IMMUNIZATION WITH SV40-TRANSFORMED CELLS YIELDS MAINLY MHC-RESTRICTED MONOCLONAL ANTIBODIES

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The B cell repertoire has been classically defined as comprising recognition specificities for antigens per se, as opposed to the T cell repertoire, which is dedicated to the recognition of antigens primarily as they are presented on cell surfaces in the context of self MHC-encoded antigens (self + X recognition) (1–3). Recently, evidence from several sources has indicated that self + X recognition may also be characteristic of a component of humoral antibody responses (4–11). These studies have shown not only that, in certain instances, stimulation of B cells requires antigen presenting cells of an appropriate MHC haplotype (4–6), but also that some antibodies produced in response to antigens present on cell surfaces recognize those antigens specifically in the context of the appropriate MHC alloantigens (7–11). A previous report from this laboratory described an extensive analysis of the mAb response to influenza (PR8)-infected syngeneic cells, which showed that ~30–60% of the responding primary splenic B cells yielded antibodies that bound PR8 antigens only in the context of infected cells expressing syngeneic MHC alloantigens (9). However, the contribution of B cells with MHC-restricted recognition in immune responses in general is uncertain, as few studies have been conducted that could effectively identify and discriminate MHC-restricted antigen recognition in the presence of recognition of the antigen per se.

In this paper we describe the monoclonal B cell response to an (SV40)[1]-transformed H-2^b fibroblast cell line (SV40-B6) (12). The majority of primary splenic B cells and sIg^- (surface Ig–negative) bone marrow precursor cells from C57BL/6 (H-2^b) and from BALB/c mice (H-2^d) responding in vitro to SV40-transformed H-2^b cells exhibit MHC + X recognition in that their antibody products uniquely bind SV40-transformed H-2^b cells. Furthermore, the majority of these antibodies recognize SV40 transformation-specific antigens in association with the H-2 K^b molecule, as defined by their ability to bind SV40-transformed A(5R) cells (K^bD^d), but not SV40-transformed HTG cells (K^dD^b).

To study the fine specificities of these MHC-restricted antibodies, nine hybridomas secreting mAb specific for the SV40-transformed H-2 syngeneic fibroblast cell line (SV40-B6) were derived from the fusion of SP2/0 cells with the progeny of antigenically stimulated primary C57BL/6 splenic B cells. The reactivity patterns of these nine mAb were consistent with antibodies obtained from the

[1] *Abbreviations used in this paper:* DMEF, supplemented DMEM; sIg, surface Ig; SV40, simian virus 40.

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splenic fragment culture system, in that the majority of the antibodies (seven of the nine) were specific for SV40-transformed cells of the H-2^b haplotype, and in particular, that the recognition of six of these antibodies was restricted by the K^b molecule.

Materials and Methods

Mice. C57BL/6 and BALB/c mice were obtained from the mouse breeding colony at Scripps Clinic and Research Foundation. Male and female mice 6–12 wk of age were used in the splenic fragment culture system and for construction of hybridomas.

Cell Cultures. SV40-transformed fibroblast cell lines of the following origins were a generous gift from Dr. Barbara Knowles (The Wistar Institute, Philadelphia, PA): C57BL/6 (K^bD^b), A(5R) (K^bD^d), HTG (K^dD^b), bm1, bm3, bm8, bm10, bm11 (12–14). The chemically transformed cell line MC57 (K^bD^b) is an independently derived fibroblast cell line originating from C57BL/6 mice. These cell lines were grown in DMEM (M. A. Bioproducts, Los Angeles, CA) supplemented with 10% FCS, 2% L-glutamine (Irvine Scientific, Irvine, CA) and 100 U/ml penicillin-streptomycin (Irvine Scientific) (DMEF) in a 5% CO_2 atmosphere at 37°C. Cells were grown in DMEF with 5% Con A supernatant for 24 h before they were used to enhance the expression of cell surface MHC molecules.

The Y3 hybridoma cell line, which produces anti-H-2^b antibody, was derived by Dr. Charles Janeway (Yale University, New Haven, CT) and was obtained from Dr. Stanley Nathenson (Albert Enstein Medical College, Bronx, NY).

Splenic Fragment Cultures. mAb specific for SV40-transformed B6 (SV40-B6) fibroblasts were produced in splenic fragment cultures as previously described, with some modifications (8, 9). Briefly, 6–8-wk-old C57BL/6 or BALB/c mice were primed by i.p. injection of 10^7 SV40-B6 cells in alum. The SV40-B6 cells were grown in DMEF plus 5% Con A supernatant, harvested by scraping (rather than by trypsinization, to preserve surface antigens), washed three times in sterile PBS and irradiated at 3,000 rad before use. 5.0×10^7 donor spleen or sIg^- bone marrow cells (see below) from naive 6–12-wk-old C57BL/6 or BALB/c mice were transferred intravenously into SV40-B6 primed syngeneic recipient mice 4–6 wk after priming. The recipient mice received 1,300 rad whole body irradiation from a cesium source 1–4 h before donor cell transfer. 2–4 h after receiving donor cells, recipient mice were injected i.p. with 10^7 irradiated (3,000 rad) washed SV40-B6 fibroblasts in saline. Fragment cultures were prepared from recipient spleens 16–20 h after cell transfer. Cultures were incubated at 37°C in an atmosphere of 93% O_2 and 7% CO_2. Medium was changed every 2–4 d, and culture fluids were individually collected from days 9–30 and assayed for antibody activity.

