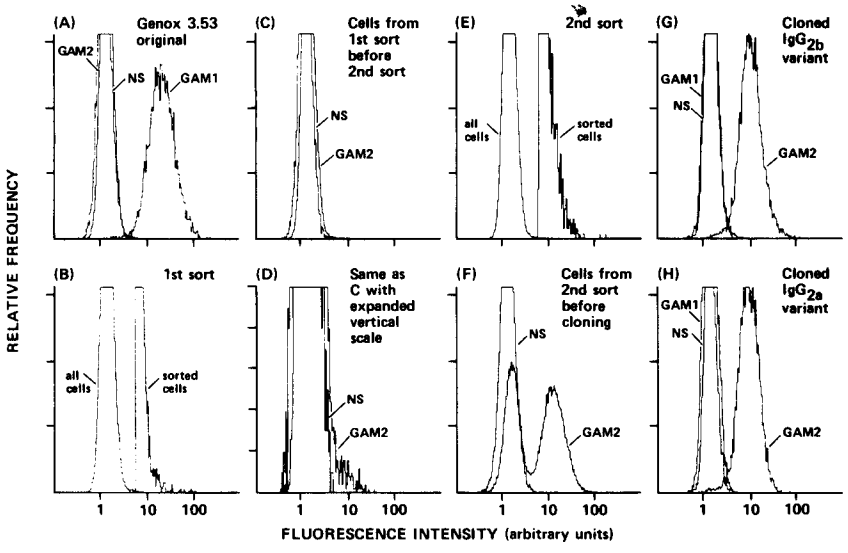
Culture and cloning of switch variant hybridoma cells. During the initial rounds of variant selection, sorted Genox 3.53 cells were collected into a single well of a 24 well culture plate (Costar), containing culture medium. After sorting 10^7 BALB/c thymocytes were added to this well to provide a feeder cell layer. Thymocyte suspensions were prepared as described [17] and washed three times with medium. For cell cloning, an electronic modification of the FACS was used to sort single cells into individual wells of a 96-well culture plate (Falcon) containing 50 μl of medium [18]. After cloning, 50 μl of medium containing 10^6 washed thymocytes was added to each well.

RESULTS AND DISCUSSION

FITC-GAM1 and FITC-GAM2 were titrated against a panel of mouse hybridomas of known immunoglobulin isotype. Cell surface staining was analyzed with the FACS. FITC-GAM2 specifically stained IgG_{2a} or IgG_{2b} producing hybridoma cells with amounts of 1.5 $\mu g/10^6$ cells at a concentration of 50 μg/ml (Table 1). No specific staining of hybridomas producing immunoglobulin of other isotypes was observed. Similarly FITC-GAM1 specifically stained IgG_1 producing hybridomas with amounts of 1.0 $\mu g/10^6$ cells at a concentration of 50 μg/ml.

Genox 3.53 hybridoma cells stained specifically with FITC-GAM1 and not with FITC-GAM2 as shown in Figure 2(A). This was to be expected as it was known that the cells secreted an IgG_1. Careful analysis of the population stained

FIGURE 2 Logarithmic FACS histograms showing the derivation of IgG_{2a} and IgG_{2b} variants of Genox 3:53. Traces are of cells stained with FITC-GAM1 (GAM1), FITC-GAM2 (GAM2), or not stained (NS). (A) The original IgG_1 secreting population of Genox 3.53 cells; (B) the population sorted from the whole population stained with FITC-GAM2; (C), (D) cells that grew out from first sorted population just before the second sort; (E) the population sorted at the second sort compared to the whole population stained with FITC-GAM2; (F) cells that grew out from the second sorted population just before cloning; (G) cloned IgG_{2b} variant 3.532b; and (H) cloned IgG_{2a} variants 3.532a.



with FITC-GAM2 revealed a small subpopulation of cells with a level of fluorescence similar to that of IgG_2 producing cells stained with FITC-GAM2. As listed in Table 2, 0.15% of the original Genox 3.53 population stained to a level to be included in the sort window shown in Figure 2(B). No cells were detected with this level of fluorescence in the unstained population of cells.

1.6×10^7 Genox 3.53 cells were stained with FITC-GAM2. Cells having fluorescence significantly above background were sorted into a single well of a 24 well culture plate. These cells were allowed to grow for one week and were then reanalyzed [Figs. 2(C),(D)]. As seen in Table 2 only a small fraction (0.26%) of the previously sorted cells stained specifically with FITC-GAM2. However, this subpopulation was enriched compared to the 0.15% found in the parent population. 6.4×10^6 cells were sorted for the second time [Fig. 2(E)] and 1539 cells (0.23% of total) collected in a single well of a 24 well plate. After several days of growth the cells were reanalyzed and at this stage: 52% of the cells stained specifically with FITC-GAM2 [Fig. 2(F)]. Individual, positively stained cells from this subpopulation were directly cloned with the FACS into individual wells of a 96 well culture plate [18].

Two weeks after cloning large colonies were seen in 35% of the wells. Supernatants from 48 colonies were assayed for indirect trace binding with ^{125}I-RAM against LB (DR6, DC1 positive) and Mich (DR5, DC1 negative) cells. Forty-seven gave strong binding to LB and no binding to Mich indicating they were still secreting antibodies with the original specificity. Supernatants from 28 of the active clones were then assayed on LB cells with ^{125}I-GAM1 and ^{125}I-GAM2. All bound 20–60,000 cpm ^{125}I-GAM2, and with one exception bound only 200–700 cpm of ^{125}I-GAM1. To distingish between clones secreting IgG_{2a} and IgG_{2b} the assay was repeated using two monoclonal antibodies that recognize allotypic determinants of mouse immunoglobulin as the iodinated second step reagent. 16.3 reacts with both IgG_{2a} and IgG_{2b}, 8.3 only reacts with IgG_{2a} of C3H mice from which Genox 3.53 was derived [16]. In total, nine clones secreting IgG_{2a} and 56 secreting IgG_{2b} were identified. Six clones of each type were expanded and stocks of frozen cells made. One clone of each type was selected on the basis of growth and antibody production for further study. These were designated G2a.5 for one that secretes IgG_{2a} and G2b.2 for one that secretes IgG_{2b}. The variant cells secrete similar amounts of IgG as the parent hybridoma and can be similarly passaged as tumors in (BALB/c × C3H) F1 or irradiated BALB/c mice to give high titred ascites fluids [19]. These cells have been sent to the American Type Culture Collection hybridoma cell bank. (For information write to Dr. Anne Hamburger, Cell Culture Department, American Type Culture Collection, 12301 Park Lawn Drive, Rockville, MD 20852.)

TABLE 2 Isolation of IgG_2 switch variants of Genox 3.53

Population	Percent Number of cells in sort window	Number of cells selected	Total number of cells sorted	Percent selected
Parent	0.15	8.6×10^3	1.6×10^7	0.054
1st sorted culture	0.26	1.5×10^3	6.4×10^5	0.23
2nd sorted culture	52.0			

TABLE 3 Monoclonal antibodies of specificity and isotype as indicated were tested on separated B and T lymphocytes of 19 HLA typed individuals.[a]