Depletion of sIg^+ B Cells from Bone Marrow. sIg^- cell populations from adult bone marrow were prepared by a modification of the rosetting techniques of Walker et al. (15). In brief, 10^7–10^9 washed bone marrow cells were incubated with a 5–10-fold excess of tanned, glutaraldehyde-fixed SRBC coated with goat anti-mouse IgM and IgG H and L chain–specific antibodies (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Rosetted bone marrow cells (sIg^+) were removed by centrifugation at 4°C through Ficoll-Hypaque density gradients. 30–50% of the input bone marrow cells were generally recovered in the interface band. At least 90% of sIg^+ lymphocytes were depleted by this method, and the resulting preparations possessed <2% sIg^+ lymphocytes, as assessed by direct fluorescence analysis employing fluorescein-labeled anti-mouse (IgM + IgG + IgA) rabbit Fab'_2 fragments (Zymed Laboratories, Burlingame, CA) on a Becton-Dickenson FACS IV.

Transfer Fusions. Hybridoma lines were established using the SP2/0 cell line originally described by Shulman et al. (16) in a transfer fusion procedure similar to that previously described (17, 18). Normal B cell fusion partners were obtained by the following protocol. Recipient C57BL/6 mice were primed 1–2 mo before use by the i.p. injection of 10^7 irradiated (3,000 rad) SV40-transformed H-2^b fibroblasts (SV40-B6) in alum, prepared as described above. Recipient mice were given 10 mg tetracycline i.p. 12 d before fusion. 5 d before fusion, recipient mice were irradiated at 800 rad, and 2–4 h later, they were

given 10^8 donor spleen cells intravenously. Donor spleen cells were obtained from 8–16-wk-old, conventionally reared naive C57BL/6 mice that had been given 10 mg tetracycline i.p. 7 d earlier. 2 h after the transfer of donor spleen cells, recipient mice received 10^7 irradiated (3,000 rad) SV40-B6 fibroblasts in saline i.p., and 2 h later, 10 mg tetracycline i.p. Recipient mice were fed water treated with puromycin.

5 d after cell transfers, the recipient mice were sacrificed, their spleens were removed, and a cell suspension was prepared. $8–40 \times 10^6$ nucleated cells were recovered per spleen, and two spleens were generally pooled per fusion. The spleen cells were fused with 10^7 SP2/0 cells at room temperature using 50% wt/vol PEG (3,500 mol wt) in DMEM containing 15% DMSO (19). Cells were washed after fusion and plated out to three 96-well microtiter plates in HAT selective media. Hybrids were kept in HAT medium for 10 d, then maintained in HT only for a further 10 d. Usually 10–14 d after fusion, wells containing hybrid cells required splitting, and at this stage the supernatants were collected and assayed for the presence of antibody to SV40-transformed fibroblasts. Positive hybrids were cloned twice at limiting dilution, and subclones of interest were grown in large quantities, culture fluids were saved and cells were transferred to (BALB/c × C57BL/6)F_1 pristane-primed mice for the production of ascites fluid.

Preparation of Cellular Immunoabsorbents. Target cells (SV40-transformed fibroblast cell lines and MC57) were harvested by treating with 0.25% trypsin EDTA (Flow Laboratories, Ingelwood, CA) for 4–7 min at 37°C, and washed with DMEM. Cells were then transferred to flat-bottom polyvinyl chloride tissue culture–treated 96-well plates (Costar 2596) at 2×10^6 cells/well in 200 μl of DMEF. The plates were incubated in 10% CO_2 for 12–24 h, or until a confluent monolayer was obtained in all wells. They were then fixed in 0.15% glutaraldehyde at room temperature for 15 min, washed with PBS, and treated with 0.2 M glycine for 10 min. The wells were washed and 10% horse serum (HS) in PBS was added as a blocking agent.

Primary kidney fibroblast solid-phase RIA plates were prepared by a modification of a previously described technique (9). Kidneys were sterilely removed from C57BL/6 mice 1 wk or less after birth, teased apart, and incubated with 2.5 ml 0.25% trypsin per kidney for 10 min at room temperature with stirring. The cells were then incubated for 4–6 h in 5 ml 0.25% trypsin per kidney in the cold. The resultant turbid cell suspension was filtered through nylon mesh, washed twice with DMEF, and transferred into tissue culture–treated, flat-bottom, polyvinyl chloride 96-well plates, grown and fixed as described above.

Solid Phase RIA. Supernatants or ascites fluids were assayed for the presence of antibody by solid-phase RIA as previously described (8, 9, 20). Briefly, 25 μl of culture fluids or appropriate dilutions of ascites fluid were added to the cellular immunoabsorbent plates described above and allowed to incubate at room temperature for 2–3 h. The plates were rinsed with 10% HS, rabbit anti–mouse Ig reagent was added for 2 h, the plates were rinsed again with 10% HS, and incubated with ^{125}I-labeled affinity-purified goat anti–rabbit Ig antibody overnight at room temperature. The plates were then washed and counted in a gamma counter. 5F1, an mAb specific for K^b, and PAB 100, an mAb specific for SV40 large T molecule were used as standards in the assays to assure equal expression of these antigens on the surface of the cellular immunoabsorbent plates. 5F1 was obtained from Dr. Linda Sherman (Scripps Clinic and Research Foundation) and PAB 100 was obtained from The American Type Tissue Culture Collection, Bethesda, MD.

The L chain and H chain isotype of the mAb were determined in an RIA similar to that described above but with rabbit anti–mouse class-specific antibodies in place of the rabbit anti–mouse Ig.

This RIA method was modified to use biotinylated SV40-B6-specific hybridoma antibodies (see below) and ^{125}I-labeled streptavidin for additional studies of the binding specificities of these antibodies.

Antibody-dependent Complement Mediated Cytotoxicity. Hybridoma SV40-B6-specific mAb were tested for fine specificity by a standard antibody-dependent complement-

TABLE I
Monoclonal Responses to SV40-B6

Donor cells*	Recipient‡	Total cells ($\times 10^6$)§	Overall frequency per 10^6 cells‖	SV40.B6	+¶	+	+	+
				MC57	−	−	−	+
				SV40.A(5R)	+	+	−	+ or −
				SV40-HTG	+	−	+	− or +
				Specificity:	SV40	SV40-K^b	SV40-D^b	K^b or D^b
C57BL/6 Sp	C57BL/6	250	0.276		14** (20)‡‡	37 (54)	12 (17)	6 (9)
C57BL/6 sIg^-	C57BL/6	750	0.071		21 (40)	23 (43)	8 (15)	1 (2)
BALB/c Sp	BALB/c	1,900	0.212		66 (16)	180 (45)	74 (18)	82 (20)
BALB/c sIg^-	BALB/c	1,200	0.030		7 (19)	15 (42)	7 (19)	7 (19)

* Donor mice were 2–4 mo old.

‡ Recipient mice were primed with 10^7 irradiated (3,000 rad) SV40-B6 cells in alum 4–6 wk before use. They received 1,300 rad 1–4 h before donor cell transfer, and 10^7 irradiated (3,000 rad) SV40-B6 cells in saline 1–2 h after donor cell transfer.

§ Each recipient mouse received 5.0×10^7 donor cells.

‖ Antibody-producing clones were detected by RIA of culture fluids collected from days 9–24 of culture. They were considered positive if their binding to SV40-B6–coated plates was 2 SD above background binding to plates coated with 10% horse serum.

¶ An mAb was considered positive if subsaturating amounts of antibody bound target cell plates at least 50% as well as to SV40-B6 cell plates, and negative if it bound target cell plates <20% as well as SV40.B6 cell plates.

** Total number of monoclonal responses of the given specificity.

‡‡ Numbers in parentheses are percentages of the total SV40-B6 responses producing antibodies of the given specificity.

mediated cytotoxicity procedure using trypan blue exclusion and cell visualization to assess killing efficiency (21).

Antibody Biotinylation and Fluorescence Analysis. Hybridoma SV40-B6-specific mAb were precipitated with 50% saturated ammonium sulfate (SAS), resuspended, and passed over a Sepharose 6 B column. Purified antibodies were then biotinylated as described by Katonia et al. (22). Biotinylated antibodies were then used in conjunction with fluoresceinated avidin (Sigma Chemical Co., St. Louis, MO), for visual screening using a fluorescence microscope.

Results

Frequency of C57BL/6 Splenic and sIg^- Bone Marrow Precursor Cells Responsive to Antigens Expressed on SV40-B6. Results of an analysis of SV40-B6-responsive cells in C57BL/6 spleen and sIg^- bone marrow cells in the splenic fragment culture system are summarized in Table I. The frequency of C57BL/6 primary splenic B cells responsive to SV40-B6 stimulation in the syngeneic C57BL/6 environment was 2.76 per 10^7 injected cells. In other antigen systems, responses are markedly enhanced by transferring donor cells to recipients that are allogeneic to the donor cells in the allotype-idiotype locus (IgH), thereby avoiding idiotype-specific suppression induced by the priming of recipient mice (20). However, the response to SV40-B6 by C57BL/6 primary splenic B cells was not enhanced by stimulation in the IgH allogeneic C3H.SW environment (data not shown); therefore, all further studies were carried out in the syngeneic environment. Assuming that 40% of the injected spleen cells are B cells, and that the efficiency of homing and stimulation in splenic fragment culture is 4%, as has been previously determined (23), and since 2.76 of every 10^7 spleen cells responded to SV40-B6, it can be calculated that 1 of every 5.3×10^4 C57BL/6 splenic B cells was specific for SV40-B6 cells.