		HLA types					Antibodies							
							DC1/MB1/MT1			A2	Monomorphic ABC		β_2–m	Monomorphic DR
							Genox 3.53	G2a.1–6	G2b.1–6	BB7.2	BB7.7	W6/32	BBM.1	CA2.206
Cells	DC1	DR	A	B	C	Bw4/Bw6	γ_1	γ_2	γ_{2b}	γ_{2b}	γ_{2b}	γ_{2a}	γ_{2b}	γ_2
SSH	+	1	1,w24	7,w62	w3	w6	−/−[b]	+/−	+/−	−/−	+/+	+/+	+/+	+/−
LeH	+	1,3	w30,w34	14,w63	w2,w5	w4,w6	−/−	+/−	+/−	−/−	+/+	+/+	+/+	+/−
DDK	+	1,3	1,26	8,w35	w4	w6	−/−	+/−	+/−	+/−	+/+	+/+	+/+	+/−
PeC	+	2,7	3	7,w47	w6	w4,w6	−/−	+/−	+/−	−/−	+/+	+/+	+/+	+/−
SFR	+	2,w6	25,w30	18,w38	–	w4,w6	−/−	+/−	+/−	−/−	+/+	+/+	+/+	+/−
DaW	+	w6,7	28,w31	w35,w44	w4	w4,w6	−/−	+/−	+/−	−/−	+/+	+/+	+/+	+/−
MWS	+	w6,3	25,29	18,w55	w3,w5	w4,w6	−/−	+/−	+/−	−/−	+/+	+/+	+/+	+/−
FEW	+	w6,3	1,1	8,8	w7	w6	−/−	+/−	+/−	−/−	+/+	+/+	+/+	+/−
LRG	+	w10,4	1,26	8,w38	w7	w4,w6	−/−	+/−	+/−	−/−	+/+	+/+	+/+	+/−
BeR	+	2,5	2,26	w38,w52	–	w4	−/−	+/−	+/−	+/+	+/+	+/+	+/+	+/−
WMc	+	2,w9	2,w30	w45,w57	–	w4,w6	−/−	+/−	+/−	+/+	+/+	+/+	+/+	+/−
BiG	+	w6,4	2,w23	w49,w58	w3	w4	−/−	+/−	+/−	+/+	+/+	+/+	+/+	+/−
PGED	+	w10	2	27,37	w2	w4	−/−	+/−	+/−	+/+	+/+	+/+	+/+	+/−
JHC	−	3,8	2,1	8,w45	w7	w6	−/−	−/−	−/−	+/+	+/+	+/+	+/+	+/−
CWh	−	7	2,29	17,w44	w6	w4	−/−	−/−	−/−	+/+	+/+	+/+	+/+	+/−
RC	−	7,8w13	3,25	w44	w5	w4	−/−	−/−	−/−	−/−	+/+	+/+	+/+	+/−
DHo	−	5,5	11,w30	w35	w4	w6	−/−	−/−	−/−	−/−	+/+	+/+	+/+	+/−
JFH	−	4,8	1	8,w39	w5	w6	−/−	−/−	−/−	−/−	+/+	+/+	+/+	+/−
LT	−	3	w31,w36	w35,w42	w4	w6	−/−	−/−	−/−	−/−	+/+	+/+	+/+	+/−

[a] All cytotoxic reactions were clear out and for clarity are indicated as + (lysis) or − (no lysis). Supernatants from six IgG_{2a} and six IgG_{2b} clones of Genox 3.53 switch variants were tested. As all clones gave identical results they are summarized under G2a.1–6 and G2b.1–6 respectives.

[b] Reactivity with B cells/reactivity with T cells.

Cytotoxic Reactions of G2a and G2b

Supernatants were prepared by allowing hybridoma cells to overgrow and eventually die for seven days after they had reached optimal maximum density (5–10 × 10^5 cells/ml). Cells and cellular debris were removed by centrifugation and 0.02% sodium azide added to the supernatants which were transported from Stanford University to Duke University at ambient temperature.

Hybridoma supernatants were tested for complement mediated cytotoxicity [20] on separated B and T lymphocytes [21] from 19 individuals well characterized for HLA antigens. Supernatants from the 12 IgG_2 secreting clones of Genox 3.53 were tested at three dilutions (neat, 1/2, 1/10). Control supernatants were from the parent IgG_1 secreting Genox 3.53; CA2.206, a monomorphic anti-DR (IgG_{2a}) [22]; BB7.2, anti-HLA-A2 (IgG_{2b}) [23]; BBM.1, anti-β_2-m (IgG_{2b}) [24]; W6/32 (IgG_{2a}) [25]; and BB7.7 (IgG_{2b}) [19] monomorphic anti-HLA-A,B,C. The results are shown in Table 3. The Genox 3.53 IgG_1 was not cytotoxic to any cells. In contrast all the IgG_2 switch variants killed cells with the same specificity that the IgG_1 has by binding assay. Only B lymphocytes of DC1 positive individuals were killed by the IgG_2 variants of Genox 3.53. Complete cytotoxicity was seen at all dilutions tested and no differences were found between IgG_{2a} and IgG_{2b}. The control supernatant also killed cells according to their specificity as determined by binding assay [19,22–25]. As expected BBM1, BB7.7, and W6/32 killed all cells from all individuals. BB7.2 killed B and T lymphocytes from HLA-A2 positive individuals and CA2.206 killed B cells but not T cells from all individuals.

These results show that spontaneous isotype switching in hybridoma cells can be used to advantage in converting noncytotoxic anti-HLA monoclonal antibodies of useful specificity into cytotoxic reagents. It is not clear if antigen density will affect the cytotoxicity of monoclonal antibodies. However, as this method worked for DC1, an HLA antigen with relatively low levels of expression, it seems likely to be applicable to all anti-HLA. The availability of pairs of antibody of identical specificity, one that is cytotoxic and one that is not, will also provide useful tools for in vivo and in vitro studies of MHC antigens.

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