To further define the specificity of mAb produced by C57BL/6 primary splenic B cells in response to SV40-B6 stimulation, we assessed their binding to SV40-B6 (K^bD^b), MC57 (chemically transformed K^bD^b), SV40-A(5R) (K^bD^d), and SV40-HTG (K^dD^b) (see Table I). 20% of the mAb bound SV40-B6, SV40-A(5R),

and SV40-HTG, but not MC57 cells, and therefore were potentially specific for antigens associated with SV40 transformation per se, or for these antigens in conjunction with nonpolymorphic cell surface antigens. A small percentage (9%) of the antibodies bound both the H-2^b cell lines, SV40-B6, and MC57, and either, but not both, the SV40-A(5R) or the SV40-HTG cell lines. Therefore, these antibodies might have been specific for self H-2 antigens per se, or for H-2 antigens in conjunction with other cell surface antigens. Alternatively, these antibodies might actually be specific for antigens associated with SV40 transformation in conjunction with MHC antigens, but have sufficient affinity for self MHC per se to bind in a solid-phase RIA. In any case, since these mAb apparently have specificity for self MHC alloantigens, they are currently being characterized in greater detail.

The vast majority (71%) of C57BL/6 primary splenic B cells responsive to SV40-transformed syngeneic cells produced antibodies that bound SV40-B6 but not MC57, and only one of the two SV40-transformed H-2 recombinant cell lines, SV40-A(5R) or SV40-HTG, but not both. These antibodies, therefore, apparently were MHC restricted in their recognition of antigens associated with SV40 transformation. The majority of these mAb, representing 54% of the total response, bound SV40-A(5R) but not SV40-HTG, suggesting K^b-restricted recognition. The minority (17% of the total response) bound SV40-HTG but not SV40-A(5R), indicating a D^b-ended restriction.

When C57BL/6 sIg^- bone marrow cells were stimulated with SV40-B6, the frequency of responsive cells was 7.1 per 10^8 injected cells. The ratio of the frequencies of SV40-B6-responsive cells in C57BL/6 sIg^- bone marrow vs. spleen was 0.26, which is within the range of ratios of frequencies of responses of sIg^- vs. spleen cell populations for numerous antigens studied to date, including DNP, PC, and the hemagglutinin of the PR8 influenza virus (24–26). The percentage of SV40-B6-specific C57BL/6 sIg^- bone marrow cells with each recognition specificity defined above is similar to the percentage of responsive spleen cells of similar fine specificity. Thus, the frequency and specificity distribution of C57BL/6 splenic B cells responsive to SV40-B6 cells appear to reflect the specificities of newly generated bone marrow precursor cells and do not evidence marked effects of environmental selective processes.

Frequency of BALB/c Splenic and sIg^- Bone Marrow B Cells Responsive to Antigens Expressed on SV40-B6. The frequencies of BALB/c primary spleen and sIg^- bone marrow cells responsive to SV40-B6 cells in splenic fragment culture were 21.2 and 3.0 per 10^8 injected cells, respectively (see Table I). Therefore, the overall frequency of B cells or B cell precursors responsive to SV40-B6 cells in spleen cell and sIg^- bone marrow cell populations is similar for mice allogeneic to the stimulating cell, and for mice syngeneic to the stimulators. Furthermore, an analysis of the fine specificity of mAb from SV40-B6-responsive BALB/c cells from both spleen and sIg^- bone marrow cell populations showed a similar representation of the different specificities as found in the precursor cells of syngeneic C57BL/6 mice. The majority (61–63%) of the antibodies bound SV40-B6 but not MC57 and only bound either SV40-A(5R) or SV40-HTG and therefore appeared to be specific for cells bearing both SV40-specific transformation antigens and either K^b or D^b molecules. Of those, most were specific for

TABLE II
Hybridomas Specific for SV40-transformed Cells

Hybrid*	Isotype‡	Specificity§	SV40 fibroblasts (determined by RIA)‖				
			bm1	bm3	bm8	bm10	bm11
SV4	μ, κ	SV40-K^b (1)	−	+	−	−	+
SV27	μ, κ	SV40-K^b (1, 3)	−	−	+/−	+	+
SV30	μ, κ	SV40-K^b (1)	−	+	−	−	−
SV36	μ, κ	SV40-D^b (1, 2, 3)	ND	ND	ND	ND	ND
SV37	$\gamma 2b\kappa$	SV40-K^b (1, 3)	−	−	+/−	+	+
SV101	$\gamma 2b\kappa$	SV40-K^b (1, 2, 3)	−	+	+	+	+
SV103	$\gamma 2b\kappa$	SV40 (1, 2, 3)	+	+	+	+	+
SV154	$\gamma 2b\kappa$	SV40-K^b (1, 3)	−	−	−	+	−
SV311	μ, λ	SV40 (1, 2, 3)	ND	ND	ND	ND	ND

* Hybridoma cell lines produced by the transfer fusion protocol from C57BL/6 primary spleen cells.
‡ Isotype detected by RIA.
§ Cell surface molecule(s) apparently necessary for the binding of that mAb as determined by discrimination between SV40-B6, MC57, SV40-A(5R), and SV40-HTG in the following assay systems: 1, RIA; 2, antibody-dependent complement-mediated cytotoxicity; 3, biotin-avidin fluorescence.
‖ SV40-transformed fibroblast cell lines with the given MHC haplotype obtained from Dr. B. Knowles.

cells bearing the K^b molecule in that they bound SV40-A(5R) and not SV40-HTG. A small percentage (16–19%) of all responsive cells produced antibodies that bound all three SV40-transformed cell lines but did not bind the chemically transformed line MC57, and therefore appeared to require only SV40 transformation–associated antigens for recognition and binding. The remaining responsive cells (~20%) bound both the H-2^b cell lines (SV40-B6, MC57) and either the SV40-A(5R) or the SV40-HTG cells, but not both, and therefore appeared to be specific for H-2^b molecules per se.

Characteristics of SV40-B6-specific Hybridoma Antibodies. Since the quantities of mAb available from fragment cultures are limited (0.1–2 μg), we constructed hybridoma cell lines that secrete SV40-B6-specific antibodies for use in extensive specificity, function, and structure analyses. These hybridomas were obtained by transfer fusions (see Materials and Methods) in which irradiated antigen-primed recipient mice provided an appropriate milieu for responses of transferred primary B cells. Hybridomas and their subclones were selected on the basis of the capacity of their antibody products to bind SV40-B6 fibroblasts but not MC57 fibroblasts, thus excluding all antibodies that bound nonspecifically to cultured cell monolayers, as well as rare antibodies that bound both H-2^b cell lines equally and therefore did not require SV40 transformation for binding. In all, nine hybridomas were selected from four separate fusions (see Table II). The participation of antigens associated with SV40 transformation in the specificity of all of these antibodies was further confirmed by the relatively poor binding of these antibodies to H-2^b primary kidney fibroblasts under conditions that permitted binding by the anti-H-2^b antibody product of the Y3 hybridoma cell line (data not shown).

Two of the nine mAb, SV103 and SV311, bound equally well to SV40-A(5R) and SV40-HTG cells, as well as to the stimulating cell line SV40-B6. The other seven preferentially bound either SV40-A(5R) or SV40-HTG, but not both. Therefore, for seven of the nine hybridoma antibodies studied, the H-2 haplotype

of the target cell apparently contributed to antigen recognition. SV103 and SV311 may represent antibodies specific for either SV40 transformation–associated antigens per se, or for these antigens in the context of cell surface antigenic determinants common to both H-2^b and H-2^d cells.

Table II shows that six of the mAb bound SV40-A(5R) as well as or better than the immunizing cell line SV40-B6, and failed to bind significantly to SV40-HTG. This strongly suggests that the K^b glycoprotein is the significant H-2^b-encoded molecule for the recognition specificities of these antibodies. In contrast, SV36 bound well to SV40-HTG but not to SV40-A(5R), suggesting that the D^b glycoprotein is the significant H-2^b-encoded molecule for the recognition specificity of this antibody. Thus, consistent with the findings described above for splenic fragment culture experiments, while some C57BL/6 B cells do respond to antigens associated with SV40 transformation per se, the recognition of the majority of responding B cells is associated with the expression of H-2^b molecules on the transformed cells, and most frequently with the expression of the H-$2K^b$ molecule.

To further confirm the recognition specificities of these mAb, many of them were used in two additional assay systems: antibody-dependent complement-mediated cytotoxicity, and biotin-avidin fluorescence staining. In each case, when an antibody was tested by multiple assay systems, its recognition specificity as determined by each assay was the same (see Table II). In all three assay systems used, a quantitative effect on specificity was identified. At high antibody concentrations, some but not all of the tested antibodies bound non–SV40-transformed B6 cells (MC57). To further analyze this, a fourth assay system was devised using biotinylated purified SV40-B6-specific hybridoma antibodies in an RIA in which ^{125}I-labeled streptavidin was used to detect binding. This assay proved beneficial for two reasons. First, both the consistency and signal to noise ratio were enhanced, presumably because of the elimination of nonspecific binding of the rabbit anti–mouse and goat anti–rabbit antibodies to the cellular immunoabsorbents. Second, it allowed for competitive inhibition studies using nonbiotinylated antibodies as inhibitors.

Fig. 1 shows two characteristic binding curves of SV40-K^b-specific hybridoma mAb on SV40-B6 or MC57 cells. SV101 bound SV40-B6 cells significantly better than MC57, and did not show increased binding to MC57 with higher concentrations of antibody. In contrast to this, SV30 at high concentrations bound both SV40-B6 and MC57 cells, however at lower concentrations, its binding was markedly better on SV40-B6 than on MC57.

Fine Specificity of SV40-B6-specific Hybridoma Antibodies. Using a panel of SV40-transformed cell lines bearing mutational variants of the H-$2K^b$ molecule, the fine specificity of the SV40-B6-specific hybridoma antibodies was studied by RIA (Table II). One of the two antibodies that failed to discriminate between SV40-A(5R) and SV40-HTG cells, SV103, also failed to discriminate between the SV40-transformed K^b mutant cells in that it bound all tested SV40-transformed cells equally well. The other antibody specific for SV40 transformation antigens per se, SV311, and mAb SV30, which required both SV40 transformation and D^b molecules for cell binding, were not tested on the panel of SV40-transformed K^b mutants. The remaining six mAb, which bound only SV40-

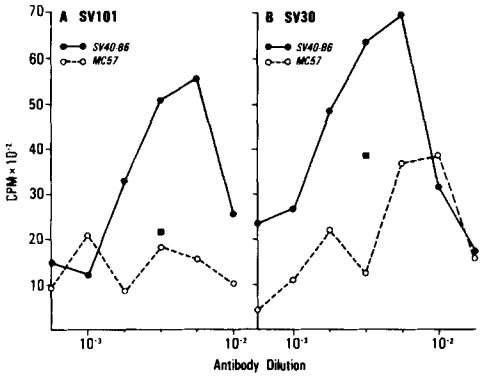

FIGURE 1. Solid phase RIA of biotinylated SV40-B6-specific hybridoma antibodies on SV40-B6 (●) and MC57 (○) fibroblast target cells. The biotinylated antibodies were added in several dilutions, and binding was detected by the addition of ^{125}I-labeled streptavidin and counting in a gamma counter. Inhibition of binding by a 10-fold excess of unbiotinylated homologous antibody (■) is given at one dilution point. *A*, SV101; *B*, SV30.

transformed cells bearing the H-2K^b molecule, all discriminated among the different SV40-transformed K^b mutant cell lines and defined five distinct patterns of reactivity. Two of the mAb, SV27 and SV37, which were derived from separate fusions, displayed a similar reactivity pattern in that they bound SV40-bm10 and SV40-bm11 well, SV40-bm8 moderately well, and failed to bind SV40-bm1 or SV40-bm3. The remaining four mAb each defined a unique reactivity pattern on the five tested K^b mutant SV40-transformed cell lines. However, none of the six K^b-restricted antibodies bound SV40-transformed cells bearing the bm1 K^b mutation. Interestingly, the binding of many of the monoclones is disrupted by mutations in more than one of the domains of the K^b molecule (27). For example, SV40 does not bind bm1 or bm10, both of which have mutations only in the second domain, but it also does not bind bm8, which has mutations only in the first domain. It does, however, bind bm3 and bm11, which also have only first-domain mutations.

Discussion

Recognition of cell surface antigens in the context of self MHC alloantigens, and therefore immune surveillance for virally infected or neoplastic cells has long been considered the province of T cells (1–3). Findings previously reported by this laboratory (8, 9) showed that a significant proportion of the B cells responsive to influenza (PR8)-infected MHC-syngeneic cells produced mAb uniquely specific for MHC-syngeneic infected cells (self + X recognition). The present studies were designed to assess the role of self + X recognition in the B cell response to SV40-transformed H-2^b cells.

SV40-transformed cells present a complex cell surface antigenic mosaic to the immune system. In addition to normal cell surface proteins, including self MHC molecules, these cells express cell surface antigens unique to SV40-transformed cells (e.g., large T protein and nonviral T proteins [NVT or p53]), and possibly others common to transformed cells in general (28–32). Studies using cytotoxic T cell clones have delineated the necessity for the association of MHC-encoded

proteins with transformation-associated proteins for effective T cell recognition of these cells (12–14, 33–35). Studies of the humoral antibody response to SV40-transformed cells have generally been conducted under conditions that only allow for recognition of antibody responses to transformation-associated antigens per se (36–39). Recently, however, a hybridoma antibody generated in response to SV40-B6 cells has been shown to effectively block a cytolytic T cell clone specific for large T protein and the H-2K^b molecule (40).

The frequency of primary splenic H-2^b B cells responsive to SV40-transformed H-2^b fibroblasts in splenic fragment culture and whose antibody products bind only SV40-transformed cells bearing MHC gene products of the immunizing cell line is 1.2 per 10^5 B cells. This is similar to the previously observed (8, 9) frequency of MHC-restricted responses to PR8-infected H-2^b fibroblasts. Significantly, while a majority of B cells responsive to PR8-infected H-2^b cells required viral antigen expression on syngeneic cells, an even greater proportion (71%) of B cells responsive to SV40-B6 produced antibodies that bound only SV40-transformed syngeneic cells. 20% of the C57BL/6 mAb produced in response to SV40-B6 antigens bound both SV40-A(5R) and SV40-HTG. These antibodies include all antibodies specific for SV40 transformation–associated antigens per se, as well as any that recognize these antigens in conjunction with cell surface determinants common to the H-2^b and H-2^d fibroblast target cells. Therefore, at most, 20% of primary C57BL/6 splenic B cells responsive to SV40-B6 fibroblasts recognize SV40 transformation–associated antigens per se. Another 9% of the responsive cells recognized cell surface antigens associated with H-2^b cells, did but did not require SV40 transformation–specific antigens for binding.

Because the above findings indicated that the in vitro monoclonal B cell response to transformed cells may be dominated by self + X recognition, we carried out an independent assessment of the primary C57BL/6 splenic B cell recognition of SV40-B6 as defined by SV40-B6-specific hybridomas. The results of an analysis of the specificities of nine hybridoma antibodies from four separate fusions, selected only on the basis of their capacity to discriminate SV40-transformed H-2^b cells from non–SV40-transformed H-2^b cells, was entirely consistent with the much larger survey of specificities obtained by fragment culture technology. Of the nine hybridoma antibodies analyzed, the specificity of seven could be attributed to complex recognition of SV40 transformation–specific antigens in association with self MHC products. Six of these seven recognized only SV40-transformed cells bearing the K^b molecule, and furthermore, these six antibodies differentiated among several SV40-transformed H-2K^b mutant cell lines, thereby defining at least five different fine specificity patterns. These data confirm that the recognized self components are encoded by the MHC, and indicate that the B cell response to SV40-transformed syngeneic cells is dominated by a diverse population of B cells whose Ig receptors and potential antibody products are specific for self MHC + foreign X recognition.

The present data extend to a second cell surface antigen complex the unexpected observation of a predeliction of B cells for MHC-restricted antigen recognition. Other laboratories have described (7, 10, 11) human myeloma proteins with restricted recognition for male antigens and murine serum responses to male antigens and Sendai virus–infected cells that exhibit varying

degrees of self MHC–restricted recognition. Taken as a whole, these findings suggest that self + X recognition, rather than being atypical, may in fact be a significant and integral characteristic of the B cell repertoire in its response to cell surface antigens, and that these specificities have not been identified more frequently due to the lack of experiments adequately constructed for this purpose.

mAb obtained from both in vitro stimulation and hybridomas displayed a clear preference for recognition of SV40-associated transformation antigens in conjunction with K^b molecules. This preferential K^b-ended restricted recognition may reflect any of a number of underlying mechanisms similar to the proposed mechanisms for the immune response (*Ir*) gene effect on T cell repertoire (41–43). These include environmental repertoire determination or differing associative capacities of given class I molecules with SV40 transformation antigens. Since SV40-A(5R) and SV40-HTG are recognized equally well by SV40-B6-specific CTL (12, 13), the relatively poor recognition by H-2^b B cells of SV40-HTG may be construed to reflect environmental determination of restricted recognition by B cells. However, two findings presented in this paper suggest that this preference for K^b-ended restricted recognition does not reflect environmental molding of the B cell repertoire.

First, the distribution of recognition specificities and the overall frequency of SV40-B6-responsive primary splenic B cells is similar in BALB/c (H-2^d) and C57BL/6 (H-2^b) mice. In both cases, the majority (63 and 71%, respectively) of responsive B cells are H-2 restricted in their recognition of SV40 transformation–associated antigens, and most commonly, the restriction appears associated with the K^b molecule. Therefore, unlike many instances in T cell repertoire expression, wherein preference for self + X recognition restricted by given MHC alloantigens appears dependent on the developmental environment of T cells (12, 43, 44), there is no obvious *Ir* gene effect due to the MHC developmental environment of the B cells in their response to SV40-B6 cells in the two strains studied.

Second, evidence for the lack of environmental modulation of this response is provided by the results from the analyses of sIg^- bone marrow cells from C57BL/6 and BALB/c mice in splenic fragment cultures. In both strains, the frequency ratio of sIg^- bone marrow to splenic SV40-B6-responsive cells is consistent with the similar ratio for several other antigens studied to date, including influenza virus PR8 antigens, PC, and DNP (24–26) suggesting that little modulation of the response has taken place during maturation of the B cells in vivo. Furthermore, the distribution of specificities is similar in the responsive sIg^- bone marrow and splenic precursor cells, again suggesting limited environmental effects on the establishment of the expressed SV40-B6-specific repertoire.

The seven hybridoma antibodies that recognize SV40-associated antigens in conjunction with H-2^b molecules should prove extremely useful in defining the molecular basis of complex antigen interactions, and studies to pursue these questions have been initiated. Some conclusions can already be drawn from the findings presented in this paper. The structure of antibody-combining sites is well established, and although the determinant interaction sites can be quite large, they represent a single binding structure (45–47). Therefore, the identi-

fication of MHC-restricted antibodies serves as an unambiguous demonstration that a single combining site can accomplish complex antigen recognition.

Postulates for the molecular basis of MHC-restricted antigen recognition by a single combining site include (*a*) the corecognition of determinants on both the foreign antigen and the MHC molecules, (*b*) the recognition of conformations of the foreign antigen induced by its interaction with an MHC molecule, and (*c*) the recognition of conformations of an MHC molecule induced by its interaction with the foreign antigen (48–50). Evidence has been presented by several laboratories (12, 13, 51–53) showing the potential for physical association of MHC molecules and antigens, a finding consistent with all three of the above postulates. Since the cell surface antigenic determinants associated with SV40 transformation are poorly defined, the findings presented herein are relatively uninformative concerning the first two postulates. However, several of our findings are of interest in this question. First, the binding of some hybridoma antibodies to SV40-transformed cells is disrupted by spatially separated H-2K^b mutations (e.g., SV4, SV27; see Table II). This may imply participation of conformational H-2 determinants in either antigen presentation or immune recognition (50). Second, none of the SV40-K^b-specific hybridoma antibodies bind SV40-transformed cells bearing the bm1 mutant. This finding is similar to the findings (12, 13) with SV40-K^b specific CTLs, which have been attributed to bm1 being nonassociative with SV40 transformation–associated antigens. Another possibility, however, is that the interaction of SV40 transformation antigens and K^b molecules bearing the bm1 mutations do not result in conformational changes necessary for recognition by lymphocytes. Finally, at high concentrations, some SV40-K^b-specific mAb are capable of variable degrees of binding to H-2K^b in the absence of SV40 transformation antigens. It is possible that this binding may reflect the ability of these antibodies to induce a conformation of the H-2 molecule similar to a conformation induced by association with SV40-associated antigens. This induced fit model has previously been proposed to explain the low-affinity binding of anti–synthetic peptide antibodies to the native protein from which the peptide sequence was derived (54, 55). As studies with these antibodies progress, these and similar issues could be resolved at the molecular level.

Taken as a whole, the results of studies using mAb derived from both splenic fragment cultures and transfer fusions are compatible in that they show an extraordinary predilection for recognition of SV40 transformation–associated antigens in conjunction with K^b molecules, and only a very low proportion of B cells that recognize cell surface antigens associated with SV40 transformation per se, or these antigens in conjunction with cell surface determinants common to H-2^b and H-2^d cell lines. The two hybridoma antibodies that display this latter recognition are currently being tested for their capacity to bind isolated SV40 transformation–associated antigens. The availability of large amounts of anti-SV40-B6 hybridoma antibodies should also ultimately provide a means for: (*a*) studying the idiotypic composition of an anti–self + X repertoire, (*b*) evaluating the biological effects of anti–self + X antibodies on T cell repertoire development, stimulation, and effector function, and (*c*) the affinity purification and analysis of the cell surface components contributing to the recognition site. It is

clear from the studies presented herein that, for certain cell surface antigens including antigens associated with transformation, self + X recognition is the primary mode of recognition for B cells and antibody as well as for T cells. Given the obvious physiological significance of such recognition, it will be important to assess the role of self + X recognition in both the evolutionary and somatic shaping of B cell repertoire expression.

Summary

Recognition of antigens on cell surfaces only in the context of the MHC-encoded alloantigens of the presenting cell (self + X) has classically been considered the province of T cells. However, evidence from several sources has indicated that B cells and antibodies can exhibit self + X–restricted recognition as well. This report concerns the mAb response to SV40-transformed H-2^b fibroblast cell lines. The specificities of the antibodies obtained have been analyzed for binding to a panel of SV40-transformed H-2-syngeneic, H-2-allogeneic, and H-2^b mutant fibroblast cell lines, as well as cell lines not bearing cell surface SV40 transformation–associated antigens. A large proportion of primary C57BL/6 (71%) and BALB/c (68%) splenic B cells responding to in vitro stimulation with SV40-transformed H-2^b cells recognize cell surface antigens associated with SV40 transformation only when coexpressed with MHC antigens of the immunizing cell, particularly the K^b molecule, on transformed cells. To extensively define the nature of antigen recognition by these antibodies, we have generated and characterized nine hybridoma antibodies specific for SV40-transformed H-2-syngeneic cell lines. Seven of these hybridoma antibodies recognize SV40-associated transformation antigens in the context of H-2^b molecules. Six of these are restricted by the K^b molecule and discriminate among a panel of SV40-transformed K^b mutant cell lines, thus confirming the participation of class I MHC–encoded molecules in the recognition by B cells of cell surface antigens.

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